Toxic Cyanobacteria in Water:

A guide to their public health consequences, monitoring and management

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Foreword

Concern about the effects of cyanobacteria on human health has grown in many countries in recent years for a variety of reasons. These include cases of poisoning attributed to toxic cyanobacteria and awareness of contamination of water sources (especially lakes) resulting in increased cyanobacterial growth. Cyanobacteria also continue to attract attention in part because of well-publicised incidents of animal poisoning.

Outbreaks of human poisoning attributed to toxic cyanobacteria have been reported in Australia, following exposure of individuals to contaminated drinking water, and in the UK, where army recruits were exposed while swimming and canoeing. However, the only known human fatalities associated with cyanobacteria and their toxins occurred in Caruaru, Brazil, where exposure through renal dialysis led to the death of over 50 patients. Fortunately, such severe acute effects on human health appear to be rare, but little is known of the scale and nature of either long-term effects (such as tumour promotion and liver damage) or milder short-term effects, such as contact irritation.

Water and health, and in particular drinking water and health, has been an area of concern to the World Health Organization (WHO) for many years. A major activity of WHO is the development of guidelines which present an authoritative assessment of the health risks associated with exposure to infectious agents and chemicals through water. Such guidelines already exist for drinking water and for the safe use of wastewater and excreta in agriculture and aquaculture, and are currently being prepared for recreational uses of water. In co-operation with the United Nations Educational, Scientific and Cultural Organization (UNESCO), United Nations Environment Programme (UNEP) and the World Meteorological Organization (WMO), WHO is also involved in the long-term monitoring of water through the GEMS/Water Programme; and in the monitoring of water supply and sanitation services in co-operation with the United Nations Children's Fund (UNICEF). The World Health Organization supports the development of national and international policies concerning water and health, and assists countries in developing capacities to establish and maintain healthy water environments, including legal frameworks, institutional structures and human resources.

The first WHO publication dealing specifically with drinking water was published in 1958 as *International Standards for Drinking-Water.* Further editions were published in 1963 and 1971. The first edition of WHO's *Guidelines for Drinking-Water Quality* was published in 1984-1985. It comprised three volumes: Volume 1: Recommendations; Volume 2: Health criteria and other supporting information; Volume 3: Drinking-water quality control in small-community supplies. The primary aim of the *Guidelines for Drinking-Water Quality* is the protection of public health. The guidelines provide an assessment of the health risks associated with exposure to micro-organisms and chemicals in drinking water. Second editions of the three volumes of the guidelines were published in 1993, 1996 and 1997 respectively and addenda to Volumes 1 and 2 were published in 1998.

Through ongoing review of the *Guidelines for Drinking-water Quality,* specific micro-organisms and chemicals are periodically evaluated and documentation relating to

protection and control of drinking-water quality is prepared. The Working Group on Protection and Control of Drinking-Water Quality identified cyanobacteria as one of the most urgent areas in which guidance was required. During the development by WHO of the *Guidelines for Safe Recreational-water Environments,* it also became clear that health concerns related to cyanobacteria should be considered and were an area of increasing public and professional interest.

This book describes the present state of knowledge regarding the impact of cyanobacteria on health through the use of water. It considers aspects of risk management and details the information needed for protecting drinking water sources and recreational water bodies from the health hazards caused by cyanobacteria and their toxins. It also outlines the state of knowledge regarding the principal considerations in the design of programmes and studies for monitoring water resources and supplies and describes the approaches and procedures used.

The development of this publication was guided by the recommendations of several expert meetings concerning drinking water (Geneva, December 1995; Bad Elster, June 1996) and recreational water (Bad Elster, June 1996; St Helier, May 1997). An expert meeting in Bad Elster, April 1997, critically reviewed the literature concerning the toxicity of cyanotoxins and developed the scope and content of this book. A draft manuscript was reviewed at an editorial meeting in November 1997, and a further draft was reviewed by the working group responsible for updating the *Guidelines for Drinking-water Quality* in March 1998.

Toxic Cyanobacteria in Water is one of a series of guidebooks concerning water management issues published by E & FN Spon on behalf of WHO. Other volumes in the series include:

Water Quality Assessments (D. Chapman, Ed., Second Edition, 1996)
Water Quality Monitoring (J. Bartram and R. Ballance, Eds, 1996)
Water Pollution Control (R. Helmer and I. Hespanhol, Eds, 1997)
It is hoped that this volume will be useful to all those concerned with cyanobacteria and health, including environmental and public health officers and professionals in the fields of water supply and management of water resources and recreational water. It should also be of interest to postgraduates in these fields as well as to those involved in freshwater ecology and special interest groups.

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Chapter 1. INTRODUCTION

This chapter was prepared by Jamie Bartram, Wayne W. Carmichael, Ingrid Chorus, Gary Jones, and Olav M. Skulberg

"A pet child has many names". This proverb is well illustrated by such expressions as blue-greens, blue-green algae, myxophyceaens, cyanophyceans, cyanophytes, cyanobacteria, cyanoprokaryotes, etc. These are among the many names used for the organisms this book considers. This apparent confusion in use of names highlights the important position that these organisms occupy in the development of biology as a science. From their earliest observation and recognition by botanists (Linné, 1755; Vaucher, 1803; Geitler, 1932), and onwards to their treatment in modem textbooks (Anagnostidis and Komárek, 1985; Staley *et al.,* 1989), the amazing combination of properties found in algae and bacteria which these organisms exhibit, have been a source of fascination and attraction for many scientists.

The cyanobacteria also provide an extraordinarily wide-ranging contribution to human affairs in everyday life (Tiffany, 1958) and are of economic importance (Mann and Carr, 1992). Both the beneficial and detrimental features of the cyanobacteria are of considerable significance. They are important primary producers and their general nutritive value is high. The nitrogen-fixing species contribute globally to soil and water fertility (Rai, 1990). The use of cyanobacteria in food production and in solar energy conversion holds promising potential for the future (Skulberg, 1995). However, cyanobacteria may also be a source of considerable nuisance in many situations. Abundant growth of cyanobacteria in water reservoirs creates severe practical problems for water supplies. The development of strains containing toxins is a common experience in polluted inland water systems all over the world, as well as in some coastal waters. Thus cyanobacterial toxins, or "cyanotoxins", have become a concern for human health.

Prior to the first acute cyanotoxin poisoning of domestic animals documented in the scientific literature (Francis, 1878), reports of cyanobacteria poisonings were largely anecdotal. Perhaps one of the earliest is from the Han dynasty of China. About 1,000 years ago, General Zhu Ge-Ling, while on a military campaign in southern China, reported losing troops from poisonings whilst crossing a river. He reported that the river was green in colour at the time and that his troops drank from the green water (Shun Zhang Yu, Pers. Comm.). Codd (1996) reported that human awareness of toxic blooms existed in the twelfth century at the former Monasterium Virdis Stagni (Monastery of the Green Loch), located near the eutrophic, freshwater Soulseat Loch near Stranraer in south west Scotland. In more recent times, several investigators have noted that local people in China, Africa, North and South America and Australia, who use water from

water bodies where green scums are present, will dig holes (soaks) near the water's edge in order to filter the water through the ground and thus prevent the green material from contaminating drinking-water supplies. This practice is similar to that of developing wells next to surface waters in order to use the filtering capacity of the soil to remove organisms and some chemicals from the surface waters - a technique known as bankside filtration.

1.1 Water resources

The hydrological cycle represents a complex interconnection of diverse water types with different characteristics, each subject to different uses. Recent developments have shown the importance of water resource management in an integrated manner and of recognising interconnections, especially between human activities and water quality.

Most of the world's available freshwater (i.e. excluding that in polar ice-caps, snow and glaciers) exists as groundwater. This ready supply of relatively clean and accessible water has encouraged use of this resource, and in many regions groundwater provides drinking water of excellent quality. However, in some areas, geological conditions do not allow the use of groundwater or the supplies are insufficient. Thus, where groundwater supplies are insufficient or of unsuitable quality, surface water must be used for purposes such as drinking-water supply. Compared with surface waters, groundwaters have a high volume and low throughput. Over-abstraction is therefore common.

This book is concerned principally with inland, surface freshwaters, and to a lesser extent with estuarine and coastal waters where cyanobacteria can grow, and under suitable conditions, form water blooms or surface scums. Cyanobacteria are a frequent component of many freshwater and marine ecosystems. Those species that live dispersed in the water are part of the phytoplankton whilst those that grow on sediments form part of the phytobenthos. Under certain conditions, especially where waters are rich in nutrients and exposed to sunlight, cyanobacteria may multiply to high densities - a condition referred to as a water bloom (see Chapter 2).

The composition of freshwaters is dependent on a number of environmental factors, including geology, topography, climate and biology. Many of these factors vary over different time scales such as daily, seasonally, or even over longer timespans. Large natural variations in water quality may therefore be observed in any given water system.

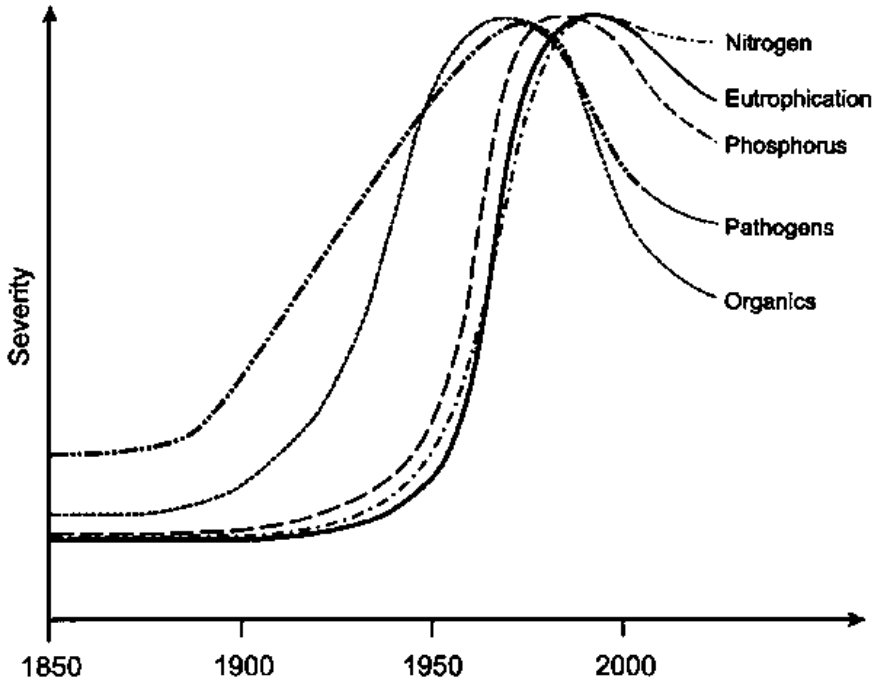
Eutrophication is the enhancement of the natural process of biological production in rivers, lakes and reservoirs, caused by increases in levels of nutrients, usually phosphorus and nitrogen compounds. Eutrophication can result in visible cyanobacterial or algal blooms, surface scums, floating plant mats and benthic macrophyte aggregations. The decay of this organic matter may lead to the depletion of dissolved oxygen in the water, which in turn can cause secondary problems such as fish mortality from lack of oxygen and liberation of toxic substances or phosphates that were previously bound to oxidised sediments. Phosphates released from sediments accelerate eutrophication, thus closing a positive feedback cycle. Some lakes are naturally eutrophic but in many others the excess nutrient input is of anthropogenic origin, resulting from municipal wastewater discharges or run-off from fertilisers and manure spread on agricultural areas. Losses of nutrients due to erosion and run-off from soils may be low in relation to agricultural input and yet high in relation to the eutrophication

they cause, because concentrations of phosphorus of less than 0.1 mg l^{-1} are sufficient to induce a cyanobacterial bloom (see Chapter 8).

Hydrological differences between rivers, impoundments and lakes have important consequences for nutrient concentrations and thus for cyanobacterial growth. Rivers generally have a significant flushing rate. The term "self-purification" was adopted to describe the rapid degradation of organic compounds in rivers where turbulent mixing effectively replenishes consumed oxygen. This term has been applied, mistakenly, to any process of removing undesirable substances from water but does not actually eliminate the contaminants, including processes such as adsorption to sediments or dilution. Substances bound to sediments may accumulate, be released back into the water, and may be carried downstream. This process is important for phosphorus. Lakes generally have long water retention times compared with rivers, and by their nature lakes tend to accumulate sediments and the chemicals associated with them. Sediments therefore act as sinks for important nutrients such as phosphorus, but if conditions change the sediments may also serve as sources, liberating the nutrient back into the water where it can stimulate the growth of cyanobacteria and algae.

Surface water systems world-wide are now often highly regulated in efforts to control water availability, whether for direct use in irrigation, hydropower generation or drinking water supplies or to guard against the consequences of floods and droughts. Many major rivers (such as the Danube in Europe or the Murray in Australia) may be viewed as a cascade of impoundments. This trend in regulation of flow has an impact upon the quality and the quantity of water. It alters sediment transport and, as a result, the transport of substances attached to sediments, such as plant nutrients which may enhance cyanobacterial growth. By increasing retention times and surface areas exposed to sunlight, impoundments change the growth conditions for organisms and promote opportunities for cyanobacterial growth and water-bloom formation through modifications to river discharges. For many estuarine and coastal systems, human impact on hydrological conditions and nutrient concentrations is also now extensive.

Figure 1.1 Schematic representation of the development of surface water pollution with pathogens, oxygen-consuming organic matter, phosphorus and cyanobacteria in north-western Europe and in North America



Changes in the nature and scale of human activities have consequences both for the qualitative and quantitative properties of water resources. Historically, the development of society has involved a change from rural and agricultural to urban and industrial water uses, which is reflected in both water demands and water pollution as illustrated in Figure 1.1. The general trend has been an increase in concentrations of pollutants in surface waters together with increases in urbanisation. Construction of sewerage first enhanced this trend by concentrating pollutants from latrines (which can leak into groundwater or surface waters). After some decades, construction of sewage treatment systems began extensively in the 1950s. Originally these systems comprised only a biological step which degraded the organic material which otherwise had led to dramatic oxygen depletion in the receiving water bodies. Pathogens were also reduced to some extent, but phosphate remained unaffected. Upgrading treatment systems to remove phosphorus only began in the 1960s and also had the side-effect of further reducing pathogens. A resultant decline in eutrophication, and thus of cyanobacterial blooms, is lagging behind the decline of phosphorus inputs to freshwaters because phytoplankton growth becomes nutrient-controlled only below threshold concentrations (see Chapter 8).

It is unclear whether the historical shift in water demand from rural to urban will continue in the future, although a number of influences are apparent. The anticipated food crises of the early twenty first century will place increasing demands upon irrigated agriculture - a process that already accounts for about 70 per cent of water demand world-wide. By contrast, many industries have successfully developed processes with substantial water economy measures, and their demand upon water resources per unit of activity is now decreasing in some countries. Domestic water consumption tends to increase with population and affluence, but development of lower consumption appliances and control

of losses from water mains may stabilise, or even reduce, demand in the future. Nevertheless, overall trends point to an increasing total demand for water, driven principally by global population growth.

1.2 Eutrophication, cyanobacterial blooms and surface scums

Eutrophication was recognised as a pollution problem in many western European and North American lakes and reservoirs in the middle of the twentieth century (Rohde, 1969). Since then, it has become more widespread, especially in some regions; it has caused deterioration in the aquatic environment and serious problems for water use, particularly in drinking-water treatment. A recent survey showed that in the Asia Pacific Region, 54 per cent of lakes are eutrophic; the proportions for Europe, Africa, North America and South America are 53 per cent, 28 per cent, 48 per cent and 41 per cent respectively (ILEC/Lake Biwa Research Institute, 1988-1993). Eutrophication also affects slow flowing rivers, particularly if they have extended low-flow periods during a dry season. Practical measures for prevention of nutrient loading from wastewater and from agriculture have been developed. In some regions preventative measures are being implemented more and more. During the 1990s, increasing introduction of nutrient removal during sewage treatment in North America and in north western Europe has begun to show success in reducing phosphorus concentrations; in a few water bodies, algal and cyanobacterial blooms have actually declined. Technical measures for reduction of nutrients already present in lakes are also available but have not been widely applied (see Chapter 8).

Wherever conditions of temperature, light and nutrient status are conducive, surface waters (both freshwater and marine) may host increased growth of algae or cyanobacteria. Where such proliferation is dominated by a single (or a few) species, the phenomenon is referred to as an algal or cyanobacterial bloom. Problems associated with cyanobacteria are likely to increase in areas experiencing population growth with a lack of concomitant sewage treatment and in regions with agricultural practices causing nutrient losses to water bodies through over-fertilisation and erosion.

There are important differences in algal and cyanobacterial growth between tropical and temperate areas. A characteristic pattern of seasonal succession of algal and cyanobacterial communities is, for example, diatoms in association with rapidly growing small flagellates in winter and spring, followed by green algae in late spring and early summer, and then by species which cannot easily be eaten by zooplankton, such as dinoflagellates, desmids and large yellow-green algae (in moderately turbulent waters also diatoms) in late summer and autumn. In eutrophic and hypertrophic waters, cyanobacteria often dominate the summer phytoplankton. As winter approaches, in most water bodies, increasing turbulence and the lack of light during the winter leads to their replacement by diatoms. In the tropics, seasonal differences in environmental factors are often not great enough to induce the replacement of cyanobacteria by other phytoplankton species. If cyanobacteria are present or even dominant for most of the year, the practical problems associated with high cyanobacterial biomass and the potential health threats from their toxins increase. High cyanobacterial biomass may also contribute to aesthetic problems, impair recreational use (due to surface scums and unpleasant odours), and affect the taste of treated drinking water.

Phosphorus is the major nutrient controlling the occurrence of water blooms of cyanobacteria in many regions of the world, although nitrogen compounds are sometimes relevant in determining the amount of cyanobacteria present. However, in contrast to planktonic algae, some cyanobacteria are able to escape nitrogen limitation by fixing atmospheric nitrogen. The lack of nitrate or ammonia, therefore, favours the dominance of these species. Thus, the availability of nitrate or ammonia is an important factor in determining which cyanobacterial species become dominant.

Cyanobacterial blooms are monitored using biomass measurements coupled with the examination of the species present. A widely-used measure of algal and cyanobacterial biomass is the chlorophyll *a* concentration. Peak values of chlorophyll *a* for an oligotrophic lake are about 1-10 µg l^{-1}, while in a eutrophic lake they can reach 300 µg l^{-1}. In cases of hypereutrophy, such as Hartbeespoort Dam in South Africa, maxima of chlorophyll *a* can be as high as 3,000 µg l^{-1} (Zohary and Roberts, 1990).

Trophic state classifications, such as that adopted by the Organisation for Economic Co-operation and Development (OECD), combine information concerning nutrient status and algal biomass (OECD, 1982). They provide a basis for the evaluation of status and trends for management and they facilitate international information exchange and comparison.

1.3 Toxic cyanobacteria and other water-related health problems

The contamination of water resources and drinking water supplies by human excreta remains a major human health concern, just as it has been for centuries. By contrast, the importance of toxic substances, such as metals and synthetic organic compounds, has only emerged in the latter half of the twentieth century. Although eutrophication has been recognised as a growing concern since the 1950s, only recently have cyanobacterial toxins become widely recognised as a human health problem arising as a consequence of eutrophication. The importance of such toxins, relative to other water-health issues, can currently only be estimated. A significant proportion of cyanobacteria produce one or more of a range of potent toxins (see Chapter 3). If water containing high concentrations of toxic cyanobacteria or their toxins is ingested (in drinking water or accidentally during recreation), they present a risk to human health (see Chapter 4). Some cyanobacterial substances may cause skin irritation on contact.

The relationship between water resources and health is complex. The most well recognised relationship is the transmission of infectious and toxic agents through consumption of water. Drinking water has therefore played a prominent role in concerns for water and human health. Diseases arising from the consumption of contaminated water are generally referred to as "waterborne". Globally, the waterborne diseases of greatest importance are those caused by bacteria, viruses and parasites, such as cholera, typhoid, hepatitis A, cryptosporidiosis and giardiasis. Most of the pathogens involved are derived from human faeces and the resulting diseases are generally referred to as "faecal-oral" diseases; however they can also be spread by means other than contaminated water, such as by contaminated food. Waterborne diseases also include some caused by toxic chemicals, although many of these may only cause health effects some time after exposure has occurred and may therefore be difficult to associate directly with the cause.

The second major area of interaction between water and human health concerns its role in personal and domestic hygiene, through which it contributes to the control of disease. Because hygiene is a key measure in the control of faecal-oral disease, such diseases are also "water hygiene" diseases. Other water hygiene diseases include skin and eye infections and infestations, such as tinea, scabies, pediculosis and trachoma. All of these diseases occur less frequently when adequate quantities of water are available for personal and domestic hygiene. It is important to note that the role of water in control of water hygiene diseases depends on availability and use, and water quality is therefore a secondary consideration in this context.

"Water contact diseases" are the third group of water-related diseases and occur through skin contact. The most important example world-wide is schistosomiasis (bilharzia). In infected persons, eggs of *Schistosoma* spp. are excreted in faeces or urine. The schistosomes require a snail intermediate host and go on to infect persons in contact with water by penetrating intact skin. The disease is of primary importance in areas where collection of water requires wading or direct contact with contaminated surface waters such as lakes or rivers. The water contact diseases also include those diseases arising from non-infectious agents in the water, that may give rise, for example, to allergies and to skin irritation or to dermatitis.

The fourth principal connection between water and human health concerns "water habitat vector" diseases. These are diseases transmitted by insect vectors that spend all or part of their lives in or near water. The best-known examples are malaria (transmitted by mosquito bites and caused by *Plasmodium* spp.) and filariasis (transmitted by mosquito bite and caused by microfilaria).

The classification of water-related disease into four groups (waterborne disease, water hygiene disease, water contact disease and water habitat vector disease) was originally developed in order to associate groups of disease more clearly with the measures for their transmission and control and has contributed greatly to furthering this understanding. Because of its importance to the global burden of disease, the classification is based upon infectious disease. Nevertheless, the principal groups of diseases related to chemicals occurring in water may also be categorised in a similar way. However, there are a number of water-health associations that fall outside these categories. These include deficiency-related diseases and recreational uses of water. For recreational water use, the principal area of concern relating to faecal-oral disease transmission may be classified reasonably alongside other waterborne disease transmission. However, concern related to transmission of, for example, eye and ear infections does not readily fit into the classification system, nor does the increased transmission of diseases arising from the effect of immersion compromising natural defence systems (such as those of the eye).

Public health concern regarding cyanobacteria centres on the ability of many species and strains of these organisms to produce cyanotoxins. Cyanotoxins may fall into two of the four groups of water-related diseases. They may cause waterborne disease when ingested, and water contact disease primarily through recreational exposure. In hospitals and clinics, exposure through intravenous injection has led to human fatalities from cyanotoxins (see Chapter 4). These toxins pose a challenge for management. Unlike most toxic chemicals, cyanotoxins only sometimes occur dissolved in the water - they are usually contained within cyanobacterial cells. In contrast to pathogenic bacteria,

these cells do not proliferate within the human body after uptake, only in the aquatic environment before uptake.

Cyanotoxins belong to rather diverse groups of chemical substances (see Chapter 3), each of which shows specific toxic mechanisms in vertebrates (see Chapter 4). Some cyanotoxins are strong neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins), others are primarily toxic to the liver (microcystins, nodularin and cylindrospermopsin), and yet others (such as the lipopolysaccharides) appear to cause health impairments (such as gastroenteritis) which are poorly understood. Microcystins are geographically most widely distributed in freshwaters. Recently, they have even been identified in marine environments as a cause of liver disease in net-pen reared salmon, although it is not clear which organism in marine environments contains these toxins. As with many cyanotoxins, microcystins were named after the first organism found to produce them, *Microcystis aeruginosa,* but later studies also showed their occurrence in other cyanobacterial genera.

The hazard to human health caused by cyanotoxins can be estimated from toxicological knowledge (see section 4.2) in combination with information on their occurrence (see section 3.2). However, although the information clearly indicates hazards, there are few documented cases of human illness unequivocally attributed to cyanotoxins (see section 4.1). In a number of cases, investigation of cyanobacteria and cyanotoxins was carried out only several days after patients had been exposed and had developed symptoms. This was because diagnosis moved on to considering cyanobacteria only after other potential causative agents had proved negative, or even years later when knowledge of cyanobacterial blooms in a water body was connected with the information on an outbreak of symptoms of unidentified cause.

The number of quantitative surveys on cyanotoxin occurrence is low, and the level of cyanotoxin exposure through drinking water or during recreational activities largely unknown. Surveys on cyanobacteria and cyanotoxins have been primarily ecological and biogeographical. Early surveys in a number of countries including Australia, Canada, Finland, Norway, South Africa, Sweden, the UK and the USA involved toxicity testing of scum samples by mouse bioassay. Surveys during the 1990s have tended to employ more sensitive and definitive methods for characterisation of the toxins, such as chromatographic or immunological methods (see Chapter 3). These studies provide an improving basis for estimating the range of concentrations to be expected in a given water body and season. However, monitoring cyanotoxin concentration is more difficult than many other waterborne disease agents, because variations in cyanobacterial quantities, in time and space, is substantial, particularly if scum-forming species are dominant (see section 2.2). Wind-driven accumulations and distribution of surface scums can result in concentrations of the toxin by a factor of 1,000 or more (or even result in the beaching of scums) and such situations can change within very short time periods, i.e. the range of hours. Therefore, discontinuous samples only provide a fragmentary insight into the potential cyanotoxin dose for occasional swimmers and into the amount entering drinking water intakes.

Very few studies of cyanotoxin removal by drinking water treatment processes have been published (see Chapter 9), although some water companies have carried out unpublished studies. Thus, a reliable basis for estimation of cyanotoxin exposure through drinking water is lacking. In regions using surface waters affected with

cyanobacteria as a source for drinking water, actual toxin exposure will depend strongly on method of water abstraction and treatment.

In comparing the available indications of hazards from cyanotoxins with other water-related health hazards, it is conspicuous that cyanotoxins have caused numerous fatal poisonings of livestock and wildlife, but no human fatalities due to oral uptake have been documented. Human deaths have only been observed as a consequence of intravenous exposure through renal dialysis. Cyanotoxins are rarely likely to be ingested by humans in sufficient amounts for an acute lethal dose. Thus, cyanobacteria are less of a health hazard than pathogens such as *Vibrio cholerae* or *Salmonella typhi.* Nevertheless, dose estimates indicate that a fatal dose is possible for humans, if scum material is swallowed. However, swallowing such a repulsive material is likely to be avoided. The combination of available knowledge on chronic toxicity mechanisms (such as cumulative liver damage and tumour promotion by microcystins) with that on ambient concentrations occurring under some environmental conditions, shows that chronic human injury from some cyanotoxins is likely, particularly if exposure is frequent or prolonged at high concentrations.

1.4 Present state of knowledge

Research into developing further understanding of the human health significance of cyanobacteria and individual cyanotoxins, and into practical means for assessing and controlling exposure to cyanobacteria and to cyanotoxins, is a priority. A major gap also lies in the synthesis and dissemination of the available information.

Information concerning the efficiency of cyanotoxin removal in drinking water treatment systems is limited. Especially, simple, low-cost techniques for cyanobacterial cell removal, such as slow sand filtration, should be investigated and developed further. More information is also needed on the capability of simple disinfection techniques, such as chlorine, for oxidising microcystins and cylindrospermopsin (Nicholson *et at.,* 1994). If this is found to be applicable, or if "conventional" treatments are found to be effective if properly operated, these approaches would provide a practical tool for removing cyanotoxins in many situations.

Whilst cyanobacterial blooms remain sporadic or occasional events, most emphasis is still placed upon the protection of drinking water supplies through the preparation of contingency plans and their activation when appropriate. Early warning systems and predictive models can facilitate this and should be based upon available information on the conditions leading to cyanobacterial bloom development and on occurrence, localisation and movement of scums.

Epidemiological evidence is of particular value in determining the true nature and severity of human health effects (and therefore the appropriate response) but is generally lacking in relation to human exposures to cyanobacteria. The limited studies undertaken to date in relation to recreational exposure require further substantiation. Opportunistic studies into exposures through drinking water may provide further valuable insights. Information from experimental toxicology also needs to be strengthened. In particular, long-term exposure studies (of at least one year or longer) should be carried out to assess the chronic toxicity of microcystins and cylindrospermopsins. Uptake routes (e.g. through nasal tissues and mucous membranes) require further investigation.

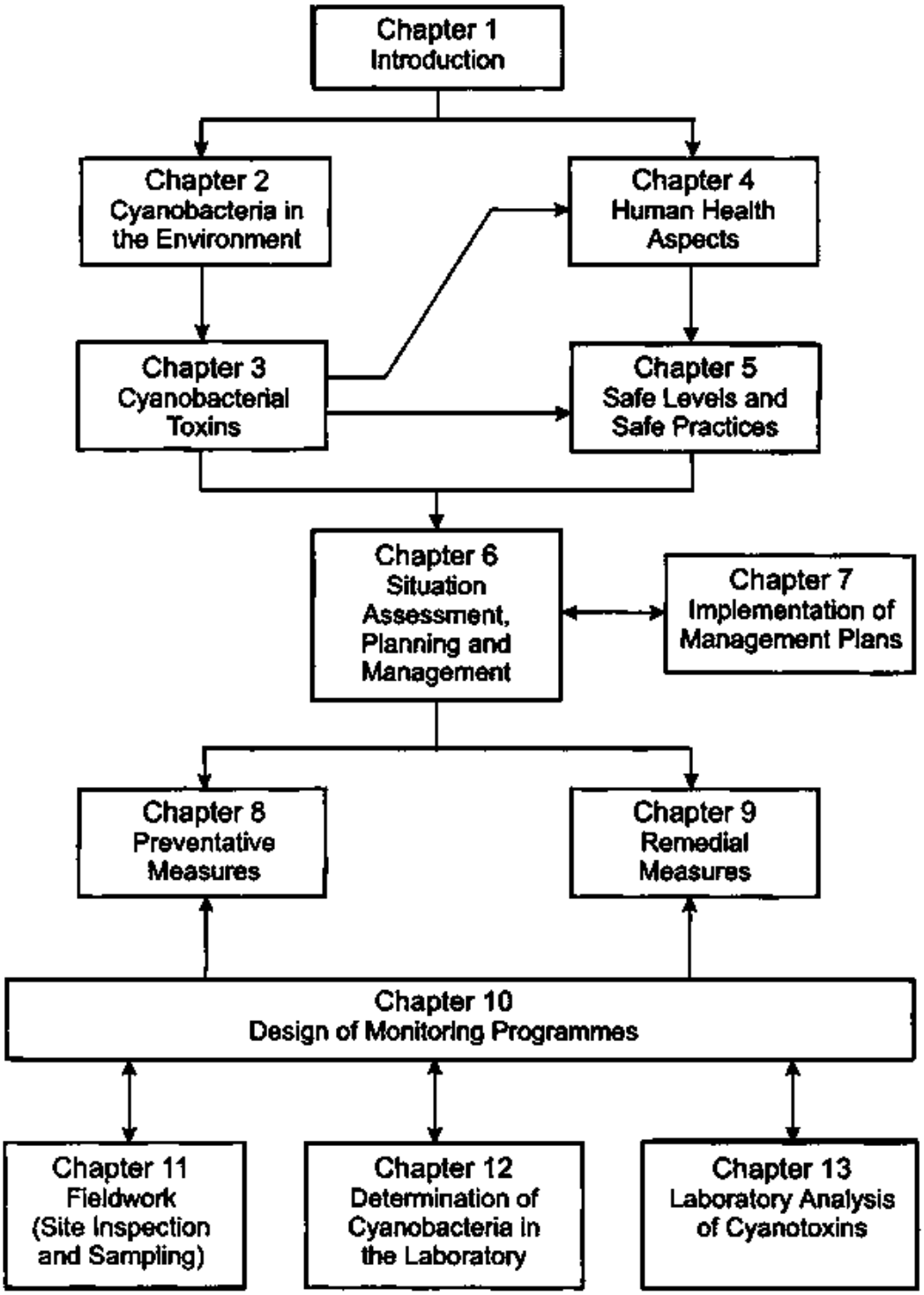
Further systematic studies are also required into the suggested tumour-promoting effects of some cyanotoxins, particularly in the dose range of potential oral uptake with drinking or bathing water.

Lipopolysaccharide (LPS) endotoxins from cyanobacteria pose a potential health risk for humans, but knowledge of the occurrence of individual LPS components, their toxicology, and their removal in drinking water treatment plants, is so poor that guidelines cannot be set at present. Further bioactive cyanobacterial metabolites are also identified frequently and the health significance of these requires investigation.

1.5 Structure and purpose of this book

The structure of this book follows a logical progression of issues as outlined in Figure 1.2. Because of the lack of comprehensive literature in the field of cyanotoxins, this book aims to give background information as well as practical guidance. Some parts of the text will mainly be of interest to particular readers. Chapters 2 and 3 provide the background for understanding the behaviour of cyanobacteria and their toxin production in given environmental conditions. Chapter 4 reviews the evidence regarding health impacts, primarily for public health experts establishing national guidelines or academics identifying and addressing current research needs. Chapters 5-7 provide guidance on safe practices in the planning and management of drinking water supplies and recreational resorts. Readers who access the book with specific questions regarding prevention of cyanobacterial growth or their removal in drinking water treatment will find Chapters 8 and 9 of direct relevance. Guidance on the design and implementation of monitoring programmes is given in Chapter 10, and Chapters 11-13 provide field and laboratory methods for monitoring cyanobacteria, their toxins and the conditions which lead to their excessive growth. As far as is possible, individual chapters have been written to be self-contained and self-explanatory. However, substantial cross-referencing, particularly between Chapters 10 to 13, requires that these chapters should be used jointly. Where chapters call upon information presented elsewhere in the text, this has been specifically noted.

Figure 1.2 Aspects of monitoring and managing toxic cyanobacteria in water as discussed in the various chapters of this book



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Chapter 2. CYANOBACTERIA IN THE ENVIRONMENT

This chapter was prepared by Luuc R. Mur, Olav M. Skulberg and Hans Utkilen

For management of cyanobacterial hazards to human health, a basic understanding of the properties, the behaviour in natural ecosystems, and the environmental conditions which support the growth of certain species is helpful. This chapter provides information on how cyanobacteria are structured and the abilities which they posses that support their proliferation in aquatic ecosystems.

2.1 Nature and diversity

2.1.1 Systematics

Plants and animals possess consistent features by which they can be identified reliably and sorted into recognisably distinct groups. Biologists observe and compare what the organisms look like, how they grow and what they do. The results make it possible to construct systematic groupings based on multiple correlations of common characters and that reflect the greatest overall similarity. The basis for such groupings is the fact that all organisms are related to one another by way of evolutionary descent. Their biology and phylogenetic relationships makes the establishment of systematic groupings possible (Minkoff, 1983).

However, microbial systematics has long remained an enigma. Conceptual advances in microbiology during the twentieth century included the realisation that a discontinuity exists between those cellular organisms that are prokaryotic (i.e. whose cells have no nucleus) and those that are eukaryotic (i.e. more complexly structured cells with a nucleus) within the organisation of their cells. The microalgae investigated by phycologists under the International Code of Botanical Nomenclature (ICBN) (Greuter *et al.,* 1994) included organisms of both eukaryotic and prokaryotic cell types. The blue-green algae (Geitler, 1932) constituted the largest group of the latter category. The prokaryotic nature of these organisms and their fairly close relationship with eubacteria made work under provisions of the International Code of Nomenclature of Bacteria (ICNB) (Sneath, 1992) more appropriate (Rippka *et al.,* 1979; Waterbury, 1992).

The prevailing systematic view is that comparative studies of the genetic constitution of the cyanobacteria will now contribute significantly to the revision of their taxonomy. Relevant classification should reflect as closely as possible the phylogenetic

relationships as, for example, encoded in 16S or 23S rRNA sequence data (Woese, 1987). The integration of phenotypic, genotypic and phylogenetic information render possible a consensus type of taxonomy known as polyphasic taxonomy (Vandamme *et al.,* 1996).

The names "cyanobacteria" and "blue-green algae" (Cyanophyceae) are valid and compatible systematic terms. This group of micro-organisms comprises unicellular to multicellular prokaryotes that possess chlorophyll *a* and perform oxygenic photosynthesis associated with photosystems I and II (Castenholz and Waterbury, 1989).

2.1.2 Occurrence in nature

The majority of cyanobacteria are aerobic photoautotrophs. Their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism. In the natural environment, however, it is known that some species are able to survive long periods in complete darkness. Furthermore, certain cyanobacteria show a distinct ability for heterotrophic nutrition (Fay, 1965).

Cyanobacteria are often the first plants to colonise bare areas of rock and soil. Adaptations, such as ultraviolet absorbing sheath pigments, increase their fitness in the relatively exposed land environment. Many species are capable of living in the soil and other terrestrial habitats, where they are important in the functional processes of ecosystems and the cycling of nutrient elements (Whitton, 1992).

The prominent habitats of cyanobacteria are limnic and marine environments. They flourish in water that is salty, brackish or fresh, in cold and hot springs, and in environments where no other microalgae can exist. Most marine forms (Humm and Wicks, 1980) grow along the shore as benthic vegetation in the zone between the high and low tide marks. The cyanobacteria comprise a large component of marine plankton with global distribution (Wille, 1904; Gallon *et al.,* 1996). A number of freshwater species are also able to withstand relatively high concentrations of sodium chloride. It appears that many cyanobacteria isolated from coastal environments tolerate saline environments (i.e. are halotolerant) rather than require salinity (i.e. are halophilic). As frequent colonisers of euryhaline (very saline) environments, cyanobacteria are found in salt works and salt marshes, and are capable of growth at combined salt concentrations as high as 3-4 molar mass (Reed *et al.,* 1984). Freshwater localities with diverse trophic states are the prominent habitats for cyanobacteria. Numerous species characteristically inhabit, and can occasionally dominate, both near-surface epilimnic and deep, euphotic, hypolimnic waters of lakes (Whitton, 1973). Others colonise surfaces by attaching to rocks or sediments, sometimes forming mats that may tear loose and float to the surface.

Cyanobacteria have an impressive ability to colonise infertile substrates such as volcanic ash, desert sand and rocks (Jaag, 1945; Dor and Danin, 1996). They are extraordinary excavators, boring hollows into limestone and special types of sandstone (Weber *et al.,* 1996). Another remarkable feature is their ability to survive extremely high and low temperatures. Cyanobacteria are inhabitants of hot springs (Castenholz, 1973), mountain streams (Kann, 1988), Arctic and Antarctic lakes (Skulberg, 1996a) and snow and ice (Kol, 1968; Laamanen, 1996). The cyanobacteria also include species that run through the entire range of water types, from polysaprobic zones to katharobic waters (Van Landingham, 1982).

Cyanobacteria also form symbiotic associations with animals and plants. Symbiotic relations exist with, for example, fungi, bryophytes, pteridophytes, gymnosperms and angiosperms (Rai, 1990). The hypothesis for the endosymbiotic origin of chloroplasts and mitochondria should be mentioned in this context. The evolutionary formation of a photosynthetic eukaryote can be explained by a cyanobacteria being engulfed and co-developed by a phagotrophic host (Douglas, 1994).

Fossils of what were almost certainly prokaryotes are present in the 3,450 million year old Warrawoona sedimentary rock of north-western Australia. Cyanobacteria were among the pioneer organisms of the early earth (Brock 1973; Schopf, 1996). These photosynthetic micro-organisms were, at that time, probably the chief primary producers of organic matter, and the first organisms to release elemental oxygen into the primitive atmosphere. Sequencing of deoxyribonucleic acid (DNA) has given evidence that the earliest organisms were thermophilic and thus able to survive in oceans that were heated by volcanoes, hot springs and bolide impacts (Holland, 1997).

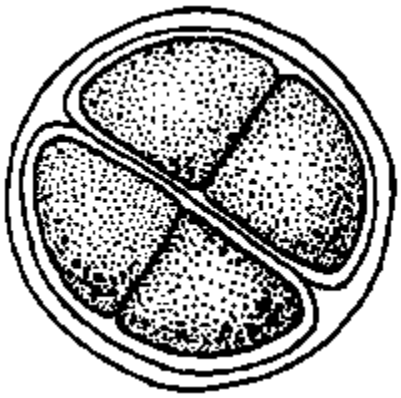
2.1.3 Organisation, function and behaviour

The structure and organisation of cyanobacteria are studied using light and electron microscopes. The basic morphology comprises unicellular, colonial and multicellular filamentous forms (Figure 2.1).

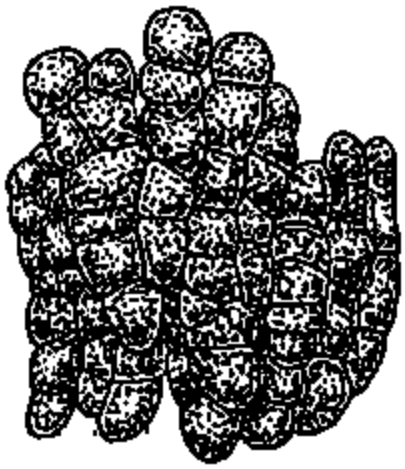
Unicellular forms, for example in the order Chroococcales, have spherical, ovoid or cylindrical cells. They occur singly when the daughter cells separate after reproduction by binary fission. The cells may aggregate in irregular colonies, being held together by the slimy matrix secreted during the growth of the colony. By means of a more or less regular series of cell division, combined with sheath secretions, more ordered colonies may be produced.

Figure 2.1 Basic morphology of cyanobacteria

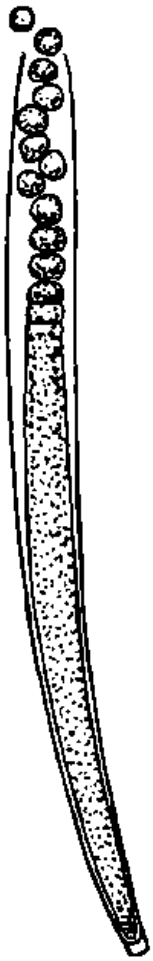
Unicellular, isopolar (Order: *Chroococcales)*



Pseudoparenchymatous (Order: *Pleurocapsales)*

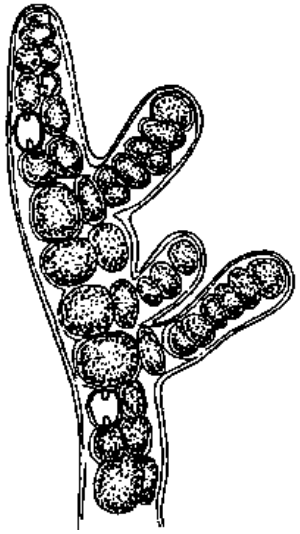


Unicellular, heteropolar (Order: *Chamaesiphonales)*



Multicellular, trichal, heterocysts not present (Order: *Oscillatoriales)*

Multicellular, trichal, with branches, heterocysts present (Order: *Stigonematales)*



Multicellular, trichal, heterocysts present (Order: *Nostocales)*

A particular mode of reproduction, which may supplement binary fission, distinguishes cyanobacteria in the order Chamaesiphonales and Pleurocapsales. In the Chamaesiphonales exospores are budded off from the upper ends of cells. In the second order, the principal mode of replication is by a series of successive binary fissions converting a single mother cell into many minute daughter cells (baeocytes or endospores).

Filamentous morphology is the result of repeated cell divisions occurring in a single plane at right angles to the main axis of the filament. The multicellular structure consisting of a chain of cells is called a trichome. The trichome may be straight or coiled. Cell size and shape show great variability among the filamentous cyanobacteria. Species in the order Oscillatoriales, with unseriated and unbranched trichomes, are composed of essentially identical cells. The other orders with a filamentous organisation

(orders Nostocales and Stigonematales) are characterised with trichomes having a heterogeneous cellular composition. Vegetative cells may be differentiated into heterocysts (having a thick wall and hyaline protoplast, capable of nitrogen fixation) and akinetes (large thick-walled cells, containing reserve materials, enabling survival under unfavourable conditions). In the order Stigonematales, the filaments are often multiseriated, with genuine branching. Both heterocysts and akinetes are present.

The only means of reproduction in cyanobacteria is asexual. Filamentous forms reproduce by trichome fragmentation, or by formation of special hormogonia. Hormogonia are distinct reproductive segments of the trichomes. They exhibit active gliding motion upon their liberation and gradually develop into new trichomes.

In contrast to eukaryotic microalgae, cyanobacteria do not possess membrane-bound sub-cellular organelles; they have no discrete membrane-bound nucleus; they possess a wall structure based upon a peptidoglycan layer; and they contain 70 S rather than 80 S ribosomes (Fay and Van Baalen, 1987; Bryant, 1994).

The photosynthetic pigments of cyanobacteria are located in thylakoids that lie free in the cytoplasm near the cell periphery. Cell colours vary from blue-green to violet-red. The green of chlorophyll *a* is usually masked by carotenoids (e.g. beta-carotene) and accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin (phycobiliproteins). The pigments are embodied in phycobilisomes, which are found in rows on the outer surface of the thylakoids (Douglas, 1994). All cyanobacteria contain chlorophyll *a* and phycocyanine.

The basic features of photosynthesis in cyanobacteria have been well described (Ormerod, 1992). Cyanobacteria are oxygenic phototrophs possessing two kinds of reaction centres, PS I and PS II, in their photosynthetic apparatus. With the accessory pigments mentioned above, they are able to use effectively that region of the light spectrum between the absorption peaks of chlorophyll *a* and the carotenoids. The ability for continuous photo-synthetic growth in the presence of oxygen, together with having water as their electron donor for CO_2 reduction, enables cyanobacteria to colonise a wide range of ecological niches (Whitton, 1992). Phycobiliprotein synthesis is particularly susceptible to environmental influences, especially light quality. Chromatic adaptation is largely attributable to a change in the ratio between phycocyanin and phycoerythrin in the phycobilisomes. Thus, cyanobacteria are able to produce the accessory pigment needed to absorb light most efficiently in the habitat in which they are present.

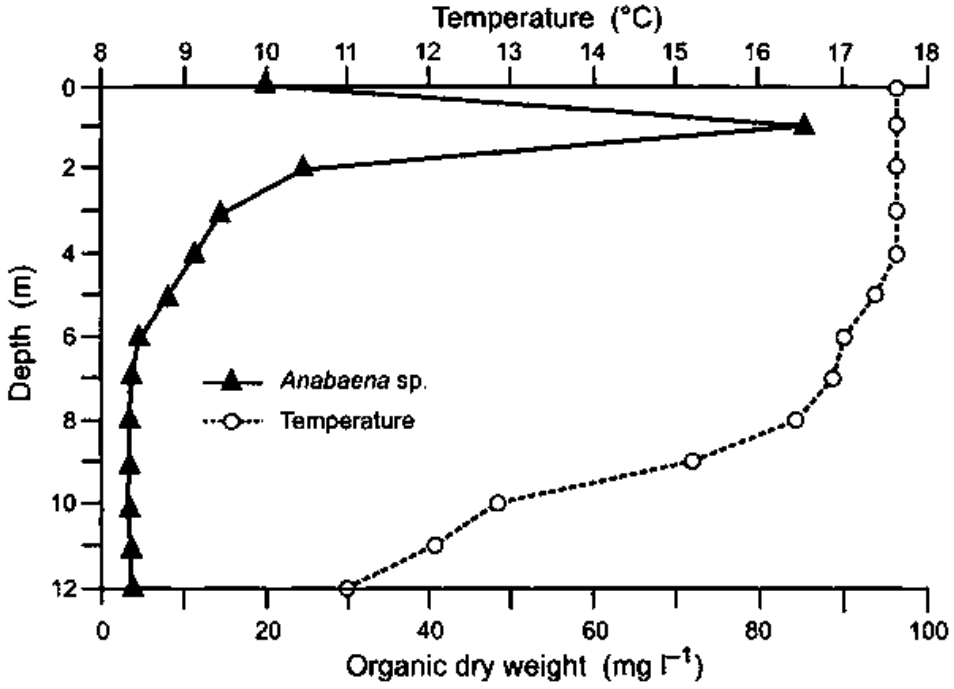
Cyanobacteria have a remarkable ability to store essential nutrients and metabolites within their cytoplasm. Prominent cytoplasmic inclusions for this purpose can be seen with the electron microscope (e.g. glycogen granules, lipid globules, cyanophycin granules, polyphosphate bodies, carboxysomes) (Fay and Van Baalen, 1987). Reserve products are accumulated under conditions of an excess supply of particular nutrients. For example, when the synthesis of nitrogenous cell constituents is halted because of an absence of a usable nitrogen source, the primary products of photosynthesis are channelled towards the synthesis and accumulation of glycogen and lipids.

Dinitrogen fixation is a fundamental metabolic process of cyanobacteria, giving them the simplest nutritional requirements of all living organisms. By using the enzyme nitrogenase, they convert N_2 directly into ammonium (NH_4) (a form through which

nitrogen enters the food chain) and by using solar energy to drive their metabolic and biosynthetic machinery, only N_2, CO_2, water and mineral elements are needed for growth in the light. Nitrogen-fixing cyanobacteria are widespread among the filamentous, heterocyst forming genera (e.g. *Anabaena, Nostoc)* (Stewart, 1973). However, there are also several well documented examples of dinitrogen fixation among cyanobacteria not forming heterocysts (e.g. *Trichodesmium)* (Carpenter *et al.,* 1992). Under predominantly nitrogen limited conditions, but when other nutrients are available, nitrogen fixing cyanobacteria may be favoured and gain growth and reproductive success. Mass developments (often referred to as "blooms") of such species in limnic (e.g. eutrophic lakes, see Figure 2.2 in the colour plate section) and marine environments (e.g. the Baltic Sea) are common phenomena world-wide.

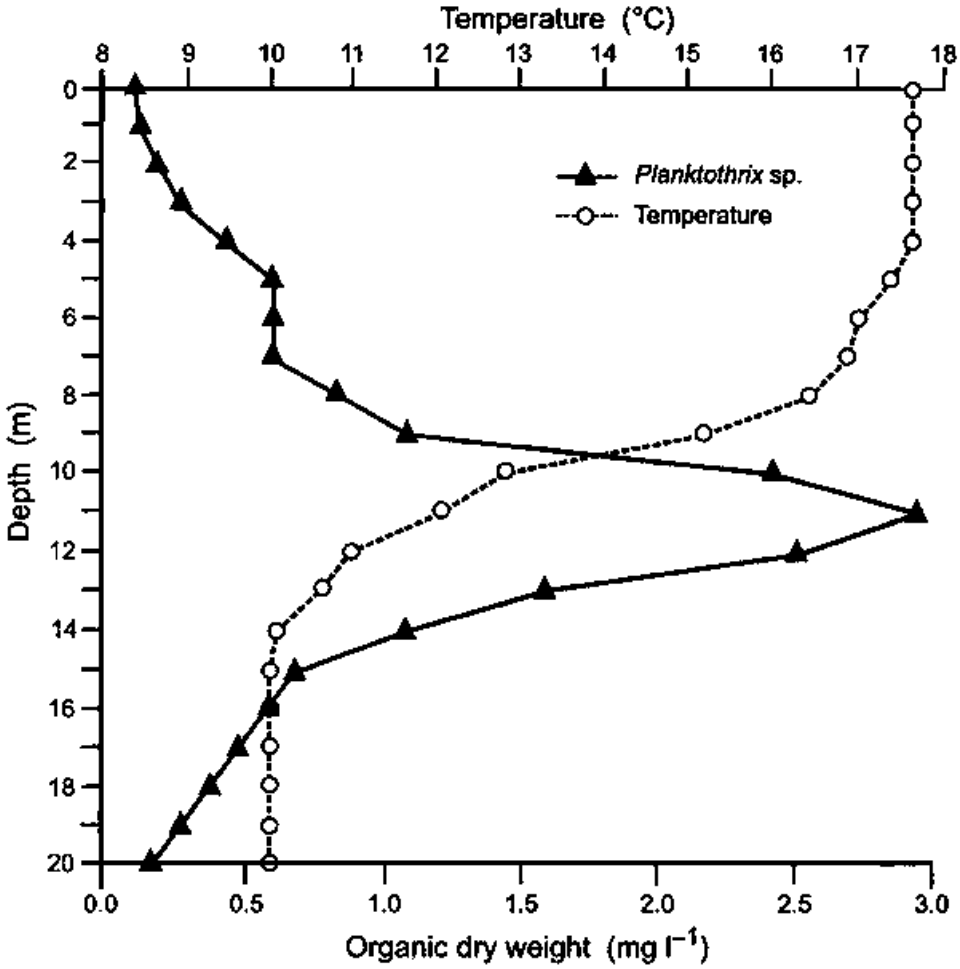
Many species of cyanobacteria possess gas vesicles. These are cytoplasmic inclusions that enable buoyancy regulation and are gas-filled, cylindrical structures. Their function is to give planktonic species an ecologically important mechanism enabling them to adjust their vertical position in the water column (Walsby, 1987). To optimise their position, and thus to find a suitable niche for survival and growth, cyanobacteria use different environmental stimuli (e.g. photic, gravitational, chemical, thermal) as clues. Gas vesicles become more abundant when light is reduced and the growth rate slows down. Increases in the turgor pressure of cells, as a result of the accumulation of photosynthate, cause a decrease in existing gas vesicles and therefore a reduction in buoyancy. Cyanobacteria can, by such buoyancy regulation, poise themselves within vertical gradients of physical and chemical factors (Figures 2.3A and 2.3B). Other ecologically significant mechanisms of movement shown by some cyanobacteria are photomovement by slime secretion or surface undulations of cells (Häder, 1987; Paerl, 1988).

Figure 2.3A Vertical distribution of *Anabaena* sp. in a thermally stratified eutrophic lake during bloom conditions



The presence of very small cells of cyanobacteria (in the size range 0.2-2 µm) has been recognised as a potentially significant source of primary production in various freshwater and marine environments. These cyanobacteria constitute a component of the picoplankton in pelagic ecosystems. Cells can be recognised and estimates of their abundance made by using epifluorescence microscopy (e.g. observing the orange fluorescence due to phycoerythrin). The unicellular genus *Synechococcus* is one of the most studied, and geographically most widely distributed, cyanobacteria in the picoplankton. Toxigenic strains of *Synechococcus* have been reported (Skulberg *et al*., 1993).

Figure 2.3B Vertical distribution of *Planktothrix* sp. in a thermally stratified meso-oligotrophic lake during bloom conditions



2.1.4 Biological diversity

Although cyanobacteria probably evolved as a group of organisms about 2,000 million years before the advent of eukaryotes, they comprise fewer taxa than eukaryotic microalgae (Bisby, 1995). The concept of species in the cyanobacteria has, however, no distinct boundaries. The situation is similar for most organisms, except for those that are sexually reproductive. Depending on the classification system used, the number of species recognised varies greatly. Based on the International Code of Botanical

Nomenclature the class Cyanophyceae, for example, contains about 150 genera and 2,000 species (Hoek *et al.,* 1995).

Chemotaxonomic studies include the use of markers, such as lipid composition, polyamines, carotenoids and special biochemical features. The resulting data support the more traditional examinations of phenotypic and ecological characteristics. Physiological parameters are conveniently studied using laboratory cultures (Packer and Glazer, 1988).

The diversity of cyanobacteria can be seen in the multitude of structural and functional aspects of cell morphology and in variations in metabolic strategies, motility, cell division, developmental biology, etc. The production of extracellular substances and cyanotoxins by cyanobacteria illustrates the diverse nature of their interactions with other organisms (i.e. allelopathy) (Rizvi and Rizvi, 1992).

A molecular approach to the systematics of cyanobacteria may be most fruitful for inferring phylogenetic relationships. Macromolecules, such as nucleic acids and proteins, are copies or translations of genetic information. The methods applied involve direct studies of the relevant macromolecules by sequencing, or indirectly by electrophoresis, hybridisation, or immunological procedures (Wilmotte, 1994). Nucleic acid technologies, especially the polymerase chain reaction (PCR), have advanced to the point that it is feasible to amplify and sequence genes and other conserved regions from a single cell. To date, 16S rRNA has given the most detailed information on the relationships within the cyanobacteria (Rudi *et al.,* 1997). However, the molecular results obtained should be integrated with other characteristics as the base for a polyphasic taxonomy (Vandamme, *et al.,* 1996). A considerable morphological, as well as a genotypical, polymorphy exists in the cyanobacteria, although as data from rRNA sequencing indicates they are correlated to a high degree.

The phylogenetic relationship of cyanobacteria is the rationale behind the meaningful systematic groupings. However, it is difficult to set up a system of classification that serves both the everyday need for practical identification, and offers an expression of the natural relationship between the organisms in question (Mayr, 1981). Meanwhile, it will be necessary to use the available manuals and reference books to help in these investigations and with the proper identification of the cyanobacteria. Table 12.1 shows examples of how cyanobacteria with toxigenic strains are treated for determinative purposes according to the prevailing classification systems.

Because they are photoautotrophs, cyanobacteria can be grown in simple mineral media. Vitamin B_{12} is the only growth factor that is known to be required by some species. Media must be supplemented with the essential nutrients needed to support cell growth, including sources of nitrogen, phosphorus, trace elements, etc. Toxigenic strains of cyanobacteria are deposited in international-type culture collections (Rippka, 1988; Sugawara *et al.,* 1993). Clonal cultures are distributed for research, taxonomic work and teaching purposes.

2.1.5 Practical scope

The cyanobacteria have both beneficial and detrimental properties when judged from a human perspective. Their extensive growth can create considerable nuisance for

management of inland waters (water supply, recreation, fishing, etc.) and they also release substances into the water which may be unpleasant (Jüttner, 1987) or toxic (Gorham and Carmichael, 1988). The water quality problems caused by dense populations of cyanobacteria are intricate, many and various (Skulberg, 1996b) and can have great health and economic impacts. As a consequence, the negative aspects of cyanobacteria have gained research attention and public concern.

The properties that make the cyanobacteria generally undesirable are also the qualifications for possible positive economic use. Blue-greens are the source of many valuable products (Richmond, 1990) and carry promising physiological processes, including light-induced hydrogen evolution by biophotolysis (Skulberg, 1994). Extensive research has taken place in the relevant fields of biotechnology. Cyanobacteria may be used for food or fodder because some strains have a very high content of proteins, vitamins and other essential growth factors and vital pigments of interest can also be produced (Borowitzka and Borowitzka, 1988). Cyanobacteria are also sources for substances of pharmaceutical interest (such as antibiotics) (Falch *et al.,* 1995). These examples are only a few of the possible applications of cyanobacteria for economic development and their exploitation is among the many challenges for biotechnology for the next millennium. Also in this context, their secondary metabolites and health relationships will become important.

2.2 Factors affecting bloom formation

Cyanobacteria have a number of special properties which determine their relative importance in phytoplankton communities. However, the behaviour of different cyanobacterial taxa in nature is not homogeneous because their ecophysiological properties differ. An understanding of their response to environmental factors is fundamental for setting water management targets. Because some cyanobacteria show similar ecological and ecophysiological characteristics, they can be grouped by their behaviour in planktonic ecosystems as "ecostrategists" typically inhabiting different niches of aquatic ecosystems. A number of properties and reactions to environmental conditions are discussed below in order to describe these ecostrategists and to aid the understanding of their specific behaviour.

2.2.1 Light intensity

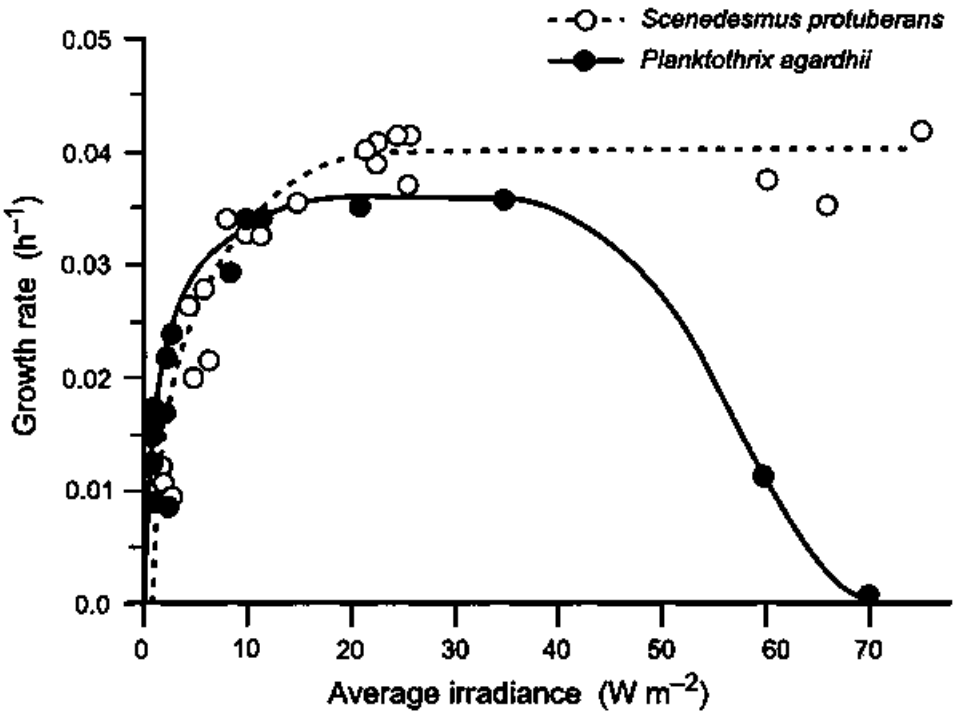
Like algae, cyanobacteria contain chlorophyll *a* as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments such as the phycobiliproteins which include allophycocyanin (blue), phycocyanin (blue) and sometimes phycoerythrine (red) (Cohen-Bazir and Bryant, 1982). These pigments harvest light in the green, yellow and orange part of the spectrum (500-650 nm) which is hardly used by other phytoplankton species. The phycobiliproteins, together with chlorophyll *a,* enable cyanobacteria to harvest light energy efficiently and to live in an environment with only green light.

Many cyanobacteria are sensitive to prolonged periods of high light intensities. The growth of *Planktothrix* (formerly *Oscillatoria) agardhii* is inhibited when exposed for extended periods to light intensities above 180 µE m^{-2} s^{-1}. Long exposures at light intensities of 320 µE m^{-2} s^{-1} are lethal for many species (Van Liere and Mur, 1980). However, if exposed intermittently to this high light intensity, cyanobacteria grow at their

approximate maximal rate (Loogman, 1982). This light intensity amounts to less than half of the light intensity at the surface of a lake, which can reach 700-1,000 µE m^{-2} s^{-1}. Cyanobacteria which form surface blooms seem to have a higher tolerance for high light intensities. Paerl *et al.* (1983) related this to an increase in carotenoid production which protects the cells from photoinhibition.

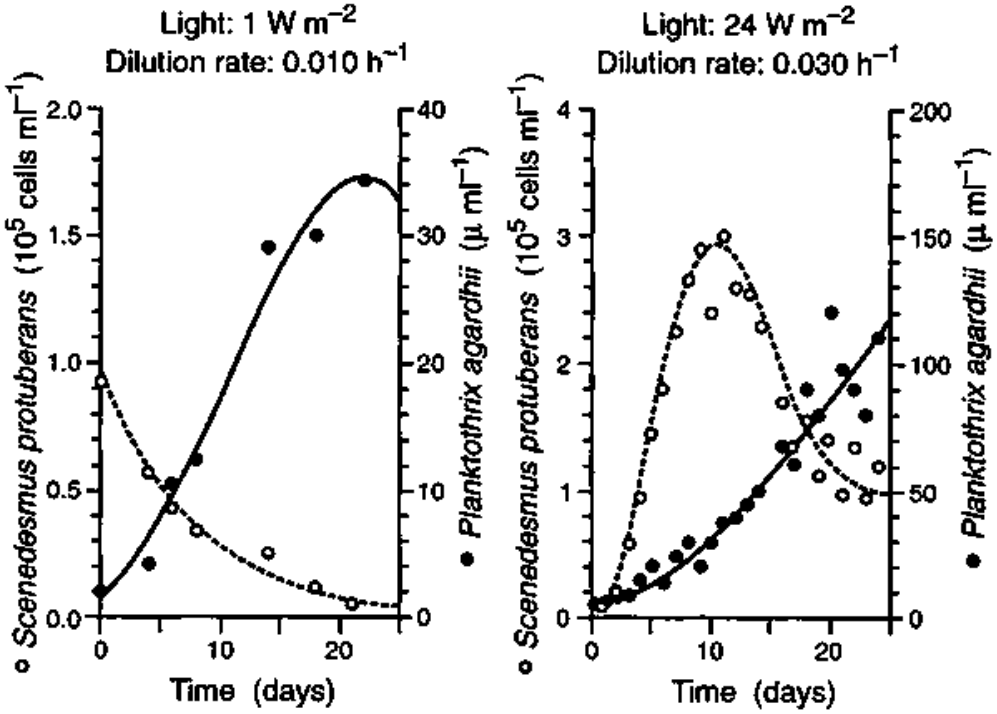
Cyanobacteria are further characterised by a favourable energy balance. Their maintenance constant is low which means that they require little energy to maintain cell function and structure (Gons, 1977; Van Liere *et al.,* 1979). As a result of this, the cyanobacteria can maintain a relatively higher growth rate than other phytoplankton organisms when light intensities are low. The cyanobacteria will therefore have a competitive advantage in lakes which are turbid due to dense growths of other phytoplankton. This was demonstrated in an investigation measuring growth of different species of phytoplankton at various depths in a eutrophic Norwegian lake (Källqvist, 1981). The results showed that the diatoms *Asterionella, Diatoma* and *Synedra* grew faster than the cyanobacterium *Planktothrix* at 1 m depth, while the growth rate was about the same for all these organisms at 2 m depth. At the very low light intensities below 3 m only *Planktothrix* grew. The ability of cyanobacteria to grow at low light intensities and to harvest certain specific light qualities, enables them to grow in the "shadow" of other phytoplankton. Van Liere and Mur (1979) demonstrated competition between cyanobacteria and other phytoplankton. Whereas the green alga *(Scenedesmus protuberans)* grew faster at high light intensities, growth of the cyanobacterium *(Planktothrix agardhii)* was faster at low light intensities (Figures 2.4A and 2.4B). If both organisms were grown in the same continuous culture at low light intensity, *Planktothrix* could out-compete *Scenedesmus* (Figure 2.4A). At high light intensities, the biomass of the green alga increased rapidly, causing an increase in turbidity and a decrease in light availability. This increased the growth rate of the cyanobacterium, which then became dominant after 20 days (Figure 2.4B). Although cyanobacteria cannot reach the maximum growth rates of green algae, at very low light intensities their growth rate is higher. Therefore, in waters with high turbidity they have better chances of out-competing other species. This can explain why cyanobacteria which can grow under very poor nutritional conditions (see section 2.2.4) often develop blooms in nutrient-rich eutrophic waters.

Figure 2.4A Competition for light between a cyanobacterium and a green alga. Growth rates of *Planktothrix agardhii* Gomont and *Scenedesmus protuberans* Fritsch as a function of average light intensities at pH 8.0, 20 °C with continuous illumination (Redrawn after Van Liere, 1979)



The light conditions in a given water body determine the extent to which the physiological properties of cyanobacteria will be of advantage in their competition against other phytoplankton organisms (Mur *et al.,* 1978). The zone in which photosynthesis can occur is termed the euphotic zone (Z_{eu}). By definition, the euphotic zone extends from the surface to the depth at which 1 per cent of the surface light intensity can be detected. It can be estimated by measuring transparency with a Secchi disk (see Chapter 11) and multiplying the Secchi depth reading by a factor of 2-3. The euphotic zone may be deeper or more shallow than the mixed, upper zone of a thermally stratified water body, the depth of which is termed the epilimnion (Z_m) (Figure 2.5). Many species of planktonic algae and cyanobacteria have little, or only weak, means of active movement and are passively entrained in the water circulation within the epilimnion. Thus, they can be photosynthetically active only when the circulation maintains them in the euphotic zone. In eutrophic waters, phytoplankton biomass is frequently very high and causes substantial turbidity. In such situations, the euphotic zone is often more shallow than the epilimnion, i.e. the ratio Z_{eu}/Z_m is < 1, and phytoplankton spend part of the daylight period in the dark. Thus, the Z_{eu}/Z_m ratio is a reasonable (and easy to measure) approach to describing the light conditions encountered by the planktonic organisms.

Figure 2.4B Competition for light between a cyanobacterium and a green alga. Outcome of competition between *Planktothrix agardhii* Gomont and *Scenedesmus protuberans* Fritsch in continuous cultures at two different light intensities and dilution rates (Redrawn after Van Liere, 1979)



2.2.2 Gas vesicles

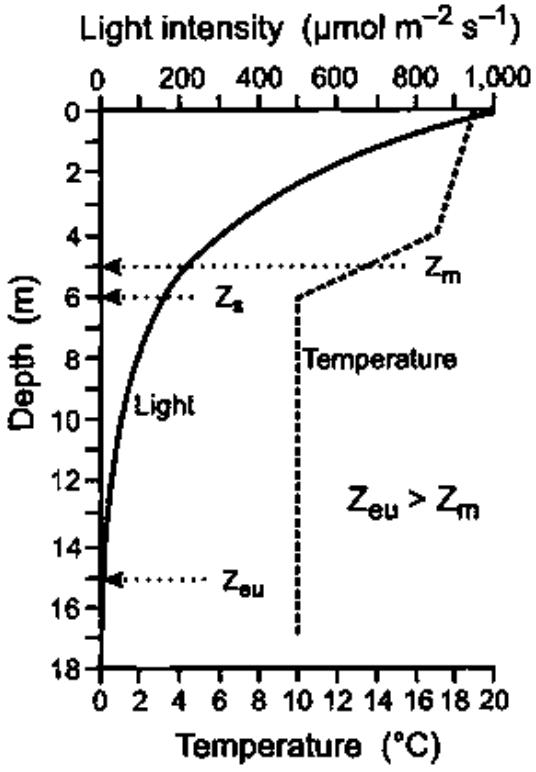
Many planktonic cyanobacteria contain gas vacuoles (Walsby, 1981). These structures are aggregates of gas-filled vesicles, which are hollow chambers with a hydrophilic outer surface and a hydrophobic inner surface (Walsby, 1978). A gas vesicle has a density of about one tenth that of water (Walsby, 1987) and thus gas vesicles can give cyanobacterial cells a lower density than water.

2.2.3 Growth rate

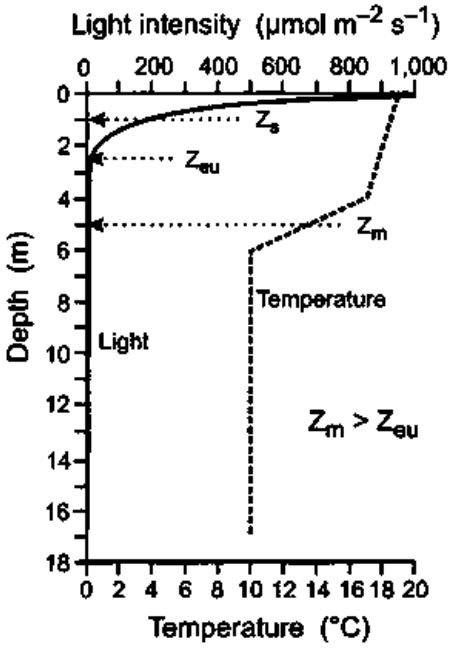
The growth rate of cyanobacteria is usually much lower than that of many algal species (Hoogenhout and Amesz, 1965; Reynolds, 1984). At 20 °C and light saturation, most common planktonic cyanobacteria achieve growth rates of 0.3-1.4 doublings per day, while diatoms reach 0.8-1.9 doublings per day and growth rates of up to 1.3-2.3 doublings per day have been observed for single-celled green algae (Van Liere and Walsby, 1982). Slow growth rates require long water retention times to enable a bloom of cyanobacteria to form. Therefore cyanobacteria do not bloom in water with short retention times. A. comprehensive overview of mechanisms determining the growth rates of planktonic algae and cyanobacteria under different field conditions is available in Reynolds (1997).

Figure 2.5 Vertical extension of the euphotic zone (Z_{eu}) in relation to depth of the epilimnion (Z_m) in situations with different turbidity.

A. Euphotic zone is deeper than epilimnion;



B. Euphotic zone is not as deep as epilimnion. Secchi depth (Z_s) is included as rough measure of euphotic depth (Z_{eu}) ($Z_s \times 2.5 \cong Z_{eu}$)



2.2.4 Phosphorus and nitrogen

Because cyanobacterial blooms often develop in eutrophic lakes, it was originally assumed that they required high phosphorus and nitrogen concentrations. This assumption was maintained even though cyanobacterial blooms often occurred when concentrations of dissolved phosphate were lowest. Experimental data have shown that the affinity of many cyanobacteria for nitrogen or phosphorus is higher than for many other photosynthetic organisms. This means that they can out-compete other phytoplankton organisms under conditions of phosphorus or nitrogen limitation.

In addition to their high nutrient affinity, cyanobacteria have a substantial storage capacity for phosphorus. They can store enough phosphorus to perform two to four cell divisions, which corresponds to a 4-32 fold increase in biomass. However, if total phosphate rather than only dissolved phosphate is considered, high concentrations indirectly support cyanobacteria because they provide a high carrying capacity for phytoplankton. High phytoplankton density leads to high turbidity and low light availability, and cyanobacteria are the group of phytoplankton organisms which can grow best under these conditions.

A low ratio between nitrogen and phosphorus concentrations may favour the development of cyanobacterial blooms. A comparison between the optimum N:P ratios for eukaryotic algae (16-23 molecules N:1 molecule of P) with the optimum rates for bloom-forming cyanobacteria (10-16 molecules N: 1 molecule P), shows that the ratio is lower for cyanobacteria (Schreurs, 1992).

2.2.5 Population stability

While many planktonic algae are grazed by copepods, daphnids and protozoa, cyanobacteria are not grazed to the same extent, and the impact of grazing by some specialised ciliates and rhizopod protozoans is usually not substantial. Cyanobacteria are attacked by viruses, bacteria and actino-mycetes, but the importance of these natural enemies for the breakdown of populations is not well understood. Because they have few natural enemies, and their capacity for buoyancy regulation prevents sedimentation, the loss rates of cyanobacterial populations are generally low. Thus, their slow growth rates are compensated by the high prevalence of populations once they have been established.

2.2.6 Temperature

Maximum growth rates are attained by most cyanobacteria at temperatures above 25 °C (Robarts and Zohary, 1987). These optimum temperatures are higher than for green algae and diatoms. This can explain why in temperate and boreal water bodies most cyanobacteria bloom during summer.

2.3 Cyanobacterial ecostrategists

The physiological properties of cyanobacteria discussed above vary between different species. As a consequence, different "ecostrategists" inhabit different types of water bodies. A preliminary approach to describing these ecostrategists, based on

ecophysiological laboratory work together with field observations (largely from north-western Europe), is described below. This information may be useful for management, because it helps to predict which cyanobacteria can be expected to occur under certain conditions. Further development of this approach will be possible as more data on occurrence of cyanobacteria under different growth conditions are collected from other continents.

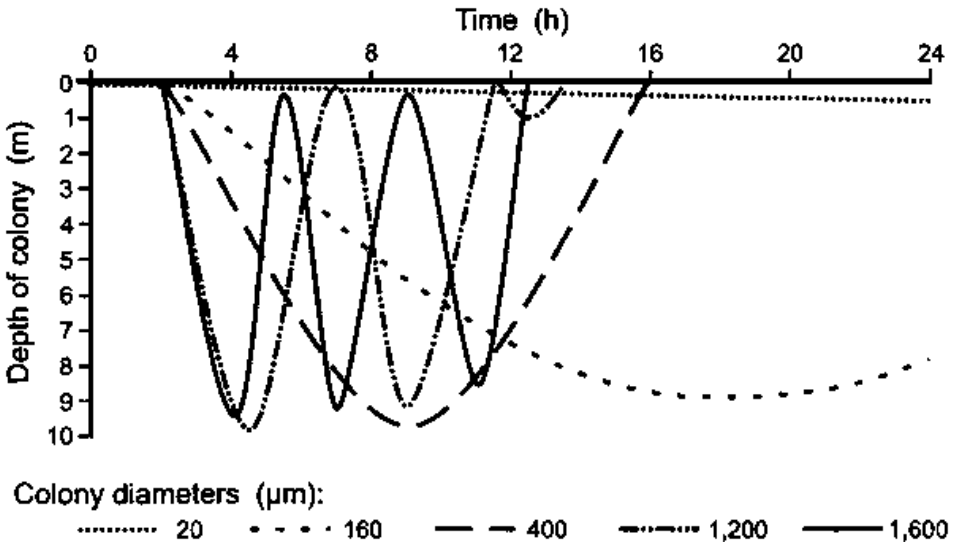
2.3.1 Scum-forming ecostrategists

During the vegetation period, a number of cyanobacteria develop large aggregates (colonies) of coccoid cells or filaments which are not homogeneously distributed over the water column. Important genera showing this development are *Microcystis, Anabaena* and *Aphanizomenon.* At the water surface the rate of photosynthesis of the colonies is high and the cells store large quantities of carbohydrates. Although the cells contain gas vesicles, the heavy carbohydrates acts as ballast and induce sinking within the colonies. According to Stoke's Law the sinking rate is dependent on the difference in density between the water and the cells, and on the square of the colony size (d^2). Large colonies sink faster than small ones, and single cells hardly show any vertical migration. By sinking, colonies move out of the euphotic zone into the deeper, dark water layers, where they use their carbohydrates during respiration and synthesise new gas-vesicles (Utkilen *et al.,* 1985). They then become buoyant again and return to the euphotic zone. Buoyancy regulation enables the colonies to position themselves in light conditions which are optimal for their growth. A prerequisite is that the water body is not too turbulent. During the night, all colonies may become buoyant and some of the population may be accumulated on the water surface where they can be blown together by wind, forming stable scums along downwind shores. Vertical movement by buoyancy regulation is illustrated in Figure 2.6. The frequency of vertical migration is dependent upon colony size.

In temperate regions, as temperatures decline in the autumn, photosynthesis becomes more rapid than respiration, and the carbohydrate "ballast" is not consumed. The colonies therefore sink to the bottom of the water body where they may survive the winter, gradually consuming their carbohydrate stores by respiration or fermentation. Cells which re-ascend from the bottom in the spring are unicellular or formed into very small colonies. During this period *Microcystis* spp. is difficult to recognise in plankton samples, and only becomes more conspicuous when the colonies increase in size during early summer.

Buoyancy regulation can be a substantial advantage in competition with other phytoplankton organisms. However, this type of regulation is only possible in water bodies with a shallow euphotic zone in relation to the depth of vertical mixing ($Z_{eu} < Z_m$). Therefore, in temperate climates, blooms of *Microcystis* spp. are found particularly in water bodies deeper than 3 m, because the euphotic zone is likely to be substantially more shallow than the mixed depth. However, even in shallow lakes, where they do not have the competitive advantage of vertical migration, *Microcystis* spp. may become dominant and form substantial blooms, as has been reported from Hungary, Australia, and particularly from subtropical and tropical regions. Reynolds (1997) characterises *Microcystis* spp. as notoriously and overwhelmingly dominant in some lakes of the lower latitudes that exhibit diel stratification.

Figure 2.6 Effect of colony size on vertical movement of *Microcystis aeruginosa* by buoyancy regulation (simulation). Colonies with diameters <20 µm scarcely migrate, colonies with diameters <160 µm accomplish less than one migration per day, and colonies up to 1,600 µm diameter can migrate down to 10 m depth and back up to the surface three times per day

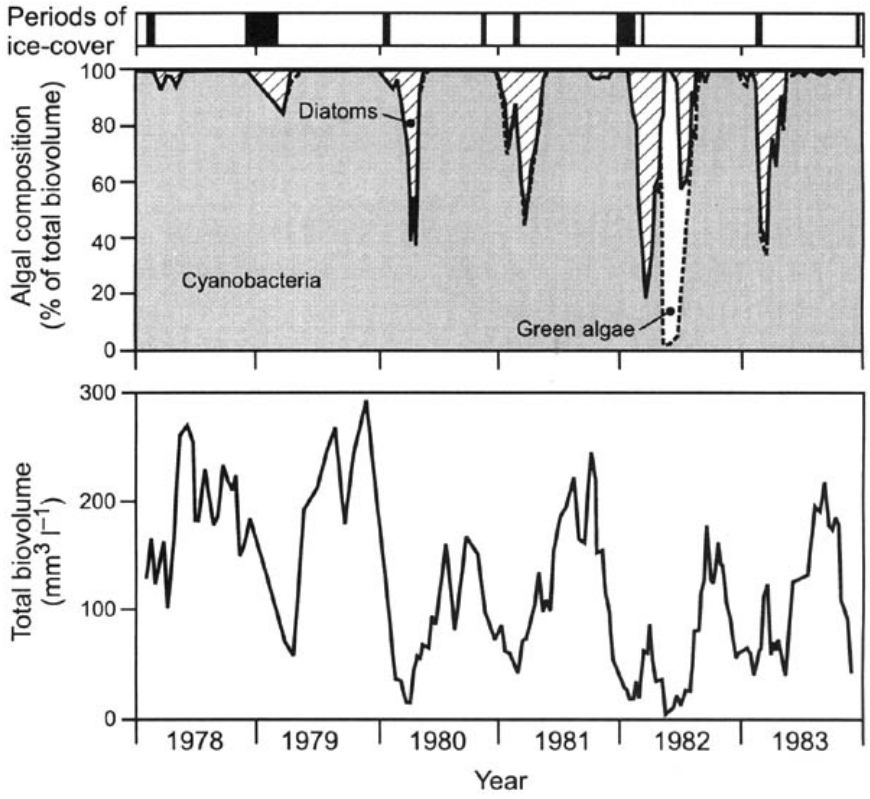


Many cyanobacteria cannot survive high light intensities over longer periods. This may limit their distribution to more turbid, eutrophic ecosystems. However, *Microcystis* species are less sensitive to high light intensities because buoyancy regulation enables them to find light conditions that are optimal for their growth. This means that the presence of *Microcystis* cannot be related strictly to the level of eutrophication. The genus is therefore found in mesotrophic, eutrophic and in hypertrophic waters. However, the amount of biomass that this species can attain depends on the level of eutrophication. Most *Microcystis* blooms are found in lakes with an average summer chlorophyll *a* concentration of 20-50 µg l^{-1} and a Secchi transparency of 1-2 m.

2.3.2 Homogeneously dispersed ecostrategists

This ecotype comprises filamentous species, such as *Planktothrix (Oscillatoria) agardhii* and *Limnothrix (Oscillatoria) redekei.* These species are extremely sensitive to high light intensities and do not form colonies (Reynolds, 1987). Because the filaments are quite small, vertical migration by buoyancy regulation is less pronounced than their passive entrainment by water circulation. Therefore, these species are homogeneously dispersed throughout the epilimnion.

Figure 2.7 Distribution of phytoplankton in Lake Veluwe, The Netherlands, showing an almost permanent bloom of cyanobacteria during the years 1978-83. The dominant bloom of *Planktothrix agardhii* was only interrupted by periods of ice cover (Redrawn from RIZA, 1996)



This type of ecostrategist is found in eutrophic and hypertrophic shallow lakes. Many lakes with blooms of dispersed ecotypes have a depth of not more than 3 m and chlorophyll concentrations of 50 µg l^{-1} and, in extreme cases, greater than 200 µg l^{-1}. The filaments are hardly grazed and do not sediment. Blooms of this type often lead to virtual monocultures which can prevail year-round for many years (Figure 2.7). Population dynamics in such lakes can be limited. In temperate regions, the autumn population can even survive under ice in winter. In such situations, the spring population starts growth with a relatively high density and thus has an advantage in competition with other species (Visser, 1990). By causing high turbidity, these cyanobacterial populations effectively suppress the growth of other phytoplankton species. Thus, the next summer population establishes itself almost without any seasonal succession between different species of phytoplankton. This high stability of the population precludes any redistribution of phosphorus and nitrogen to other components of the ecosystem and this can cause a resilience effect in lake restoration projects (see Chapter 8).

Figure 2.2A **Micrograph of *Planktothrix (Oscillatoria) agardhii***

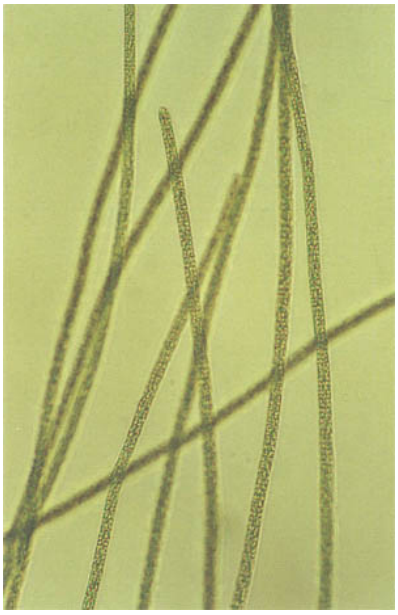


Figure 2.2B **Micrograph of *Anabaena lemmermannii***

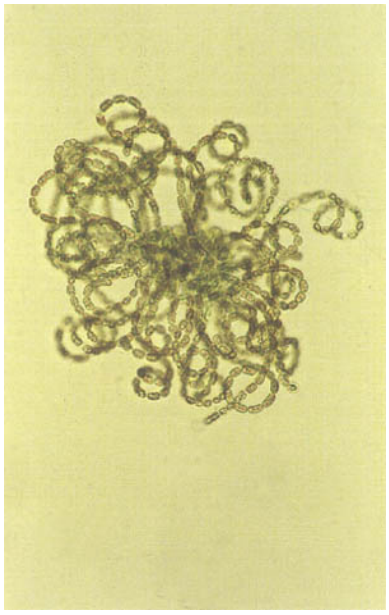


Figure 2.2C **Micrograph of *Microcystis aeruginosa***

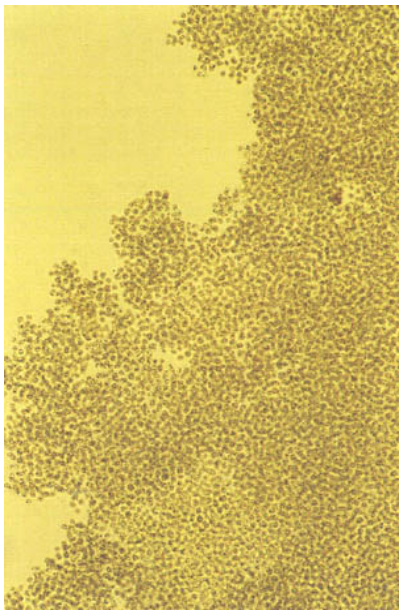


Figure 2.2D **Surface bloom of *Planktothrix (Oscillatoria)***

Figure 2.2E **Surface bloom of *Microcystis***

Figure 2.2F **Aerial photograph with infrared colour film of a freshwater bloom of cyanobacteria**

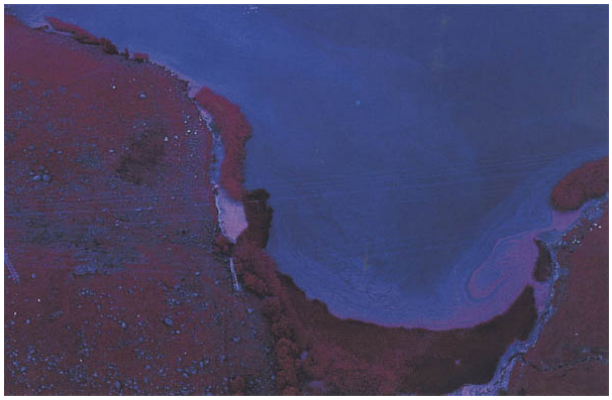


Figure 2.2G **Use of a barrier or boom to keep surface scums of algae and cyanobacteria away from water offtake structures (Photograph courtesy of Peter Baker, Australian Water Quality Centre)**

2.3.3 Stratifying ecostrategists

Representatives of this ecotype develop stable summer populations in the intermediate zone of thermally stratified lakes and reservoirs known as the metalimnion see (see Figure 2.3). The organisms contain the red pigment phycoerythrin to absorb the green light, which is the prevailing wave length at this depth. The most common of these species is *Planktothrix (Oscillatoria) rubescens,* but red varieties of other *Planktothrix* species can also form metalimnic populations (Aune *et al.,* 1997).

The single filaments of these species hardly show any vertical migration. However, in late autumn at the end of the growing season, the cells can become buoyant and then form red surface scums (Walsby *et al.,* 1983). The niche of this type of *Planktothrix* is very limited. It needs sufficient light in the metalimnetic zone, but may be inhibited by too much light. Most metalimnetic blooms are found at light intensities of 1-5 per cent of the surface irradiance and in a range of Z_{eu}/Z_m between 0.7 and 1.2.

2.3.4 Nitrogen fixing ecostrategists

The mass development of species capable of fixation of atmospheric nitrogen (species of the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Nodularia,* and *Nostoc)* can often be related to periodic nitrogen limitation. Examples are found in deep, as well as in shallow, systems. However, while these ecostrategists often dominate in ecosystems with low levels of inorganic dissolved nitrogen, the reverse does not necessarily apply. Numerous lakes with clear nitrogen limitation are not dominated by nitrogen-fixing cyanobacteria. Low light availability may be the reason for this, because nitrogen fixation requires high amounts of energy. In turbid lakes, insufficient light energy may be available for effective nitrogen fixation (Zevenboom and Mur, 1980). A number of nitrogen fixing species can form colonies and possess gas vesicles. This means that they can regulate buoyancy, like *Microcystis,* and can form stable scums along downwind shores.

Restoration measures which simultaneously reduce phosphate and nitrogen loading (sewage diversion, isolation) may strengthen prevailing nitrogen-limiting conditions and hence the probability of large populations of nitrogen-fixing cyanobacteria.

2.3.5 Small, colony-forming taxa

Cases of large populations of the small, colony-forming genus *Aphanothece* have been reported. Little information is available on buoyancy regulation and scum formation by the species involved. In several water bodies, *Aphanothece* dominance has occurred after a decrease of *Planktothrix rubescens* populations. Reynolds (1997) reported them as the only cyanobacteria present in the summer plankton of small, intermittently flushed lakes in England. The dominance of this group is not strictly related with phosphate or nitrogen limitation, and there are no obvious relationships that can explain the sudden dominance of these cyanobacteria. They seem to dominate in an intermediate state during lake recovery after restoration measures have been taken; their ecology is unknown.

2.3.6 Benthic cyanobacteria

Besides the planktonic ecostrategists described above, cyanobacteria may grow on the bottom sediments of water bodies which are sufficiently clear to allow light penetration to these surfaces. These benthic species may form coherent mats. Especially high rates of photosynthesis by such mats sometimes leads to trapping of the photosynthetically produced oxygen as bubbles within the mats; parts of the mats may then become sufficiently buoyant to tear loose and rise to the surface. For monitoring and management of toxic cyanobacteria, awareness of these is important because cyanotoxin problems are usually not expected in clear, oligotrophic waters. However, toxic benthic cyanobacteria have caused animal deaths in Scotland, where beached mats along the shore of a clear loch were scavenged by dogs (Gunn *et al.,* 1992), and in Switzerland where toxic benthic populations of *Oscillatoria limosa* were ingested by cattle drinking from pristine mountain lakes (Metz *et al.,* 1997, 1998).

2.4 Additional information

It is beyond the scope of this book to give a detailed account of the taxonomy and ecology of cyanobacteria. However, in addition to the references cited in the previous sections of this chapter there are many useful texts that are widely available. Taxonomy and species identification are covered in some detail by Anagnostidis and Komárek (1985), Staley *et al.* (1989), Larsen and Moestrup (1990) and Waterbury (1992). Detailed accounts of plankton ecology, including cyanobacteria, are available in Sommer (1989) and Reynolds (1997) and cyanobacterial ecophysiology is described by Mur (1983).

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Chapter 3. CYANOBACTERIAL TOXINS

This chapter was prepared by Kaarina Sivonen and Gary Jones

The cyanotoxins are a diverse group of natural toxins, both from the chemical and the toxicological points of view. In spite of their aquatic origin, most of the cyanotoxins that have been identified to date appear to be more hazardous to terrestrial mammals than to aquatic biota. Cyanobacteria produce a variety of unusual metabolites, the natural function of which is unclear, although some, perhaps only coincidentally, elicit effects upon other biota. Research has primarily focused on compounds that impact upon humans and livestock, either as toxins or as pharmaceutically useful substances. Further ranges of non-toxic products are also being found in cyanobacteria and the biochemical and pharmacological properties of these are totally unknown. An overview of the currently identified cyanotoxins is given in section 3.1 and their toxicological properties are discussed in Chapter 4.

Studies on the occurrence, distribution and frequency of toxic cyanobacteria were conducted in a number of countries during the 1980s using mouse bioassay. Analytical methods suitable for quantitative toxin determination only became available in the late 1980s, but studies of specific cyanotoxins have been increasing since then. The results of both approaches indicate that neurotoxins are generally less common, except perhaps in some countries where they frequently cause lethal animal poisonings. In contrast, the cyclic peptide toxins (microcystins and nodularins) which primarily cause liver injury are more widespread and are very likely to occur if certain taxa of cyanobacteria are present. Section 3.2 presents an overview of the data currently available on the occurrence of cyanotoxins. It is noteworthy, however, that current knowledge is clearly biased by the inconsistent distribution of research effort around the world, with studies from Asia, Africa and South America beginning to appear in the 1990s. Because the ecological role of the toxins is unclear, it is not possible to use a functional approach to study the factors that enhance toxicity. Section 3.3 looks at the available data on relationships between environmental factors and toxin content and at the emerging understanding of genetic regulation of toxin production. Research into toxin production by cyanobacteria is increasing, and a better understanding of toxin function may provide a basis for predicting occurrence of toxicity in the future.

For assessing the health risk caused by cyanotoxins, an understanding of their persistence and degradation in aquatic environments is of crucial importance. Section 3.4 gives an overview of the current understanding of these processes. Because effects on aquatic biota may be relevant issues for water managers, and because public

concern could raise questions in this field for practitioners, section 3.5 briefly introduces the limited state of knowledge of cyanotoxin impacts on other aquatic organisms.

3.1 Classification

Mechanisms of cyanobacterial toxicity currently described and understood are very diverse and range from hepatotoxic, neurotoxic and dermatotoxic effects to general inhibition of protein synthesis. To assess the specific hazards of cyanobacterial toxins it is necessary to understand their chemical and physical properties, their occurrence in waters used by people, the regulation of their production, and their fate in the environment.

Cyanotoxins fall into three broad groups of chemical structure: cyclic peptides, alkaloids and lipopolysaccharides (LPS). An overview of the specific toxic substances within these broad groups that have been identified to date from different genera of cyanobacteria, together with their primary target organs in humans, is given in Table 3.1.

3.1.1 Hepatotoxic cyclic peptides - microcystins and nodularins

Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide toxins of the microcystin and nodularin family. They pose a major challenge for the production of safe drinking water from surface waters containing cyanobacteria with these toxins. In mouse bioassays, which traditionally have been used to screen toxicity of field and laboratory samples, cyanobacterial hepatotoxins (liver toxins) cause death by liver haemorrhage within a few hours of the acute doses (see Chapter 4). Microcystins have been characterised from planktonic *Anabaena, Microcystis, Oscillatoria (Planktothrix), Nostoc,* and *Anabaenopsis* species, and from terrestrial *Hapalosiphon* genera. Nodularin has been characterised only from *Nodularia spumigena.*

The cyclic peptides are comparatively large natural products, molecular weight (MW) ≈ 800-1,100, although small compared with many other cell oligopeptides and polypeptides (proteins) (MW > 10,000). They contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound. They are water soluble and, except perhaps for a few somewhat more hydrophobic microcystins, are unable to penetrate directly the lipid membranes of animal, plant and bacterial cells. Therefore, to elicit their toxic effect, uptake into cells occurs through membrane transporters which otherwise carry essential biochemicals or nutrients. As will be outlined in section 4.2, this restricts the target organ range in mammals largely to the liver. In aquatic environments, these toxins usually remain contained within the cyanobacterial cells and are only released in substantial amounts on cell lysis. Along with their high chemical stability and their water solubility, this containment has important implications for their environmental persistence and exposure to humans in surface water bodies (see section 3.4).

able 3.1 General features of the cyanotoxins

Toxin group[1]	Primary target organ in mammals	Cyanobacterial genera[2]
Cyclic peptides		
Microcystins	Liver	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*
Nodularin	Liver	*Nodularia*
Alkaloids		
Anatoxin-a	Nerve synapse	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon*
Anatoxin-a(S)	Nerve synapse	*Anabaena*
Aplysiatoxins	Skin	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Cylindrospermopsins	Liver[3]	*Cylindrospermopsis, Aphanizomenon, Umezakia*
Lyngbyatoxin-a	Skin, gastro-intestinal tract	*Lyngbya*
Saxitoxins	Nerve axons	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides (LPS)	Potential irritant; affects any exposed tissue	All

[1] Many structural variants may be known for each toxin group - see section 3.1 for details

[2] Not produced by all species of the particular genus

[3] Whole cells of toxic species elicit widespread tissue damage, including damage to kidney and lymphoid tissue

The first chemical structures of cyanobacterial cyclic peptide toxins were identified in the early 1980s and the number of fully characterised toxin variants has greatly increased during the 1990s. The first such compounds found in freshwater cyanobacteria were cyclic heptapeptides (that is they contain seven peptide-linked amino acids) with the general structure of:
cyclo-(D-alanine[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-glutamate[6]-Mdha[7])
in which X and Z are variable L amino acids, D-MeAsp[3] is *D-erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine (Figure 3.1 A). The amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyanobacterial cyclic peptide toxins.

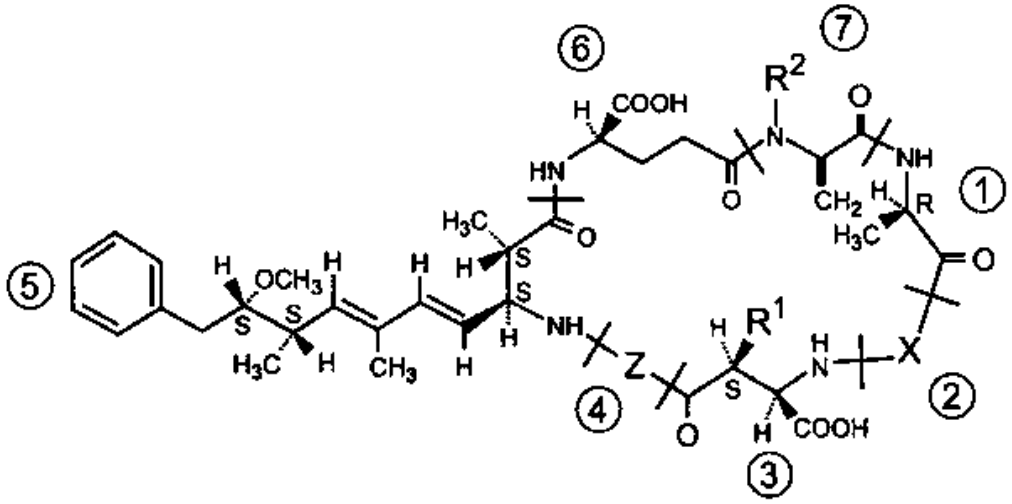
These compounds were first isolated from the cyanobacterium *Microcystis aeruginosa* and therefore the toxins were named microcystins (Carmichael *et al.,* 1988). Structural variations have been reported in all seven amino acids, but most frequently with substitution of L-amino acids at positions 2 and 4, and demethylation of amino acids at positions 3 and/or 7 (Figure 3.1A). About 60 structural variants of microcystins have been characterised so far from bloom samples and isolated strains of cyanobacteria (Table 3.2).

In one species of brackish water cyanobacterium, an identically acting and structurally very similar, cyclic pentapeptide occurs. It has been named as nodularin after its producer, *Nodularia spumigena.* The chemical structure of nodularin is cyclo-(D-MeAsp[1]-L-arginine[2]-Adda[3]-D-glutamate[4]-Mdhb[5]), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Figure 3.1B). A few naturally occurring variations of nodularins have been found: two demethylated variants, one with D-Asp[1] instead of D-MeAsp[1], the other with DMAdda[3] instead of Adda[3]; and the non-toxic nodularin which has the 6Z-stereoisomer of Adda[3] (Namikoshi *et al.,* 1994). The equivalent 6Z-Adda[3] stereoisomer of microcystins is also non-toxic. In the marine sponge, *Theonella swinhoei,* a nodularin analogue called motuporin has been found. It differs from nodularin only by one amino acid, having hydrophobic L-Val in place of the polar L-Arg in nodularin (de Silva *et al.,* 1992). The toxin might be cyanobacterial in origin because the sponge is known to harbour cyanobacterial symbionts.

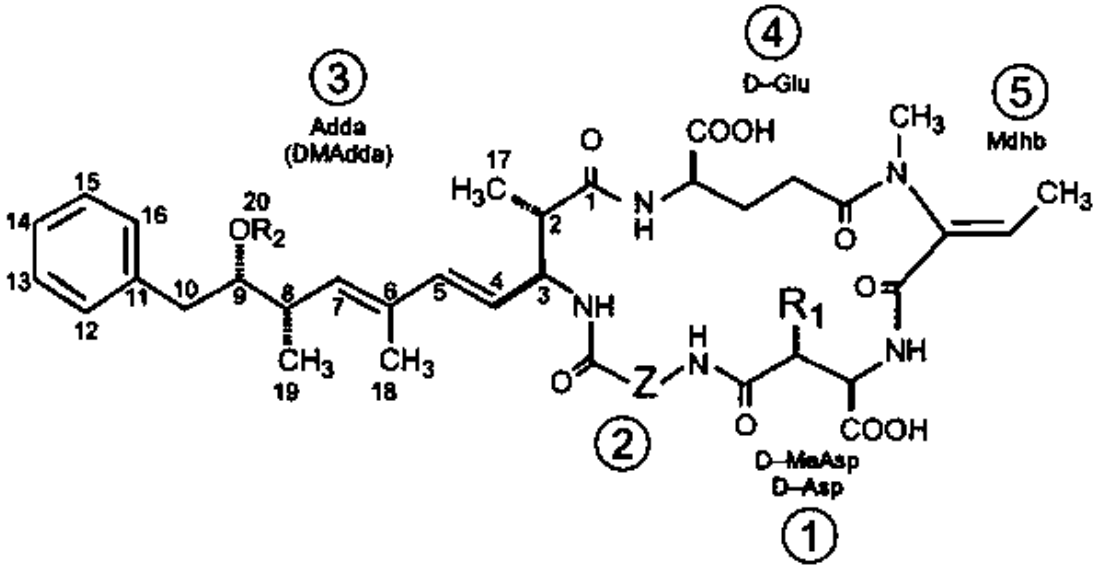
The mammalian toxicity of microcystins and nodularins is mediated through their strong binding to key cellular enzymes called protein phosphatases (see Chapter 4). In solution, microcystins and nodularins adopt a chemical "shape" that is similar, especially in the Adda-glutamate part of the cyanotoxin molecule (Rudolph-Böhner *et al.,* 1994; Annila *et al.,* 1996). Recent studies have shown that this region is crucial for interaction with the protein phosphatase protein molecule, and hence it is crucial for the toxicity of these cyanotoxins (Barford and Keller, 1994; Goldberg *et al.,* 1995). Microcystins show an additional characteristic of forming a covalent bond between the Mdha residue and the protein phosphatase molecule.

Figure 3.1 The structure of cyclic peptide toxins and cylindrospermopsin.

A. General structure of microcystins (MCYST), cyanobacterial heptapeptide hepatotoxins, showing the most frequently found variations. X and Z are variable L-amino acids (in MCYST-LR, X = L-Leusine (L) and Z = L-Arginine (R)); R^1 and R^2 are H (demethylmicrocystins) or CH_3; D-MeAsp is D-*erythro*-β-methylaspartic acid; Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid and Mdha is N-methyldehydroalanine (Dha = dehydroalanine) (see Table 3.2 for known microcystins); General structure of microcystins cyclo-(D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7])



B. Structures of nodularins (Z = L-arginine) and motuporin (Z = L-Valine). Mdhb is N-methyldehydrobutyrin; Nodularin R_1,R_2 = CH_3; D-Asp[1]Nodularin R_1 = H, R_2 = CH_3; DMAdda[3] Nodularin R_1 = CH_3, R_2 = H; Structure of nodularins cyclo-(D-MeAsp[1]-Z[2]-Adda[3]-D-Glu[4]-Mdhb[5])



C. Structure of cylindrospermopsin; Cylindrospermopsin MW 415; $C_{15}H_{21}N_5O_7S$

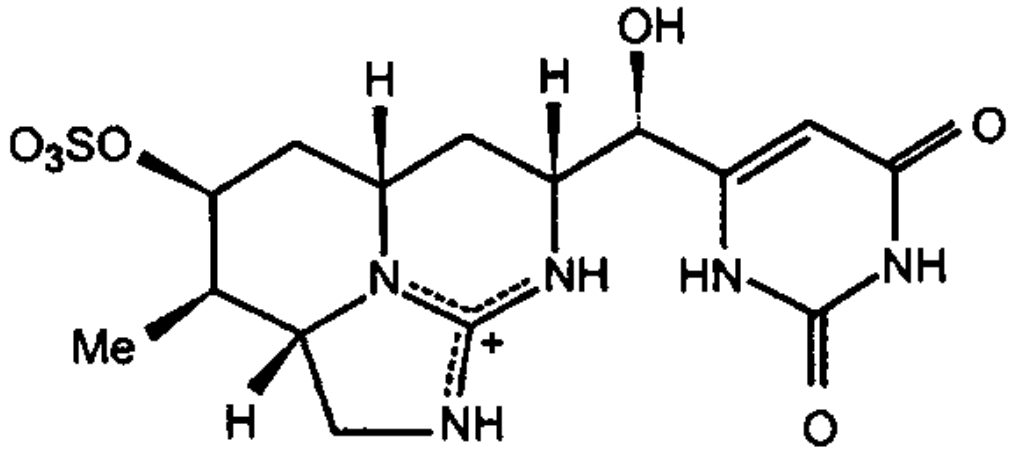


Table 3.2 The microcystins (MCYST) reported in the scientific literature

Microcystin	Molecular weight	Toxicity LD_{50}[2]	Organism[3]	Reference(s)
MCYST-LA	909	50	*M. aeruginosa*[s], *M. viridis*[s]	Botes *et al*., 1984; Kaya and Watanabe, 1990
MCYST-LAba	923	NR	*M. aeruginosa*[s]	Gathercole and Thiel, 1987
MCYST-LL	951	+	*M. aeruginosa*[b]	Craig *et al*., 1993
MCYST-AR	952	250	*Microcystis* spp.[b]	Namikoshi *et al.,* 1992a
MCYST-YA	959	NR	*M. aeruginosa*[s]	Botes *et al.,* 1985
[D-Asp[3],Dha[7]]MCYST-LR	966	+	*M. aeruginosa*[s], *Anabaena* sp.[s]	Harada et *al.,* 1991b; Sivonen *et al.,* 1992a
[D-Asp[3],Dha[7]]MCYST-EE(OMe)	969	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
MCYST-VF	971	NR	*M. aeruginosa*[s]	Bateman *et al.,* 1995
(D-Asp[3]]MCYST-LR	980	160-300	*A. flos-aquae*[s], *M. aeruginosa*[s], *M. viridis*[b], *O. agardhii*[s]	Krishnamyrthy *et al.,* 1989; Cremer and Henning, 1991; Harada *et al.,* 1990b; 1991a; Luukkainen *et al.,* 1993
[Dha[7]]MCYST-LR	980	250	*M. aeruginosa*[s], *Anabaena sp.*[s], *O. agardhii*[s]	Harada *et al.,* 1991b; Sivonen *et al*., 1992a; Luukkainen *et al.,* 1993
[DMAdda[5]]MCYST-LR	980	90-100	*Microcystis* spp.[b], *Nostoc* sp.[s]	Namikoshi *et al.,* 1992a; Sivonen *et al.,* 1992b
[Dha[7]]MCYST-EE(OMe)	983	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
[D-Asp[3],Dha[7]]MCYST-E(OMe)E(OMe)	983	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
MCYST-LF	985	+	*M. aeruginosa*[s]	Azevedo *et al.,* 1994

MCYST-LR	994	50	*M. aeruginosa*[s], *A. flos-aquae*[s] *M. viridis*[s]	Botes *et al.,* 1985; Rinehart *et al.,* 1988; Krishnamyrthy *et al.,* 1989; Watanabe *et al.,* 1988
[D-Asp[3],D-Glu(OCH_3)[6]]MCYST-LR	994	NR	*A. flos-aquae*[s]	Sivonen *et al.,* 1992d
[(6Z)-Adda[5]]MCYST-LR	994	>1,200	*M. viridis*[b]	Harada *et al.,* 1990a,b
[Dha[7]]MCYST-E(OMe)E(OMe)	997	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
[L-Ser[7]]MCYST-LR	998	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1992c
MCYST-LY	1,001	90	*M. aeruginosa*[s]	Stoner *et al.,* 1989
[L-Ser[7]]MCYST-EE(OMe)	1,001	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
[D-Asp[3],Ser[7]]MCYST-E(OMe)E(OMe)	1,001	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
MCYST-HilR	1,008	100	*Microcystis* spp.[b]	Namikoshi *et al.,* 1995
[D-Asp[3],ADMAdda[5]]MCYST-LR	1,008	160	*Nostoc* sp.[s]	Sivonen *et al.,* 1990a; Namikoshi *et al.,* 1990
[D-Glu(OCH_3)[6]]MCYST-LR	1,008	>1,000	*A. flos-aguae*[s], *Microcystis sp.*[s]	Sivonen *et al.,* 1992d; Bateman *et al.,* 1995; Rinehart *et al*., 1994
[D-Asp[3],Dha[7]]MCYST-RR	1,009	+	*O. agardhii*[b], *Anabaena* sp.[s], *M. aeruginosa*[s]	Krishnamyrthy *et al.,* 1989; Sivonen *et al.,* 1992a; Luukkainen *et al.,* 1994
[D-Asp[3], ADMAdda[5], Dhb[7]]MCYST-LR	1,009	+	*Nostoc sp.*[s]	Beattie *et al.,* 1998
[L-MeSer[7]]MCYST-LR	1,012	150	*Microcystis* spp.[b]	Namikoshi *et al., 1992a;* 1995
[Dha[7]]MCYST-FR	1,014	NR	*Microcystis sp.*[s]	Luukkainen *et al.,* 1994
[L-Ser[7]]MCYST-E(OMe)E(OMe)	1,015	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1998
[ADMAdda[5]]MCYST-LR	1,022	60	*Nostoc* sp.[s]	Sivonen *et al.,* 1990a; Namikoshi *et al.,* 1990
[D-Asp[3],ADMAdda[5]]MCYST-LHar	1,022	+	*Nostoc* sp.[s]	Sivonen *et al.,* 1992b
[D-Asp[3]]MCYST-RR	1,023	250	*O. agardhii*[s], *Anabaena* sp.[s], *M. aeruginosa*[s]	Meriluoto *et al.,* 1989; Sivonen *et al.,* 1992a; Luukkainen *et al.,* 1994
[Dha[7]]MCYST-RR	1,023	180	*M. aeruginosa*[s], *Anabaena* sp.[s], *O. agardhii*[s]	Kiviranta *et al.,* 1992; Sivonen *et al.,* 1992a; Luukkainen *et al.,* 1993
MCYST-LW	1,024	NR	*M. aeruginosa*[s]	Bateman *et al.,* 1995
MCYST-FR	1,028	250	*Microcystis* spp.[b]	Namikoshi *et al.,* 1992a
MCYST-M(O)R	1,028	700-800	*Microcystis* spp.[b]	Namikoshi *et al.,* 1992a
[Dha[7]]MCYST-HphR	1,028	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1992b
[D-Asp[3], Dha[7]]MCYST-HtyR	1,030	+	*Anabaena* sp.[s]	Namikoshi *et al.,* 1992b

[Dha[7]]MCYST-YR	1,030	+	*M. aeruginosa*[s]	Sivonen *et al.,* 1992c
[D-Asp[3]]MCYST-YR	1,030	+	*Microcystis* spp.[b]	Namikoshi *et al.,* 1992d
MCYST-YM(O)	1,035	56	*M. aeruginosa*[b]	Botes *et al.,* 1985; Elleman *et al.,* 1978
[ADMAdda[5]]MCYST-LHar	1,036	60	*Nostoc* sp.[s]	Sivonen *et al.,* 1990a; Namikoshi *et al.,* 1990
MCYST-RR	1,037	600	*M. aeruginosa*[s], *M. viridis*[s], *Anabaena* sp.[s]	Kusumi *et al.*, 1987; Painuly *et al.,* 1988; Watanabe *et al.,* 1988; Sivonen *et al., 1992a*
[(6Z)-Adda[5]]MCYST-RR	1,037	>1,200	*M. viridis*[b]	Harada *et al.,* 1990a,b
[D-Ser[1], ADMAdda[5]]MCYST-LR	1,038	+	*Nostoc* sp.[s]	Sivonen *et al.,* 1992b
[ADMAdda[5],MeSer[7]]MCYST-LR	1,040	+	*Nostoc* sp.[s]	Sivonen *et al.,* 1992b
[L-Ser[7]]MCYST-RR	1,041	+	*Anabaena* sp.[s], *M. aeruginosa*[s/b]	Namikoshi *et al.,* 1992c; Luukkainen *et al.,* 1994
[D-Asp[3],MeSer[7]]MCYST-RR	1,041	+	*O. agardhii*[s]	Luukkainen *et al.* 1993
MCYST-YR	1,044	70	*M. aeruginosa*[s], *M. viridis*[s]	Botes *et al.,* 1985; Watanabe *et al.*, 1988
[D-Asp[3]]MCYST-HtyR	1,044	160-300	*A. flos-aquae*[s]	Harada *et al.*, 1991a
[Dha[7]]MCYST-HtyR	1,044	+	*Anabaena* sp.[s]	Namikoshi *et al.*, 1992b
MCYST-(H_4)YR	1,048	NR	*Microcystis* spp.[b]	Namikoshi *et al.*, 1995
[D-Glu-$OC_2H_3(CH_3)OH$[6]]MCYST-LR	1,052	>1,000	*Microcystis* spp.[b]	Namikoshi *et al.*, 1992a
[D-Asp[3],ADMAdda[5], Dhb[7]]MCYST-RR	1,052	+	*Nostoc* sp.[s]	Beattie *et al.*, 1998
MCYST-HtyR	1,058	80-100	*A. flos-aquae*[s]	Harada *et al.*, 1991a
[L-Ser[7]]MCYST-HtyR	1,062	+	*Anabaena* sp.[s]	Namikoshi *et al.*, 1992b
MCYST-WR	1,067	150-200	*Microcystis* spp.[b]	Namikoshi *et al.*, 1992a
[D-Asp[3],ADMAdda[5],Dhb[7]]MCYST-HtyR	1,073	+	*Nostoc* sp.[s]	Beattie *et al.*, 1998
[L-MeLan[7]]MCYST-LR	1,115	1,000	*Microcystis* spp.[b]	Namikoshi *et al.*, 1995

Aba	Aminoisobutyric acid
ADMAdda	*O*-Acetyl-*O*-demethylAdda
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DMAdda	*O*-DemethylAdda
E(OMe)	Glutamic acid methyl ester Δ
(H_4)Y	1,2,3,4,-tetrahydrotyrosine
Har	Homoarginine

Hil	Homoisoleucine
Hph	Homophenylalanine
Hty	Homotyrosine
MeLan	*N*-Methyllanthionine
M(O)	Methionine-*S*-oxide
MeSer	*N*-Methylserine
(6Z)-Adda	Stereoisomer of Adda at the Δ^6 double bond

[1] Several partial structures of microcystins have been reported in addition to those shown in this table (see Boland *et al.*, 1993; Craig *et al*., 1993; Jones *et al.,* 1995; Sivonen *et al.*, 1995)

[2] Toxicity determined i.p. mouse (µg kg^{-1}); the LD_{50} value is the dose of toxin that kills 50% of exposed animals; a '+' denotes a toxic result in a non-quantitative mouse bioassay or inhibition of protein phosphatase and 'NR' denotes 'Not reported'

[3] An '*s*' denotes toxins isolated from culture samples and a *'b'* denotes toxins isolated from bloom samples

Most of the structural variants of microcystin and nodularin are highly toxic within a comparatively narrow range (intra-peritoneal (i.p.) mouse toxicities largely in the range 50-300 µg kg^{-1} body weight (bw); see Table 3.2 and section 4.2). Only a few non-toxic variants have been identified. In general, any structural modifications to the Adda-glutamate region of the toxin molecule, such as a change in isomerisation of the Adda-diene (6(E) to 6(Z)) or acylation of the glutamate, renders microcystins and nodularins non-toxic (Harada *et al.*, 1990 a,b; Rinehart *et al*., 1994). Linear microcystins and nodularin are more than 100 times less toxic than the equivalent cyclic compounds. The linear microcystins are thought to be microcystin precursors and/or bacterial breakdown products (Choi *et al.*, 1993; Rinehart *et al.*, 1994; Bourne *et al.,* 1996).

Microcystins and nodularin have been characterised from axenic cyanobacterial strains (i.e. strains free of contaminating bacteria) and thus the cyanobacterial origin of these compounds is clear. At the present time, it is known that microcystins are produced by bloom forming species of *Microcystis, Anabaena, Oscillatoria (Planktothrix),* and *Nostoc* (see Table 3.2), by a species of *Anabaenopsis* and by a soil isolate of *Haphalosiphon hibernicus.* Nodularins have been found, with the exception of the marine sponge *Theonella* already mentioned, only in *Nodularia spumigena* (see section 3.2 for more details). Further species may yet be demonstrated to produce microcystin.

3.1.2 Neurotoxic alkaloids - anatoxins and saxitoxins

Mass occurrences of neurotoxic cyanobacteria have been reported from North America, Europe and Australia, where they have caused animal poisonings. In mouse bioassays death by respiratory arrest occurs rapidly (within 2-30 minutes) (see Chapters 4 and 13). Three families of cyanobacterial neurotoxins are known:

- anatoxin-a and homoanatoxin-a, which mimic the effect of acetyl choline,

- anatoxin-a(S), which is an anticholinesterase, and

- saxitoxins, also known as paralytic shellfish poisons (PSPs) in the marine literature, which block nerve cell sodium channels.

Anatoxin-a has been found in *Anabaena, Oscillatoria* and *Aphanizomenon,* homoanatoxin-a from *Oscillatoria,* anatoxin-a(S) from *Anabaena,* and saxitoxins from *Aphanizomenon, Anabaena, Lyngbya* and *Cylindrospermopsis.* Sixteen confirmed saxitoxins from cyanobacterial samples have been reported, some of which (e.g. the decarbamoyl-gonyautoxins) may be chemical breakdown products in some species (see section 3.4.2).

The alkaloid toxins are diverse, both in their chemical structures and in their mammalian toxicities. Alkaloids, in general, are a broad group of heterocyclic nitrogenous compounds (i.e. they contain ring structures with at least one carbon-nitrogen bond) usually of low to moderate molecular weight (< 1,000). They are produced, in particular, by plants and by some bacteria, and are invariably bioactive and commonly toxic. The non-sulphated alkaloid toxins of freshwater cyanobacteria (anatoxins and saxitoxin) are all neurotoxins. The sulphated PSPs, C-toxins and gonyautoxins (sulphated derivatives of saxitoxin) are also neurotoxins, but the sulphated alkaloid cylindrospermopsin blocks protein synthesis with a major impact on liver cells. Some marine cyanobacteria also contain alkaloids (lyngbyatoxins, aplysiatoxins) which are dermatoxins (skin irritants), but have also been associated with gastro-enteritis and more general symptoms such as fever (see Chapter 4).

Alkaloids have varying chemical stabilities, often undergoing spontaneous transformations to by-products which may have higher or lower potencies than the parent toxin. Some are also susceptible to direct photolytic degradation (see section 3.4).

Anatoxin-a

Anatoxin-a is a low molecular weight alkaloid (MW = 165), a secondary amine, 2-acetyl-9-azabicyclo(4-2-1)non-2-ene (Figure 3.2) (Devlin *et al.,* 1977). Anatoxin-a is produced by *Anabaena flos-aquae, Anabaena* spp. *(flos-aquae-lemmermannii* group), *Anabaena planktonica, Oscillatoria, Aphanizomenon* and *Cylindrospermum* (see section 3.2 for details). Homoanatoxin-a (MW = 179) is an anatoxin-a homologue isolated from an *Oscillatoria formosa (Phormidium formosum)* strain (Figure 3.2). It has a propionyl group at C-2 instead of the acetyl group in anatoxin-a (Skulberg *et al.,* 1992). The LD_{50} (lethal dose resulting in 50 per cent deaths) of anatoxin-a and homoanatoxin-a are 200 - 250 µg kg^{-1} bw (Devlin *et al.,* 1977; Carmichael *et al.,* 1990; Skulberg *et al.,* 1992).

Anatoxin-a(S)

Anatoxin-a(S) is a unique phosphate ester of a cyclic N-hydroxyguanine (MW = 252) (Figure 3.2) produced by *Anabaena flos-aquae* strain NRC 525-17 (Matsunaga *et al.,* 1989). It has more recently been identified in blooms and isolated strains of *Anabaena lemmermannii* (Henriksen *et al.,* 1997; Onodera *et al.,* 1997a). The LD_{50} of anatoxin-a(S) is 20 µg kg^{-1} bw (i.p. mouse) (Carmichael *et al.,* 1990). Structural variants of anatoxin-a(S) have not been detected.

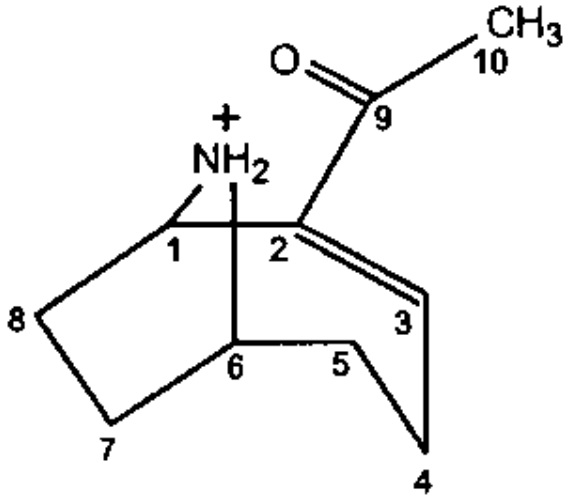
Saxitoxins

Saxitoxins are a group of carbamate alkaloid neurotoxins which are either non-sulphated (saxitoxins - STX), singly sulphated (gonyautoxins - GTX) or doubly sulphated (C-toxins)

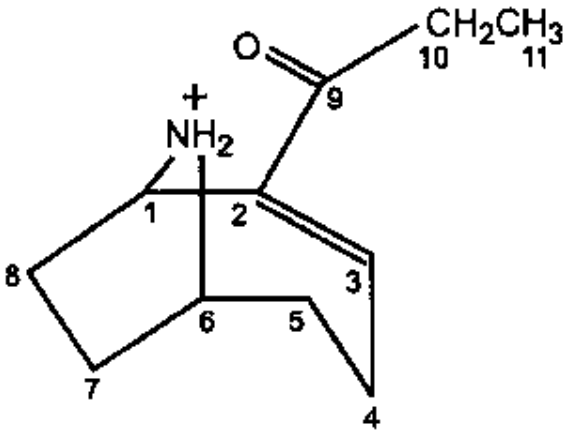
(Figure 3.2 and Table 3.3). In addition, decarbamoyl variants and several new toxins have been identified in some species.

Figure 3.2 The chemical structures of cyanobacterial neurotoxins, anatoxin-a, homoanatoxin-a, anatoxin-a(S), and the general structure of saxitoxins. Sixteen different saxitoxins have been reported from cyanobacteria (for details see Table 3.3). MW = molecular weight

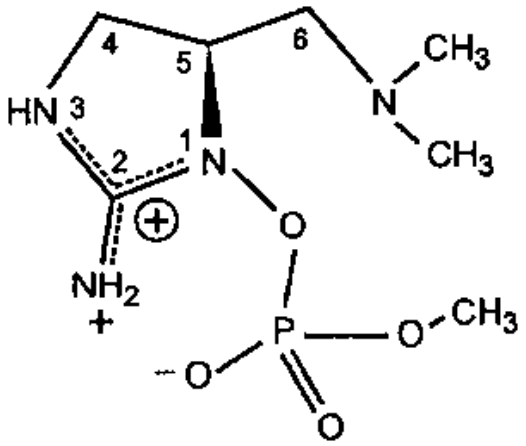
Anatoxin-a MW 165; $C_{10}H_{15}NO$



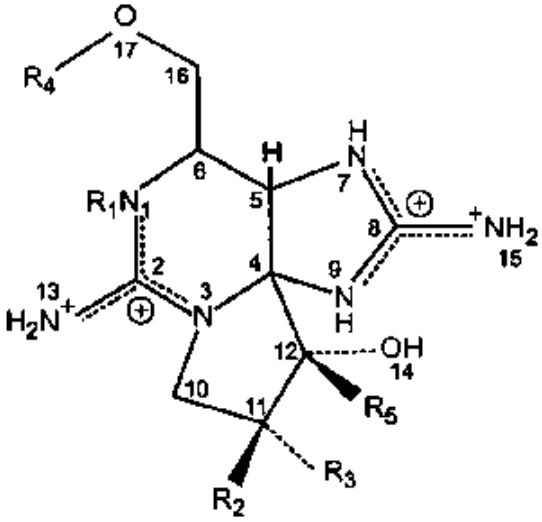
Homoanatoxin-a MW 179; $C_{11}H_{17}NO$



Anatoxin-a(S) MW 252; $C_7H_{17}N_4O_4P$



General structure of saxitoxins



Saxitoxins were originally isolated from shellfish where they are concentrated from marine dinoflagellates (so called "red tide" algae) and have caused deaths in humans (Anderson, 1994) (see section 4.1). Saxitoxins have been found in the cyanobacteria *Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii.* The North American *Aphanizomenon flos-aquae* strains NH-1 and NH-5 contain mostly neosaxitoxin and less saxitoxin (plus a few unidentified neurotoxins). *Anabaena circinalis* strains (from Australia) contain mostly C1 and C2 toxins, with lesser amounts of gonyautoxins 2 and 3. The freshwater cyanobacterium *Lyngbya wollei* produced three known and six new saxitoxin analogues. *Cylindrospermopsis raciborskii* in Brazil was found to contain mostly neosaxitoxin and a smaller amount of saxitoxin.

Table 3.3 Saxitoxins reported from cyanobacterial strains and bloom samples (for the chemical structure see Figure 3.2)

Name of toxin	Variable chemical groups in toxins					Cyanobacteria			
	R_1	R_2	R_3	R_4	R_5	Aph[1]	Ana[2]	Lyn[3]	Cyl[4]
STX	H	H	H	$CONH_2$	OH	+	+		+
GTX2	H	H	OSO_3^-	$CONH_2$	OH		+		
GTX3	H	OSO_3^-	H	$CONH_2$	OH		+		
GTX5	H	H	H	$CONHSO_3^-$	OH		+		
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH		+		
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH		+		
NEO	OH	H	H	$CONH_2$	OH	+			+
GTX1	OH	H	OSO_3^-	$CONH_2$	OH		*		
GTX4	OH	OSO_3^-	H	$CONH_2$	OH		*		
GTX6	OH	H	H	$CONHSO_3^-$	OH		*		
dcSTX	H	H	H	H	OH		+	+	
dcGTX2	H	H	OSO_3^-	H	OH		+	+	
dcGTX3	H	OSO_3^-	H	H	OH		+	+	
LWTX1[3]	H	OSO_3^-	H	$COCH_3$	H			+	
LWTX2[3]	H	OSO_3^-	H	$COCH_3$	OH			+	
LWTX3[3]	H	H	OSO_3^-	$COCH_3$	OH			+	
LWTX4[3]	H	H	H	H	H			+	
LWTX5[3]	H	H	H	$COCH_3$	OH			+	
LWTX6[3]	H	H	H	$COCH_3$	H			+	

STX Saxitoxin
GTX Gonyautoxins
C C-toxins
dcSTX Decarbamoylsaxitoxin
LWTX Lyngbya-wollei-toxins

[1] Toxins found in *Aphanizomenon flos-aquae,* New Hampshire, USA (Ikawa *et al.,* 1982; Mahmood and Carmichael, 1986)

[2] Toxins reported in an *Anabaena circinalis* strain and bloom samples, Australia (Humpage *et al.*, 1994; Negri *et al.,* 1995; Negri *et al.,* 1997). dcGTX2 and dcGTX3 are probably break down products of C1 and C2 in this species (Jones and Negri, 1997). An asterisk in this column denotes toxins reported by Humpage *et al.,* 1994 for *Anabaena circinalis* based on retention time data, but not confirmed by mass spectrometry, and not found in subsequent studies

[3] Toxins detected in *Lyngbya wollei,* USA (Onodera *et al.*, 1997b)

[4] Toxins thus far found in *Cylindrospermopsis raciborskii,* Brazil (Lagos *et al.,* 1997)
Other neurotoxic cyanobacteria

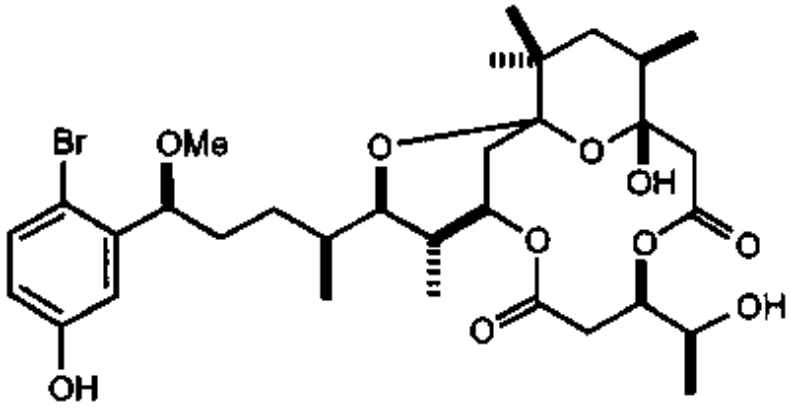
In marine *Trichodesmium* blooms from the Virgin Islands, a neurotoxic factor has been reported which was not anatoxin-a or anatoxin-a(S) but remains to be characterised (Hawser *et al.,* 1991).

3.1.3 Cytotoxic alkaloids

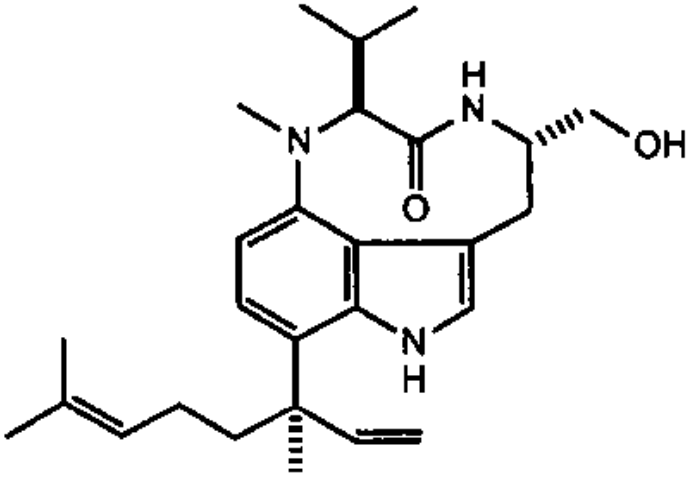
In tropical and subtropical waters of Australia, the alkaloid hepatotoxin cylindrospermopsin with a completely different mechanism of toxicity has caused health problems in drinking water supplies (see section 4.1). It is a cyclic guanidine alkaloid with a molecular weight of 415 (Figure 3.1C). It is produced by *Cylindrospermopsis raciborskii* (Hawkins *et al.,* 1985, 1997), *Umezakia natans* (Harada *et al.,* 1994) and *Aphanizomenon ovalisporum* (Banker *et al.,* 1997). In pure form, cylindrospermopsin mainly affects the liver, although crude extracts of *C. raciborskii* injected or given orally to mice also induce pathological symptoms in the kidneys, spleen, thymus and heart (see Chapter 4 for more details). Pure cylindrospermopsin has an LD_{50} in mice (i.p.) of 2.1 mg kg l^{-1} bw at 24 h and 0.2 mg kg l^{-1} bw at 5-6 days (Ohtani *et al.,* 1992). Recently, new structural variants of cylindrospermopsin have been isolated from an Australian strain of *C. raciborskii,* with one being identified as demethoxy-cylindrospermopsin (Chiswell *et al.,* 1999).

Figure 3.3 The chemical structures of debromoaplysiatoxin and lyngbiatoxin-a

Debromoaplysiatoxin



Lyngbyatoxin A



3.1.4 Dermatotoxic alkaloids - aplysiatoxins and lyngbyatoxin

Benthic marine cyanobacteria such as *Lyngbya, Oscillatoria* and *Schizothrix* may produce toxins causing severe dermatitis among swimmers in contact with the filaments (see section 4.2). The inflammatory activity of *Lyngbya* is caused by aplysiatoxins and debromoaplysiatoxin (Figure 3.3) which are potent tumour promoters and protein kinase C activators (Mynderse *et al.,* 1977; Fujiki *et al.,* 1990). Another strain of *Lyngby*a *majuscula* has caused dermatitis and severe oral and gastrointestinal inflammation. It was found to contain lyngbyatoxin-a (see Figure 3.3) (Cardellina *et al.,* 1979). Debromoaplysiatoxin along with other toxic compounds has also been isolated from other Oscillatoriaceae, such as *Schizothrix calcicola* and *Oscillatoria nigroviridis.*

3.1.5 Irritant toxins - lipopolysaccharides

Weise *et al.* (1970) were the first to isolate LPS from the cyanobacterium *Anacystis nidulans* and numerous reports of endotoxins in cyanobacteria have followed. Lipopolysaccarides are generally found in the outer membrane of the cell wall of Gram negative bacteria, including cyanobacteria, where they form complexes with proteins and phospholipids. They are pyrogenic and toxic (Weckesser and Drews, 1979). Lipopolysaccarides, as the name implies, are condensed products of a sugar, usually a hexose, and a lipid, normally a hydroxy C_{14}-C_{18} fatty acid. The many structural variants of LPS are generally phylogenetically conserved, i.e. particular orders, genera and occasionally species, have identical or similar fatty acid and sugar components contained in their cell wall LPS. It is generally the fatty acid component of the LPS molecule that elicits an irritant of allergenic response in humans and mammals.

Lipopolysaccharides are an integral component of the cell wall of all Gram negative bacteria, including cyanobacteria, and can elicit irritant and allergenic responses in human and animal tissues that come in contact with the compounds. There is considerable diversity of LPS composition among the cyanobacteria, but differences are largely related to phylogeny. Thus, different genera typically have distinct LPS compositions that are largely conserved within that genus (Kerr *et al.,* 1995). Cyanobacterial LPS are considerably less potent than LPS from pathogenic gram-

negative bacteria such as, for example, *Salmonella* (see Chapter 4). The chemical stability of cyanobacterial LPS in surface waters is unknown.

Structurally, LPS is a complex polymer composed of four regions. Region I, the O-antigen region, consists of repeating oligosaccharide units that may vary in structure, with numerous combinations of different sugar residues and associated glycosidic linkages. As suggested by its name, the O-antigen also exhibits several antigenic determinants that constitute the receptor sites for a number of lysogenic bacteriophages. Regions II and III are the outer core and backbone of a core polysaccharide. There is generally only minor variation in core structure between species. The backbone of the polysaccharide is connected to a glycolipid, lipid A (Region IV), via a short link normally composed of 3-deoxy-D-mannoocmiosonic acid (KDO). Lipid A is a disaccharide of glucosamines highly substituted with phosphate, fatty acids and KDO, although the proportion of KDO is low or absent in cyanobacteria compared with other bacterial LPS. The lipid A component is also acetylated with amide and ester-linked hydroxy fatty acids.

Recent studies of the fatty acid composition of Australian species of cyanobacteria (Kerr *et al.,* 1995) show a range of β-OH fatty acids ranging in size from C_{10} to C_{22}. Normal, saturated and branched chain acids have been detected. There was a stark predominance of straight chain 14:0 and 18:0 β-OH acids in *Microcystis* strains that was quite distinct from *Anabaena* and *Nodularia* strains where 16:0 β-OH predominated the LPS fatty acid fraction.

Although comparatively poorly studied, cell wall components, particularly LPS endotoxins from cyanobacteria may contribute to human health problems associated with exposure to mass occurrences of cyanobacteria. The few results available indicate that cyanobacterial LPS is less toxic than the LPS of other bacteria, such as *Salmonella* (Keleti and Sykora, 1982; Raziuddin *et al.,* 1983) (see also section 4.2). More studies are needed to evaluate the chemical structures and health risks of cyanobacterial LPS.

3.1.6 Other bioactive compounds

Cyanobacteria are known to produce several other bioactive compounds, some of which are of medical interest, as well as compounds toxic to other cyanobacteria, bacteria, algae and zooplankton (see section 3.5). Severe intoxication of fish embryos by crude extracts of *Planktothrix agardhii* has been reported by Oberemm *et al.* (1997). Skulberg *et al.* (1994) reported the presence of an unidentified "protracted toxic effect" in cyanobacterial samples that caused death within 4-24 hours in mice. Whether this effect was due to a specific cyanotoxin is unclear.

Cyanobacteria have been found to be a rich source of biomedically interesting compounds and therefore screening programmes for new bioactivities are underway. Cyanobacteria are known to produce antitumour, antiviral, antibiotic and antifungal compounds. Of the cyanobacterial extracts screened by a Hawaiian research group, 0.8 per cent showed solid tumour selective cytotoxicity (Moore *et al.,* 1996). Depsipeptides (peptides with an ester linkage) called cryptophycins isolated from a cyanobacterium, *Nostoc* sp. strain GSV 224, are promising candidates for an anticancer drug (Trimurtulu *et al.,* 1995). Recently, several new cyclic or linear peptides and depsipeptides from cyanobacteria have been characterised. Some are protease inhibitors, but the biological activity of the others remains to be characterised (Namikoshi and Rinehart, 1996). Many

of the cyanobacterial bioactive compounds possess structural similarities to natural products from marine invertebrates.

3.2 Occurrence of cyanotoxins

3.2.1 Mass occurrences of toxic cyanobacteria

The toxicity of cyanobacterial mass occurrences (blooms) was originally brought to the attention of scientists through reports of animal poisonings by farmers and veterinarians, with the first well documented case being reported from Australia in 1878 (Francis, 1878). In most, if not all, reported cases since that time, afflicted animals consumed water from water bodies where there was an obvious presence of a cyanobacterial scum on the water surface (see Ressom *et al.* (1994) and Yoo *et al.* (1995) for a list of reported animal poisonings). More recent measurements of cyanobacterial toxins using sensitive modem analytical methods have often revealed high frequencies of toxic blooms even when animal poisonings have not been reported (Table 3.4).

Throughout the world, it appears that liver-toxic (hepatotoxic, microcystin-containing) freshwater blooms of cyanobacteria are more commonly found than neurotoxic blooms. Liver-toxic blooms have been reported from all continents and almost every part of the world where samples have been collected for analysis. Nevertheless, mass occurrences of neurotoxic cyanobacteria are common in some countries and these have been reported from North America, Europe and Australia, where they have caused several animal poisonings. Blooms which have caused both liver and kidney damage due to the toxin cylindrospermopsin (and possibly related cyanotoxins) have been reported in Australia, Japan, Israel and Hungary.

In recent years, surveys have been carried out in a number of countries in South America, Africa, Australasia, Asia and Europe. The conclusion that can be drawn from these surveys is that toxic cyanobacteria are internationally ubiquitous, and that as further surveys are carried out more toxic cyanobacterial blooms and new toxic species will be discovered. This is particularly true of tropical and subtropical regions that are currently under-represented in the literature. It seems likely that every country in the world will have water bodies which support blooms of toxic cyanobacteria at some time or another. It is also important to note that mass occurrences of toxic cyanobacteria are not always associated with human activities causing pollution or "cultural eutrophication" (see Chapter 2). For example, massive blooms of toxic cyanobacteria have been reported in Australian reservoirs with pristine or near-pristine catchments (watersheds), and toxic benthic cyanobacteria have killed cattle drinking from oligotrophic, high-alpine waters in Switzerland.

3.2.2 Species composition and variation among toxic blooms

Cyanobacterial populations may be dominated by a single species or be composed of a variety of species, some of which may not be toxic. Even within a single-species bloom there may be a mixture of toxic and non-toxic strains. A strain is a specific genetic subgroup within a particular species, and each species may encompass tens or hundreds of strains, each with slightly different traits. Some strains are much more toxic than others, sometimes by more than three orders of magnitude. This can mean that one highly toxic strain, even when occurring in minor amounts amongst larger numbers of

non-toxic strains, may render a bloom sample toxic (Sivonen *et al.,* 1989a,b; Bolch *et al.,* 1997; Vezie *et al.,* 1998).

Table 3.4 Frequencies of mass occurrences of toxic cyanobacteria in freshwaters

Country	No of samples tested	% of toxic samples	Type of toxicity	Reference
Australia	231	42	Hepatotoxic	Baker and Humpage, 1994
			Neurotoxic	
Australia	31	84[1]	Neurotoxic	Negri *et al.*, 1997
Brazil	16	75	Hepatotoxic	Costa and Azevedo, 1994
Canada, Alberta	24	66	Hepatotoxic	Gorham, 1962
			Neurotoxic	
Canada, Alberta	39	95	Hepatotoxic	Kotak *et al.,* 1993
Canada, Alberta (3 lakes)	226	74[1]	Hepatotoxic	Kotak *et al.*, 1995
Canada, Saskatchewan	50	10	Hepatotoxic	Hammer, 1968
			Neurotoxic	
China	26	73	Hepatotoxic	Carmichael *et al.,* 1988b
Czech and Slovak Rep.	63	82	Hepatotoxic	Maršálek *et al.,* 1996
Denmark	296	82	Hepatotoxic	Henriksen *et al.,* 1996b
			SDF	
			Neurotoxic	
Former German	10	70	Hepatotoxic	Henning and Kohl, 1981
Dem. Rep.			SDF	
Germany	533	72[1]	Hepatotoxic	Fastner, 1998
Germany	393	22	Neurotoxic	Bumke-Vogt, 1998
Greece	18	?	Hepatotoxic	Lanaras *et al.,* 1989
Finland	215	44	Hepatotoxic	Sivonen, 1990a
			Neurotoxic	
France, Brittany	22	73[1]	Hepatotoxic	Vezie *et al.*, 1997
Hungary	50	66	Hepatotoxic	Törökné, 1991
Japan	23	39	Hepatotoxic	Watanabe and Oishi, 1980
Netherlands	10	90	Hepatotoxic	Leeuwangh *et al.,* 1983
Norway	64	92	Hepatotoxic	Skulberg *et al.*, 1994
			Neurotoxic	
			SDF	

Portugal	30	60	Hepatotoxic	Vasconcelos, 1994
Scandinavia	81	60	Hepatotoxic	Berg *et al.*, 1986
Sweden	331	47	Hepatotoxic	Willén and Mattsson, 1997
			Neurotoxic	
UK	50	48	Hepatotoxic	Codd and Bell, 1996
		28[1]		
USA, Minnesota	92	53	Unspecified	Olson, 1960
			Neurotoxic	
USA, Wisconsin	102	25	Hepatotoxic	Repavich *et al.,* 1990
			Neurotoxic	
Mean		59		

[1] HPLC was used to detect the toxin content of the samples

Some of the studies shown in the table have been conducted over several years while others lasted only one season. The relative share of cyanobacteria in the samples varied; low frequency of cyanobacteria led to low percentages of toxic samples in some studies. In most of the studies the method used to detect toxicity is mouse bioassay, normally with a 4-hour time limit (or longer when slow death factors (SDF) have been included). SDF may indicate low hepatotoxicity of samples or other unknown toxicity.

Toxic and non-toxic strains from the same cyanobacterial species cannot be separated by microscopic identification. The use of molecular genetic methods, in particular the use of molecular probes and primers that target specific toxin production genes, will lead to the development of more precise identification methods for toxic cyanobacteria in the future. To confirm that a particular cyanobacterial strain is a toxin-producer, it is important to isolate a pure culture of that strain, preferably free of other bacteria; then to detect and quantify toxin concentrations in the pure culture (either by bioassay or chemical analysis); and, where possible, to purify and characterise fully the toxins (for such examples see Tables 3.2 and 3.3). It is likely that the list of confirmed toxic species will increase in the future due to the isolation of new species and strains, and because of the use of improved isolation, culturing and analytical methods.

Microcystis sp., commonly *Microcystis aeruginosa,* are linked most frequently to hepatotoxic blooms world-wide (see Tables 3.2 and 3.5 for details and references for all toxic species). *Microcystis viridis* and *Microcystis botrys* strains also have been shown to produce microcystins. As noted in section 2.2, *Microcystis* is a non-nitrogen-fixing genus which is often dominant under nutrient-rich conditions (especially where there is a significant supply of ammonia), although it also forms blooms in less polluted waters. Microcystin-producing *Anabaena* sp. have been reported from Canada, Denmark, Finland, France and Norway. A recent study from Egypt revealed that 25 per cent of 75 *Anabaena* and *Nostoc* strains isolated from soil, rice fields and water bodies contained microcystins. *Planktothrix agardhii* and *Planktothrix rubescens* (previously called *Oscillatoria agardhii* and *O. rubescens)* are common microcystin producers in the Northern Hemisphere; toxic strains of these have been isolated from blooms in Denmark, Finland and Norway. In addition, these species were frequently shown to be dominant in microcystin containing blooms in China, in Germany and in Sweden. In Swiss alpine lakes, *Oscillatoria limosa,* which is benthic (i.e. it grows attached to sediments and rocks), is a microcystin producer. In spite of the widespread occurrence of

cyanobacterial blooms in Australia, *Planktothrix* blooms are rare there. This may be due to the higher temperature and tendency for elevated clay-derived turbidity in Australian water bodies.

Table 3.5 Toxic cyanobacteria species and their geographical distribution

Toxic species	Cyanotoxin	Location	Reference(s)
Anabaena flos-aquae	Microcystins	Canada	Krishnamurthy *et al.,* 1989; Harada *et al*., 1991
Anabaena ?	Microcystins	Denmark	Henriksen *et al.*, 1996b
Anabaena spp.	Microcystins	Egypt	Yanni and Carmichael, 1997
Anabaena spp. *(flos-aquae, lemmermannii, circinalis)*	Microcystins	Finland	Sivonen *et al.,* 1990b; 1992a
Anabaena circinalis	Microcystins	France	Vezie *et al*., 1998
Anabaena flos-aquae	Microcystins	Norway	Sivonen *et al*., 1992a
Microcystis aeruginosa	Microcystins	Worldwide	Several; see Rinehart *et al*., 1994 for a summary
M. viridis	Microcystins	Japan	Kusumi *et al.,* 1987; Watanabe *et al*., 1986
M. botrys	Microcystins	Denmark	Henriksen *et al.,* 1996b
Planktothrix agardhii	Microcystins	China	Ueno *et al*., 1996a
P. agardhii	Microcystins	Denmark	Henriksen *et al*., 1996b
P. mougeotii	Microcystins	Denmark	Henriksen *et al*., 1996b
P. agardhii	Microcystins	Finland	Sivonen, 1990b; Luukkainen *et al.,* 1993
P. agardhii	Microcystins	Norway	Krishnamurthy *et al.*, 1989; Meriluoto *et al*., 1989
Oscillatoria limosa	Microcystins	Switzerland	Mez *et al*., 1996
Nostoc sp.	Microcystins	Finland	Sivonen *et al*., 1990a, 1992b
Nostoc sp.	Microcystins	England	Beattie *et al*., 1998
Anabaenopsis millerii	Microcystins	Greece	Lanaras and Cook, 1994
Haphalosiphon hibernicus (soil isolate)	Microcystins	USA	Prinsep *et al*., 1992
Nodularia spumigena	Nodularins	Australia	Baker and Humpage, 1994; Jones *et al.,* 1994
N. spumigena	Nodularins	Baltic Sea	Sivonen *et al*., 1989b
N. spumigena	Nodularins	New Zealand	Carmichael *et al*., 1988a; Rinehart *et al*., 1988
Aphanizomenon ovalisporum	Cylindrospermopsin	Israel	Banker *et al*., 1997
Cylindrospermopsis raciborskii	Cylindrospermopsin	Australia	Hawkins *et al*., 1985; 1997
C. raciborskii	Cylindrospermopsin	Hungary	Törökné, 1997
Umezakia natans	Cylindrospermopsin	Japan	Harada *et al*., 1994

Anabaena flos-aquae	Anatoxin-a	Canada	Carmichael *et al*., 1975; Devlin *et al*., 1977
Anabaena spp.	Anatoxin-a	Finland	Sivonen *et al*., 1989a
Anabaena blooms	Anatoxin-a	Germany	Bumke-Vogt, 1998
Anabaena sp.	Anatoxin-a	Ireland	James *et al*., 1997
Anabaena sp.	Anatoxin-a (minor amounts)	Japan	Park *et al.,* 1993a
Anabaena planctonica bloom	Anatoxin-a	Italy	Bruno *et al.,* 1994
Aphanizomenon sp.	Anatoxin-a	Finland	Sivonen *et al.,* 1989a
Aphanizomenon blooms	Anatoxin-a	Germany	Bumke-Vogt, 1998
Cylindrospermum sp.	Anatoxin-a	Finland	Sivonen *et al*., 1989a
Microcystis sp.	Anatoxin-a (minor amounts)	Japan	Park *et al*., 1993a
Oscillatoria sp. benthic	Anatoxin-a	Scotland	Edwards *et al*., 1992
Oscillatoria sp. ?	Anatoxin-a	Ireland	James *et al.,* 1997
Planktothrix sp.	Anatoxin-a	Finland	Sivonen *et al.,* 1989a
Planktothrix formosa	Homoanatoxin-a	Norway	Skulberg *et al*., 1992
Anabaena flos-aquae	Anatoxin-a(S)	Canada	Matsunaga *et al.*, 1989; Mahmood and Carmichael, 1987
A. lemmermannii	Anatoxin-a(S)	Denmark	Henriksen *et al.,* 1997; Onodera *et al.,* 1997a
Anabaena circinalis	Saxitoxins	Australia	Humpage *et al*., 1994; Negri *et al*., 1995; 1997
Aphanizomenon flos-aquae	Saxitoxins	USA	Jackim and Gentile, 1968; Ikawa *et al*., 1982; Mahmood and Carmichael, 1986
Cylindrospermopsis raciborskii	Saxitoxins	Brazil	Lagos *et al.,* 1997
Lyngbya wollei	Saxitoxins	USA	Carmichael *et al*., 1997; Onodera *et al*., 1997b

The toxicity of the species listed in the table is in most cases verified by laboratory studies with isolated strains. A few bloom samples are also included from the new areas of occurrence where toxicity of the species is not verified by strain isolation but the toxins are determined in the bloom samples. The authors have suggested the listed species as the probable toxin producer (based on their dominance) but these reports should be treated as tentative until pure strains are studied.
Nostoc rivulare blooms in Texas, USA have caused poisoning of domestic and wild animals (Davidson, 1959) and, more recently, two unidentified *Nostoc* strains were shown to produce microcystins (Table 3.5).

The hepatotoxin, cylindrospermopsin, has been found in *Cylindro-spermopsis raciborskii* in Australia and Hungary, in *Umezakia natans* in Japan, and in *Aphanizomenon ovalisporum* in Israel (Table 3.5). In spite of their occurrence in Europe, it appears that cylindrospermopsin-producing genera most commonly form toxic blooms in subtropical,

tropical or arid zone water bodies. However, there have been reports of increasing occurrences of *Cylindrospermopsis raciborskii in* Europe and the USA (Padisák, 1997).

The neurotoxin, anatoxin-a, was first shown to be produced by *Anabaena flos-aquae* strains originating from Canada, and later by Finnish strains of unidentified *Anabaena* species, and in individual species of *Oscillatoria, Aphanizomenon,* and *Cylindrospermum,* by benthic *Oscillatoria* from Scotland, and by *Anabaena* and *Oscillatoria* in Ireland. It also was present in *Anabaena planctonica* blooms in Sardinia, Italy, in *Anabaena* and *Aphanizomenon* blooms in Germany, and in minor amounts in some Japanese bloom samples, as well as in *Anabaena* strains. Homoanatoxin-a has been characterised from an *Oscillatoria formosum (Phormidium formosum)* strain from Norway (see Table 3.5). To date, anatoxin-a(S) has been found only from *Anabaena* species: *A. flos-aquae* in the USA and Scotland, and *A. lemmermannii* in Denmark.

Aphanizomenon flos-aquae blooms and strains were found to contain saxitoxins in the USA and this species was for a long time the only known saxitoxin producer amongst the cyanobacteria. More recently, saxitoxins have been shown to be common in Australian rivers and reservoirs and to be produced by *Anabaena circinalis.* In North America, a benthic freshwater *Lyngbya wollei* was found to produce saxitoxins, as was a strain of *Cylindrospermopsis raciborskii* in Brazil (Table 3.5).

3.2.3 Cyanotoxin patterns in strains and species of cyanobacteria

Cyanobacteria may produce several toxins simultaneously. In general, more than one microcystin has been characterised from the strains listed in Table 3.2. Among neurotoxic strains, several PSPs are found in the same strain, although there are considerable variations between species (Table 3.3). Furthermore, simultaneous neurotoxin and hepatotoxin production has been noted; the best example studied being the *Anabaena flos-aquae* strain NRC 525-17 which produces anatoxin-a(S) (Matsunaga *et al.,* 1989) and several microcystins (Harada *et al.,* 1991a).

Microcystin

Although many strains produce several microcystins simultaneously, usually only one or two of them are dominant in any single strain. Qualitative variation in the microcystins present is most frequently found among strains of *Anabaena* but also in *Microcystis* (Sivonen *et al.,* 1995). Some taxa have a number of microcystins in common, such as planktonic *Anabaena, Microcystis* and *Planktothrix (Oscillatoria).* However, there is evidence of microcystin variants that are typical for certain cyanobacterial taxa.

Planktothrix and some strains of *Anabaena* produce only demethylmicrocystins (Table 3.2). *Planktothrix (Oscillatoria)* isolates from Finland (13 strains studied and toxins fully characterised) seem to produce one of two types of microcystin (D-Asp^3-RR or Dha^7-RR) (Sivonen *et al.,* 1995).

German field samples dominated by *Planktothrix* have also shown these microcystins, with dominance of one major demethylated microcystin in populations of *P. rubescens,* and two or three of these variants in populations of *P. agardhii* (Fastner *et al.,* 1998).

Microcystis strains from Japan appear to contain chiefly microcystin-LR, -RR and -YR, with some cultures showing all three variants, and some strains being dominated by one of them. These three microcystins are the only variants reported in several studies on *M. aeruginosa* and *M. viridis* (e.g. Watanabe, 1996). Many of the microcystins listed in Table 3.2 have been found only in minor amounts or, to date, have been found to be produced only by individual isolates.

In natural samples which usually contain many strains, or more than one toxin-producing species, different combinations of microcystins can be found. For example, in a *Microcystis* bloom from Homer Lake, USA, 19 different microcystins were characterised (Namikoshi *et al.,* 1992a, 1995) and in one Australian bloom of *Microcystis aeruginosa,* 23 microcystins were detected by high pressure liquid chromatography (HPLC), none of which were microcystin-LR (Jones *et al.,* 1995).

Microcystin-LR is often mentioned as the most frequently occurring microcystin, although such observations may be biased by the fact that a chemical standard for the analysis of microcystin-LR was the earliest to be commercially available (see Chapter 13). It has been reported to be the major toxin in bloom and strain samples from Portugal (Vasconcelos *et al.,* 1995. 1996), France (Vezie *et al.,* 1997), Canada (Kotak *et al.,* 1993) and frequently co-occurring with microcystin-RR and -YR in Japan (Watanabe *et al.,* 1988, 1989).

Geographical patterns are indicated by some survey results. Wide variation among the L-amino acids of microcystins has been reported for South Africa (Scott, 1991), frequent presence of demethylmicrocystins (RR and LR) has been seen in Finnish strains (Sivonen *et al.,* 1995) and microcystins in Danish samples show wide variation (Henriksen, 1996a). In part, these patterns probably reflect regional differences in dominance of cyanobacterial species or strains. Water bodies with regular dominance of specific taxa are likely to exhibit characteristic patterns of microcystin variants.

Nodularin

The cyanotoxin, nodularin, is found in waters where *Nodularia spumigena* is present; the most prominent areas being the Baltic Sea and brackish water estuaries and coastal lakes of Australia and New Zealand. However, the best known *Nodularia spumigena* bloom location, Lake Alexandrina, Australia, has salinities which are only slightly elevated above normal river water and at levels still suitable for drinking water. The presence of variants of nodularin in environmental samples is usually rather insignificant. In the Baltic Sea, the collection of samples for several years has shown nodularin to be the major compound present. The same is true for the almost 90 hepatotoxic *Nodularia* strains isolated from the same source (Sivonen *et al.,* 1989b; Lehtimäki *et al.,* 1997). Analyses of several strains isolated from blooms across Australia have revealed similar results, with nodularin variants being found rarely, and then only at low relative abundance (Jones *et al.,* 1994; Blackburn *et al.,* 1997).

Saxitoxins

There is much diversity of saxitoxin distribution in cyanobacteria from around the world (Table 3.3). In addition, the lack of analytical standards for many saxitoxins has probably restricted findings in some countries. Saxitoxin-producing *Anabaena circinalis* blooms

are widespread in Australian rivers and reservoirs, and the relative abundance of individual toxins is remarkably constant in toxin-producing strains, which is quite opposite to the microcystin-producing cyanobacteria. In all healthy *Anabaena circinalis* cultures examined in Australia, the relative composition of individual saxitoxins is very similar and dominated by C-toxins (Blackburn *et al.,* 1997). As blooms and cultures age, the proportion of decarbamoyl-gonyautoxins breakdown products increases at the expense of the C-toxins (see section 3.4). In North American *Aphanizomenon flos-aquae* and in Brazilian *Cylindrospermopsis raciborskii* samples, mostly neosaxitoxin and smaller amounts of saxitoxin have been detected. In the case of the *Aphanizomenon flos-aquae* from North America, only bloom samples and strains from New Hampshire, USA have been found to contain saxitoxins. Mat-forming *Lyngbya wollei* from Alabama, USA was found to produce decarbamoyl saxitoxin (dcSTX), decarbamoylgonyautoxin-2 and-3 (dcGTX2 and dcGTX3) and six new saxitoxins (see section 3.1).

3.2.4 Concentrations of cyanotoxins in surface waters

Information on the concentrations of cyanotoxins in surface waters has been appearing only recently in the international literature. In early studies (pre-1990s), the toxicity of bloom samples was determined by mouse bioassays, but this method is unsuitable for measuring the low concentrations of cyanotoxins that usually prevail in cyanobacterial populations when they do not accumulate in scums. The development of better analytical methods, in the first instance HPLC and more recently enzyme linked immunosorbent assay (ELISA), (and for microcystins and nodularins also the protein phosphatase assay) has made the quantification of total and individual toxins possible (see Box 3.1 and Chapter 13).

The quantitative determination of toxin concentrations is mostly performed from lyophilised (freeze-dried) cultures, bloom samples or seston (particulate material suspended in water, which contains not only cyanobacterial cells but usually other algae, some zooplankton, and possibly inorganic material such as soil and sediment particles) (see Chapter 13). Results are usually expressed as milligrams or micrograms of toxin per gram dry weight (dw). Whereas in cultures and bloom samples, the dry weight originates from cyanobacteria, it will encompass further particles (seston) in plankton samples taken outside of scum areas. The highest published concentrations of cyanotoxins from cyanobacterial bloom samples, measured by HPLC, are (see Table 3.6):

- microcystin - 7,300 µg g^{-1} dw from China and Portugal,
- nodularin - 18,000 µg g^{-1} dw from the Baltic Sea,
- cylindrospermopsin - 5,500 µg g^{-1} dw from Australia,
- anatoxin-a - 4,400 µg g^{-1} dw from Finland,
- saxitoxins - 3,400 µg g^{-1} dw from Australia,
- anatoxin-a(S) - 3,300 µg g^{-1} dw from the USA.

Box 3.1 Toxins and toxicity: what's in a name?

There is often a misunderstanding of the terms "toxicity" or "toxin content" when applied to cyanobacteria or a water sample that contains cyanotoxins. In particular, the terms are often taken to be synonymous, which they are not. Furthermore, the measurement units in which data are reported are not always carefully considered.

To some extent, the problem lies in the different ways in which toxin data can be expressed, and the way in which people think about the cyanobacterial or water sample, particularly in the context of their own professional background. For example, a water treatment plant operator may assume or expect a "toxicity" value to refer to the toxin concentration per litre of drinking water, a biologist may think of the same term to mean the amount of toxin per mass of cyanobacteria, whereas a toxicologist will normally take the term to reflect the amount of cyanobacteria needed to kill an animal.

Toxicity. In the strict sense, toxicity refers only to animal testing data, and is expressed as the amount of cyanobacteria lethal to an animal (usually normalised per kilogram of body weight). The commonly reported LD_{50} value, a measure of toxin potency, is the amount of cyanobacteria or pure toxin needed to kill 50 per cent of animals in an experimental trial, again normalised per kilogram of body weight. Thus, the lower the LD_{50} the more potent the cyanobacterial sample or pure toxin (see Table 3.2 for examples). Note, however, that cell growth assays may express results as EC_{50} (concentration reducing growth rate by 50 per cent).

Toxin concentration and toxin content. Toxin concentration can refer either to the amount of toxin per litre of water or the amount of toxin per mass of cyanobacterial bloom material. Therefore, particular attention must be paid to the units in which the data are reported. In the published literature on toxic cyanobacteria, the amount of toxin per mass of bloom material is often referred to as the toxin content. Strictly speaking, this is incorrect; the correct term should be the gravimetric, or per cyanobacterial mass, toxin concentration.

Toxin quota. This refers to the amount (mass or moles) of toxin per cyanobacteria cell.

Cell bound versus free toxin. In most circumstances almost all toxins are cell-bound (within the cells) and little or no toxin is found in solution except where a bloom is senescent or where an algicide has been applied.

Higher concentrations have been reported in unpublished studies and may be expected under some circumstances.

For the purposes of water treatment and public health management, toxin concentration per litre of water is often a more relevant unit, for example for relating ambient concentrations to guideline levels. Concentration per litre usually refers to toxins contained within the cells as well as dissolved in water, and taken from a defined volume of water. Very high concentrations of microcystins per litre of water (rather than per mass of cyanobacteria), have been reported up to 25,000 µg l^{-1} microcystin and up to 3,300 µg l^{-1} anatoxin-a(S) (Table 3.6). It should be noted, however, that these very high concentrations of toxins would be from scums or from very dense accumulations of cyanobacteria.

In one or two studies where many toxin concentration measurements have been made from more or less randomly collected plankton samples, the distribution of data revealed

a small number of samples with high toxin concentrations, some samples with moderate concentrations, and many samples with low or zero concentrations (Figure 3.4). However, such surveys tend to include a large proportion of samples where the particle content is not dominated by cyanobacteria. If cyanobacteria constitute only a small fraction of the total dry weight, toxin concentration per gram dry weight will necessarily be low. In contrast, if mass developments of toxic cyanobacterial species are investigated, cyanobacteria will constitute a large fraction of the total dry weight. In these cases, toxin content is often high. Recent German data show that the relative frequency of high, moderate and low toxin concentrations per gram dry weight is dependent on the particular species that is dominant (Figure 3.5).

For management, the option of estimating toxin content from the biomass of a dominant cyanobacterial species (i.e. the "toxin quota" per cell or biovolume) can be helpful. Survey data from Germany show that microcystin toxin quotas differ between taxa, but variation within most of the samples dominated by the same taxon is only moderate (two- to fivefold) (Figure 3.5). In contrast, an Australian investigation with other species of cyanobacteria showed substantial variation of toxin concentrations per dry weight, even in cases with dominance of the same cyanobacterial species (Negri *et al.,* 1997).

Table 3.6 Toxin concentrations reported in cyanobacterial bloom or water samples

Location	Period of study	No. of toxic samples (total no. of samples)	Toxins identified	Range of total concentrations (µg g^{-1} dw, unless otherwise indicated)	Analysis method	Reference
Microcystins						
Australia	1991	4	Microcystins, 24 unidentified	2,100-4,100[2]	HPLC	Jones *et al*., 1995
Canada, Alberta	1990	37(50)	Microcystin-LR	4-610	HPLC	Kotak *et al*., 1993
Canada, Alberta (3 lakes)	1990-93	168(226)	Microcystin-LR	1-1,550	HPLC	Kotak *et al*., 1995
China	1988	5(10)	Microcystin-RR, -LR	200-7,300	HPLC	Zhang *et al*., 1991
Czech and Slovak Rep.	1995-96	(63)	Microcystin-LR	4-6,835	HPLC	Maršálek *et al*., 1996
Denmark	1992-94		Microcystin-RR, -LR	3-2,800	HPLC	Christoffersen, 1996
Denmark	1993-95	198(296)	Microcystins	5-1,900	HPLC	Henriksen *et al*., 1996b
Finland	1994-95	17(20)	Microcystin-LR	> 10-800	HPLC	Lahti *et al*., 1997
France	1994	16(22)	Microcystins	70-3,970	HPLC	Vezie *et al*., 1997
France, L.	1994	19(30)	Microcystins	30-230	HPLC	Vezie *et al*.,

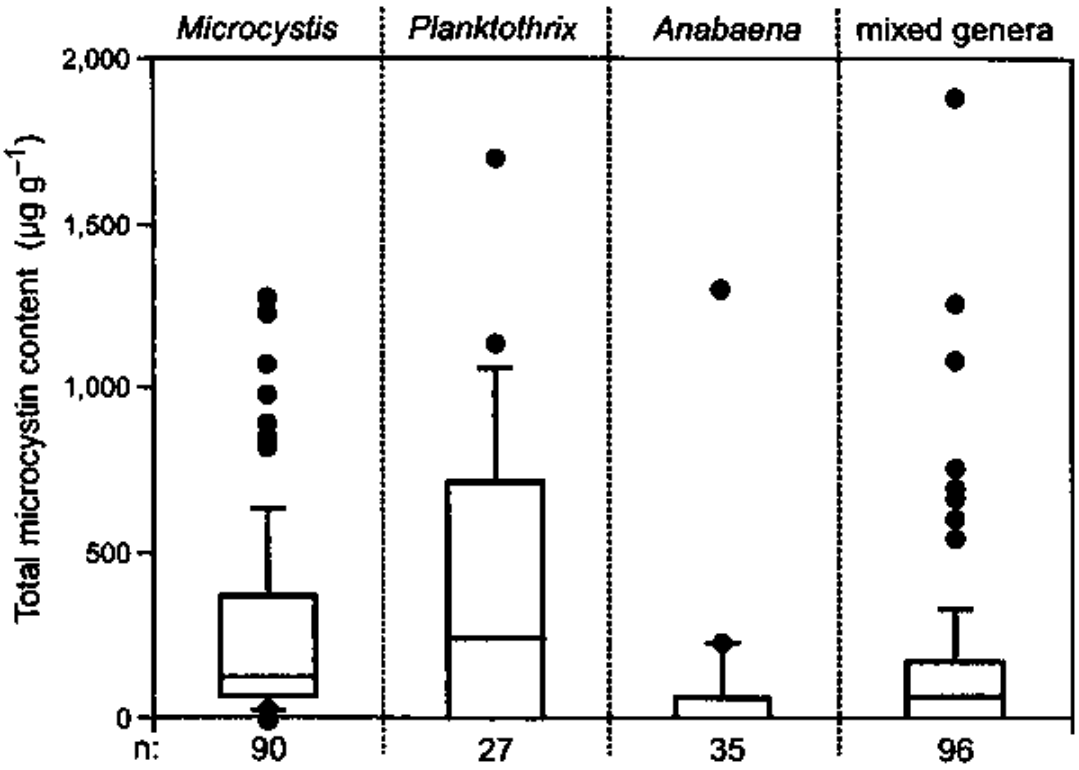
Grand-Lieu						1998
Germany	1992	8(15)	Microcystin-LR	36-360	HPLC	Fastner, 1994
Germany	1993	17(18)	Microcystins	0.15-36[1,2]	ELISA	Ueno *et al*., 1996b
Germany	1995-96	385(533)	Microcystins	1-5,000	HPLC	Fastner, 1998
Germany	1997	34	Microcystins, several	1-25,000 [1]	HPLC	Chorus *et al*., 1998
Japan	1990	12(14)	Microcystin-RR, -YR, -LR	160-950	HPLC	Watanabe *et al*., 1992
Japan	1988-92	11(19)	Microcystin-RR, -YR, -LR, [Dha[7]] -LR	70-1,610	HPLC	Park *et al*., 1993a
Japan, Lake Suwa	1980-91	13	Microcystin-RR, -YR, -LR	30-2,100	HPLC	Park *et al*., 1993b
Japan	1986-88	4(4)	Microcystin-RR, -YR, -LR	100-860	HPLC	Shirai *et al*., 1991
Japan	1992-95	18(22)	Microcystin-RR, -YR, -LR	0.04-480 [1]	HPLC	Tsuji *et al*., 1996
Japan	1993-95	46(57)	Microcystins	0.05-1,300 [1,2]	ELISA	Ueno *et al*., 1996b
Japan	1993-94	12(17)	Microcystins	0.06-94 [1,2]	ELISA	Nagata *et al*., 1997
Japan	1989-94	10(10)	Microcystins	300-15,600 [1,2]	ELISA	Nagata *et al*., 1997
				330-19,500 [1,2]	HPLC	
Portugal	1989-92	12(12)	Microcystin-LR plus six known and three unidentified microcystins	1,000-7,100	HPLC	Vasconcelos *et al*., 1996
Portugal	1994-95	28(29)	Microcystins	0.1-37 [1,2]	ELISA	Ueno *et al*., 1996b
South Africa	1985-86		Microcystin-FR, -LR, -YR, -LA, -YA, -LAba	5-420	HPLC	Wicks and Thiel, 1990
South Africa	1988-89	9(9)	Microcystin-YR, -LR, -FR, -YA, -LA, -LAba	40-630	HPLC	Scott, 1991
UK	1992	3(3)	Microcystins, 3	17-131 [1,2]	HPLC	Codd *et al*., 1995
USA, Wisconsin	1993	9	Microcystins	1,900-12,800 [2]	ELISA	McDermott *et al*., 1995
Nodularins						

Baltic Sea	1985-87	17(23)	Nodularin	< 100-2,400	HPLC	Sivonen *et al*., 1989b
Baltic Sea	1990-91	6(16)	Nodularin	300-18,000	HPLC	Kononen *et al*., 1993
Tasmania, Orielton Lagoon	1992-93	7(9)	Nodularin	2,000-3,500	HPLC	Jones *et al*., 1994
Anatoxins						
Denmark	1993-95	9(10)	Anatoxin-a(S)	4-3,300 [3]	ChE inhibition assay	Henriksen *et al*., 1997
Finland	1985-87	13(30)	Anatoxin-a	10-4,400	GC/MS	Sivonen *et al*., 1989a
Finland and Japan		3(3)	Anatoxin-a	0.4-2,600	LC/MS	Harada *et al*., 1993
Germany	1995-96	10(45)	Anatoxin-a	0.02-0.36 [1]	GC-ECD	Bumke-Vogt, 1998
Ireland	1995	2(2)	Anatoxin-a	10-100	HPLC (fluorimetric)	James *et al*., 1997
Japan	1988-92	9(14)	Anatoxin-a	0.3-16	TSP-LC/MS	Park *et al*., 1993a
Saxitoxins						
Australia	1990-92	11(11)	Saxitoxins	85-2,040	HPLC and FAB-MS	Humpage *et al*., 1994
Australia	1992-94	24(31)	Saxitoxins	50-3,400	HPLC	Negri *et al*., 1997
USA	1994	7(8)	Saxitoxins	5-60 [4]	HPLC/AOAC	Carmichael *et al*., 1997

dw	Dry weight
HPLC	High pressure liquid chromatography
ELISA	Enzyme linked immunosorbent assay
GC/MS	Gas chromatography/mass spectrometry
LC/MS	Liquid chromatography/mass spectrometry
GC-ECD	Gas chromatography-electron capture detection
TSP-LC/MS	Thermospray-liquid chromatography/mass spectrometry
FAB-MS	Fast atom bombardment-mass spectrometry
AOAC	Mouse bioassay done according to the Association of Official Analytical Chemists

[1] Given as µg l^{-1}
[2] Microcystin-LR used as standard
[3] Measured by enzyme inhibition
[4] Micrograms of STX equivalents

Figure 3.4 Cell-bound total microcystin content (measured by HPLC) of samples taken in Denmark between 1993 and 1995 and dominated by different cyanobacteria. Boxes show median values and the values within the 50 percentile range; bars indicate the 10th and 90th percentile; n = number of samples (Modified from Henriksen, 1996)



Further regional investigations are needed to clarify the toxins and species for which toxin quotas may be reasonably stable and, thus, predictable.

The further development of quantitative, pre-concentration methods coupled with the highly sensitive analytical methods noted above (see also Chapter 13) has also enabled the measurement of very low concentrations of extracellular, dissolved toxins in water. Data for dissolved toxin concentration, as reported in the scientific literature, are given in Table 3.7. Prevalence and degradation of dissolved toxins in water are discussed in section 3.4. Concentrations of microcystins dissolved in water vary from trace concentrations up to 1,800 µg l^{-1} or higher, following the collapse of a large, highly toxic bloom.

3.2.5 Seasonal variations in bloom toxin concentration

The timing and duration of the bloom season of cyanobacteria depends largely on the climatic conditions of the region. In temperate zones, mass occurrences of cyanobacteria are most prominent during the late summer and early autumn and may last 2-4 months. In regions with more Mediterranean or subtropical climates, the bloom season may start earlier and persist longer.

Figure 3.5 Cell-bound total microcystin content (measured by HPLC) of samples taken in Germany between 1995 and 1996 dominated by different cyanobacteria. A. On a dry weight basis; B. On a chlorophyll a basis; C. On a volume basis. *(Micro.* spp = *Microcystis* spp.; *Plankto. agardhii = Planktothrix agardhii; Plankto. rubescens = Planktothrix rubescens)* Boxes show median values and the values within the 50 percentile range; bars indicate the 10th and 90th percentile; n = number of samples (Modified from Fastner *et at.,* 1998)

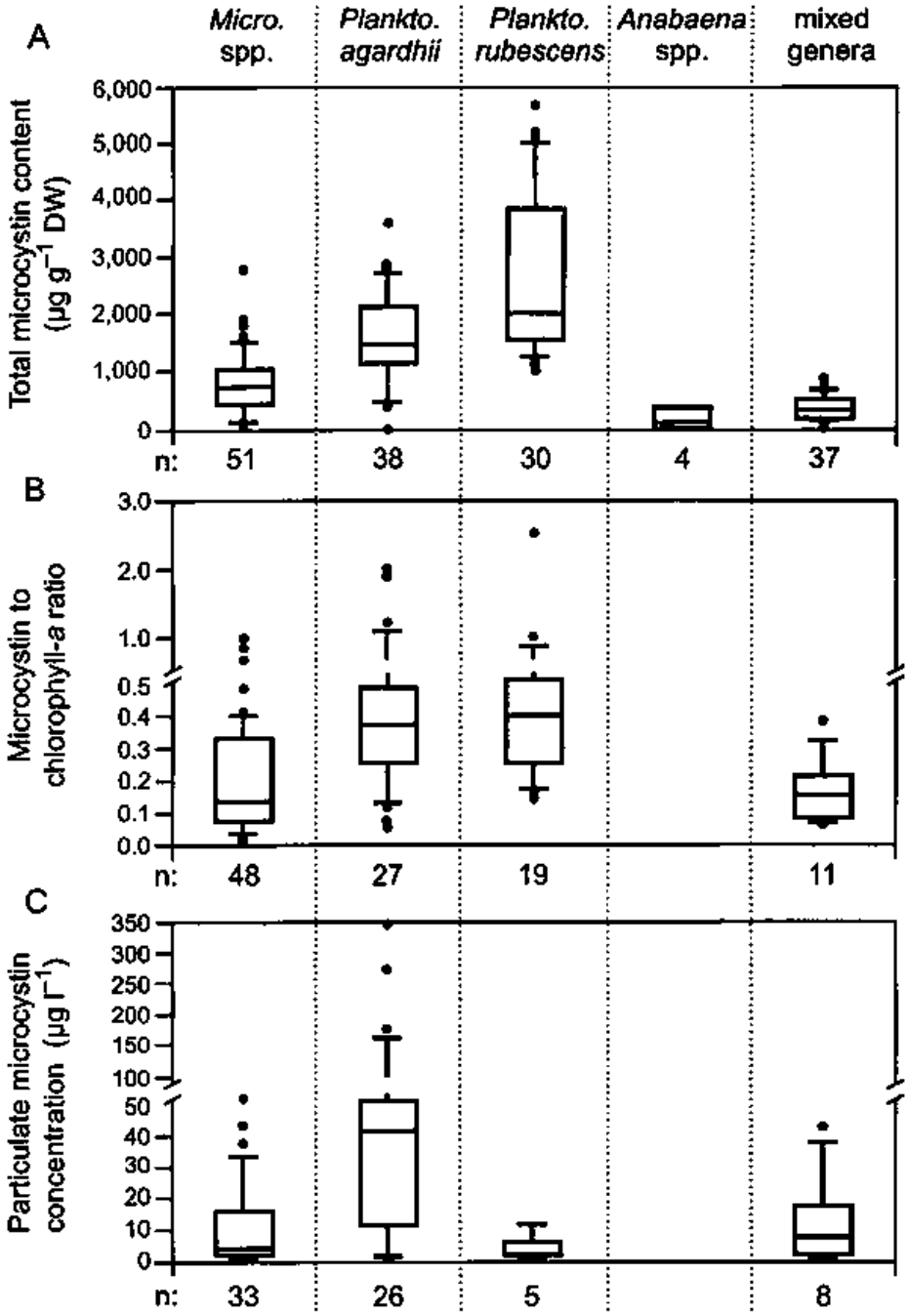


Table 3.7 Dissolved (extracellular) toxin concentrations measured in water samples

Location	Period of study	No. of toxic samples (total no. of samples)	Toxin identified	Concentration (µg l^{-1})	Analysis method	Reference
Australia		24	Microcystins	3-1.800[1]	HPLC	Jones and Orr, 1994
China	1993-94	130(835)	Microcystins	0.05-1.6	ELISA	Ueno *et al*., 1996a
Finland	1993-94	38(38)	Microcystin-LR	0.06-0,21	HPLC and ELISA	Lahti *et al*., 1997
Germany	1993-94	11(19)	Microcystins	0.07-0.76	ELISA	Ueno *et al*., 1996b
Japan	1992-95	9(22)	Microcystin-RR, -YR, -LR	0.02-3.8	HPLC	Tsuji *et al*., 1996
Japan	1993-95	26(38)	Microcystins	trace-5.6	ELISA	Ueno *et al*., 1996b
Japan	1993-94	4(13)	Microcystins	0.08-0.8	ELISA	Nagata *et al*., 1997
Thailand	1994	7(10)	Microcystins	0.08-0.35	ELISA	Ueno *et al*., 1996b
USA, Wisconsin	1993	27(27)	Microcystins	0.07-200	ELISA	McDermott *et al.,* 1995

HPLC High pressure liquid chromatography

ELISA Enzyme linked immunosorbent assay

[1] High range concentrations following treatment of a large bloom with algicide, which released intracellular microcystins (see section 3.4)

In France, four months is not uncommon, and in Japan, Portugal, Spain, South Africa and southern Australia blooms may occur for up to six months or longer. By contrast, in dry years, in tropical or subtropical areas of China, Brazil and Australia, cyanobacterial blooms may occur almost all year round, perhaps waning only briefly during reservoir overturn.

In shallow lakes, particularly in north-western Europe, populations of *Planktothrix agardhii (Oscillatoria agardhii)* may prevail perennially for many years. In deeper, thermally stratified lakes and reservoirs with moderate nutrient pollution, *Planktothrix rubescens (Oscillatoria rubescens)* may form blooms at the interface between the warmer upper and colder deeper layers of water during summer, but maintain high, evenly distributed density throughout the entire water body during winter. Both *Planktothrix* species may contain high amounts of microcystins (see Figure 3.5). Blooms of cyanobacteria, especially *Planktothrix agardhii,* have been found in winter under ice in Scandinavian and German lakes and can thus be an all year round problem.

Although toxic cyanobacteria occur in a large number of lakes, reservoirs and rivers in the world, quantitative reports on seasonal variation of cyanobacterial species composition and toxin concentration are rare. Only a few studies on seasonal, spatial and diel (day to night) variations in lakes have been published. Carmichael and Gorham (1981) showed a high degree of spatial variation of bloom toxicity that was due mostly to variations in the relative amounts of toxic *Microcystis aeruginosa* throughout the lake, rather than to substantial variations in cell toxin content. Other measurements of toxin concentrations in lakes have revealed similar trends; samples taken at the same time from different parts of the lake may show wide divergence in cyanotoxin content (Ekman-Ekebom *et al.,* 1992; Kotak *et al.,* 1995; Vezie *et al.,* 1998). A study in Alberta, Canada, showed considerable variation in toxin concentrations among the three lakes studied, both within and between years, even though the lakes were located within the same climatic region (Kotak *et al.,* 1995).

In any year or season, individual water bodies have their own populations of cyanobacteria and algae, the dominance of which is dependent not only on the weather, but on the specific geochemical conditions of the lake. If there are no major changes in these conditions, toxic blooms are likely to recur annually in those lakes that have a history of toxic blooms (Wicks and Thiel, 1990; Ekman-Ekebom *et al.,* 1992). Certain species, including the highly toxic *Planktothrix agardhii* and *P. Rubescens,* are known to produce maximum mass occurrences deep in the water column and which may be overlooked by surface monitoring of waters. Such situations may also cause problems for water treatment (see Chapters 2 and 9) (Lindholm and Meriluoto, 1991).

Studies over prolonged periods usually show that toxin concentration per gram dry weight may vary substantially over a time scale of weeks to months, but rarely from day to day as is sometimes reported. The maximum toxin concentration per gram dry weight is usually reported in summer or autumn, when cyanobacterial biomass dominates dry matter (Wicks and Thiel, 1990; Watanabe *et al.,* 1992; Park *et al.,* 1993b; Kotak *et al.,* 1995; Maršálek *et al.,* 1995; Vezie *et al.,* 1998). However, the time of toxin concentration maximum and biomass maximum are not necessarily coincident. Thus, there can be significant variation in the amount of toxin per mass of cyanobacteria over time, independently of changes in the size of the cyanobacterial population. The explanations for this are twofold. Firstly, there may be a waxing and waning of species or strains of quite different toxin quotas (i.e. toxin content per cell). Secondly, the toxin quotas may change up to five-fold in response to changes in environmental conditions (see section 3.3). A study by Kotak *et al.* (1995) found substantially higher concentrations of microcystin in blooms during the day than at night, whereas a study from Australia found no variation in microcystin content when samples were incubated during 24 hours at different depths in a reservoir (Jones and Falconer, 1994). Both findings need to be explored further.

High regional, seasonal, spatial, temporal and diel variations of toxin concentrations indicate that predicting or modelling the occurrence of toxin concentrations requires a comprehensive understanding of population (strain) development in different types of aquatic ecosystems, as well as of the variability of their toxin quotas. Data bases for such predictive models have yet to be compiled.

3.3 Production and regulation

Laboratory studies with pure strains of cyanobacteria have found that environmental factors can induce changes in toxicity or toxin concentration (on a per unit biomass basis), but usually by a factor of no more than three or four. On a per cell basis, the changes in toxin content are probably even smaller. These environmentally-induced changes are far less than the range of more than three orders of magnitude in toxin content measured between individual strains grown in culture under identical conditions. This lends support to the assumption that much, if not most, of the variation in toxicity of "monospecific" natural blooms is the waxing and waning of strains of the same species, but with varying toxin quotas. The factors that control the growth and toxin content of individual strains are as yet unknown, but clearly the genetic regulation of cyanotoxin production is an important area for further study and understanding (see section 3.3.3).

Both toxigenic (toxin producing) and non-toxigenic strains exist within many species of cyanobacteria. When grown in the laboratory, particular strains always produce much greater amounts of toxins than others. Indeed, the difference may be as much as three orders of magnitude or more (Bolch *et al.,* 1997). Several attempts to differentiate toxin producing (toxigenic) from non-toxic strains of the same species using microscopic methods have failed. The use of molecular biological methods to characterise toxic and non-toxic planktonic isolates has been initiated recently and will, in future, help to clarify the taxonomic status of these organisms, as well as the ecology of individual strain types (see section 3.3.3).

3.3.1 Regulation by chemical and physical factors

The production of toxin by a single cyanobacterial strain seems to be consistent and the spontaneous and permanent loss of toxin production has been seldom reported. The effects of several environmental factors on growth and toxin production by cyanobacteria have been studied in batch and continuous culture experiments. Culture age in batch cultures, and temperature, are the parameters most frequently examined, followed by light, nutrients, salinity, pH and micronutrient concentrations. Studies have been done with hepatotoxic *Microcystis, Oscillatoria (Planktothrix), Anabaena* and *Nodularia;* anatoxin-a producing *Anabaena, Aphanizomenon* and *Planktothrix;* and saxitoxin producing *Aphanizomenon* and *Anabaena circinalis* (Table 3.8). Microcystins and anatoxin-a are largely retained within cells when the conditions for the growth of the organism are favourable. The amount of microcystin in a culture increases during the logarithmic growth phase, being highest in the late logarithmic phase. The maximum anatoxin-a concentration is found during the logarithmic growth phase (Sivonen, 1996; Watanabe, 1996). While the variants of microcystins produced by a particular strain are rather constant, the ratios of individual microcystins may change with time, or under conditions of different temperatures and light. Environmental factors affect toxin content of cyanobacteria, but only within a range of less than an order of magnitude. The majority of studies indicate that cyanobacteria produce most toxins under conditions which are most favourable for their growth. For example, different cyanobacterial species have different light requirements: *Planktothrix* prefers low light intensities for growth, *Anabaena* moderate and *Aphanizomenon* high light intensities. All strains produce most toxin when grown under their optimum light conditions (Table 3.8). Two- to

threefold differences in toxin content (on a per unit biomass basis) have been reported in relation to light conditions.

Strains and species also differ slightly in their optimum growth temperatures. The toxin content in most studies was highest at temperatures between 18 °C and 25 °C, whereas low (10 °C) or very high temperatures (30 °C) decreased toxin content. Temperature gradients caused two- to threefold differences in toxin content.

In a study using mouse bioassay to detect effects of pH on toxin production, cells were found to be more toxic when grown at high and low pH (Van der Westhuizen and Eloff, 1983).

Table 3.8 Laboratory studies on cellular toxin concentrations in cyanobacteria

Parameter	Organism	Toxin(s)/ analysis method	Changes in toxin concentrations (dw)	Highest/lowest toxin production	Reference(s)
Temperature (°C)					
12.5-30	*Anabaena* spp. (2 strains), batch cultures	Microcystins HPLC	3.5-30 fold[1]	Highest at 25, lowest at 30; different toxins at different temperatures	Rapala *et al.,* 1997
10-28	*Anabaena* spp. (2 strains), continuous cultures	Microcystins HPLC	3-10 fold	Lowest at 10, highest at 25	Rapala and Sivonen, 1998
15-30	*Anabaena* spp. (2 strains), batch cultures	Anatoxin-a HPLC	3 fold	Lowest at 30, highest at 20	Rapala *et al.,* 1993
10-28	*Anabaena* spp. (2 strains), continuous cultures	Anatoxin-a HPLC	4-7 fold	Highest at 19-21, lowest at 10 and 28	Rapala and Sivonen, 1998
15-30	*Aphanizomenon* sp. (1 strain), batch cultures	Anatoxin-a HPLC	3 fold	Lowest at 30, highest at 20	Rapala *et al.,* 1993
10,25,34	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins mouse bioassay	5 fold	Highest toxicity at 25, lowest at 10	Codd and Poon, 1988
15-35	*Microcystis aeruginosa* (1 strain) batch cultures	Microcystins mouse bioassay HPLC	4 fold	Highest toxicity at 20; different toxins at different temperatures	van der Westhuizen and Eloff, 1985; van der Westhuizen *et al.,* 1986
18,25,35	*Microcystis aeruginosa* (1	Microcystins mouse	1.4 fold	Highest toxicity at 18, lowest at	Watanabe and Oishi,

	strain), batch cultures	bioassay		32	1985
10-30	*Nodularia spumigena* (2 strains), batch cultures	Nodularin HPLC	3-4 fold	Highest at 20, lowest at 10 or 30	Lehtimäki *et al.,* 1994
7-28	*Nodularia spumigena* (1 strain), batch cultures	Nodularin HPLC	3 fold	Highest at 19	Lehtimäki *et al.,* 1997
15-30	*Oscillatoria agardhii* (2 strains), batch cultures	Microcystins HPLC	7 fold	Strain dependent; lowest at 30	Sivonen, 1990b
Light (µmol m^{-2} s^{-1})					
2-100 continuous	*Anabaena* spp. (2 strains), batch cultures	Microcystins HPLC	3 fold	Highest at 25	Rapala *et al*., 1997
7, 19, 42 continuous	*Anabaena* spp. (2 strains), continuous cultures	Microcystins HPLC	2.5-15	Lowest at 10, highest at 25	Rapala and Sivonen, 1998
2-128 continuous	*Anabaena* spp. (2 strains), batch cultures	Anatoxin-a HPLC	3 fold	Highest at 26-44, lowest at 2	Rapala *et al.,* 1993
7, 19, 42 continuous	*Anabaena* spp. (2 strains), continuous cultures	Anatoxin-a HPLC	No effect	Highest at 19, lowest at 7	Rapala and Sivonen, 1998
2-128 continuous	*Aphanizomenon* sp. (1 strain), batch cultures	Anatoxin-a HPLC	4 fold	Highest at 128, lowest at 2	Rapala *et al.,* 1993
5-50 continuous	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins mouse bioassay	2.4 fold	Highest toxicity at 20	Codd and Poon, 1988
20-75 continuous	*Microcystis aeruginosa* (1 strain), continuous cultures	Microcystins HPLC	2.5 fold	Highest at 40	Utkilen and Gjølme, 1992
21-205 continuous	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins mouse bioassay	1.2 fold	Highest toxicity at 142, lowest at 21	van der Westhuizen and Eloff, 1985
7.5, 30, 75 continuous	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins mouse bioassay	3.8 fold	Highest toxicity at 30, lowest at 7.5	Watanabe and Oishi, 1985
25, 50, 80	*Nodularia*	Nodularin	No difference		Lehtimäki *et*

continuous	*spumigena* (2 strains), batch cultures	HPLC			*al*., 1994
2-155 continuous	*Nodularia spumigena* (1 strain), batch cultures	Nodularin HPLC	50 fold[1]	Higher at high irradiances, minimal at 2	Lehtimäki *et al*., 1997
12-95 continuous	*Oscillatoria agardhii* (2 strains), batch cultures	Microcystins HPLC	2.5 fold	Highest at 12-44	Sivonen, 1990b
Phosphorus (mg P l^{-1})					
0.05-5.5	*Anabaena* spp. (2 strains), batch cultures	Microcystins HPLC	5 fold	Highest at 5.5, lowest at 0.05	Rapala *et al*., 1997
0.05-5.5	*Anabaena* spp. (2 strains), batch cultures	Anatoxin-a HPLC	No difference	No statistically significant differences	Rapala *et al*., 1993
0.05-5.5	*Aphanizomenon* sp. (2 strains), batch cultures	Anatoxin-a HPLC	2 fold	Lowest at 0.05-0.1, highest at 0.5-5.5	Rapala *et al*., 1993
BG-11 and medium without P	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins Mouse bioassay	1.7 fold	Higher toxicity without P	Codd and Poon, 1988
0.0025, 0.025	*Microcystis aeruginosa* (1 strain), continuous cultures	Microcystins HPLC	2.3 fold	More toxin at 0.025	Utkilen and Gjølme, 1995
MA medium 1/1; dilutions 1/10, 1/20	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins Mouse bioassay	Less than 1	Highest toxicity with the original medium	Watanabe and Oishi, 1985
0.1-5.5	*Oscillatoria agardhii* (2 strains), batch cultures	Microcystins HPLC	1.8-2.5 fold	Lowest toxin at 0.1	Sivonen, 1990b
0.3, 0.6, 1.0	*Nodularia spumigena* (2 strains), batch cultures	Nodularin HPLC	Less than 1 fold	Lowest at 0.3	Lehtimäki et al., 1994
0-5.5	*Nodularia spumigena* (1 strain), batch cultures	Nodularin HPLC	4 fold	Lowest at 0-0.02, highest at 0.2-5.5	Lehtimäki *et al*., 1997
Nitrogen (mg N l^{-1})					
BG-11 medium, medium	*Microcystis aeruginosa* (1 strain), batch	Microcystins mouse bioassay	5 fold	Higher toxicity with the medium containing N	Codd and Poon, 1988

without N	cultures				
0.05-1	*Microcystis aeruginosa* (1 strain), continuous cultures	Microcystins HPLC	3 fold	Higher at high N	Utkilen and Gjølme, 1995
MA medium 1/1; dilutions 1/10, 1/20	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins mouse bioassay	2.5 fold	Highest toxicity with the original medium	Watanabe and Oishi, 1985
0.42-84	*Oscillatoria agardhii* (2 strains), batch cultures	Microcystins HPLC	5 fold	Higher at high N, lowest at low N	Sivonen, 1990b
Micronutrients					
Al, Cd, Cr, Cu, Fe, Mn, Ni, Sn, Zn; various concentrations	*Microcystis aeruginosa* (1 strain), batch cultures	Microcystins HPLC	1.7 fold	Less toxins at low Fe concentrations	Lukac and Aegerter, 1993
0.1-3.4 µg Fe l^{-1}	*Microcystis aeruginosa* (1 strain), continuous cultures	Microcystins HPLC	1.5 fold	More toxin at high Fe concentrations	Utkilen and Gjølme, 1995
0.03-1.2 µg Fe l^{-1}	*Microcystis aeruginosa* (1 strain), continuous cultures	Microcystins HPLC	0-3 fold	Less toxin at low Fe concentrations	Lyck *et al*., 1966
Salinity (‰)					
3, 5, 8, 11	*Nodularia spumigena (2* strains), batch cultures	Nodularin HPLC	No difference	No statistical difference	Lehtimäki *et al.,* 1994
0-30	*Nodularia spumigena* (1 strain), batch cultures	Nodularin HPLC	8 fold	Highest at 15, lowest at 0 and 30	Lehtimäki *et al.,* 1997
0-35	*Nodularia spumigena* (6 strains), batch cultures	Nodularin HPLC	5 fold	Highest at 12, lowest at 35	Blackburn *et al*., 1996
CO_2					
BG-11 medium, medium without CO_2	*Microcystis aeruginosa* (1 strain), batch culture	Microcystins mouse bioassay	6 fold	Higher toxicity with the medium containing CO_2	Codd and Poon, 1988
pH					
1-14	*Microcystis*	Microcystins	1.8 fold	Toxicity highest	van der

	aeruginosa (1 strain), batch cultures	mouse bioassay		at low and high pH	Westhuizen and Eloff, 1983

dw Dry weight

HPLC High performance liquid chromatography

[1] When the growth of the strains was poor the amount of toxins was also very low (less than 0.1 mg g^{-1} dw of cells); when these cases were compared to maximal toxin production more than ten-fold differences could be seen

In high concentrations of phosphorus, hepatotoxic strains produced more toxins, but for anatoxin-a production phosphorus had no effect (Table 3.8). The differences induced by low and high phosphorus concentrations vary between two- and fourfold. Similarly, in field studies, a positive correlation of total phosphorus with microcystin-LR concentration in cells of *Microcystis aeruginosa* (Kotak *et al.,* 1995) or in bloom material of *Microcystis* spp. (Lahti *et al.,* 1997b) has been found. Non-nitrogen fixing species, like *Microcystis* and *Oscillatoria,* produce more toxins under nitrogen-rich conditions. Nitrogen fixing species are not dependent on the nitrogen in the media for their toxin production (Rapala *et al*., 1993; Lehtimäki *et al*., 1997). In continuous cultures when the toxins were expressed in relation to cell protein rather than to dry weight, Utkilen and Gjølme (1995) found that nitrogen and phosphorus limited conditions had no effect on the toxin content of *Microcystis aeruginosa.*

Indications regarding the role of iron are contradictory (Lukac and Aegerther, 1993; Utkilen and Gjølme, 1995; Lyck *et al.,* 1996). While studying the effect of trace metals on growth and on toxin content of *Microcystis aeruginosa,* Lukac and Aegerter (1993) found that in batch cultures only zinc was required for both optimal growth and toxin production.

Orr and Jones (1998) have unified many of these rather disparate studies on the effect of growth conditions on cyanotoxin production. They showed that the rate of microcystin production by a cyanobacterial population in culture is directly proportional to its growth rate, no matter what environmental factor was limiting growth. Moreover, they showed that the amount of microcystin contained by a single cell of *Microcystis aeruginosa* (i.e. on a per cell or cell quota basis) is constant within a narrow range (two- to threefold).

3.3.2 Biosynthesis

To understand how cyanotoxins are produced, it is necessary to study the biochemical and genetic basis of toxin production. Knowledge of the biosynthetic pathways of cyanotoxins is in its early stage and no complete biochemical pathways are known. Biosynthesis of several cyanotoxins has been studied by feeding labelled precursors to a cyanobacterial culture and following their incorporation into the carbon skeleton of the toxins. Shimizu *et al.* (1984) used an *Aphanizomenon flos-aquae* strain to study biosynthesis of saxitoxin analogues. They proposed a new pathway for neosaxitoxin biosynthesis, the key steps of which are the condensation of an acetate unit, or its derivative, to the amino group bearing an α-carbon of arginine or an equivalent, and a subsequent loss of the carboxyl carbon and imidazole ring formation on the adjacent

carbonyl carbon. They established the origin of all the carbons in the toxin alkaloid ring system. The side-chain carbon was derived from methionine (Shimizu, 1986).

Anatoxin-a is related structurally to the tropane class of alkaloids found in higher plants. Based mainly on ^{14}C-labelled precursors and enzymatic studies, Gallon *et al.* (1990) and Gallon *et al.* (1994) suggested the biosynthesis of anatoxin-a to be analogous to that of tropanes. Anatoxin-a was proposed to be formed from ornithine/arginine via putrescine, which is oxidised to pyrroline, a precursor of anatoxin-a. Labelling experiments using ^{13}C NMR (nuclear magnetic resonance spectrometry) indicated that the carbon skeleton of anatoxin-a is derived from acetate and glutamate. The studies showed that C-1 of glutamic acid is retained during the transformation of anatoxin-a and not lost by decarboxylation, a finding incompatible with the tropane alkaloid theory (Hemscheidt *et al.,* 1995b). All of the carbons of anatoxin-a(S) are derived from amino acids. Three methyl carbons arise from L-methionine or other donors to the tetrahydrofolate C1 pool. L-arginine accounts for C-2, C-4, C-5 and C-6 carbons of the toxin (Moore *et al.,* 1992, 1993). The intermediate in the biosynthesis of anatoxin-a(S) from L arginine is (2*S*,4*S*)-4 hydroxyarginine (Hemscheidt *et al.,* 1995a). The structure of the cylindrospermopsin suggests a polyketide origin for the toxin (Moore *et al.,* 1993).

The origin of carbons in microcystin (Moore *et al.,* 1991) and in nodularin (Choi *et al.,* 1993; Rinehart *et al.,* 1994) have been studied by following the incorporation of labelled precursors into the toxins by NMR. Carbons C1-C8 of Adda in nodularin are acetate derived and the remaining carbons presumably originate from phenylalanine. Methyl groups in carbons 2, 4, 6, 8, and the O-methyl group in the Adda unit, originated from methionine. The D-Glu and L-Arg carbons C4-C5 were acetate derived, with C1-C2 being from glutamate. Methyldehydrobutyrine was possibly formed from threonine, its methyl group coming from methionine. The β-methylaspartic acid was found to originate from condensation of pyruvic acid (C3-C4) and acetyl-CoA (C1-C2) (Rinehart *et al.,* 1994). The studies on the carbon skeleton of nodularin, with some minor differences, agree with work on microcystin-LR by Moore *et al.* (1991). In their study, L-Leu and D-Ala units in microcystin had acetate incorporation. The dehydroamino acid in microcystin has been proposed to be formed from serine rather than from threonine (Rinehart *et al.,* 1994). Rinehart's group found linear nodularin, which was shown by culture experiments to be a precursor of cyclic nodularin. Three additional linear peptides were isolated from a bloom sample, one of them was possibly a precursor of cyclic microcystin-LR and the others possibly degradation products (Rinehart *et al.,* 1994).

3.3.3 Genetic regulation of cyanotoxin production

The genes and enzymes involved in cyanotoxin production are still mostly unknown. The first molecular biological studies on toxic cyanobacteria investigated the possible involvement of plasmids in toxin production. Four toxic strains of *Microcystis aeruginosa* contained plasmids, and no plasmid could be shown in one toxic and in several non-toxic strains (Schwabe *et al.,* 1988). More recently, a similar study in Australia found no evidence for plasmid involvement in microcystin synthesis (Bolch *et al.,* 1997). Gallon *et al.* (1994) studied an anatoxin-a producing *Anabaena* strain NCR 44-1, which spontaneously became non-toxic. They found that the size of a plasmid was reduced in that non-toxic clone, but this work has not been repeated or confirmed.

Multi-enzyme complexes and peptide synthetase genes are involved in hepatotoxin production. Several cyclic and linear peptides, often with D-amino acids, are known to be produced, non-ribosomally, by multi-domain peptide synthetases via the so-called thiotemplate mechanism in bacteria and lower eukaryotes. The best characterised are the synthesis of gramicidin S and tyrocidin by *Bacillus.* Peptide synthetase genes have been detected and sequenced (partly) in *Microcystis aeruginosa* (Meissner *et al.,* 1996) and in *Anabaena* (Rouhiainen *et al.,* 1994). Analogous polymerase chain reaction (PCR) products to the peptide synthetase genes have been shown by using DNA from *Microcystis* (Jacobs *et al.,* 1995; Arment and Carmichael, 1996) and *Nodularia* as a template. Dittman *et al.* (1997) showed, in knockout experiments, that peptide synthetase genes are responsible for microcystin production.

At least some strains which produce hepatotoxins also produce other small cyclic peptides (Namikoshi and Rinehart, 1996; Weckesser *et al.,* 1996) which are likely to be produced by nonribosomal peptide synthesis.

3.4 Fate in the environment

3.4.1 Partitioning between cells and water

It appears likely that cyanotoxins are produced and contained within the actively growing cyanobacterial cells (i.e. they are intracellular or particulate). Release to the surrounding water, to form dissolved toxin, appears to occur mostly, if not exclusively, during cell senescence, death and lysis, rather than by continuous excretion.

In laboratory studies, where both intracellular and dissolved toxins (microcystins/nodularin and saxitoxins) have been measured, it is generally the case that in healthy log phase cultures, less than 10-20 per cent of the total toxin pool is extracellular (Sivonen, 1990b; Lehtimäki *et al.,* 1997; Negri *et al.,* 1997; Rapala *et al.,* 1997). As cells enter stationary phase the increased rate of cell death may lead to an increase in the extracellular dissolved fraction. Even during log-phase cell growth in culture, a small percentage of cells in the population may be dying and lysing (and releasing intracellular toxins), even though there is an overall positive population growth. There are some indications that anatoxin-a may leak out of cells during growth especially in low light conditions. High concentrations of anatoxin-a, sometimes exceeding the intracellular pool of toxins, have been found in media in a batch culture study (Bumke-Vogt *et al.,* 1996).

In the field, healthy bloom populations produce little extracellular toxin. The range of measured concentrations for dissolved cyanotoxins, in all cases except those where a major bloom is obviously breaking down, is 0.1-10 µg l^{-1} (Lindholm and Meriluoto, 1991; Jones and Orr, 1994; Tsuji *et al.,* 1996; Ueno *et al.,* 1996b; Lahti *et al.,* 1997b) Cell-bound concentrations are several orders of magnitude higher (see Tables 3.6 and 3.7). In lakes or rivers, toxins liberated from cells are rapidly diluted by the large mass of water, especially if mixing of water by wind action or currents is vigorous (Jones and Orr, 1994). However, the concentration of dissolved toxins may be much higher in ageing or declining blooms. This is an important consideration for water treatment plant operators,

because it means that removal of healthy cyanobacterial cells intact from the raw water supply may obviate or substantially reduce the need for additional adsorptive (activated carbon) or oxidative (ozone or chlorine) toxin removal processes (see Chapter 9).

The release of toxins from cells is enhanced by chemical treatments for the eradication of cyanobacteria, especially the use of algicides (either copper-based or organic herbicides). Treatment of a bloom with copper sulphate, for example, may lead to complete lysis of the bloom population within three days and release of all the toxins into the surrounding water (Berg *et al.,* 1987; Kenefick *et al.,* 1992; Jones and Orr, 1994). The efficacy of copper sulphate treatment is, however, very much dependent on water chemistry, especially alkalinity, pH and dissolved organic content (see sections 8.5 and 9.2).

3.4.2 Chemical breakdown

The four main groups of cyanotoxins: microcystins, anatoxins, PSPs and cylindrospermopsins, exhibit quite different chemical stabilities and biological activities in water.

Microcystins

Microcystins, being cyclic peptides, are extremely stable and resistant to chemical hydrolysis or oxidation at near neutral pH. Microcystins and nodularin remain potent even after boiling. In natural waters and in the dark, microcystins may persist for months or years. At high temperatures (40 °C) and at elevated or low pH, slow hydrolysis has been observed, with the times to achieve greater than 90 per cent breakdown being approximately 10 weeks at pH 1 and greater than 12 weeks at pH 9 (Harada *et al.,* 1996). Rapid chemical hydrolysis occurs only under conditions that are unlikely to be attained outside the laboratory, e.g. 6M HCl at high temperature.

Microcystins can be oxidised by ozone and other strong oxidising agents, and degraded by intense ultra violet (UV) light. These processes have relevance for water treatment and are discussed in Chapter 9, although they are unlikely to contribute to degradation occurring in the natural environment.

In full sunlight, microcystins undergo slow photochemical breakdown and isomerisation, with the reaction rate being enhanced by the presence of water-soluble cell pigments, presumably phycobiliproteins (Tsuji *et al.,* 1993). In the presence of pigments the photochemical breakdown of microcystin in full sunlight can take as little as two weeks for greater than 90 per cent breakdown, or longer than six weeks, depending on the concentration of pigment (and presumably toxin, although this has not been tested). A more rapid breakdown under sunlight has been reported in the presence of humic substances (which can act as photosensitisers) in field concentrations ranging from 2-16 mg l^{-1} dissolved organic carbon (DOC). Approximately 40 per cent of the microcystins was degraded per day under summer conditions of insolation (Welker and Steinberg, 1998). In deeper or muddy waters, the rate of breakdown is likely to be considerably slower.

Anatoxins

Anatoxin-a is relatively stable in the dark, but in pure solution in the absence of pigments it undergoes rapid photochemical degradation in sunlight. Breakdown is further accelerated by alkaline conditions (Stevens and Krieger, 1991). The half-life for photochemical breakdown is 1-2 hours. Under normal day and night light conditions at pH 8 or pH 10, and at low initial concentrations (10 µg l^{-1}), the half-life for anatoxin-a breakdown was found to be approximately 14 days (Smith and Sutton, 1993). Anatoxin-a(S) decomposes rapidly in basic solutions but is relatively stable under neutral and acidic conditions (Matsunaga *et al.,* 1989).

Saxitoxins

In the dark at room temperature, saxitoxins undergo a series of slow chemical hydrolysis reactions. The C-toxins lose the N-sulphocarbamoyl group to form decarbamoyl gonyautoxins (dc-GTXs); while the dc-GTXs, GTXs and STXs slowly degrade to, as yet unidentified, non-toxic products. The half-lives for the breakdown reactions are in the order of 1-10 weeks, with more than three months often being required for greater than 90 per cent breakdown (Jones and Negri, 1997). Because dc-GTXs are much more toxic than C-toxins (by a factor of 10-100), a solution or water body containing a natural mixture of C-toxins and GTXs, for example from the lysis of an Australian bloom of *Anabaena circinalis,* will actually increase in toxicity over a period of up to three weeks, before toxicity begins to abate during the succeeding 2-3 months. Boiling an extract of *Anabaena* with predominant C-toxins may also substantially increase toxicity. Similar transformation reactions occur in living cells as they age in culture or in a natural bloom (Negri *et al.,* 1997). No detailed studies have been carried out on saxitoxin breakdown in sunlight, either with or without pigments.

Cylindrospermopsins

Cylindrospermopsin is relatively stable in the dark, with slow breakdown occurring at elevated temperature (50 °C) (Chiswell *et al.,* 1999). In sunlight and in the presence of cell pigments, breakdown occurs quite rapidly being more than 90 per cent complete within 2-3 days (Chiswell *et al.,* 1999). Pure cylindrospermopsin is relatively stable in sunlight.

3.4.3 Removal on natural sediments and soils

Microcystins appear to be retained only weakly on natural suspended solids in rivers and reservoirs; usually no more than 20 per cent of the total microcystin concentration is adsorbed. In a laboratory experiment, some of the dissolved anatoxin-a and microcystins were reported by Rapala *et al.* (1993) to be adsorbed on lake sediments. Percolation through clay soils may provide some cyanotoxin removal, but this will depend greatly on the type of clay, surface charge, cation concentration of the water, etc. Cyanobacterial cells and microcystins were retained in soil columns, but less efficiently in sediment columns, in laboratory experiments simulating the fate of cyanobacterial toxins in artificial recharge of groundwater and bank filtration (Lahti *el al.,* 1996). No data are available for other cyanobacterial toxins, but some removal may be expected, again depending on the chemical conditions of soil and water.

Sedimentation of living cells without lysis, for example through grazing by zooplankton and sinking of faecal pellets, may lead to accumulation and persistence of toxin material in sediments, although this process has received little scientific attention. As discussed in more detail below, microcystins retained in intact cells may persist for several months. Cells deposited in sediments may be subject to fairly rapid breakdown by sediment bacteria and protozoa, with the resultant release of toxins.

3.4.4 Biodegradation

Microcystins

In spite of their chemical stability and resistance to eucaryotic and many bacterial peptidases, microcystins are susceptible to breakdown by aquatic bacteria found naturally in rivers and reservoirs. These bacteria appear to be reasonably common and widespread. Degradative bacteria have been found in sewage effluent (Lam *et al.,* 1995), lake water (Jones *et al.,* 1994; Cousins *et al.,* 1996; Lahti *et al.,* 1997a), lake sediment (Rapala *et al.,* 1994; Lahti *et al.,* 1997a) and river water (Jones *et al.,* 1994). Nonetheless, one Finnish study showed a complete lack of degradation of microcystin over a three-month period by an inoculum taken in winter from the Vantaanjoki River (Kiviranta *et al.,* 1991). There is usually an initial lag phase with little loss of microcystin and this period can be as short as two days or more than three weeks, depending on the water body, climatic conditions, the concentration of dissolved microcystin and in some cases, although not all, the previous bloom history of a lake (Jones *et al.,* 1994; Rapala *et al.,* 1994; Lahti *et al.,* 1997b). Once the biodegradation process commences, removal of microcystin can be more than 90 per cent complete within 2-10 days. This may vary depending on the water body, initial microcystin concentration and water temperature (Jones *et al.,* 1994; Lahti *et al.,* 1997b).

Jones *et al.* (1994) isolated a species of aquatic *Sphingomonas* that initiated ring-opening of microcystin-LR to produce linear (acyclo-)microcystin-LR as a transient intermediate (Bourne *et al.,* 1996). This compound was nearly 200 times less toxic than the parent toxin. The products of complete bacterial degradation were non-toxic to mice at doses up to 500 µg kg^{-1} (compared with an LD_{50} for microcystin-LR of about 60 µg kg^{-1}). The same bacterium, however, did not degrade the closely related cyclic pentapeptide nodularin. In a strain of *Pseudomonas aeruginosa* from a Japanese lake, microcystin degradation appeared to proceed by attack on the Adda side chain of microcystin (Takenaka and Watanabe, 1997). Several bacteria were isolated from lake water and sediment in Finland capable of degradation of microcystins and some strains also degraded nodularin. One strain was identified as a *Sphingomonas* sp. and two of the strains belonged to the beta-subgroup of *Proteobacteria,* although the genera remains to be determined (Lahti *et al.,* 1997a).

Other cyanobacterial toxins

Little work has been undertaken on the biodegradation of anatoxins, saxitoxins or cylindrospermopsin. Anatoxin-a may be readily degraded by bacteria associated with cyanobacterial filaments. Laboratory studies using non-axenic strains of cyanobacteria found low concentrations of dissolved anatoxin-a in the culture medium (Kiviranta *et al.,* 1991; Rapala *et al.,* 1993) whereas high concentrations of anatoxin-a were found in the medium of a continuous culture using an axenic strain (free of contaminating bacteria) of

the same species (Rapala and Sivonen, 1998). A *Pseudomonas* sp. strain able to degrade anatoxin-a at a rate of 6-10 µg ml^{-1} per three days was isolated by Kiviranta *et al.* (1991). In the presence of lake sediment and natural bacteria, the half-life for breakdown of anatoxin-a in the laboratory was about five days (Smith and Sutton, 1993). In a recent study by Jones and Negri (1997) no bacterially-mediated degradation of saxitoxins from *Anabaena circinalis* was observed in a range of surface water samples.

3.4.5 Bioaccumulation

Microcystins bioaccumulate in common aquatic vertebrates and invertebrates, including fish (Carbis *et al.,* 1997; Beattie *et al.,* 1998), mussels (Eriksson *et al.,* 1989; Falconer *et al.,* 1992; Prepas *et al.,* 1997; Watanabe *et al.,* 1997) and zooplankton (Watanabe *et al.,* 1992). In mussels, the highest microcystin concentrations are found in the hepatopancreas, and in vertebrates they are found in the liver. Williams *et al.,* (1997) have shown covalent binding and accumulation of microcystin-LR in salmon liver and crab larvae. Whether the levels of microcystin accumulation are sufficient to pose a risk to humans is uncertain, and will depend on levels of consumption and the severity of toxic blooms in the area where fish or shellfish are caught or collected. Common advice given by water authorities is that the viscera of the fish should not be eaten, but caution should be taken in all cases where major toxic blooms occur.

Saxitoxins from marine "red tide" dinoflagellates are well known for their propensity to bioaccumulate in marine vertebrates and invertebrates, often with disastrous consequences for animals and humans that consume them. Similarly, saxitoxins from the freshwater cyanobacterium *Anabaena circinalis* may bioaccumulate in an Australian species of freshwater mussel to concentrations exceeding international guidelines (Shumway *et al.,* 1995) during as little as seven days exposure to a cell density of 100,000 cells per ml of a toxigenic strain (Negri and Jones, 1995). This cell density is commonly encountered in natural blooms of this species.

3.4.6 Persistence and stability in cells

Culture studies indicate that microcystins and nodularin degrade only very slowly (time scale of weeks), if at all, whilst contained within living cells (Sivonen, 1990b; Lehtimäki *et al.,* 1994, 1997; Rapala *et al.,* 1997; Orr and Jones, 1998). Similarly, scums of *Microcystis aeruginosa* that dry on the shores of lakes may contain high concentrations of microcystin for several months (Jones *et al.,* 1995). These toxins are released back into the water body when re-immersed. Thus there is the potential for significant localised concentrations of dissolved microcystin even in the absence of living cells or a recently collapsed bloom.

In a lake study carried out over two summer - autumn periods, Lahti *et al.* (1997b) found that dissolved microcystin was more persistent than particulate toxin, with 30 and 15 days respectively required for 90 per cent degradation to occur.

3.5 Impact on aquatic biota

Direct cyanobacterial poisoning of animals can occur by two routes: through consumption of cyanobacterial cells from the water, or indirectly through consumption of other animals that have themselves fed on cyanobacteria and accumulated cyanotoxins. As was outlined in section 3.4.5, cyanotoxins are known to bioaccumulate in common aquatic vertebrates and invertebrates, including fish, mussels and zooplankton. Consequently, there is considerable potential for toxic effects to be magnified in aquatic food chains. Such toxicity biomagnification is well known for anthropogenic pollutants such as heavy metals and pesticides. There is no reason to suspect that the situation would be any different with natural cyanotoxins.

It is difficult to ascribe the deaths of natural populations of aquatic animals, especially fish, unequivocally to cyanotoxin poisoning. One of the main reasons for this is because the collapse of a large cyanobacterial bloom can lead to very low concentrations of oxygen in the water column as a consequence of bacterial metabolism; consequent fish deaths may be due to the anoxia. The best evidence for the potential for toxic effects on aquatic organisms comes from controlled laboratory trials with exposure of animals to toxic cyanobacteria or cell-free solutions of cyanotoxins.

3.5.1 Effects on aquatic bacteria

The influence of cyanobacterial toxins on bacteria is not fully understood and the scientific literature gives a number of contradictory statements. According to some authors neither an extract of *Microcystis aeruginosa* nor pure microcystin-LR have a biocidal effect on *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* or *Pseudomonas hydrophila* (Foxall and Sasner, 1988). However, these limited tests should not be seen as general indicators of the potential impacts of cyanotoxins on aquatic bacteria. The majority of aquatic bacteria are yet to be cultured, and studies with common mammalian pathogens or "laboratory" bacteria should not be taken as all encompassing. It is quite possible that cyanotoxins impact on some species of aquatic bacteria and not others. Certainly, microcystins are not toxic to all bacteria because several species are known to degrade quite high concentrations of these toxins (see section 3.4.4). It is even possible that the slow release of cyanotoxins from the cell surface or from senescent cells may stimulate associations of particular bacterial types (see section 3.4.4) which may even act as symbionts.

Attempts have been made to use bacterial toxicity tests (based on inhibition of bacterial phosphorescence) to screen for the presence of cyanotoxins, especially microcystins. However it appears that the inhibition of bacterial phosphorescence is not related to the commonly known cyanotoxins. It has been suggested that the negative effect may be related to the presence of unidentified LPS endotoxins in the cell wall of the cyanobacterial cells (see sections 3.1 and 13.3).

3.5.2 Effects on zooplankton

Evidence of the potential effects of cyanotoxins on zooplankton from numerous studies, mostly in laboratory situations, is complex and inconsistent. The vast majority of published studies has been based on mouse bioassay data describing cyanobacterial toxicity, with only a few more recent studies having used analytical methods such as HPLC (see section 13.4) to quantify individual toxins. Overall, it appears that cyanobacteria may exhibit a deleterious effect on zooplankton, but the effect is highly variable between genera and species, and even between clones of individual zooplankton species. One of the main questions yet to be resolved is whether the observed inhibitory effects are due to the putative poor nutritional value of cyanobacteria, to the known cyanotoxins, or to other unidentified compounds. There is evidence in the literature to support all three effects as being significant, at least with particular species under experimental growth conditions. A major difference in study design is whether organisms are exposed to cyanotoxins dissolved in water, or fed with toxic cyanobacteria. The latter is likely to lead to a substantially higher dose. Furthermore, Jungmann and Benndorf (1994) reported that exposure of *Daphnia* to dissolved microcystins showed effects only at concentrations several orders of magnitude above those found in field samples. They did, however, observe toxicity to *Daphnia* by unidentified metabolites other than microcystins from *Microcystis.*

There is dramatic variation among zooplankton species in their response to toxic (and even non-toxic) cyanobacteria. For example, DeMott *et al.* (1991) showed that the four species of zooplankton differed in their sensitivity to hepatotoxins by almost two orders of magnitude, but toxicity was observed only at very high concentrations that are scarcely encountered in natural water bodies (48 h LC_{50} ranging from 450 to 21,400 µg of microcystin per litre). Snell (1980) found that there was a genotype-dependent response of the rotifer *Asplanchna girodi* to toxic *Anabaena flos-aquae* and *Lyngbya* sp. Hietala *et al.* (1997) observed a variation in susceptibility of more than three orders of magnitude in the acute toxicity of *Microcystis aeruginosa* to 10 clones of *Daphnia pulex.* Both DeMott *et al.* (1991) and Laurén-Määttä *et al.* (1997) suggested that clone and species differences between zooplankton susceptibilities to toxic cyanobacteria may lead to selection pressures in favour of resistant strains or species in water bodies where toxic cyanobacteria occur frequently.

Benndorf and Henning (1989) found that the toxicity of a field population of *Microcystis* was increased by the feeding activity of *Daphnia galeata* over a period of a few months. A possible explanation for this phenomenon is offered by DeMott *et al.* (1991) who demonstrated that a number of zooplankton species will avoid grazing on toxic cyanobacteria, but continue to graze on non-toxic species. Similar results have also been shown for grazing by the phytoplanktivorous fish *Tilapia* and silver carp. Thus, grazing pressure from zooplankton and some fish may lead to the selective enrichment of toxic cyanobacterial strains over time.

It is likely that under natural conditions in water bodies, certain species and strains of zooplankton may be affected by cyanotoxins, whereas others will be unaffected. As such, cyanotoxins may influence the zooplankton community structure, especially during times when cyanobacteria are dominant within the phytoplankton.

3.5.3 Effects on fish

If fish are dosed with cyanotoxins by i.p. injections or by force-feeding, they develop similar symptoms of intoxication as laboratory mammals. The question relevant for field exposure is whether cyanotoxins enter healthy fish. For example, Tencalla *et al.* (1994) showed that gastrointestinal uptake by gavage (force-feeding) caused massive hepatic necrosis followed by fish deaths, whereas immersion of adults and juveniles in contaminated water did not cause toxic effects. Other reported evidence suggests that immersion in toxic cyanobacteria or cyanotoxins may be harmful to fish. Differences in sensitivity may be pronounced between species: goldfish were found to be nearly 30 times less susceptible to i.p. microcystin than mice (Sugaya *et al.,* 1990). Release of toxic compounds from mass developments of cyanobacteria was considered to be the cause of fish kills by Penaloza *et al.* (1990). Histopathological investigations offish deaths during cyanobacterial blooms in the UK, indicated that the cause of death was mostly due to damage of the gills, digestive tract and liver (Rodger *et al.,* 1994). The gill damage was probably caused by the high pH induced by cyanobacterial photosynthesis activity prior to the bloom collapse, together with the higher level of ammonia arising from the decomposition of the cyanobacteria. However, gill damage may have enhanced microcystin uptake and thus led to liver necrosis. Damage to gills by dissolved microcystin-LR has been shown experimentally in *Tilapia* and trout (Garcia, 1989; Gaete *et al.,* 1994; Bury *et al.,* 1996).

Other pathological symptoms ascribed to toxic cyanobacterial blooms include damage to the liver, heart, kidney, gills, skin and spleen (Garcia, 1989; Råbergh *et al.,* 1991). Garcia (1989) and Rodger *et al.* (1994) carried out experiments on trout, while Råbergh *et al.* (1991) experimented on carp. The latter study highlighted degenerative changes in kidney tubules and glomeruli. The effect of microcystins on European carp, *Cyprinus carpio,* under natural field conditions in Australia has been described by Carbis *et al.* (1997) as atrophy of hepatocytes, gills with pinpoint necrosis, epithelial ballooning, folded lamellar tips, exfoliation of the lamellar epithelium, elevated asparate aminotransferase activity and serum bilirubin concentrations. Laboratory studies indicate that dissolved microcystins may affect fish embryos (Oberemm *et al.,* 1997) and behaviour of fish (Baganz *et al.,* 1998).

The most definitive effect of microcystin on fish concerns Atlantic Salmon reared in net pens in coastal waters of British Columbia and Washington State, USA. As yet unidentified microcystin-producing organisms produce a progressive degeneration of the liver in salmon smolts placed into open-water net pens (Anderson *et al.,* 1993). The disease, referred to as Net Pen Liver Disease (NPLD), has resulted in significant economic losses for the mariculture industry.

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Chapter 4. HUMAN HEALTH ASPECTS

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Evidence for adverse human health effects from cyanotoxins derives from three principal sources: epidemiological evidence including human poisonings, animal poisonings, and toxicological studies.

Epidemiological evidence results from studies of human populations that have shown symptoms of poisoning or injury attributed to the presence of cyanotoxins in drinking water or other sources of water. This type of evidence depends upon good case definition, good characterisation of exposure and a reporting system that enables these data to be compared. Such evidence, discussed later, includes the fatal intoxication by microcystin of 50 dialysis patients in Brazil, and the hospitalisation of 140 children supplied with water from a dam containing *Cylindrospermopsis raciborskii* in Australia. Reports of gastro-enteritis after the appearance of cyanobacterial blooms in drinking water sources come from North and South America, Africa and Europe. Most cases of human injury attributed to cyanobacterial toxins have been studied retrospectively, and complete epidemiological data, especially regarding exposure (number of organisms, type and concentration of cyanotoxins), are rarely available. Nevertheless, epidemiological evidence is of special importance in directly demonstrating the link between toxin exposures and human health outcomes, which otherwise cannot be derived directly from animal experiments.

A large number of animal poisonings have been recorded, some of which have been reported in detail, while others are anecdotal. These are important in the overall body of knowledge on cyanobacterial toxicity, because they demonstrate effects under natural conditions. However, cyanotoxin exposure is rarely quantified and cannot be directly extrapolated to human populations.

Although animal toxicity tests are not performed under conditions of natural exposure and are undertaken with different species, they are of particular importance because they are conducted under controlled laboratory conditions. They provide plausibility for the role of cyanotoxins in human and animal poisonings, and provide information on their comparative toxicity. Oral toxicity testing is especially relevant, and has been carried out on large animals as well as on rodents.

The overall case for the human health relevance of exposure to cyanotoxins can only be assessed properly by combining all available evidence and understanding its strength and its weaknesses.

Of all the cyanobacterial toxins currently known, the cyclic peptides represent the greatest concern to human health because of the potential risk of long-term exposure to comparatively low concentrations of the toxins in drinking water supplies. As will be discussed in sections 4.2.1 and 4.2.2, the cyclic peptide microcystins and nodularins are specific liver poisons in mammals. Following acute exposure to high doses, they cause death from liver haemorrhage or from liver failure, and they may promote the growth of liver and other tumours following chronic exposures to low doses.

The alkaloid neurotoxins (anatoxins and saxitoxins/PSP toxins) have shown only acute effects in mammals. Risk assessment will, therefore, be limited to acute exposure. However, there are a number of complicating factors. Firstly, while there is a great deal of published information on the mammalian and human toxicity and toxicology of saxitoxins/PSP toxins, the animal data for anatoxins is rather sparse, and completely lacking for human exposure. Secondly, some of the alkaloid toxins, PSP toxins in particular, are known to accumulate to high concentrations in marine and freshwater biota (see section 3.4.5). In many areas of the world, the local human population will be consuming fish, shellfish and animals that have grown in, or nearby, water bodies contaminated with toxic cyanobacteria. Thus there may be several sources of oral exposure for neurotoxins in addition to drinking water or recreational water. The potential for transfer of PSP toxins, and possibly other neurotoxins, to humans via these routes may be significant in freshwaters.

The toxicity of pure cylindrospermopsin has recently been studied following the identification of the toxin in 1992 (Ohtani *et al.,* 1992). These studies have helped to characterise the histopathology of cylindrospermopsin after intraperitoneal (i.p.) injection (Hawkins *et al.,* 1997; Falconer *et al.,* 1999; Seawright *et al.,* 1999). It is not known whether cylindrospermopsin will elicit histopathological damage following chronic (long-term) exposure at low concentrations.

Lipopolysaccharides (LPS) from cyanobacteria can elicit both allergic and toxic responses in humans, although little is known about their acute or chronic effects. The lipid part of the molecule is believed to cause the deleterious response in humans, while the sugar moiety is important in determining LPS solubility and micelle properties, and hence is important in modulating toxicity. A lack of knowledge regarding the occurrence and toxicity of cyanobacterial LPS, combined with the diversity of LPS structures within the cyanobacteria, should be considered by health officials and water managers, particularly when gastrointestinal and respiratory symptoms are reported from exposed humans.

4.1 Human and animal poisonings

Water supplies have been associated with gastrointestinal illness throughout human history with cholera, dysentery, and typhoid responsible for much human misery and death. The epidemiological evidence for human illness due to cyanobacterial toxins therefore has to be viewed against a background of alternative causes, with bacterial, viral or protozoal infections being the first causes to be investigated.

4.1.1 Short-term effects in humans

The recorded cases of gastrointestinal and hepatic illness that can be reliably attributed to cyanobacterial toxins in water supplies have all been coincident with either the breakdown of a natural cyanobacterial bloom or with the artificial lysis of a bloom by application of copper sulphate. Both mechanisms lead to cyanotoxin release from decomposing cells. Whereas treatment procedures might have removed cyanotoxins bound in intact cells, they were not effective in removing the dissolved cyanotoxins in these cases. The earliest reported cases of gastro-enteritis from cyanobacteria were in the population of a series of towns along the Ohio River in 1931. In these cases low rainfall had caused the water of a side branch of the river to develop a cyanobacterial bloom which was then washed into the main river. As this water moved downstream a series of outbreaks of illness were reported, which could not be attributed to infectious agents (Tisdale, 1931). In Harare, Zimbabwe, children living in an area of the city supplied from a particular water reservoir, developed gastro-enteritis each year at the time when a natural bloom of *Microcystis* was decaying in the reservoir. Other children in the city with different water supplies were not affected and no infectious agent was identified (Zilberg, 1966). The most lethal outbreak attributed to cyanobacterial toxins in drinking water occurred in Brazil, when a newly flooded dam developed an immense cyanobacterial bloom. Eighty-eight deaths, mostly children, were reported to have occurred (Teixera *et al.,* 1993) (Box 4.1).

Examples of illness following the use of copper sulphate to destroy a cyanobacterial bloom in a water storage reservoir have been described in the USA and in Australia. In each of these instances the cyanobacterial genera involved were identified, either at the time or subsequently. In one incident, 62 per cent of the population connected to a filtered, chlorinated water supply developed symptoms of gastro-enteritis within a period of five days. No pathogens were found, and it was concluded that abloom of cyanobacteria in an open storage reservoir which had over 100,000 cells per ml of *Schizothrix calcola, Plectonema, Phormidium* and *Lyngbya* was responsible. The reservoir had just been treated with copper sulphate (Lippy and Erb, 1976).

Box 4.1 Gastro-enteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil

A severe gastro-enteritis epidemic in the Paulo Afonso region of Bahia State in Brazil followed the flooding of the newly constructed Itaparica Dam reservoir in 1988. Some 2,000 gastro-enteritis cases, 88 of which resulted in death, were reported over a 42-day period.

Clinical data and water sample tests were reviewed, blood and faecal specimens from gastro-enteritis patients were subjected to bacteriological, virological and toxicological testing and drinking water samples were examined for micro-organisms and heavy metals. The results demonstrated that the source of the outbreak was water impounded by the dam and pointed to toxin produced by cyanobacteria present in the water as the responsible agent. No other infectious agent or toxin was identified, and cases occurred in patients who had been drinking only boiled water. The cases were restricted to areas supplied with drinking water from the dam.

Cyanobacteria of the *Anabaena* and *Microcystis* genera were present in untreated water at 1,104 to 9,755 units per ml (conversion of colony units to cells per ml depends on colony size, but a minimum of 100 cells per colony is likely in a mixed bloom of these genera).

In Armidale, Australia, the water supply reservoir had been monitored for blooms of toxic *Microcystis* for several years, when a particularly dense bloom occurred. Within three weeks the water supply authority treated the reservoir with 1 ppm (part per million) of copper sulphate, which killed the bloom. An epidemiological study of the local population indicated liver damage occurring simultaneously with the termination of the bloom (see Box 4.2).

A more severe outbreak of cyanobacterial toxicity in a human population occurred on an island off the north-eastern coast of Australia. Due to complaints of bad taste and odour in the water supply, which were attributed to a cyanobacterial bloom, the authorities treated the reservoir with copper sulphate. Within a week numerous children developed severe hepato-enteritis, and a total of 140 children and 10 adults required hospital treatment (Byth, 1980). *Cylindrospermopsis raciborskii* was identified as the cyanobacterium responsible for this episode (see Box 4.3).

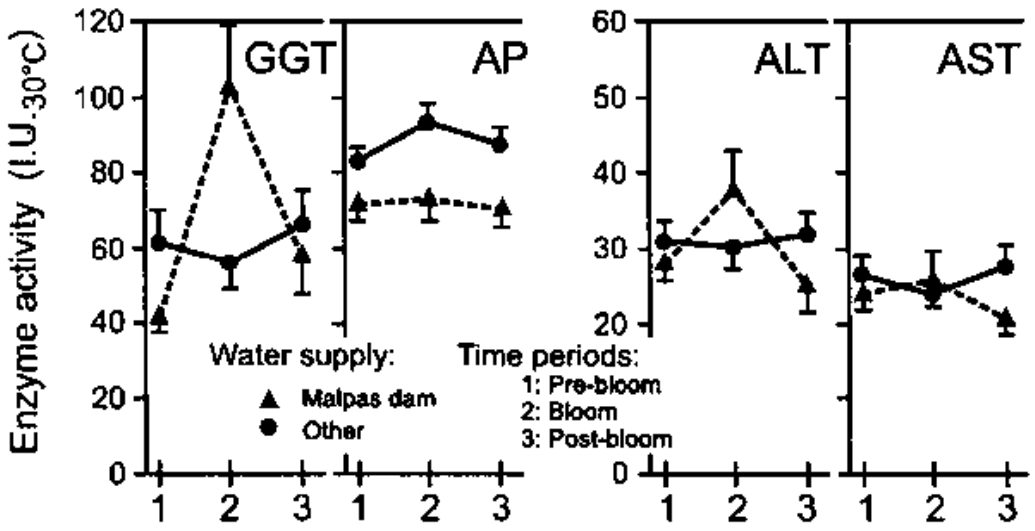
Within human populations, for a variety of reasons, there will be individuals who are at a much greater risk of injury from cyanotoxins than the population as a whole. Children are the most obvious example, because they drink a higher volume of water in proportion to their body weight than adults. Individuals who already have injury to organs susceptible to cyanobacterial toxins are also at increased risk, such as people with hepatitis, liver cirrhosis, toxic liver injury from other sources, or kidney damage. Kidney dialysis patients, if exposed to microcystins in the water used for dialysis, are especially vulnerable because treatment exposes them intravenously to large volumes of water. In a disastrous incident in Caruaru, Brazil, 117 patients developed cholestatic liver disease and at least 47 deaths were attributed to dialysis with water containing cyanobacterial toxins. Examination of the carbon filter from the dialysis unit demonstrated microcystin-LR, as did the blood and liver tissue of deceased patients (Jochimsen *et al.,* 1998) (see Box 4.4).

Box 4.2 Toxic *Microcystis* in the Armidale water supply reservoir and public health

The city of Armidale, New South Wales, Australia has a drinking water supply from a eutrophic reservoir which has experienced repeated blooms of *Microcystis aeruginosa* from the early 1970s to the present day. The reservoir, Malpas Dam, is on a plateau 150 m higher than the treatment plant to which it is connected by about 20 km of pipeline. The plant treated water by pre-chlorination, alum flocculation, sedimentation, rapid sand filtration and post chlorination and fluoridation. The geography of the water storage results in toxic scums accumulating around the offtake area through wind-drift. The local water authority use copper sulphate dosing to control blooms, with aerial distribution of 1 ppm of copper in the top metre of water. The cyanobacterial blooms have been monitored for toxicity, and the toxin has been characterised as microcystin-YM (Botes *et al.,* 1985).

In 1981, a particularly extensive toxic bloom of *Microcystis* was monitored during its development and subsequent termination with copper sulphate. At the time of this bloom, complaints of bad taste and odour were received, leading to the copper sulphate treatment of the reservoir. This event was used as the basis for a retrospective epidemiological study of liver function in the population consuming the water, compared with a population in the same region supplied from other reservoirs. The data for the activity of plasma enzymes measuring liver function were obtained for patients having blood samples at the Regional Pathology Laboratory for the six weeks prior to the bloom, the six weeks of peak bloom and its termination, and for six weeks after

that. The data were then separated into analyses from patients having the Malpas drinking water supply, and those using other supplies.



Serum enzymes measuring liver function in patients consuming drinking water from Malpas Dam or from other supplies during a heavy bloom of *Microcystis aeruginosa* and its termination with copper. GGT = gamma glutamyl transferase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AP = alkaline phosphatase (From The Medical Journal of Australia, 1983; 1511-1514)

As shown in the figure above, a statistically significant increase in gamma glutamyl transferase in the blood was seen with the group using the Malpas water supply during the peak of the bloom and its lysis with copper sulphate, compared with the same population before and after, or the other population on independent water supplies. The clinical record gave no evidence of an infectious hepatitis outbreak or disproportionate alcoholism (Falconer *et al.*, 1983). While the mean increase in enzyme activity was indicative of minor liver toxicity, individuals within the population studied had highly elevated enzyme activity, indicating substantial liver damage. The serum enzyme showing this change has also been used as an indicator of *Microcystis* toxicity in experimental studies with pigs and rodents, where it is an effective marker for liver injury (Fawell *et al.*, 1993; Falconer *et al.,* 1994).

4.1.2 Chronic effects in humans

While acute toxicity is the most obvious problem in cyanobacterial poisoning, a long-term risk may also be present. Short exposures to toxins may result in long-term injury, and chronic low-level exposure may cause adverse health effects. Animal experiments have shown chronic liver injury from continuing oral exposure to microcystins. In particular the possibility of carcinogenesis and tumour growth promotion need careful evaluation, because both have been shown in animal experimentation.

The incidence of human hepatocellular carcinoma in China is one of the highest in the world, and studies have explored whether cyanobacterial toxins are part of a complex of risk factors which increase the incidence of this disease. The distribution of hepatocellular carcinoma varies geographically. In south-east China, rates of less than 15 incidents per 100,000 people are seen in some districts, compared with over 60

incidents per 100,000 people in adjacent localities. Two proven risk factors are infection with hepatitis B virus and intake of aflatoxin B_1 from food items, such as corn. The third significant element of association was the source of drinking water. On a village basis, lower cancer mortality rates were seen when the water was drawn from deep wells, compared with much higher rates when the water came from ponds and ditches. Cyanobacteria are abundant in surface waters in south east China where the incidence of hepatocellular carcinoma is highest, and it has been proposed that microcystins in the drinking water are responsible for the higher incidences of cancer among drinkers of pond and ditch water (Yu, 1989, 1995). Very low levels of cyanotoxins were found in one study with limited sampling of some drinking water sources in China (Ueno *et al.,* 1996), but a more representative study would be needed to investigate this association. At present, all three risk factors are being reduced concurrently in China and liver cancer rates appear to be decreasing (Yu, 1995) (see also Box 5.3).

Box 4.3 Palm Island Mystery Disease

In 1979, there was a major outbreak of hepato-enteritis among the children of an Aboriginal community living on a tropical island off the coast of Queensland, Australia. Altogether 140 children and 10 adults required treatment, which was provided by the local hospital for less severe cases and by the regional hospital on the mainland for severe cases possibly requiring intensive care. Diagnostic information included a detailed clinical examination showing malaise, anorexia, vomiting, headache, painful liver enlargement, initial constipation followed by bloody diarrhoea and varying levels of severity of dehydration. Urine analysis showed electrolyte loss together with glucose, ketones, protein and blood in the urine, demonstrating extensive kidney damage. This was the major life-threatening element of the poisoning. Blood analysis showed elevated serum liver enzymes in some children, indicating liver damage. Sixty-nine percent of patients required intravenous therapy and in the more severe cases the individuals went into hypovolaemic/acidotic shock. After appropriate treatment all the patients recovered (Byth, 1980).

Examination of faecal samples and foods eliminated a range of infectious organisms and toxins as possible causes for the outbreak, hence the name "Palm Island Mystery Disease". The affected population, however, all received their drinking water supply from one source, Solomon Dam. Families on alternative water supplies on the island were not affected by the disease.

Prior to the outbreak of the illness an algal bloom occurred in Solomon Dam. The bloom discoloured the water and gave it a disagreeable odour and taste. When the bloom became dense, the dam reservoir was treated with 1 ppm of copper sulphate (Bourke *et al.*, 1983). Clinical injury among consumers on that water supply was reported the following week.

The organisms from the dam were cultured and administered to mice, following which the mice slowly (over several days) developed widespread tissue injury involving the gastrointestinal tract, the kidney and liver (Hawkins *et al.,* 1985). The widespread tissue damage and delayed effects are quite different to those following *Microcystis aeruginosa* administration (Falconer *et al.,* 1981; see section 4.2.1).

Subsequent monitoring of the algal blooms in the dam identified the cyano-bacterium *Cylindrospermopsis raciborskii* as the cause of the blooms, with seasonal cell concentrations of up to 300,000 cells per ml of water. This organism does not form scums, and has the highest cell concentrations well below the water surface. In order to reduce bloom formation, the responsible authorities later introduced destratification of the reservoir (Hawkins and Griffiths, 1993).

Subsequent research on toxins produced by *Cylindrospermopsis raciborskii has* identified the cytotoxic alkaloid cylindrospermopsin which is toxic after i.p. and oral administration. It is possible that other toxins will be isolated from this organism (Hawkins *et al.,* 1997, see also section 4.2.7).

Box 4.4 Outbreak of severe hepatitis following haemodialysis in Caruaru, Brazil

In February 1996, an outbreak of severe hepatitis occurred at a Brazilian haemodialysis centre in Caruaru, Brazil, located 134 km from Recife, the state capital of Pernambuco. At this clinic 117 of 136 patients (86 per cent) experienced visual disturbances, nausea, vomiting, muscle weakness and painful hepatomegaly, following routine haemodialysis treatment. Subsequently, 100 patients developed acute liver failure and 50 of these died. As of October 1997, 49 of the deaths could be attributed to a common syndrome now called "Caruaru Syndrome". This syndrome includes:

- *Symptoms.* Painful huge hepatomegaly, jaundice and a bleeding diathesis manifested by ecchymosis, epistaxis and metrorrhagia.
- *Laboratory picture.* Elevated transaminases, variable hyperbilirubinemia, prolonged prothrombin time and severe hypertriglyceridemia.
- *Histopathology.* Light microscopy - disruption of liver plates, liver cell deformity, necrosis, apoptosis, cholestasis, cytoplasmic vacuolisation, mixed leukocyte infiltration and multinucleated hepatocytes; electron microscopy - intracellular oedema, mitochondrial changes, rough and smooth endoplasmic reticulum injuries, lipid vacuoles and residual bodies.

The pattern of liver plate disruption was identical to that found with previous laboratory animal experiments involving microcystin exposure.

Initial reports, at the beginning of March 1996, from the medical and public health profession in Pernambuco, implicated several xenobiotic or microbial sources as possibly responsible for a cluster of human fatalities. However, comparison with previous knowledge about cyanotoxicosis, from other parts of the world, suggested that cyanobacteria toxins might be responsible. Since phytoplankton counts were not being made by the city's water utility at the time of the outbreak, the presence of toxic cyanobacteria or microcystin could not be confirmed or denied. Examination of previous years' phytoplankton counts showed that cyanobacteria had been dominant in the reservoir during the summer months since at least 1990, with the most common genera present being *Microcystis, Anabaena* and *Anabaenopis (Cylindrospermopsis).* Samples collected on March 29, 1996 showed that the most common cyanobacteria present were *Aphanizomenon, Oscillatoria* and *Spirulina* (average number 24,500 cells per ml).

Carbon from the dialysis centre's in-house water treatment system showed chromatogram peaks that had characteristics of microcystins when analysed using HPLC. One of these peaks had a retention time corresponding to microcystin-LR, the most commonly found of the microcystin family. Further examination of carbon, sand and cation/anion exchange filters showed microcystin in the ppm range.

Blood sera, provided by state health officials in Pernambuco and sent through the Centers for Disease Control in Atlanta Georgia, USA, from affected and control patients, and liver tissue from deceased patients, showed a microcystin content of up to 10 ng ml^{-1} for sera and 0.1 to 0.5 ng mg^{-1} in liver tissue. All exposed patient sera and tissue were positive for microcystins. Chemical analysis of the most abundant microcystins revealed the presence of microcystins -YR, -LR and -AR. The average microcystin concentration in liver samples from 33 victims compares closely with that reported in laboratory test animals receiving acute exposure to microcystin-LR. When the analyses are completed, the resulting information on dose and related effects will be important in the hazard characterisation and risk assessment of microcystins in humans. A clinic for survivors set up by Brazilian health authorities will help monitor their progress and serve as a data base to assess long-term health effects, including cancer rates, because the microcystins are known to be potent liver tumour promoters in experimental animals.

The available biological and chemical evidence supports microcystins from the reservoir water as being the major factor in the deaths of patients at the dialysis clinic. The occurrence of microcystin in the water used for dialysis was due to insufficient treatment of the water obtained by truck from the city's water treatment system, as well as to insufficient functioning and maintenance of the clinic's dialysis water treatment system.

4.1.3 Injury from recreational exposure

There have been repeated descriptions of adverse health consequences for swimmers exposed to cyanobacterial blooms. Even minor contact with cyanobacteria in bathing water can lead to skin irritation and increased likelihood of gastrointestinal symptoms (Pilotto *et al.,* 1997), Some severe skin reactions have been reported, particularly from contact with the marine cyanobacterium, *Lyngbya majuscula,* which causes deep blistering when trapped under the bathing suit of swimmers (Grauer, 1961). In this case the organism contains a powerful dermal toxin which is further described in section 4.2.8.

Individual sensitivity to cyanobacteria in bathing waters varies greatly, because there can be both allergic reactions and direct responses to toxins. The cyanobacterial pigments can cause severe allergic reactions in sensitive individuals (Cohen and Reif, 1953). Cyanobacteria have features in common with general airborne allergens and surveys have shown allergic responses to cyanobacteria in patients with naso-bronchial allergy (Mittal *et al.,* 1979). More extensive discussion on this aspect can be found in Ressom *et al.* (1994). Illnesses from accidental swallowing of cyanobacteria during swimming are described in Box 4.5.

4.1.4 Animal poisonings

Numerous cases of animal poisoning (often lethal) substantiate the concern of health hazards for humans exposed to cyanobacteria. Therefore, the most important incidents are reported below. For impact of cyanotoxins on aquatic biota, see section 3.5.

The first report of cyanobacterial poisoning was of the deaths of cattle, sheep, dogs, horses and pigs after drinking a scum of *Nodularia spumigena* in Lake Alexandrina, Australia (Francis, 1878). Francis confirmed the source of poisoning by dosing a calf with the scum from the lake, which subsequently caused the death of the animal. Since that time there have been frequent instances of farm animal poisonings from cyanobacterial water blooms, and also deaths of pet dogs after swimming in, or eating, cyanobacteria. Other affected animals range in size from ducks, coots and other waterfowl, to skunks and mink, and even up to rhinoceros (see Carmichael, 1992). Besides the consumption of cyanobacteria from the water, it has been suggested that an additional source of intoxication for terrestrial animals is cyanotoxins that have bioaccumulated in the food chain. For example, freshwater mussels accumulate both microcystins (Prepas *et al.,* 1997) and saxitoxins (Negri and Jones, 1995), and mussels are an important food source for water rats, musk rats and birds.

An extensive list of poisoning incidents, and discussion of them, is included in Ressom *et al.* (1994). A selected group is given in Table 4.1. As with cases of suspected human poisoning by cyanobacterial toxins, in the earlier cases much of the evidence necessary for proof of poisoning is unavailable. Ideally, for such proof, the toxic organisms or toxins

need to be identified in the body of the victim, together with the evidence of access to toxic material (see also section 3.5). One of the most convincing mammalian poisonings has been the recorded deaths of sheep drinking from a farm dam contaminated with the neurotoxic *Anabaena circinalis* in Australia (Negri *et al.,* 1995). The authors recovered high concentrations of saxitoxins from cyanobacteria in the farm dam and from the rumen fluid from the dead sheep.

Box 4.5 Examples of health effects due to recreational exposure

1959 Saskatchewan, Canada. In spite of recreational use warnings and deaths in livestock, people swam in a lake infested with cyanobacteria. Thirteen persons became ill (headaches, nausea, muscular pains, painful diarrhoea). In the excreta of one patient (a medical doctor who had accidentally ingested 300 ml of water), numerous cells of *Microcystis* spp. and some trichomes of *Anabaena circinalis* were identified (Dillenberg and Dehnel, 1960).

1989 United Kingdom. Ten of 20 army recruits showed symptoms indicating intoxication (e.g. vomiting, diarrhoea, central abdominal pain, blistering of the lips, sore throats) after swimming and canoe training in water with a dense bloom of *Microcystis* spp. Two of the recruits developed severe pneumonia attributed to the aspiration of a *Microcystis* toxin and needed hospitalisation and intensive care (Turner *et al.,* 1990). The severity of illness appeared to be related to the swimming skills and amount of water ingested.

1995 Australia. Epidemiological evidence of adverse health effects after recreational water contact was established in a prospective study involving 852 participants. Results showed an elevated incidence of diarrhoea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, eye or ear irritations within seven days following exposure (Pilotto *et al.*, 1997). Symptoms increased significantly with duration of water contact and cell density of cyanobacteria.

The post mortem symptoms of toxicity can also be characteristic of the poison involved. Animal deaths from cyanobacterial toxicity have been reported from North and South America, Europe, Australia and Africa. The major injury reported is hepatotoxicosis, i.e. liver poisoning. The cyanobacteria responsible have been *Microcystis aeruginosa, Nodularia spumigena* and *Oscillatoria (Planktothrix) agardhii.* In the most recent cases, post mortem examination has shown evidence of cyanobacterial ingestion as well as characteristic tissue injury in the liver.

Table 4.1 Selected examples of animal poisoning associated with cyanobacteria

Country	Species killed	Pathology	Organism	Reference
Argentina	Cattle	Hepatotoxicity	*Microcystis aeruginosa*	Odriozola *et al.*, 1984
Australia	Sheep	Hepatotoxicity	*Microcystis aeruginosa*	Jackson *et al.,* 1984
Australia	Sheep	Neurotoxicity, PSPs	*Anabaena circinalis*	Negri *et al.,* 1995
Canada	Cattle	Neurotoxicity, anatoxin-a	*Anabaena flos-aquae*	Carmichael and Gorham, 1978
Canada	Waterfowl	Neurotoxicity, anatoxin-a	*Anabaena flos-aquae*	Pybus and Hobson, 1986
Finland	Dogs	Hepatotoxicity, nodularin	*Nodularia spumigena*	Perrson *et al.,* 1984
Finland	Waterfowl, fish, muskrats	Hepatotoxicity, gill damage	*Planktothrix agardhii*	Eriksson *et al.,* 1986
Norway	Cattle	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Skulberg, 1979
UK				
England	Shepherd dogs	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Pearson *et al.,* 1990
Scotland	Dogs	Neurotoxicity, anatoxin-a	*Oscillatoria* spp.	Gunn *et al.,* 1992
Scotland	Fish (trout)	Gill injury, microcystin	*Microcystis aeruginosa*	Bury *et al.,* 1995
USA	Dogs	Neurotoxicity, anatoxin-a(S)	*Anabaena flos-aquae*	Mahmood *et al.,* 1988

PSPs Paralytic shellfish poisons

The other main cause of livestock and pet deaths due to cyanobacterial toxins has been from acute neurotoxicity leading to respiratory failure, with no post mortem indications of organ injury. In one case (Gunn *et al.,* 1992) the neurotoxin, anatoxin-a, was isolated from the stomach contents of a dog. Henriksen *et al.,* (1997) demonstrated lethality in wild ducks due to anatoxin-a(S). The cyanobacteria associated with deaths from neurotoxicity are *Anabaena flos-aquae, Anabaena circinalis, Aphanizomenon flos-aquae* and *Oscillatoria* spp. The toxins anatoxin-a, anatoxin-a(S) and saxitoxins have been implicated in different cases.

While the reported deaths have usually occurred shortly after the animals have ingested cyanobacterial scums, lasting injury with progressive mortality has also been seen in animal poisoning by *Microcystis aeruginosa.* The characteristic symptoms are those of liver failure with secondary photo-sensitisation, i.e. severe sunburn-like reactions (McBarron and May, 1966; Carbis *et al.,* 1995).

4.2 Toxicological studies

In order to set safe levels of toxicants or contaminants in food or drinking water, it is first necessary to determine the dose level in humans that is considered to be without adverse effects when taken daily over a lifetime; this is known as the Tolerable Daily Intake (TDI). Ideally, this value is derived from human studies, but often such studies are inadequate or non-existent. Alternatively, when there is an adequate experimental database, it can be derived from animal studies. One of the major shortcomings of animal studies is that differences in sensitivity between animals and humans vary, so that safety factors need to be incorporated to deal with this uncertainty (see below).

A further important extrapolation problem associated with animal studies relates to routes of exposure. Many toxins are more toxic when given by the intravenous (i.v.) or i.p. route of administration than by the oral route. This difference is evident when LD_{50} values (single dose level that will cause death in 50 per cent of the exposed animals within 7-14 days) are examined for various routes of exposure (see Chapter 3 and Table 4.2). Studies using the i.p. or i.v. route of administration require much less toxin and can be used to indicate relative acute toxicity and may provide information on the mechanism of toxicity. Such studies are used in bioassays (see section 13.3.1), but they have little use in predicting toxicity after oral administration. Another problem with many natural toxins, including cyanotoxins, is obtaining sufficient pure toxin to conduct controlled experiments. Several studies, to be described, have used algal extracts rather than pure toxin. An advantage of this is that the extract more closely mimics the real environmental situation. A disadvantage is that unless the extract is fully characterised for all the toxins that may be present and their concentrations, the assay may have limited predictive value for scenarios involving other conditions.

Several steps are involved in determining the TDI from appropriate human or animal studies. The highest dose associated with the absence of adverse health effects (the NOAEL or No Observed Adverse Effect Level) is selected or, when no NOAEL is available, the lowest dose associated with adverse effects (the LOAEL or Lowest Observed Adverse Effect Level) is selected. In order to extrapolate from animal data to the human situation, the next step involves the application of a number of uncertainty factors. In the absence of reliable data on inter- and intraspecies differences in sensitivity to a chemical substance, standard default uncertainty factors of 10 are generally used to give a composite uncertainty factor of 100. Additional uncertainty factors may be used to deal with inadequacies in the database and the severity of effects noted (WHO, 1993; IPCS, 1994). When extrapolating from human data, it is also necessary to use some of these factors. With human data there are, in addition, often problems in determining exposure accurately and thus the dose causing adverse effects.

The expert review of the pertinent literature in April 1997, which led to the production of this book, revealed that information currently available is insufficient for calculation of a TDI for most of the cyanotoxins. For microcystin-LR more data exist and a provisional value could be derived. The following sections summarise the present health-related information on each of the cyanotoxins, in order to provide research data to health officers and others, and in order to provide a basis for the assessment of the health hazards of cyanotoxins.

4.2.1 Microcystins

In the various reported incidents of poisoning in humans and livestock caused by cyanobacteria or their toxins, *Microcystis* is the most frequently cited organism. As a consequence, extensive experimental studies have been carried out on this cyanobacterium and its toxins. Although there is qualitative evidence of cause and effect, indicating human susceptibility, these data have so far been inadequate to establish a dose response relationship that can be used for assessing human risk in a variety of exposure scenarios. The animal studies described below have been conducted in an attempt to address these issues.

Metabolic disposition

There have been no pharmacokinetic studies with orally administered microcystins. After i.v. or i.p. injection of sublethal doses of variously radiolabelled microcystins in mice and rats, about 70 per cent of the toxin was rapidly localised in the liver (Falconer *et al.,* 1986; Runnegar *et al.,* 1986; Brooks and Codd, 1987; Robinson *et al.,* 1989, 1991; Meriluoto *et al.,* 1990; Lin and Chu, 1994a; Nishiwaki *et al.,* 1994). Plasma half-lives of microcystin-LR, after i.v. administration, were 0.8 and 6.9 minutes for the first and second phases of elimination, but the concentration of radioactive (^{3}H-microcystin-LR) label in the liver did not change throughout the later part of the six-day study period (Robinson *et al.,* 1991). In this study, about 9 per cent of the dose was excreted early via the urinary route, with the remainder being excreted slowly (~1 per cent per day) via the faecal route. Based on the protective effect of microsomal enzyme inducers, it is evident that the liver plays a large role in the detoxification of microcystins (Brooks and Codd, 1987). Time-dependent appearance and disappearance of additional chromatograph peaks, thought to represent detoxification products, were seen in urine, faeces and liver cytosol fractions (Robinson *et al.,* 1991). Three metabolic products have since been identified, a glutathione conjugate, a cysteine conjugate and a conjugate with the oxidised ADDA diene (Kondo *et al.,* 1996).

Microcystin-LR does not readily cross cell membranes, and hence does not enter most tissues. After oral uptake it is transported across the ileum into the bloodstream through a bile acid type transporter (the multispecific organic ion transport system) present in hepatocytes and cells lining the small intestine (Runnegar *et al.,* 1991; Falconer *et al.,* 1992) and is concentrated in the liver as a result of active uptake by hepatocytes (Runnegar *et al.,* 1981). It is covalently bound to a 40 kdalton protein (protein phosphatase 2A and possibly protein phosphatase 1) in the hepatocyte cytosol (Holmes *et al.,* 1994; Bagu *et al.,* 1997). Some other microcystin congeners are more hydrophobic than microcystin-LR and may cross cell membranes by other mechanisms, including diffusion.

Acute and subacute toxicity

Microcystin-LR is highly toxic. The LD_{50} by the i.p. route ranges from 25 to 150 µg kg^{-1} body weight (bw) in mice (a value of 50 or 60 µg kg^{-1} bw is commonly accepted). The oral LD_{50} (administered by gavage, i.e. dosing directly into the stomach through the mouth) is 5,000 µg kg^{-1} bw in one strain of mice (Fawell *et al.,* 1994), 10,900 µg kg^{-1} bw in a different strain of mice (Yoshida *et al.,* 1997), and higher in rats (Fawell *et al.,* 1994). This indicates that, even by the oral route, microcystin-LR displays acute toxicity in

rodents. There is no evidence of hydrolysis of microcystins by peptidases in the stomach and it is apparent that a significant amount of microcystin-LR passes the intestinal barrier and is absorbed. Similarly, the oral LD_{50} of *Microcystis* extracts in mice was 50- to 170-fold higher than the i.p. LD_{50} of the same extracts (Falconer, 1991; Kotak *et al.,* 1993).

The i.p. LD_{50}s of several of the commonly occurring microcystins (microcystin-LA, -YR, and -YM) are similar to that of microcystin-LR, but the i.p. LD_{50} for microcystin-RR is about tenfold higher (see Table 3.1).

However, because of differences in lipophilicity and polarity between the different microcystins, it cannot be presumed that the i.p. LD_{50} will predict toxicity after oral administration.

The microcystins are primarily hepatotoxins. After acute exposure by i.v. or i.p. injection of microcystin, severe liver damage is characterised by a disruption of liver cell structure (due to damage to the cytoskeleton), a loss of sinusoidal structure, increases in liver weight due to intrahepatic haemorrhage, haemodynamic shock, heart failure and death. Other organs affected are the kidneys and lungs (Hooser *et al.,* 1990) and the intestines (Falconer, 1994; Falconer and Humpage, 1996).

In a recent study, a single oral dose of microcystin-LR at 500 µg kg^{-1} bw was given to 5 and 32 week old mice, and liver pathology was examined 2-19 hours later. In 62 per cent of the older mice, there was clear microscopic evidence of liver injury. Furthermore, microcystin-LR and a metabolite were detected in the livers. On the contrary, none of the young mice developed liver injury. In untreated control mice, an examination of gastric mucosa and small intestine revealed age-related disruption of surface cell structure. The authors suggested that this disruption may markedly influence gastro-intestinal absorption of microcystin-LR and hence explain the observed age-dependent liver toxicity in exposed mice (Ito *et al.,* 1997a).

Intranasal installation of microcystin-LR in mice resulted in extensive necrosis of the epithelium of the nasal mucosa of both the olfactory and respiratory zones, progressing to destruction of large areas of tissue down to levels of deep blood vessels (Fitzgeorge *et al.,* 1994). The LD_{50} by this route of administration was the same as the i.p. LD_{50}, and dose-dependent liver lesions were observed. The same authors also demonstrated cumulative liver damage after repeated dosing. While a single dose of 31.3 µg kg^{-1} bw did not result in an increase in liver weight, repeated daily administration over a period of seven days caused a 75 per cent increase in liver weight, which was almost as high as the effect of a single intranasal dose of 500 µg kg^{-1} bw.

Repeated oral administration

Pure microcystin-LR. For assessing possible chronic human health effects, studies involving repeated oral administration of pure microcystins at various dose levels are most desirable. In a 13-week gavage study in mice, conducted under good laboratory practice (GLP), pure microcystin-LR was administered orally to groups of 15 male and female mice at 0, 40, 200, or 1,000 µg kg^{-1} bw per day for 13 weeks (Fawell *et al.,* 1994). The NOAEL was 40 µg kg^{-1} bw per day. At the next highest dose level there were slight liver tissue changes in some male and female mice. At the highest dose level, all male

and most female mice showed liver changes, including chronic inflammation, focal degeneration of hepatocytes and haemosiderin deposits. In male mice at the two highest dose levels, serum transaminases were significantly elevated, serum gamma glutamyl transferase was significantly reduced, and there were small but significant reductions in total serum protein and serum albumin. In female mice, at the highest dose level, only the changes in transaminases were observed (Fawell *et al.,* 1994).

Microcystis extract. In an oral dosing study, *Microcystis aeruginosa* extract was supplied to mice at five concentrations (equivalent to 750 to 12,000 µg kg^{-1} bw per day of microcystin-YM) in their drinking water for up to one year. At the higher concentrations there was increased death, increased bronchopneumonia (which was endemic in the colony), and chronic liver injury. There was no evidence of tumourigenic changes in the liver, despite the liver injury caused by chronic oral exposure to microcystins. No clear NOAEL was established (Falconer *et al.,* 1988).

In another study in pigs, *Microcystis aeruginosa* extract was given to groups of five pigs in their drinking water for 44 days at dose levels calculated from potency estimates using the mouse i.p. bioassay to be equivalent to 280, 800 or 1,310 µg kg^{-1} bw per day of microcystins (assuming an average i.p. LD_{50} for microcystins of 100 µg kg^{-1} bw). The extract contained at least seven microcystin variants, with microcystin-YR tentatively identified by high pressure liquid chromatography (HPLC) as the major constituent. A LOAEL of 280 µg kg^{-1} bw per day of toxins was identified, with general liver injury (evident from histopathology and changes in serum enzymes) observed at the two higher dose levels. At the lowest dose level, one pig was affected. The authors determined the potency of their extract by the mouse i.p. LD_{50} bioassay, by HPLC analysis and by the *in vitro* phosphatase inhibition assay (Falconer *et al.,* 1994). Summation of the peak areas from the HPLC identification of microcystin variants, standardised against microcystin-LR (see Chapter 5), indicated that the LOAEL equated with 100 µg microcystin-LR equivalents per kg bw per day.

Developmental effects

In an investigation on the effects of microcystin-LR on embryonic and foetal development of the mouse, groups of 26 time-mated female mice were dosed once daily by gavage with aqueous solutions of pure microcystin-LR from days 6 to 15 of pregnancy, at dose levels of 0, 200, 600, or 2,000 µg kg^{-1} bw per day. Maternal clinical signs, body weights and food consumption were recorded. Only treatment at 2,000 µg kg^{-1} bw per day was associated with maternal toxicity and mortality. Seven of the 26 females died and two were sacrificed because of distress during the dosing period. On day 18 of pregnancy the remaining females were killed. At the highest dose level, there was a retardation of foetal weight and skeletal ossification, but no foetal deaths. Apart from this there was no effect of microcystin-LR on sex ratio, implantation, post-implantation loss, or on external or visceral or skeletal abnormalities. The NOAEL for any aspect of developmental toxicity was 600 µg kg^{-1} bw per day (Fawell *et al.,* 1994).

In a scoping study, eight 20-week old male and female mice which had received an extract of *Microcystis aeruginosa* in their drinking water since weaning were mated; extract exposure continued throughout pregnancy. Examination of litters showed no effects on weight, number or sex ratio of offspring, compared with control litters. However, there was some evidence of hippocampal injury and reduced brain size in 7

out of 73 of the five-day old young from parents who had received the *Microcystis* extract, and in none of the controls (Falconer *et al.,* 1988).

Carcinogenicity

Microcystin administered alone. In a recent study, microcystin-LR, administered i.p. to mice, induced neoplastic liver nodules. Animals were given the toxin at 20 µg kg^{-1} bw, 100 times over 28 weeks. At autopsy, nodules up to 5 mm in diameter were observed in the livers of all exposed mice. Some mice were kept a further two months after cessation of dosing, and autopsy showed that liver nodules persisted (Ito *et al.,* 1997b). In the same study, mice orally administered microcystin-LR, at a dose level of 80 µg kg^{-1} bw, 100 times over the same time period showed no evidence of liver injury or nodule formation. Previous work had shown that microcystin-LR given 20 times i.p. to mice, at 25 µg kg^{-1} bw, over 10 weeks did not induce liver nodules (Ohta *et al.,* 1994).

Microcystin interaction with tumour initiators. It is generally understood that some chemicals (usually DNA-damaging) can initiate the cancer process while other classes of chemicals are able to promote the appearance of cancer after initiation has occurred (Boutwell, 1974; Yamasaki, 1988; Fitzgerald and Yamasaki, 1990). Microcystins have been tested for tumour promoting activity.

In vivo animal experiments. There has been some evidence of tumour promotion in animal studies (see also section on mechanism of action). In a modified, two-stage carcinogenesis mouse skin bioassay, a single dose of 7,12-dimethyl benzanthracene (DMBA, 500 µg) was applied to the dorsal skin of groups of 20, three-month old, female mice. After one week, groups of treated and control mice received either water alone, water with *Microcystis* extract (80 mg microcystin-YM per litre; equivalent to 50 mg microcystin-LR per litre), or croton oil (as a tumour-promoting phorbol ester-containing positive control) applied to the skin (0.5 per cent in 0.1 ml acetone twice a week). At 52 days after DMBA exposure, there was a 1.6-fold increase in the number and a sevenfold increase in the mean weight of skin papillomas (a type of benign tumour) per mouse in animals given the cyanobacterial extract compared with those given water alone. It was concluded by the authors that oral consumption of *Microcystis* extract from drinking water may act directly or indirectly as a tumour promoter (Falconer, 1991). The mechanism of action is not clear because microcystin-LR, at least, has difficulty penetrating epidermal cells (Matsushima *et al.,* 1990). In this study, there was considerable liver damage, which could affect the interpretation of these findings (Falconer, 1991). In a short-term two-stage carcinogenicity bioassay, groups of 10-19 seven-week old male F344 rats were initiated by i.p. injection with 200 mg kg^{-1} bw diethylnitrosamine (DEN), followed by partial hepatectomy (performed to stimulate cell division and thus increase the sensitivity of the assay). In two separate experiments, twice-weekly i.p. doses of 1 and 10 µg kg^{-1} microcystin-LR and of 10,25 and 50 µg kg^{-1} microcystin-LR were then administered during eight weeks and this resulted in a dose-dependent increase in the number (up to threefold) and area (up to 11-fold) of GST-P-positive liver foci (GST-P is the placental form of glutathione-S transferase, a biomarker for preneoplastic changes in liver (Sato *et al.,* 1984)). The i.p. NOAEL in this study was 1 µg kg^{-1} (Nishiwaki-Matsushima *et al.,* 1992). Microcystin-LR given without DEN initiation showed no induction of liver foci. Macroscopic nodules, histologically diagnosed as neoplastic nodules, were seen in this study when DEN was followed by microcystin-LR

at 10 µg kg^{-1} (before) and 50 µg kg^{-1} (after) partial hepatectomy (Nishiwaki-Matsushima *et al.,* 1992).

In another tumour initiation and promotion assay aimed at evaluating possible tumour promoting effects in the upper small intestine, two doses of 40 mg kg^{-1} bw of the initiator N-methyl-N-nitrosourea (MNU) were orally administered to C57 black mice, one week apart, followed by drinking water containing various levels of *Microcystis* extracts, estimated to be equivalent to 0, 1.2 or 4.2 mg kg^{-1} bw per day of microcystins for up to 22 weeks. Time to 50 per cent survival was about 17 weeks in controls and 15 weeks in extract-exposed mice. No primary liver tumours were seen in any group and there was no evidence of microcystin-induced promotion of lymphoid or duodenal tumours (Falconer and Humpage, 1996).

In vitro studies on interaction of microcystins and tumour initiators are discussed in the section on *in vitro* studies below.

Genotoxicity

No mutagenic response has been observed for purified toxins (exact nature not determined) derived from *Microcystis* in the Ames *Salmonella* assay (strains TA98, TA100 and TA102) with or without S9 activation. The *Bacillus subtilis* multigene sporulation test was also negative with regard to mutagenicity using both the 168 and hcr-9 strains (Repavich *et al.,* 1990). An earlier study with purified *Microcystis* extract also elicited a negative response in the Ames test (Runnegar and Falconer, 1982).

The purified toxins from a *Microcystis* species tested against human lymphocytes suggested that the toxins may be clastogenic as indicated by dose-related increases in chromosomal breakage (Repavich *et al.,* 1990).

In vitro studies

Extracts from *Microcystis aeruginosa* blooms containing mainly microcystins (no analytical data presented) from a lake in China were tested in a two-stage cell-transformation assay using Syrian hamster embryo (SHE) cells. In this assay, the microcystin extract had no initiating activity when followed by the tumour promoter TPA (12-*O*-tetradecanoyl phorbol 13-acetate). With methylcholanthrene as the initiator (0.5 µg ml^{-1}), followed by bloom extract, a dose-related (up to sevenfold) increase in transformation frequency was observed (Wang and Zhu, 1996).

Primary hepatocyte cultures in the presence of picomolar and nanomolar concentrations of microcystin-LR showed selective cell toxicity and selective cell proliferation depending on the ploidy (chromosome copy number) of the cells (Humpage and Falconer, 1999).

Mechanism of action

Microcystin-LR was found to be a potent inhibitor of eukaryotic protein serine/threonine phosphatases 1 and 2A both *in vitro* (Honkanen *et al.,* 1990; MacKintosh *et al.,* 1990) and *in vivo* (Runnegar *et al.,* 1993), and this effect has become the basis of one of the bioassays to detect its presence. Substances that inhibit these protein phosphatases are considered to be non-phorbol ester (TPA)-type tumour promoters. Other substances that

act in a similar way to microcystins are okadaic acid, nodularin, tautomycin and calyculin (for a review see Fujiki and Suganuma, 1993). The protein phosphatases serve an important regulatory role to maintain homeostasis in the cell (Cohen, 1989). Protein phosphatase inhibition results in a shift in the balance towards higher phosphorylation of target proteins, such as tumour suppresser proteins. This is a major post-translational modification which can result in excessive signalling and may lead towards cell proliferation, cell transformation and tumour promotion (Fujiki and Suganuma, 1993). In liver cells, intermediate filaments of the cytoskeleton are hyperphosphorylated leading to cellular disruption (Falconer and Yeung, 1992). In monolayer cell cultures this leads to detachment from adjacent cells, and involves actin filament contraction (Hooser *et al.,* 1991, Ghosh *et al.,* 1995). The inhibition of protein phosphatase 2A by microcystin-LR can be effectively reversed in the presence of polyclonal antibodies against microcystin-LR (Lin and Chu, 1994b); such antibodies can also protect *in vivo* against microcystin-LR toxicity as shown with i.p. co-administration studies in mice (Nagata *et al.,* 1995).

The implications of protein phosphatase inhibition in humans, due to low level chronic exposure to microcystins, are not known.

Additional study requirements

Further short-term studies are needed to understand better the genotoxic and tumour promoting potential of microcystins. Such studies would also be useful as a preliminary to the design of appropriate chronic or other *in vivo* studies to assess their carcinogenic potential. Regarding the possible role of microcystins in tumour promotion, further studies are especially needed to establish a dose-effect relationship for nodule induction with microcystin alone using various routes of administration.

Derivation of a tolerable daily intake (TDI)

Most of the relevant data on microcystin toxicity have come from either reported human injury related to consumption of drinking water containing cyanobacteria (see section 4.2) or from limited work with experimental animals (see above). Although the cyanotoxins have not been reviewed by the International Agency for Research on Cancer (IARC), their standard evaluation procedures (IARC, 1995) lead to the conclusion that, at present, the human evidence for microcystin carcinogenicity is inadequate and the animal evidence is limited. In such instances, the current practice for deriving a TDI is to use a LOAEL or NOAEL divided by appropriate safety or uncertainty factors as described in the Addendum to the *Guidelines for Drinking Water Quality* (WHO, 1998).

A 13-week mouse oral (by gavage) study with pure microcystin-LR has been considered the most suitable for the derivation of a guideline value for microcystin-LR. In a study by Fawell *et al.* (1994) a NOAEL of 40 µg kg^{-1} bw per day was determined, based on liver histopathology and serum enzyme level changes. By applying a total uncertainty factor of 1,000 (10 for intra-species variability, 10 for inter-species variability and 10 for limitations in the database, in particular a lack of data on chronic toxicity and carcinogenicity), a provisional TDI of 0.04 µg kg^{-1} bw per day has been determined for microcystin-LR. This TDI is supported by the results of a 44-day pig study, in which pigs were given *Microcystis* extract in their drinking water, resulting in a LOAEL of 100 µg microcystin-LR equivalents per kg bw per day (Falconer *et al.,* 1994; see above). To this LOAEL an overall uncertainty factor of 1,500 was applied, arrived at by using 10 for

intra-species variability, 3 rather than 10 for inter-species variability (because pigs physiologically resemble humans more closely than rodents), 5 for extrapolating from a LOAEL to a NOAEL (10 was considered inappropriate due to the low incidence of effects in the lowest dose group and the deduced shape of the dose-response curve) and 10 for the less-than-lifetime exposure. This resulted in a provisional TDI of 0.067 µg kg^{-1} bw per day. The lower of these two values, 0.04 µg kg^{-1} bw per day, has been used in deriving a provisional guideline value (see Chapter 5).

4.2.2 Nodularin

Compared with *Microcystis* and microcystins, there have been fewer reported incidents of human and livestock disease involving *Nodularia* spp. and nodularin (see section 4.1).

Experimental animal data

The toxicity and liver pathology induced by nodularin is similar to that caused by microcystins, with hepatocyte necrosis and haemorrhagic diathesis (Runnegar *et al.*, 1988). Nodularin inhibits protein phosphatases 1 and 2A with the same potency as microcystin-LR (Yoshizawa *et al.,* 1990). In a two-stage liver carcinogenesis experiment in male F344 rats initiated with DEN and without partial hepatectomy, repeated i.p. administration of 10 µg nodularin per kg bw induced GST-P-positive foci more effectively than microcystin-LR. Nodularin alone also induced some foci. In addition, nodularin was capable of activating several proto-oncogenes of the *fos* and *jun* family, which are considered to play a role in tumour promotion (Ohta *et al.,* 1994).

Derivation of a tolerable daily intake

There are no studies available that use oral administration of nodularin, thus a TDI cannot be set. Nevertheless, several experimental studies cited above indicate that nodularin has similar toxicity to microcystin-LR. It may be appropriate, therefore, to consider nodularin in an analogous fashion to microcystins in terms of human health risk assessment.

4.2.3 Anatoxin-a

The potent neurotoxin, anatoxin-a, from *Anabaena flos-aquae* has frequently been involved in animal and wildfowl poisoning (Ressom *et al.,* 1994) (Table 4.1).

Experimental studies

Anatoxin-a is a nicotinic (cholinergic) agonist that binds to neuronal nicotinic acetylcholine receptors. It has been suggested that the activation of presynaptic nicotinic acetylcholine receptors by anatoxin-a results in an influx of Na^{+}, producing sufficient local depolarisation to open voltage sensitive Ca^{++} and Na^{+} channels. The latter may then amplify the response, activating further Ca^{++} channels (Soliakov *et al.,* 1995). As a result of this depolarisation there is a block of further electrical transmission, and at sufficiently high doses this can lead to paralysis, asphyxiation and death (Carmichael *et al.,* 1975; Carmichael, 1997). Anatoxin-a is more potent than nicotine or acetylcholine in evoking type 1A or type 2 current responses in rat hippocampal neurones (Alkondon and Albuquerque, 1995), and it is more potent than nicotine in its ability to evoke the

secretion of endogenous catecholamines from bovine adrenal chromaffin cells through their neuronal-type nicotinic receptors (Molloy *et al.,* 1995). Similar to nicotine, anatoxin-a was more potent than noradrenaline in releasing dopamine from striatal nerve terminals from rat superfused hippocampal synaptosomes (Clarke and Reuben, 1996). *In vivo* studies in the rat showed that the toxin stimulates the sympathetic system through the release of catecholamines from nerve endings (Dube *et al.,* 1996).

Acute toxicity

In mice, the i.p. LD_{10} (lowest dose causing death) of anatoxin-a was 250 µg kg^{-1} bw (Stevens and Krieger, 1989) and the i.p. LD_{50} of anatoxin-a is 375 µg kg^{-1} bw (Fitzgeorge *et al.,* 1994). The i.v. LD_{50} is less than 100 µg kg^{-1} bw (Fawell and James, 1994). The oral LD_{50} for anatoxin-a is greater than 5,000 µg kg^{-1} bw, and the intranasal LD_{50} in mice is 2,000 µg kg^{-1} bw (Fitzgeorge *et al.,* 1994).

Subacute toxicity

In two studies, anatoxin-a was administered to rats orally in the drinking water for 54 days at 0.51 or 5.1 ppm (equivalent to 51 and 510 µg kg^{-1} bw per day, respectively) or by i.p. injection at 16 µg per rat per day for 21 days (equivalent to 80 µg kg^{-1} bw per day; Astrachan *et al.,* 1980). No toxicity was observed (no deaths due to treatment; no significant changes in body weight gain, haematology or clinical chemistry).

In a dose range-finding study for a four-week oral toxicity experiment, groups of 2 male and 2 female mice were administered anatoxin-a hydro-chloride by gavage at dose levels of 1,500, 7,500 or 15,000 µg kg^{-1} per day for five days. All mice at the highest dose, and one female at the intermediate dose, died within three minutes of their first dose. Males at the intermediate dose were hyperactive after their third dose. All other animals survived, and no treatment-related signs of clinical toxicity, or changes in body weight were observed. No treatment-related changes were observed at necropsy (Fawell and James, 1994).

In a 4-week study, groups of 10 male and female mice were administered anatoxin-a hydrochloride by gavage at dose levels of 0, 120, 600 or 3,000 µg kg^{-1} bw. In the course of the experiment, one male receiving 600 µg kg^{-1} bw per day and one female receiving 3,000 µg kg^{-1} per day died. No signs of clinical toxicity or histopathological abnormalities were observed in these two animals, and no cause of death could be identified. It was concluded by the authors that the possibility that these deaths were treatment-related could not be excluded, although this was considered to be unlikely. There were no other treatment-related findings. Although the NOAEL for anatoxin-a hydro-chloride in this study was determined to be 120 µg kg^{-1} bw per day, equivalent to 100 µg kg^{-1} bw per day of anatoxin-a, the toxicological database was considered to be inadequate to use these results for setting a formal TDI.

Reproductive effects

Doses of 200 or 125 µg kg^{-1} bw anatoxin-a were given i.p. to hamsters one or three times per day, respectively, at days 12 to 14 of pregnancy (after organo-genesis), and the dams were sacrificed at day 15. The treatment given three times per day caused foetal malformation (hydrocephaly) in all foetuses in one of 10 litters, and stunted growth in

almost all litters; treatment given once per day resulted in stunted growth. No maternal toxicity was observed (Astrachan *et al*., 1980).

Groups of 10 and 12 time-mated female mice were given anatoxin-a hydrochloride by gavage at 0 or 3,000 µg kg^{-1} bw per day (equivalent to 2,460 µg kg^{-1} bw per day anatoxin-a) respectively, on days 6 to 15 of pregnancy. The anatoxin-a dose was considered the maximum dose that could be tolerated. No treatment-related effects were observed in the dams or offspring, although there was a slight decrease in foetal weight compared with controls (Fawell and James, 1994). No data on the genotoxic potential of anatoxin-a are available.

Derivation of a tolerable daily intake

Anatoxin-a is a potent acute neurotoxin. The available data indicate that significant concern with regard to chronic toxicity (Fawell and James, 1994) is unlikely, but the data base is insufficient for derivation of a TDI.

4.2.4 Homoanatoxin-a

Homoanatoxin-a is a potent neuromuscular blocking agent with an i.p. LD_{50} in mice of 250 µg kg^{-1} bw. Toxicosis in the lethal dose range leads to severe body paralysis, convulsions and death by respiratory arrest in 7-12 minutes. Experiments with rat phrenic nerve hemidiaphragm preparations demonstrated that the physiological effects of homoanatoxin-a are related to those observed for *d*-tubocurarine (Skulberg *et al.* 1992). Recent studies have shown that homoanatoxin-a enhances the influx of Ca^{++} ions in the cholinergic nerve terminals (Aas *et al.,* 1996). No TDI can be derived.

4.2.5 Anatoxin-a(S)

Anatoxin-a(S) is an organophosphate produced by the cyanobacteria *Anabaena flos-aquae* and *A. lemmermannii* (see Chapter 3). This toxin blocks acetylcholinesterase activity in a manner analogous to organophosphate insecticides. The i.p. LD_{50} in mice is 20 µg kg^{-1} bw with muscle weakness, respiratory distress (dyspnea) and convulsions (effect on seizure threshold) preceding death (Mahmood and Carmichael, 1986a; Matsunaga *et al.* 1989). There are no oral toxicity studies for this toxin.

Derivation of a tolerable daily intake

A lack of adequate experimental data or human data precludes the derivation of a TDI.

4.2.6 Saxitoxins

Effects in humans

Saxitoxin and some of its analogues are produced by *Anabaena circinalis* in Australian freshwaters and *Aphanizomenon flos-aquae* in the USA (Sawyer *et al.,* 1968; Mahmood and Carmichael, 1986b; Humpage *et al.,* 1994). The saxitoxin group has been the cause of paralytic shellfish poisoning (PSP) in people. Several species of dinoflagellates produce PSP toxins that accumulate in molluscs which filter-feed on these organisms.

People who have consumed shellfish containing high levels of PSP toxins may suffer from this acute illness. The signs and symptoms of PSP in humans may range from a slight tingling and numbness about the lips to complete paralysis and death from respiratory failure (Meyer *et al.,* 1928; Medcof *et al.,* 1947; McFarren *et al.,* 1958). More than 1,000 cases of PSP have been reported since the early 1900s in North and Central America and there have been 109 deaths. In a recent epidemic in Guatemala, the mortality rate in children was 50 per cent, while for adults it was 5 per cent (Rodrigue *et al.* 1990; for a review see Kao, 1993). No PSP-like illnesses have been reported in humans from the consumption of drinking water containing saxitoxins, although there have been fatalities in sheep after ingesting a PSP toxin-containing bloom of *A. circinalis* (Negri *et al.,* 1995).

Experimental studies

Mode of action. Of the various PSP toxins, only saxitoxin has been studied in detail for pharmacological effects, partly because the other toxins are usually not available in sufficient quantities for such studies. Nearly all the systemic actions of saxitoxin can be explained by its pharmacological effect on nerve axon membranes. This involves a wide spread blockage of sodium ion channels of the excitable membranes of nerves, thereby affecting (partially or completely, depending on dose) impulse generation in peripheral nerves and skeletal muscles (Catterall, 1980). This results in generalised nerve dysfunction as measured by electromyography (Easthaugh and Shepherd, 1989). In mammals, these effects lead to paralysis, respiratory depression and respiratory failure. Direct cardiac effects are usually minimal (see review by Kao, 1993).

Metabolism. No data are available on PSP toxin absorption, distribution, metabolism and excretion.

Table 4.2 LD_{50} values following a single dose of PSP toxin extract in the mouse in relation to the route of administration

Route of administration	**LD_{50}[1] (µg PSP kg^{-1} body weight)**	
	Male	**Female**
Intravenous	3.4 (3.2-3.6)	
Intraperitoneal	10.0 (9.7-10.5)	8.0 (7.6-8.6)
Oral	263.0 (251-267)	

PSP Paralytic shellfish poison

Source: IPCS, 1984; adapted from Wiberg and Stephenson, 1960

[1] The 95% confidence limits are given in parentheses

Acute toxicity

The principle of the standardised mouse bioassay developed by Sommer and Meyer (1937) is measurement of time to death after i.p. injection. In that study, the authors suggested that signs characteristic of PSP, such as dyspnea, could be observed after the i.p. administration of toxin. Hypotensive effects have been observed to accompany

the respiratory depression, implicating both central and peripheral nervous system actions (Watts *et al.,* 1966).

Acute toxicity studies have been conducted in several species with extracts containing PSP toxins obtained from the Alaskan butter clam (Genenah and Shimizu, 1981). Using this preparation, Wiberg and Stephenson (1960) determined the LD_{50} values for male mice, using three routes of administration (i.v., i.p. and oral). In addition, the i.p. LD_{50} was determined in female mice (Table 4.2). The PSP toxin extract was much less toxic when administered by the oral route than by the i.v. or i.p. routes (Table 4.2). Increasing the pH of the injection medium (> 3.8) or the addition of sodium ions affected i.p. toxicity, while the addition of sodium ions did not influence oral or i.v. toxicity. Similar comparative LD_{50}s for different routes of administration were obtained in rats; in addition new-born rats were about tenfold more susceptible than adults after oral administration (Watts *et al.,* 1966) (Table 4.3). For a number of animal species tested, the oral LD_{50} for PSP toxin ranged from 128 µg kg^{-1} bw PSP toxin in guinea pigs to 420 µg kg^{-1} bw in mice (IPCS, 1984).

Table 4.3 LD_{50} values following oral or intraperitoneal administration of a single dose of PSP toxin extract to rats of different ages

Age	LD_{50}[1] (µg PSP kg^{-1} body weight)	
	Oral	Intraperitoneal
New-born (24 hours)	64 (51-80)	5.5 (4.7-6.5)
Weanling (21 days)	270 (204-356)	8.3 (7.7-9.0)
Adult (60-70 days)	531 (490-576)	10.0 (8.5-11.8)

[1] The 95% confidence limits are given in parentheses

Source: IPCS, 1984; adapted from Watts *et al.,* 1966

Prior exposure to non-lethal doses of PSP toxin seems to lower the susceptibility of rats to lethal doses of PSP toxin. In a study using Sprague-Dawley rats (sex not indicated), the oral LD_{50} value for the purified PSP extract was determined (McFarren *et al.,* 1958). One group of rats was given a non-lethal dose of PSP toxin (about one-third of the LD_{50}) 14 days before the test. The LD_{50} for the pre-treated rats was about 50 per cent higher than that for untreated rats. This finding corroborates the fact noted by Prakash *et al.* (1971) that fishermen who habitually eat shellfish containing low levels of PSP toxins appear to be less susceptible to developing PSP.

There is a wide range in i.p. toxicity of the various PSP toxins (i.e. saxitoxin, neosaxitoxin, the gonyautoxins and C toxins) as tested following the Association of Official Analytical Chemists (AOAC) mouse bioassay (AOAC, 1984). Potencies of these toxins are usually expressed in mouse units (MU) per µmol of toxin. Saxitoxin is one of the most toxic of the PSP toxins (2,483 MU per µmol) and the C toxins are the least toxic (15-143 MU per µmol) (Oshima, 1995). Potencies may also be expressed as saxitoxin equivalents. Inter-conversions during storage, cooking or digestion may modify the i.p. and oral toxicity (Humpage *et al.,* 1994; see also section 3.4.2). No subchronic or chronic animal studies with PSP toxins are available.

Derivation of a tolerable daily intake
The animal toxicity data for the saxitoxins are inadequate to set a TDI.

4.2.7 Cylindrospermopsin

This cyanotoxin was initially isolated from a culture of *Cylindrospermopsis raciborskii* obtained from a water supply reservoir in tropical northern Australia. The organism was identified as a result of an outbreak of acute hepato-enteritis and renal damage among an Aboriginal population on Palm Island, off the coast of North Queensland (Hawkins *et al.,* 1985) (see Box 4.2). Intraperitoneal injection of the lysed organism to mice resulted in widespread and progressive tissue injury, with cell necrosis in the liver, kidneys, adrenals, lung, heart, spleen and thymus (Hawkins *et al.,* 1985, 1997). In mice, the i.p. LD_{50} at 24 hours was 52 mg dry weight (dw) of cells per kg bw, equivalent to 300 µg kg^{-1} bw of cylindrospermopsin, whereas the i.p. LD_{50} at seven days was approximately 32 mg cells per kg bw, equivalent to 180 µg kg^{-1} bw of toxin (Hawkins *et al.,* 1997). Administration of the pure toxin to mice showed this delayed toxicity more clearly, with the 24-hour i.p. LD_{50} being 2,100 µg kg^{-1} bw and the 5-6 day i.p. LD_{50} being 200 µg kg^{-1} bw (Ohtani *et al.,* 1992). *In vitro* studies with pure cylindrospermopsin have shown that it inhibits glutathione synthesis and protein synthesis in general (Runnegar *et al.,* 1994, 1995; Terao *et al.,* 1994). In mouse liver after i.p. administration, major changes were seen in hepatocytes, with progressive proliferation of the smooth endoplasmic reticulum and accumulation of lipid over five days (Terao *et al.,* 1994). No data on the oral toxicity of pure cylindrospermopsin are available, but studies with aqueous extracts of *Cylindrospermopsis* provide a preliminary indication. After administering to mice a single oral dose of an aqueous extract of freeze-dried *Cylindrospermopsis* cells, a median lethal dose in the range of 4.4 to 6.9 mg kg^{-1} toxin equivalent was determined (Seawright *et al.,* 1999). Because the i.p. LD_{50} is 0.2 mg kg^{-1} and the LD_{100} is likely to be double that, the oral toxicity appears to be over tenfold lower than i.p. toxicity.

At present it is not known if cylindrospermopsin is the only toxin in *Cylindrospermopsis,* because the major kidney damage seen on i.p. dosing of some toxic extracts is not similarly observed after administration of pure toxin.

Derivation of a tolerable daily intake
On the basis of present data it is not possible to derive a TDI.

4.2.8 Other cyanotoxins produced by marine cyanobacteria

Swimmers off Hawaii and Okinawa who have come into contact with the marine cyanobacterium *Lyngbya majuscula* have contracted acute dermatitis, causing "swimmers' itch" (Moikeha and Chu, 1971; Hashimoto *et al.,* 1976). The effect is a cutaneous inflammation with signs of erythema, blisters and desquamation within 12 hours of exposure to the cyanobacterium. The inflammatory activity of *Lyngbya* is caused by aplysiatoxins and debromoaplysiatoxin (Mynderse *et al.,* 1977). These toxins are potent animal skin tumour promoters and protein kinase C activators (Fujiki *et al.,* 1990). Aplysiatoxins are toxic to mice at a minimum lethal dose of about 0.3 mg kg^{-1} (Moore, 1977). Debromoaplysiatoxin, along with other toxic compounds, has also been isolated from other Oscillatoriaceae such as *Schizothrix calcicola* and *Oscillatoria nigroviridis.* The chemically different lyngbyatoxin-a (Cardellina *et al.,* 1979), found in another shallow water strain of marine Lyngbya *majuscula, has* caused dermatitis and

severe oral and gastrointestinal inflammation (Moore et al., *1993).* Its toxicity to mouse corresponds to aplysiatoxins (Moore, 1977) and it has skin tumour promoting activity similar to the well known tumour promoter, TPA (Fujiki *et al.,* 1981, 1984). *Lyngbya majuscula* sometimes grows epiphytically on edible algae, such as *Acanthophora spicifera* which is eaten in Indonesia and in the Philippines. Cyanobacteria have also been suspected to be a source of toxins in the ciguatera food chain that may lead to human poisoning (Hahn and Capra, 1992; Endean *et al*., 1993).

4.2.9 Cyanobacterial lipopolysaccharides

Lipopolysaccharides, cell wall components of gram-negative bacteria, are pyrogenic (fever-causing agents) and toxic (Weckesser and Drews, 1979). In Sewickley, Pennsylvania, an outbreak of gastro-enteritis is suspected to have been caused by cyanobacterial LPS (Lippy and Erb, 1976; Keleti *et al.,* 1979). The few studies carried out on cyanobacterial LPS indicate that they are less toxic than the LPS of other bacteria such as *Salmonella* (Keleti and Sykora, 1982; Raziuddin *et al.,* 1983). Lack of axenic cyanobacterial strains has previously hindered detailed studies on structures and toxicities of cyanobacterial LPS.

4.2.10 Other bioactive compounds in cyanobacteria

Cyanobacteria produce a wide variety of bioactive compounds in addition to the cyanotoxins described in this chapter. They include anti-tumour (cytotoxic), anti-viral, and anti-fungal compounds, antibiotics and protease inhibitors (Moore *et al.,* 1996; Namikoshi and Rinehart, 1996). Further screening of these biomedically interesting compounds is underway and is likely to lead to the discovery of many new compounds in the future, some of which may be toxic. Bioassays of cyanobacterial cell extracts have often revealed a higher toxicity than expected from the content of known toxins in the extract.

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Chapter 5. SAFE LEVELS AND SAFE PRACTICES

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Throughout their lifespan, humans are exposed to many chemical substances, both beneficial and harmful. It is not possible to eliminate exposure to all toxins in our environment. Of the harmful chemicals, some are anthropogenic and others occur naturally. Although cyanotoxins occur naturally, their presence and abundance are, to a large extent, influenced and increased by human action. The extent to which cyanotoxins pose a human health risk depends on human exposure to these toxins. Reducing human exposure to cyanotoxins may be achieved either through preventing the occurrence of hazardous cell densities of toxic cyanobacteria, or by placing barriers (such as drinking water treatment or bathing restrictions) that would reduce human exposure to the cyanobacterial hazard.

The purpose of this chapter is to describe how "safe" levels, such as guideline values, are derived and how "safe practices" are likely to assist in minimising unsafe human exposures.

Establishing and controlling safe practices and procedures to reduce or prevent microbiological problems are common in the food industry, where the process is known as Hazard Analysis of Critical Control Points (HACCP). There is also a long history of similar activities in relation to drinking water supply, where the process is referred to as "sanitary inspection". This type of approach is also starting to be applied in dealing with some natural toxins, such as fungal toxins (mycotoxins). Safe practice guidelines can assist in reducing exposure, even for substances for which a guideline value cannot be set (for example because of inadequate scientific data), or where implementation of a guideline value is very difficult (for example because of analytical or sampling problems).

Major routes of exposure to cyanotoxins include oral and dermal routes through drinking water and recreational water use. However, the very limited available information suggests that inhalation in aerosols (potentially possible while showering, water-skiing or during certain work practices) may be an equally important route given that the toxicity of microcystin following intranasal instillation approaches that for intraperitoneal (i.p.) injection (Fitzgeorge *et al.,* 1994). In some countries, cyanobacterial dietary supplements may constitute a major route of oral intake for a small sub-population, if the cyanotoxin levels in the supplements are not controlled. A specific route (intravenous) affecting a susceptible sub-population may occur in dialysis clinics (see Box 4.4).

The extent or duration of exposure throughout the year is shorter if water resources are populated by cyanobacteria at specific seasons. In temperate climates, water bodies dominated by the genus *Microcystis* usually exhibit a bloom season of 3-5 months, whereas in milder climates, such as in Australia, some of South America and South Africa, this genus may bloom for 6-10 months. Other taxa, such as *Planktothrix agardhii,* may show perennial mass development even in temperate climates. This is the case in

some lowland regions of north-western Europe, especially in years with mild winters without ice cover (see Figure 2.7).

5.1 Tolerable exposures

5.1.1 Tolerable daily intake

The repair mechanisms of the body are continuously active and ensure that cells and tissues are replaced as they are damaged by normal "wear and tear", as well as by external factors such as toxic chemicals. The tolerable daily intake (TDI) is the amount of a potentially harmful substance that can be consumed daily over a lifetime with negligible risk of adverse health effects (see Chapter 4).

Exposure to several harmful chemicals or conditions simultaneously may lead to potentiation, or to antagonistic interaction. Potentiation results in effects that are larger than the responses caused by the individual chemicals or conditions by themselves and this effect has been observed with cyanotoxins, as noted in section 4.2. However, there is experimental evidence that potentiation is unlikely to occur at low levels of exposure, such as the TDI. For cyanotoxins, the animal and human toxicity data are incomplete for the majority of the compounds. The available data have been reviewed in Chapter 4 and allow only the derivation of a provisional TDI for microcystin-LR (see section 4.2.1). Clearly, this does not imply that microcystin-LR is the only microcystin that is toxic, or that other cyanotoxins are less harmful. It merely reflects the lack of toxicological data.

5.1.2 WHO guideline values for drinking water quality

The World Health Organization (WHO) *Guidelines for Drinking-water Quality* (WHO, 1993, 1996) represent a scientific consensus, based on very broad international participation, of the health risks presented by microbes and chemicals in drinking water. This scientific consensus is used to derive "guideline values" which are associated with guidance on monitoring and management aspects. The guideline values themselves are based upon a number of assumptions that may be amended locally or nationally according to specific circumstances (Box 5.1).

A guideline value for lifetime consumption of a chemical contaminant of drinking water is usually calculated by applying the derived TDI to a typical daily water intake in litres *(L)* by an individual of a given body weight *(bw).* The proportion *(P)* of total daily intake of the contaminant which is ingested from the drinking water needs to be considered, because some intake may come from food or by inhalation from air (WHO, 1996). The guideline value is then calculated as:

$$\text{Guideline value} = \frac{\text{TDI} \times bw \times P}{L}$$

A provisional guideline value of 1.0 µg l^{-1} has been adopted by WHO for microcystin-LR (WHO, 1998). In order to derive this, an average adult body weight of 60 kg and an average water intake for adults of 2 litres per day was used, as is standard practice. The provisional TDI of 0.04 µg kg^{-1} bw per day (section 4.2) was used and the proportion of the TDI allocated to drinking water (*P*) was assumed to be 0.8. The resulting

concentration (0.96 µg l^{-1}) was rounded to 1.0 µg l^{-1}. In water containing cyanobacterial cells, this guideline value should be applied to the total cell-bound and extracellular microcystins.

There were insufficient data to derive a guideline value for cyanotoxins other than microcystin-LR. For saxitoxins (STX), the guideline of 80 µg STX equivalents per 100 g (this is the conventional way of expressing this value) shellfish, used in North America for closing shellfish growing areas for harvesting, may provide preliminary orientation (IPCS, 1984).

Exceeding the provisional guideline value of 1 µg l^{-1} for microcystin-LR can be tolerated (see Box 5.1 for an explanation of handling short-term deviations). This may occur if, for example, discontinuation of exposure is expected in the near future due to implementation of measures to eliminate cyanotoxins from drinking water or cyanobacteria from the water resource. In such instances of guideline exceedances, it may be appropriate that information is communicated to the public, and especially to particularly susceptible sub-populations (such as patients with liver disease, parents of infants, dialysis centres or dialysis patients).

5.1.3 Assessment of microcystins other than microcystin-LR in relation to the guideline value

There are more than 60 different analogues of microcystin (see section 3.1.1) and, in many regions, microcystin-LR is not the most commonly occurring microcystin. It may not even be amongst the microcystins detected. The expression and interpretation of quantitative results from analysis or assay for these toxins in relation to the WHO guideline value for microcystin-LR may, therefore, be problematic.

Box 5.1 Derivation of WHO guidelines for drinking water quality

The primary aim of the *Guidelines for Drinking-water Quality* (WHO, 1993) is the protection of public health. The Guidelines are intended to be used as the basis for the development of national standards that, if properly implemented, will ensure the safety of drinking water supplies through the elimination, or reduction to a minimum concentration, of constituents of water that are known to be hazardous to health. The guideline values recommended are not mandatory limits, they are intended to be used in the development of risk management strategies which may include national or regional standards in the context of local or national environmental, social, economic and cultural conditions.

The main reason for not promoting the adoption of international standards for drinking water quality is the advantage provided by the use of a risk-benefit approach (qualitative or quantitative) to the establishment of national standards or regulations. This approach should lead to standards and regulations that can be readily implemented and enforced and which ensure the use of available financial, technical and institutional resources for maximum public health benefit.

The judgement of safety, or what is a tolerable level of risk in certain circumstances, is a matter in which society as a whole has a role to play. It should be recalled that water is essential to sustain life and the Guidelines therefore emphasise the importance of securing water supply. They also indicate that protection of supplies from contamination is almost invariably the best method of ensuring safe drinking water and is to be preferred to treating a contaminated water supply to

render it suitable for consumption.

A principle of the Guidelines is that the potential consequences of microbial contamination are such that its control must always be of paramount importance and must never be compromised. The health risk due to toxic chemicals in drinking water differs from that caused by microbiological contaminants. There are few chemical constituents of water that can lead to acute health problems except through massive accidental contamination of a supply. Moreover, experience shows that, in such incidents, the water usually becomes undrinkable because of unacceptable taste, odour and appearance. The fact that chemical contaminants are not normally associated with acute effects places them in a lower priority category than microbial contaminants, the effects of which may be acute and widespread.

It is important that guideline values are both practical and feasible to implement as well as protective of public health. Guideline values are not set at concentrations lower than the detection limits achievable under routine laboratory operating conditions. Moreover, guideline values are recommended only when control techniques are available to remove or reduce the concentration of the contaminant to the desired level. In some instances provisional guideline values are set for constituents for which there is some evidence of a potential hazard but where the available information on health effects is limited. Provisional guideline values are also set for substances for which the calculated guideline value would be: (i) below the practical quantification level; or (ii) below the level that can be achieved through practical treatment methods. Finally, provisional guideline values are set for substances when it is likely that guideline values will be exceeded as a result of disinfection procedures.

The first edition of *Guidelines for Drinking Water Quality was* published by WHO in 1984 and 1985. The second editions of the three volumes of the guidelines were published in 1993, 1996 and 1997; and the Addenda to the second edition were published in 1997 and 1998.

- A guideline value represents the concentration of a constituent that does not result in any significant risk to the health of the consumer over a lifetime of consumption.

- The quality of water defined by the *Guidelines for Drinking-water Quality* is such that it is suitable for human consumption and for all usual domestic purposes, including personal hygiene. However, water of a higher quality may be required for some special purposes, such as renal dialysis.

- When a guideline value is exceeded, this should be a signal: (i) to investigate the cause with a view to taking remedial action; and (ii) to consult with, and seek advice from, the authority responsible for public health.

- Although the guideline values describe a quality of water that is acceptable for lifelong consumption, the establishment of these guideline values should not be regarded as implying that the quality of drinking water may be degraded to the recommended level. Indeed, a continuous effort should be made to maintain drinking water quality at the highest possible level.

- Short-term deviations above the guideline values do not necessarily mean that the water is unsuitable for consumption. The amount by which, and the period for which, any guideline value can be exceeded without affecting public health depends upon the specific substance involved. It is recommended that when a guideline value is exceeded, the surveillance agency (usually the authority responsible for public health) should be consulted for advice on suitable action, taking into account the intake of the substance from sources other than drinking water (for chemical constituents), the toxicity of the substance, the likelihood and nature of any adverse effects, the

practicability of remedial measures, and similar factors.

• In developing national drinking water standards based on these guideline values, it will be necessary to take account of a variety of geographical, socio-economic, dietary and other conditions affecting potential exposure. This may lead to national standards that differ appreciably from the guideline values.

The most widely used analytical technique for the detection and quantification of individual microcystin variants for which quantitative reference materials are available is high pressure liquid chromatography (HPLC) (see section 13.4.1 and Box 13.5). For toxin variants where reference materials are available, HPLC results can be truly quantitative. For HPLC peaks that identify microcystin variants for which no standards are available but the result has been derived from a comparison with the concentration of the standard for microcystin-LR, the estimates can be expressed as "concentration equivalents" (CE) of microcystin-LR. It is therefore possible, using HPLC, to derive an approximate concentration of total microcystins in a sample expressed in terms of microcystin-LR CE. However, some of the observed HPLC peaks may relate to toxicologically inactive or weakly toxic variants. In terms of "toxicity equivalents" (TE) of microcystin-LR the actual toxicity of an unknown sample reported as microcystin-LR equivalents (CE) is likely to be less than the same concentration of pure microcystin-LR, because microcystin-LR is one of the most potent microcystins, at least in acute terms (see Table 3.2). Thus, the microcystin CE approach would result in a "worst case" estimate of toxic microcystin concentration.

A toxin concentration measured from a water or bloom sample by a bioassay (such as the mouse bioassay or the phosphatase bioassay) may give a toxin concentration value which can be expressed as microcystin-LR TE, provided the assay has been calibrated using microcystin-LR as the quantification standard (which is usually the case). This measure of total microcystin concentration expressed as microcystin-LR TE will be closer in actual (acute) toxicity to a solution of the same concentration of pure microcystin-LR. The position of enzyme assays, such as *in vitro* protein phosphatase inhibition assays, in this context is currently unclear. Differences in toxicity of different microcystins include variation in their ability to enter intact cells and in their capacity to inhibit protein phosphatases.

In practice, it is important to report quantitative analytical results for samples containing several microcystins with the above qualifications in mind and to indicate the method and assumptions used for deriving the quantitative value. If it is necessary to calculate quantitative values for total microcystins in a sample (and it is certainly important not to ignore microcystins other than microcystin-LR), then the value should be qualified as either microcystin-LR CE or TE. This information can be used (at best) as a preliminary orientation of the hazard presented by the sample in relation to the guideline value (see section 5.1.2).

Box 5.2 Epidemiological evidence for low-level cyanobacterial hazard

The epidemiological data of Pilotto *et al.* (1997) can be used as a basis for guideline derivation for acute, non-cumulative health effects which are more likely to result in discomfort rather than serious health outcomes. These data encompass the health effects on humans of intact cyanobacterial cells and colonies and thus include effects of currently unknown substances and bacteria associated with cyanobacterial colonies. The effects measured were eye irritation, ear irritation, skin rash, as well as vomiting, diarrhoea, cold/flu symptoms, mouth ulcers and fever. An elevated "Odds Ratio" for symptoms (3.44) was shown by the people who were in water contact for more than one hour, at above 5,000 cyanobacterial cells per ml. Similar Odds Ratios were seen for symptoms in people bathing in water with 5,000-20,000 cells per ml (2.71) and above 80,000 cells per ml (2.90).

5.1.4 Recreational water exposure

Three potential routes of exposure to cyanotoxins can be distinguished: direct contact of exposed parts of the body, including sensitive areas such as the ears, eyes, mouth and throat, and the areas covered by a bathing suit (Pilotto *et al.,* 1997); accidental swallowing (Turner *et al.,* 1990); and inhalation of water. Cases of illness from accidental swallowing and inhalation of *Microcystis* have been reported (see section 4.1) and provide direct evidence of harm to recreational water users from cyanobacterial blooms in the recreational water bodies.

Health effects observed in the prospective epidemiological study of Pilotto *et al.* (1997) occurred at low cyanobacterial cell densities. These related clearly to the cyanobacterial cell population, but not to the concentration of microcystins (see Box 5.2). Thus, this hazard appears to be due to additional, or other unidentified, cyanobacterial metabolites or compounds from associated bacteria, even at moderate levels of exposure.

Intake through oral ingestion or inhalation

Incorporation of toxins through swallowing, contact with nasal mucosa, or by inhalation are likely to be important routes of exposure to cyanotoxins during water-contact sports. Well-documented evidence from one animal experiment (Fitzgeorge *et al.,* 1994) and one case of multiple human illness (Turner *et al.,* 1990) indicates that inhalation and resorption through nasal and pharyngeal mucous membranes may present a high risk in water sports involving intensive submersion of the head (jumping from diving boards, sailboarding, canoe capsizing, competitive swimming) and inhalation of aerosols (water skiing).

Experimental results indicate a hazard of cumulative liver damage by repeated microcystin intake (Fitzgeorge *et al.,* 1994, see section 4.2.1), as can occur during a holiday with daily bathing at a recreational site with a high density of microcystin-containing cyanobacteria. Sub-acute liver injury is likely to go unnoticed, because signs of liver injury are only apparent after severe injury. In addition, the dose-response curve for liver injury from microcystins is relatively steep. There may be little evidence of acute liver damage when levels are close to those that could lead to severe acute toxicity, and

thus exposure at such levels is likely to be continued by people if they are uninformed of the risk (e.g. for consecutive days of a holiday or hot spell), thereby increasing the risk of cumulative liver damage.

Risks of ingestion are particularly high for children playing in shallow near-shore water where scums tend to accumulate. Because the hazard of microcystin uptake is directly related to the levels of toxins in the water (cell-bound as well as dissolved) and the volume of water ingested or inhaled, the range in these levels needs to be recognised in deriving guidelines for recreational water safety.

Direct contact

Allergic and toxic dermal reactions of varying severity are known from cyanobacteria as well as from freshwater algae, but have not been documented extensively. Bathing suits, and particularly diving suits, tend to aggravate such effects by accumulating cyanobacterial cells, thereby enhancing the disruption of cells and hence the liberation of cell contents onto the wearer's skin. Reports from the USA have recorded allergic reactions from recreational exposure, and the cyanobacterial pigment phycocyanin was shown to be responsible in one case (Cohen and Reif, 1953). Severe dermatitis, resembling skin bums, has been reported from marine bathing in the presence of cyanobacteria dislodged from rocks, particularly after storms in tropical seas (see section 4.2.8).

5.2 Safe practices

The placing of barriers that reduce exposure to a cyanotoxin hazard is an important measure and involves identifying "critical control points" and implementing measures for their monitoring and control. In the case of cyanobacteria, critical control points might include, for example, noting the tendency of a water body to develop blooms, scums or mats. Monitoring schemes need to be developed that are capable of detecting proliferation of cyanobacteria (linked to a programme of appropriate actions) and drinking water treatment technology needs to be in existence that is capable of preventing human exposure if cyanobacteria occur in source waters.

5.2.1 Drinking water

A drinking water supply safe from cyanotoxins will either draw upon a resource which does not harbour cyanotoxins (e.g. groundwater or surface water which does not support cyanobacterial growth), or have treatment in place that is likely to remove cyanobacterial cells (without causing their disrupture) as well as removing cyanotoxins. However, in many circumstances a potential cyanotoxin hazard can be managed effectively without the necessity of advanced treatment processes, through water resource management techniques (see Chapter 8) and removal of intact cells (see Chapter 9). The critical control points for safe practices are indicated in Table 5.1.

Most of the reported incidents of human injury that have raised awareness of the importance of cyanotoxins in drinking water have involved the inappropriate treatment of water supplies, such as the use of copper sulphate in dealing with an established bloom of cyanobacteria.

A very effective approach to safe practices may involve changing the drinking water source. In a number of regions, surface waters are used for reasons of easy access and tradition, although groundwater of high quality is available. Exploring options of improving practices of drinking water abstraction with low technological input (such as drilling wells, or using bankside filtration) may lead to health benefits. In China, a high prevalence of endemic primary liver cancer was related to several factors: hepatitis B, aflatoxins in the diet, and drinking surface water polluted with cyanobacteria likely to contain microcystins. Changing the drinking water source from shallow, eutrophic ponds and ditches to groundwater was a major element of a package of measures which showed some success in improving health (Box 5.3).

5.2.2 Recreational waters

Recreational water use is likely to be a major route of exposure to cyanotoxins in some parts of the world. Whereas similar approaches to resource protection apply as for drinking water, there are very few further management options available once cyanobacteria proliferate or accumulate in a recreational water. Because adequate surveillance is sometimes difficult and management options, except precluding or discouraging use, may be scarce, a large share of the responsibility for safe practices lies with the users of a bathing site. The provision of adequate information to the public becomes, therefore, a major responsibility of public authorities.

The growth of cyanobacteria in lakes and rivers used for recreational purposes has been well recognised as a public nuisance. Water blooms of cyanobacteria may be associated with unpleasant odours and the offensive appearance of lake shores, especially when scums of the organisms accumulate and decay. Areas with extensive cyanobacterial scums or accumulated detached mats on bathing beaches may be avoided by swimmers and other water users because of the obviously unpleasant environment, particularly if locally anaerobic water conditions or cyanobacterial toxins cause fish-kills, further emphasising the unattractiveness of water contact. In temperate climates, cyanobacterial dominance is most pronounced during the summer months, when the demand for recreational water is highest. In some regions, cyanobacteria have been abundant for more than a generation and visitors have accepted this water quality as "normal" for their region. Multiple anecdotal observations of children playing with scum material have been reported.

Table 5.1 Critical control points for assessing the intrinsic safety of a drinking water supply which may contain cyanobacterial cells and/or toxins

Control point/issue	Comments
Source water type	The health risk associated with cyanobacterial contamination of groundwaters is generally negligible. An exception may occur where infiltration galleries are strongly influenced by eutrophic surface waters
Occurrence of cyanobacteria in source water and tendency for bloom formation	Many surface water sources do not support cyanobacterial growth. In others, cyanobacteria may occur occasionally at low population densities. In reservoirs and lakes with very low nutrient concentrations (total phosphorus < 10 µg P l^{-1}) or rivers and reservoirs with a hydrodynamic regime unfavourable for cyanobacteria (continuous high flows especially during summer, or deep vertical mixing), other phytoplankton species may regularly out-compete cyanobacteria. A water source which does not have a history of cyanobacterial growth or bloom formation is generally considered to present a low cyanotoxin risk, regardless of treatment type. Where bloom formation is well characterised in terms of annual cycles, the health risk may similarly be low if control measures are in place for times of bloom formation. If regular monitoring of source phytoplankton is in place, waters presenting no significant cyanotoxin risk are easily identified (see Table 6.2)
Likelihood of cell lysis in transport or treatment	Throughout cyanobacterial growth, most cyanotoxins are cell-bound. Removal of intact cyanobacterial cells therefore largely removes cyanotoxins (see section 3.4). Neurotoxins may be an exception under some circumstances. When cyanobacterial cells die and decay (lyse), toxins are released. Lysis can occur naturally or be caused by chemical treatment, hydraulic and pumping regimes in different treatment steps, and by long transport pipes for raw water. Thus, abstraction and treatment systems which lead to cell lysis present an increased risk of cyanotoxin release.
Treatment systems capable of toxin removal	Methods, such as adsorption to some types of granular activated carbon, and oxidation, can be effective in cyanotoxin removal. However, conditions of operation are critical for success. If processes are operated only periodically during cyanobacterial growth or reservoir treatment, monitoring of plant functioning must be adequate to ensure cyanotoxin removal. Substantially less is known about removal of neurotoxins and cylindrospermopsin than about microcystins, thus toxin monitoring of treatment steps and finished water is especially important if potentially neurotoxic or cylindrospermopsin-producing cyanobacteria proliferate

Box 5.3 Primary liver cancer and cyanotoxins in China

Primary Liver Cancer is one of the most common cancers in China. In 1994 and 1995, it accounted for 24 mortalities per 100,000 population in some rural counties and cities; in these areas it was ranked with stomach cancer as the two most important causes of cancer death.

The uneven geographic distribution of liver cancer was conspicuous, and "hot spots" could be related to drinking water supply, e.g. in some clearly delineated areas of Nandong District, in Jiangsu Province (particularly in Rudong, but also in Haimen and Qidong), in Nanhui (suburb of Shanghai) and Fusui (Guangxi).

- In Rudong, Nanhui and Fusui people had blocked the drainage system, causing stagnation of the water used for the drinking supply.

- In areas of Qidong-Haimen, with mortality rates 20 per 100,000, people drank water from the Yangtze River, but in areas with mortalities of 100 per 100 000, pond and ditch water was used.

- Primary liver cancer mortalities 10 per 100,000 were found in areas where water from deep wells were used for drinking.

Epidemiological study of the mortality showed strongest correlation with hepatitis B incidence, a lesser correlation with aflatoxins in the diet, and a third correlation with drinking of pond and ditch water. No correlations were found with insecticides. Samples of pond and ditch water showed microcystin present in both endemic liver cancer areas and in areas with lower liver cancer rates. Children in some endemic areas were fed corn paste and drank pond or ditch water from infancy. Further, up to 43 per cent carry the hepatitis-B virus from infection by their HBsAg positive mothers. The evidence suggests that aflatoxins from corn and microcystins from drinking water act together with the hepatitis B virus in causing and promoting primary liver cancer.

In order to alleviate this situation, attempts have been launched over the past 20 years to change the staple food and drinking habits of the people. Efforts began with the methods of harvest, following the motto *"quick to reap, quick to store, at a moisture content 12.5 per cent",* aimed at the reduction of fungal contamination. For some time, the government bought corn and exchanged it for rice to reduce aflatoxin exposure, but this function has now been transferred to a private initiative in the market economy. Recently, it has been estimated that more than 95 per cent of the population eats rice rather than corn.

Even prior to the recognition of microcystins as possible promoters of endemic primary liver cancer, the connection to poor quality surface water for drinking was observed and programmes for construction of deep wells were begun. At present, 80 per cent of the population in some of the afflicted regions have changed their water source to deep well water, and the incidence of liver cancer has dropped consistently.

The mottoes for prevention of primary liver cancer now are:

"control of water - control of crops - prevention of hepatitis"

For additional discussion, see section 4.1.2.

Health impairments from cyanobacteria in recreational waters must be differentiated between the chiefly irritative symptoms caused by unknown cyanobacterial substances (as described in Box 5.2), and the more severe hazard of exposure to high concentrations of known cyanotoxins, particularly microcystins. A single guideline therefore, is not appropriate. Rather, a series of guidelines associated with incremental severity and probability of adverse effects has been defined at three levels as described below.

1. Relatively mild and/or low probabilities of adverse health effects

For protection from health outcomes not due to cyanotoxin toxicity, but due to the irritative or allergenic effects of other cyanobacterial compounds, a guideline level of 20,000 cyanobacterial cells per ml (corresponding to 10 µg l^{-1} of chlorophyll *a* under conditions of cyanobacterial dominance) can be derived from the prospective epidemiological study by Pilotto *et al.* (1997) (see Box 5.2). Whereas the health outcomes reported in this study were related to cyanobacterial density and duration of exposure, they affected less than 30 per cent of the individuals exposed. At this cyanobacterial density, 2-4 µg l^{-1} of microcystins may be expected if microcystin-producing cyanobacteria are dominant, with 10 µg l^{-1} being possible with highly toxic blooms (regional differences in microcystin content of the cells may be substantial). This level is close to the WHO provisional drinking water guideline value of 1 µg l^{-1} for microcystin-LR (WHO, 1998) which is intended to be safe for life-long consumption. Thus, health outcomes due to microcystin are unlikely and providing information for visitors to bathing sites with this low-level risk is considered to be sufficient. Additionally, it is recommended that the authorities are informed in order to initiate further surveillance of the site.

2. *Moderate probability of adverse health effects*

At higher concentrations of cyanobacterial cells, the probability of irritative symptoms is elevated. Additionally, cyanotoxins (usually cell-bound) may reach concentrations with potential health impact. To assess risk under these circumstances the data used for the drinking water provisional guideline value may be applied. Swimmers involuntarily swallow some water while bathing and the harm from ingestion of bathing water will be comparable with that from a drinking water supply with the same toxin content. A swimmer can expect to ingest up to 100-200 ml of water in one session, sail-board riders and water skiers would probably ingest more.

A density of 100,000 cyanobacterial cells per ml (which is equivalent to approximately 50 µg l^{-1} of chlorophyll *a* if cyanobacteria dominate) is a guideline for a moderate health alert in recreational waters. At this density, 20 µg l^{-1} of microcystins are likely, if the bloom consists of *Microcystis* and has an average toxin content per cell of 0.2 pg, or 0.4 µg microcystin per µg chlorophyll *a* (up to 50 µg l^{-1} of microcystin are possible) but toxin levels may approximately double if *Planktothrix agardhii* is dominant. This toxin concentration is equivalent to 20 times the WHO provisional guideline value for microcystin-LR in drinking water, but would result in consumption of an amount close to the TDI for an adult of 60 kg consuming 100 ml of water while swimming (rather than 2 litres of drinking water). However, a child of 15 kg consuming 250 ml of water during extensive playing could be exposed to 10 times the TDI. The health risk will be

increased if the person exposed is particularly susceptible (e.g. because of chronic hepatitis B). Therefore, cyanobacterial densities likely to cause microcystin concentrations of 20 µg l^{-1} should trigger further action.

Non-scum-forming species of cyanobacteria, such as *Planktothrix agardhii,* have been observed to reach cell densities corresponding to 200 µg l^{-1} of chlorophyll *a* or even more in shallow water bodies. Transparency in such situations will be less than 0.5 m when measured with a Secchi disk (see Chapter 11). *Planktothrix agardhii* has been shown to contain a very high cell content of microcystin (1-2 µg per µg chlorophyll *a)* (see Figure 3.5) and therefore toxin concentrations of 200-400 µg l^{-1} can occur without scum formation.

An additional reason for increased alert at 100,000 cells per ml is the potential of some frequently occurring cyanobacterial species (particularly *Microcystis* spp. and *Anabaena* spp.) to form scums (see Figure 5.1). These scums may increase local cell density and thus toxin concentration by a factor of 1,000 or more in a few hours, thus rapidly changing the risk from moderate to high (see next subsection) for bathers and others involved in body-contact water sports.

Cyanobacterial scum formation presents a unique problem for routine monitoring carried out at the usual time intervals of one or two weeks, because such monitoring intervals are unlikely to detect hazardous maxima. Because of the potential for rapid scum formation at a cyanobacterial density of 100,000 cells per ml or 50 µg l^{-1} chlorophyll *a* (from scum-forming cyanobacterial taxa), intensification of surveillance and protective measures are appropriate at these levels. Daily inspection for scum formation (if scum-forming taxa are present) and measures to prevent exposure in areas prone to scum formation are the two main options.

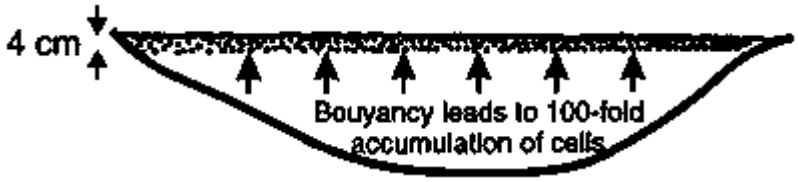
Intervention is recommended to trigger effective public information campaigns educating people on avoidance of scum contact. Furthermore, in some cases (e.g. with frequent scum formation), restriction of bathing may be judged to be appropriate. An intensified monitoring programme should be implemented, particularly looking for scum accumulations. Health authorities should be notified immediately.

Figure 5.1 Schematic illustration of scum-forming potential changing the cyanotoxin risk from moderate to very high

Lake profile

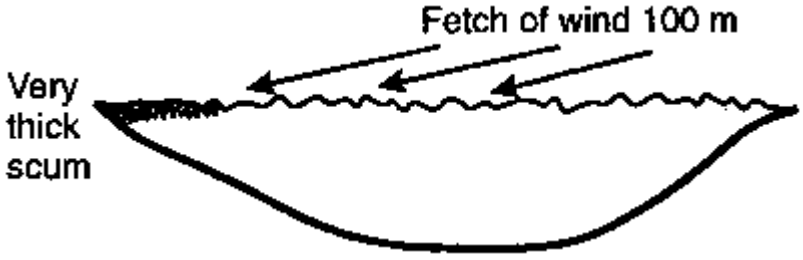
Moderate risk level:

- 50 µg l^{-1} chlorophyll *a*
- or 100,000 cells l^{-1}
- possibly 20 µg l^{-1} of microcystin in top 4 m of water body



100-fold accumulation to high risk level scum:

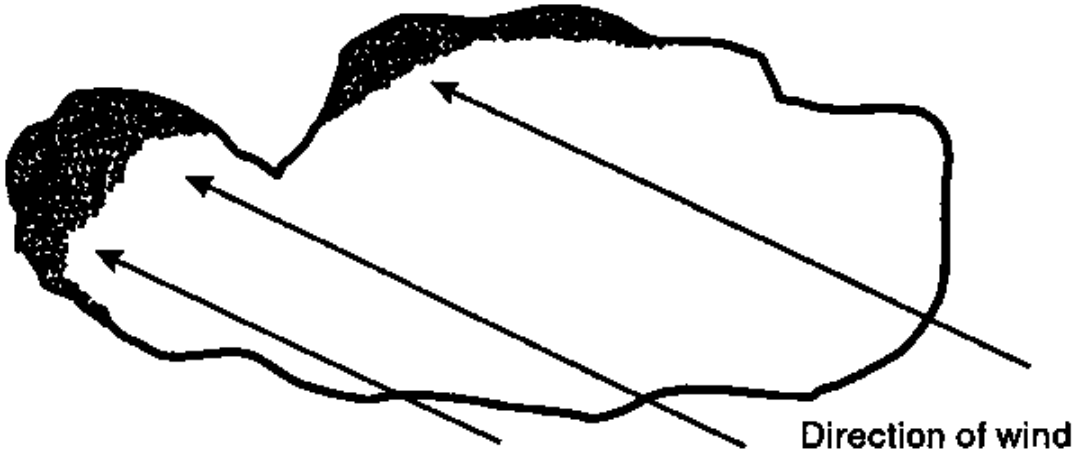
- 5,000 µg l^{-1} chlorophyll *a*
- or 10,000,000 cells l^{-1}
- possibly 2,000 µg l^{-1} of microcystin in top 4 cm of water body

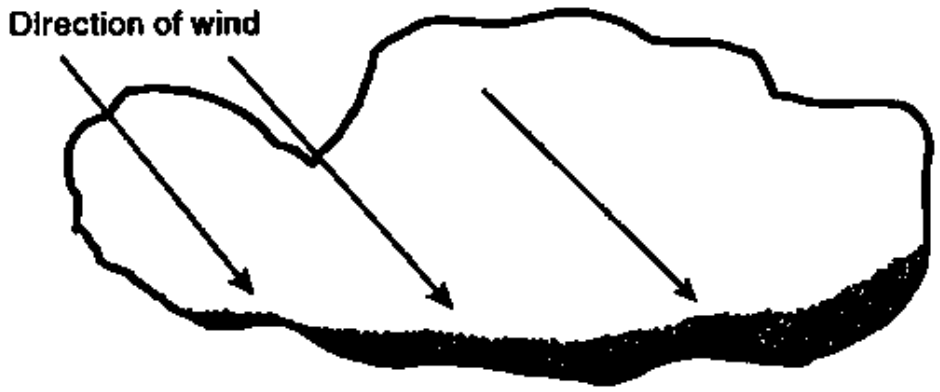


1,000-fold accumulation to very high risk level shore scum if wind sweeps scums from 100 m into 10 m:

- 50,000 µg l^{-1} chlorophyll *a*
- or 100,000,000 cells l^{-1}
- possibly 20,000 µg l^{-1} of microcystin concentrated in one bay of the water body

Lake plan





3. High risk of adverse health effects

Abundant evidence exists for potentially severe health hazards associated with scums caused by toxic cyanobacteria (see section 4.1). No human fatalities have been unequivocally associated with oral ingestion of scum, even though numerous animals have been killed by consuming water containing cyanobacterial scum material (see section 4.1). This discrepancy can be explained by the fact that animals would drink higher volumes of scum-containing water, compared with the small amounts of scum accidentally ingested by humans during bathing (resulting in a lower dose).

Cyanobacterial scums can represent a thousand-fold to million-fold concentration of cyanobacterial cell populations. It has been calculated that a child playing in a *Microcystis* scum for a protracted period and ingesting a significant volume could receive a lethal exposure, although there are no reports that this has actually occurred. Based on evidence that the oral LD_{50} of microcystin-LR in mice is 5,000-11,600 µg kg^{-1} bw (see section 4.2), for a child of 10 kg the ingestion of 2 mg of microcystin or less could be expected to cause liver injury, because concentrations of up to 24 mg l^{-1} of microcystins have been published from scum material (see section 3.2). Substantially higher enrichment of scums (up to gelatinous consistency) is occasionally observed, and accidental ingestion of smaller volumes of these could cause serious harm. Anecdotal evidence indicates that children, and even adults, may be attracted to play in scums. The presence of scums caused by cyanobacteria is a readily detected indicator of a high risk of adverse health effects for those bathers who come into contact with the scum. Immediate action to control scum contact is recommended for such situations.

The approach outlined in this section, however, does not cover all conceivable situations. Swimmers may be in contact with benthic cyanobacteria after a storm breaks off clumps of filaments, or cyanobacterial mats naturally detach from the sediment and are accumulated on the shore (Edwards *et al.,* 1992). Some marine beaches have been reported to have widespread problems due to a benthic marine cyanobacterium, *Lyngbya majuscula,* growing on rocks in tropical seas and causing severe blistering when trapped under the bathing suits of people swimming following a storm (Grauer, 1961). This response may be due to acute toxicity; *Lyngbya* can produce irritant toxins. Measures of cyanobacterial population cell density as outlined in Table 5.2, will not detect these hazards. Instead, this type of hazard calls for critical and well-informed observation of bathing sites, coupled with a flexible response.

It is difficult to define "safe" concentrations of cyanobacteria in recreational water in relation to allergenic effects or skin reactions, because individual sensitivities vary greatly. Aggravation of dermal reactions due to accumulation of cyanobacterial material and enhanced disruption of cells under bathing suits and wet suits may be a problem, even at all densities below the guidelines described above. Further information related to monitoring and management of recreational waters is available in Bartram and Rees (1999).

5.3 Other exposure routes

5.3.1 Renal dialysis

Renal dialysis patients are at great risk when water used for dialysis contains contaminants such as cyanotoxins. For these patients large volumes of water (120 litres) are used and the route of exposure is similar to the i.v. route, which allows for a much greater uptake of toxin than following oral ingestion. One serious incident, including a number of deaths arising from exposure through this route, has already been described in section 4.1.

The WHO *Guidelines for Drinking-water Quality* (WHO, 1993) do not consider the especially high quality of water needed for dialysis treatment, intravenous therapy or other clinical uses. The treatment processes used at conventional surface water treatment plants (such as coagulation, clarification and sand filtration) are normally effective in removing cyanobacterial cells, but are not effective in removing or destroying dissolved cyanotoxins, especially from water supplies with a high organic content and cyanobacterial dominance (see Chapter 9). Consequently, clinics and hospitals with special water needs, such as for dialysis treatment or for transfusions (intravenous administration), may need to provide additional water treatment to remove the cyanotoxins. Such treatment ranges from granular activated carbon filtration, followed by reverse osmosis, to more elaborate treatment including membrane filtration (e.g. 25 µm pore size filter). The extent of treatment necessary depends on the quality of the municipal water supply.

Continuous monitoring of performance and equipment is essential to ensure adequate quality of the water. On-site water treatment systems in clinics and hospitals require rigorous monitoring and regular maintenance, including back-flushing of filters and recharge of activated carbon, according to manufacturers' specifications. It is important that manufacturers' specifications should be assessed for their adequacy for maintaining performance under local conditions. Activated carbon, for example, may be exhausted for its ability to remove cyanotoxins long before it reaches saturation for removal of other organic compounds, and some manufacturers may be unaware of this.

Table 5.2 Guidelines for safe practice in managing bathing waters which may produce or contain cyanobacterial cells and/or toxins

Guidance level or situation	How guidance level derived	Health risks	Recommended action
Cyanobacterial scum formation in bathing areas	Inference from oral animal lethal poisonings Actual human illness case histories	Potential for acute poisoning Potential for long-term illness with some cyanobacterial species Short-term adverse health outcomes, e.g. skin irritations, gastrointestinal illness	Immediate action to prevent contact with scums; possible prohibition of swimming and other water-contact activities Public health follow-up investigation Inform relevant authorities
100,000 cells cyanobacteria per ml *or* 50 µg chlorophyll *a* per litre with dominance of cyanobacteria	From provisional drinking water guideline for microcystin-LR, and data concerning other cyanotoxins	Potential for long-term illness with some cyanobacterial species Short-term adverse health outcomes, e.g. skin irritations, gastrointestinal illness	Watch for scums Restrict bathing and further investigate hazard Post on-site risk advisory signs Inform relevant health authorities
20,000 cells cyanobacteria per ml *or* 10 µg chlorophyll *a* per litre with dominance of cyanobacteria	From human bathing epidemiological study	Short-term adverse health outcomes, e.g. skin irritations, gastrointestinal illness, probably at low frequency	Post on-site risk advisory signs Inform relevant authorities

5.3.2 Irrigation water

The use of water from sources containing cyanobacterial blooms and toxins for spray irrigation of crops presents potential health hazards through several exposure routes, including uptake into the food chain. Workers or passers-by may inhale toxins with spray drift, and skin contact with cyanobacteria and dissolved toxins may also occur. Questions therefore arise about the health significance of spray irrigation with water containing cyanobacterial toxins. As shown in section 4.2, animal experimentation has indicated that microcystin uptake through nasal mucosa may be considerable. When considered together with the skin irritations, respiratory distress and nasal mucosal irritations observed after recreational exposure (see sections 4.1 and 5.2.2), the indicators are that occupational exposure to spray irrigation water should be avoided (by appropriate work practices) if the water contains cyanobacterial toxins. Incidental exposure of humans and animals to such spray irrigation water, for example by downwind drift, should also be avoided.

There are several indications that terrestrial plants, including food crop plants, can take up microcystins. Mustard seedling development is inhibited if microcystin-LR is presented to the roots in aqueous solution (Kos *et al.,* 1995). Microcystins have several perturbatory effects on plant physiology and metabolism, when sufficient levels of toxin enter the plant cells. Plant protein phosphatases show high susceptibility to inhibition by microcystin-LR *in vitro* (MacKintosh *et al.,* 1990). Plant sucrose metabolism is inhibited if microcystin-LR is administered in solution by injection into the transpiration stream (Siegl *et al.,* 1990). Inhibition of whole leaf photosynthesis by French Bean plants occurred after topical exposure of the leaves to an aqueous solution of microcystin-LR during greenhouse studies (Abe *et al.,* 1996). The degree of whole leaf photosynthesis inhibition increased with subsequent brief exposures to the toxin in solution at 48-hour intervals, eventually becoming irreversible. These results were observed at dissolved microcystin-LR concentrations which can be found in untreated waters containing cyanobacterial blooms (20 μg l^{-1} of toxin); leaf necrosis occurred at higher exposure levels (Abe *et al.,* 1996). The relevance of these findings for field situations is currently unclear.

In addition to the possibility of internal accumulation of microcystins, irrigation may lead to accumulation of toxins on the external surfaces of edible plant material. The toxins are deposited when the water dries on the plant surface between irrigation periods or when the water becomes trapped in the centres of, for example, salad plants. Further research is needed into the uptake and fate of microcystins and other cyanobacterial toxins by food plants and the persistence of the toxins on plant surfaces.

5.3.3 Cyanobacteria sold as dietary supplements

In some countries cyanobacteria are sold as dietary supplements, with the number of users of these products estimated to be well over a million in North America alone. Large-scale production of cyanobacteria and microalgae started some 50 years ago. Much of the early research work dealt with the basic photosynthetic properties of microalgae, their possible therapeutic, antibiotic and toxicological properties and their potential as an agricultural commodity. The microalgae biomass industry now provides significant biomass for pigments and speciality chemicals used primarily in the food industry. The bulk of this microalgal biomass comes from two filamentous genera of cyanobacteria: *Spirulina* including *S. platensis* and *S*. *maxima* (Belay *et al.,* 1994) and *Aphanizomenon flos-aquae.* While *Spirulina* is grown in artificial outdoor ponds, mainly in southern California, Hawaii, Thailand, Taiwan and Japan, *Aphanizomenon* is at present harvested from a natural lake. Production of food-grade *Spirulina* exceeds 1×10^6 kg a^{-1} (Belay *et al.,* 1993). *Aphanizomenon* production is also substantial.

As a consequence of the consumption of these products many quality control issues arise. One such issue concerns the possible production of cyanotoxins by cyanobacterial genera used for dietary supplements. In particular, *Aphanizomenon flos-aquae* has been shown to be capable of producing saxitoxins (Mahmood and Carmichael, 1986) and the neurotoxin anatoxin-a (Bumke-Vogt *et al.,* 1999). While no saxitoxins have been detected in *Aphanizomenon flos-aquae* marketed as a dietary supplement, it is appropriate to monitor all supplements in order to ensure safety. In natural lakes, mixtures of species often occur in cyanobacterial blooms. In particular *Microcystis* and *Anabaena,* which usually contain microcystins, can both occur in association with *Aphanizomenon.* The microcystins may then become part of the biomass harvested for

human consumption. Failure to monitor and regulate these toxins in cyanobacterial biomass used as part of a human diet could lead to an increased risk for the consumer (see Box 5.4).

5.3.4 Cyanobacteria and *Vibrio cholerae*

Islam (1991) described detection of *Vibrio cholerae* inside the mucilaginous sheath of *Anabaena variabilis* for up to 15 months after artificial exposure and also noted that *V. cholerae* 01 did not lose toxigenicity during the association (Islam, 1991). Field studies have also detected *V. cholerae* in the mucilaginous sheath of *Anabaena* sp. from a pond in Dhaka when it could not be detected in association with microalgae collected from the same environment *(Euglena* sp. and *Phacus* sp. (Islam *et al.,* 1994)).

The association between *V. cholerae* and cyanobacteria remains poorly understood, but it has been noted that vibrios may produce mucinase (Schneider and Parker, 1982) and it has been suggested that exchange of oxygen (from photosynthesis for aerobic respiration) and carbon dioxide may permit a symbiotic relationship (Islam, 1987; Islam *et al.,* 1994). Other workers have noted that motile bacteria can easily discriminate heterocysts from vegetative cells and attach to the heterocyst vegetative cell junction, following which both host and epiphytes start growing (Paerl and Gallucci, 1985; Islam *et al.,* 1990). The bacteria rarely penetrate cyanobacterial cell walls (Islam *et al.,* 1990). Evidence is accumulating that association with mucilaginous cyanobacteria may be an important factor in inter-epidemic survival of *V. cholerae.* The implications of this for the control of cholera in humans remain unclear and it should be noted that the evidence relates to mucilaginous cyanobacteria, and *Anabaena* in particular. No studies have suggested or apparently investigated a relationship with cyanobacterial toxigenicity.

Box 5.4 Calculation of risk associated with consumption of cyanobacterial products contaminated with microcystins

The State of Oregon, USA, has adopted 1 µg g^{-1} (1 ppm) of microcystins as a standard for cyanobacterial products. The consumption of 2 g (as suggested by some producers) by a 60 kg person of product containing 1 µg g^{-1} would result in a microcystin intake of 0.033 µg kg^{-1} bw. This intake is slightly below the tolerable daily intake (TDI) of 0.04 µg kg^{-1} bw per day used for derivation of the WHO guideline for microcystin-LR in drinking water (WHO, 1998) (see section 4.2). However, an intake exceeding 2 g per day of a product containing microcystins at a concentration near the State of Oregon standard may exceed the TDI, and a consumption of 2 g per day by children may also exceed the TDI because of their lower body weight. In deriving its drinking water guideline value, WHO apportioned 20 per cent of intake to other sources. For persons consuming cyanobacterial products, this apportionment may be inappropriate.

5.4 Tastes and odours

Cyanobacteria have, for a long time, been recognised as a nuisance in the drinking water industry because of the ability of several taxa to produce earthy and musty smelling compounds, notably geosmin and 2-methyl isoborneol (2-MIB), for which the odour detection thresholds of less than 10 ng l^{-1} are remarkably low among sensitive individuals.

The cyanobacterial genera that are known to produce geosmin are *Anabaena, Aphanizomenon, Lyngbya, Microcystis, Oscillatoria, Phormidium, Schizothrix* and *Symploca* (Perrson, 1983). All these (except *Symploca)* are also known to include toxin-forming species and strains.

Because of this, the possibility of using odour compounds as an early warning for the development of toxin-producing cyanobacteria blooms has been considered. However, there is no evidence of a correlation between toxin production and the production of taste- and odour-producing compounds that would provide a warning of toxicity. Evidence from the literature on the capability of various cyanobacterial species to produce both toxins and taste and odour compounds has been summarised by Kenefick *et al.* (1992) and does not indicate that cyanobacterial species which produce toxins invariably also produce taste and odour. Nevertheless, some characteristic tastes and odours may indicate the presence of cyanobacteria, and toxic cyanobacteria frequently occur without noticeable tastes or odours. In Alberta, Canada, 89 bloom samples from 10 lakes were analysed for the presence of microcystin-LR, and the taste and odour compounds geosmin, 2-MIB and β-cyclocitral. The latter compound, which is only mildly odorous compared with geosmin and 2-MIB, was reported to be produced in large quantity by *Microcystis* spp. (Jüttner, 1988). The results showed that all but three of the bloom samples had detectable levels of microcystin while none had detectable levels of 2-MIB. Several samples had detectable levels of geosmin, but there was no clear relationship between the presence of geosmin and the presence of microcystin-LR. In the case of β-cyclocitral, there was a significant correlation, at the 1 per cent level. However, this relationship is of no practical use for providing early warning of the presence of microcystin-LR, because the relationship is not consistent and β-cyclocitral is not odorous enough to act as a sensitive surrogate for microcystin-LR (Hrudey *et al.,* 1993). Although there have been some rare occasions when cyanobacterial isolates have been found to produce geosmin, microcystin and anatoxin-a simultaneously, as for *Anabaena lemmermannii* from Lake Hallevann in Norway (Haneberg *et al.,* 1994), such reports are exceptions. The biochemical pathways to the biosynthesis of microcystin (Dittmann *et al.,* 1996), anatoxin (Gallon *et al.,* 1989) and saxitoxin (Skimizu *et al.,* 1984) are becoming understood. These show no connection between toxin production and the production of the alcohols geosmin and 2-MIB. It is therefore very unlikely that the production of the taste and odour compounds are biochemically connected to the production of the cyanotoxins.

Just as the presence of earthy or musty odours in water indicates the presence of cyanobacteria and/or actinomycetes, taste and odour problems can be used as a warning of the need for further investigation in the event that the occurrence of cyanobacteria could result in the presence of cyanotoxins. It is, however, important to recognise that the converse does not apply: lack of taste and odour by no means implies the absence of cyanobacteria.

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Chapter 6. SITUATION ASSESSMENT, PLANNING AND MANAGEMENT

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Chapters 3 and 4 of this volume present evidence that cyanobacterial toxins can and do cause significant adverse health effects. These effects are associated with the occurrence of cyanobacterial blooms. As described in Chapter 2, such blooms are a natural occurrence, but occur more frequently in waters which have been subject to certain forms of human interference. The most important types of interference are enrichment of waters with nutrients (eutrophication) from point sources such as municipal wastewater outlets and non-point sources such as agriculture, and the damming of rivers (which increases retention time and exposure to sunlight). Chapter 2 also describes how, although blooms are more frequent and severe in eutrophic waters, they may occur in waters which would be considered in many world regions to be of good or acceptable quality. Of more concern is the fact that the available evidence concerning trends in eutrophication indicates that the situation is severe world-wide (see Table 6.1), although it is improving in some regions whilst deteriorating in others.

The purpose of this chapter is to assist those concerned with assessing or managing the potential risks to human health arising from toxic cyanobacteria. It is intended to serve as a guide to readers who are confronted with immediate questions and issues related to risk management, whether arising from an event or because of the suspicion of a potential hazard. It also provides an axis to assist the reader in using other parts of this book and deals with four major areas:

- The overall risk management framework.

- Situation assessment (Is there a problem? Would a problem be detected if it existed? How can the severity of the problem be interpreted in relation to other demands on resources?).

- Management options (What types of management actions are available? What are their basic characteristics?).

• Planning for management (How should a management, contingency or emergency response and investigation plan be put together?).

Table 6.1 Perceived eutrophication problem in different continents and countries

Region/country	Natural lakes	Reservoirs, rivers and irrigation systems	Estuaries, lagoons and closed areas	Marine coastal waters
AFRICA				
Central	+	++	+	
North		+	++	+
South		++		
CENTRAL AMERICA				
Caribbean	+	+		+
Guatemala/Nicaragua	+			
Mexico	+	++		
NORTH AMERICA				
Canada	++	+	+	
USA	++	++	++	+
SOUTH AMERICA				
Argentina/Chile	+	++	+	+
Brazil	+	++	++	+
Columbia/Ecuador/Peru	+	++	+	+
Venezuela/Suriname	+	+	++	
ASIA				
China	++	+	+	
India/Pakistan	+	++	+	
Indochina	+	+		
Indonesia/Philippines	++	+		
Japan	++	+	++	+
OCEANIA				
Australia/New Zealand	++	++	++	+
EUROPE (EU countries)				
Belgium		+		
Denmark	++			+
France		++	+	++
F. Germany, Fed. Rep.	++	++	+	+
Greece		+	+	+
Ireland	++	+		
Italy	++	++	+	++
Netherlands		++	++	
Portugal		++	+	

Spain		++	+	
UK	++	++		
EUROPE (other countries)				
Austria	++			
Former Czechoslovakia		+		
Finland	++		+	+
Former German Dem. Rep.	+	++	+	+
Hungary	+	+		
Norway	++	+	++	+
Poland	++	+		
Romania				+
Sweden	++		+	++
Switzerland	++			
Former USSR	+	++	+	+
Former Yugoslavia				+

\+ Identified problems

++ Serious problems

Source: Adapted from Earthwatch, 1992

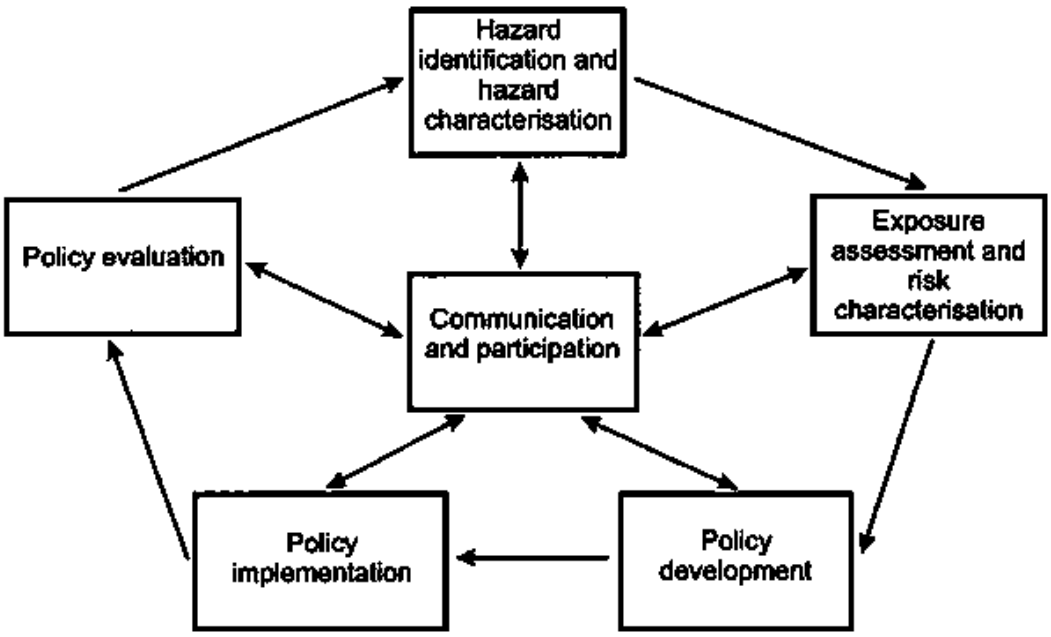
6.1 The risk-management framework

Risk management is a relatively recent discipline, in which developments are still occurring very rapidly. Various model schemes for risk management have been portrayed, most of which have some common elements. These include the need for an information base upon which to make decisions; the need to make decisions based on often inadequate information; the need to compare and "value" different and often very diverse outcomes with one another; and the need for broad participation in the process in all its stages. One schematic example is presented in Figure 6.1. This representation is different from many others because it completes a circle, showing the feedback of policy evaluation into hazard identification and prioritisation, and thereby into improved risk assessment and eventually into implementation of revised (improved) policy. It also places communication as a central and two-way process, indicating that it has an important role throughout risk management.

The implementation of risk management will vary greatly according to the political, social and economic context in which it takes place. Whilst it is often seen as a highly rational process, it should be recognised that the scientific basis for many of its elements is actually often extremely weak (the status of knowledge regarding some of the cyanobacterial toxins as outlined in Chapters 3 and 4 is a good example of this) and procedures for valuation of health effects are generally poorly developed. More importantly, no scientific assessment will support effective risk management if it fails to

address the perceptions and priorities of the society concerned. Public acceptance of cyanobacterial-related turbidity and discoloration in recreational waters in some countries illustrates this point. Thus, a commitment to communication and participation is an essential element of any effective risk management plan.

Figure 6.1 The risk management cycle (Adapted from Soby *et al.,* 1993)



The movement towards a more comprehensive approach to risk management is inhibited by four key impediments, sometimes referred to as "institutional failures" (adapted from Gerrard, 1995):

• *Data limitations* are of two kinds: a lack of historical record on which to base current change and to recognise trends, and the inadequacy of scientific understanding on which to base judgements about cause and consequence. Such data limitations are common in the management of cyanobacterial and eutrophication problems. A common question that faces water managers is whether a cyanobacterial problem is getting worse, due for example to cultural eutrophication, or whether the problem has always been present but has remained unnoticed or, perhaps, unreported. Following widespread dissemination of information concerning the potential toxicity of cyanobacteria, blooms may start to be reported more frequently by, or to, relevant authorities.

• *Poor frameworks for analysis* impede comprehensiveness in assessment and decision-making necessary to make informed judgements. Cyanobacteria are good examples. Until recently, a general lack of knowledge and poor communication by scientists were a serious impediment to sound management action - a constraint which is now being overcome in some countries.

• *Inadequate regulatory principles* which lay too much discretion at the door of the safety official and which lead to discrepancies in levels of safety provided for different groups in

society (according to their environmental circumstances and income levels). In some countries, cyanobacteria-infested drinking water sources are the only ones available to the poor or geographically isolated.

• *Insufficient consultation* procedures restrict participation of different interests that have a legitimate role to play in the determination of risk and its management (the important role of multi-agency and vested interest committees or "task forces" in the effective management of toxic cyanobacterial blooms is outlined in section 7.1). Lack of communication between government and industry sectors (including, for example, water suppliers) is especially detrimental in managing cyanotoxin issues. For example, health impact assessment generally requires health sector participation, resource management is generally under the control of environmental and/or local authorities, and abatements such as water treatment may be undertaken by local authorities or the private sector.

6.1.1 Communication and participation

In many cultures, certain characteristics lead to some risks being perceived as high priorities (that is they are little accepted or tolerated). These include:

• Risks which are "new" or unfamiliar to the population.

• Risks which are perceived to have been caused by a (especially distant and unaccountable) third party.

• Risks for which the consequences are poorly characterised and understood.

• Risks with potentially widespread or catastrophic and irreversible consequences.

• Risks outside the control of the individual.

• Risks in which the population or individual has had little possibility of influencing official responses.

Risks with these characteristics are perceived as high priorities when compared with other risks which are perhaps of equal or greater importance to health but which are voluntarily undertaken and familiar. The example of the social acceptance of smoking across much of the globe, despite widespread knowledge of the adverse health effects associated with it, is a good example of this.

A brief review of the public perception of cyanobacteria as a health hazard indicates that they will often be perceived as a high priority, perhaps higher than rational analysis of the (limited) available data would otherwise indicate. Newspaper headlines have been printed proclaiming *"Water More Toxic Than Cyanide"* after cyanobacteria were found in a particular water body. While cyanotoxins are, on a per-unit-mass basis, more toxic than cyanide, this does not mean that drinking water containing a low concentration of cyanotoxins is anywhere near as dangerous as drinking a lethal solution of cyanide, as the newspaper headline implied. Being a "new" issue, with which the general population is not familiar, the blame may be assigned to municipalities, public service utilities, or farmers. Individual choice does not enable risk avoidance (at least for drinking water

exposure) and entire communities may be affected through their drinking water supply. Experience indicates that, in some countries, public concern regarding cyanobacteria has often been greater than the corresponding concern from the professional community. In other countries exactly the opposite perception exists - the public has become accustomed to "green" or "smelly" water and may disbelieve scientific warnings of risk.

Improved understanding of risk assessment and management, and an improving understanding of effective intervention, has demonstrated that differences in public and professional perceptions are not an error by either party that needs to be corrected (for example through attempts at altering public perception or discrediting scientific attempts to assess objectively and to describe risks), but are legitimate viewpoints to be taken into account through open channels of communication and especially through enabling public participation in risk management. It should be recalled that the factors which lead to a low public tolerance of risk often include the lack of familiarity (i.e. the "newness") and the inability to exert influence. Effective communication and provision of mechanisms for participation will, therefore, often address these directly.

Several sections of this book provide guidance concerning public participation and communication in relation to cyanobacterial hazards. These actions are an important aspect of all types of planning, whether for preventive management (section 6.4.1), for contingency planning (section 6.4.2) or planning in response to an incident (section 6.4.3). They are also discussed in more detail in Chapter 7.

6.1.2 Hazard identification and hazard characterisation

In the context of risk management, a hazard is usually understood to mean the property of a substance (or activity) to cause harm. Many substances are hazardous but will not necessarily lead to harm unless circumstances lead to human exposure. Even after exposure, an adverse health outcome is not necessarily certain, but rather a probability. A hazard is therefore defined as an intrinsic property of a biological, chemical or physical agent to cause adverse health effects under specific conditions. This definition implies some certainty that under similar conditions the agent will cause similar adverse health effects.

The term "risk" refers to a probability that exposure to a hazard will lead to a specific (adverse) health outcome and is usually expressed as a frequency in a given time. Thus, for example, the WHO *Guidelines for Drinking-water Quality* (WHO, 1993) define guideline values as concentrations of specific chemicals estimated to lead to a negligible additional risk for the consuming population. Where such a point of reference is adopted, it is generally referred to as the "acceptable risk", although the term "tolerable risk" is preferred by some people (because the risk is never really acceptable, but it is tolerated).

Hazard identification involves the identification of known or potential adverse health effects associated with a particular agent, based on studies conducted under specific conditions, such as the species tested and the experimental conditions. Epidemiological studies and animal toxicity studies are ranked as providing the greatest predictive information, and this is followed by *in vitro* (test tube) assays and qualitative structure activity relationships (QSAR) predictions.

Hazard characterisation is the extrapolation phase of risk assessment aimed at making a predictive characterisation of the hazard to humans based on animal studies (species extrapolation) under low exposure conditions (extrapolation from high to low dose). The endpoint of hazard characterisation is the estimation of a "safe dose" such as a tolerable daily intake (TDI) or equivalent. In general, TDIs are only determined when there is likely to be a threshold in the relationship between dose and effect, based in part on theoretical knowledge of the mechanism of action. For genotoxic carcinogens it has been accepted that there is no threshold dose below which effects, such as initiation of the carcinogenic process, will not occur. When such chemicals cannot be completely avoided (such as some natural toxicants and contaminants), mathematical models (most of which presume linearity at low doses) have been used to estimate, through extrapolation, the possibility of adverse effects at low doses. The dose corresponding to a risk level of 1 in 1×10^5 or 1 in 1×10^6 has been considered as posing a negligible or tolerable risk.

With cyanotoxins, risk assessment is made more difficult by the paucity of scientifically-sound toxicological and epidemiological studies (see Chapter 4). The available animal data are limited, particularly in the case of chronic or long-term effects of cyanotoxins. The lack of data is reflected in the fact that a WHO guideline has been agreed only for one group of cyanotoxins (i.e. the microcystins, see Chapter 5) and only as a provisional guideline value for the toxin most commonly studied (i.e. microcystin-LR). Uncertainties about the environmental fate of cyanotoxins (for example, to what extent the toxins are accumulated in fish and shellfish that may be consumed by humans, see Chapter 3) add to the difficulty.

6.1.3 Exposure assessment and risk characterisation

Exposure assessment can relate to exposure to cyanobacterial toxins during an outbreak of disease, or it can be an assessment of the likely exposure of people through consuming drinking water or swimming in lakes known to contain certain levels of cyanotoxins or cyanobacteria.

Risk characterisation is the qualitative and/or quantitative estimation, including the attendant uncertainties, of the severity and probable occurrence or absence of known or potential adverse health effects in an exposed population. This estimation is based on hazard identification, hazard characterisation and exposure assessment. If it is calculated as the "probable risk" (such as the number of persons in a population that are expected to get cancer from exposure to a toxic chemical), the estimated risk takes on more meaning than it deserves, because of uncertainties in the process. Alternatively, risk characterisation can be taken as establishing levels of daily exposure over a lifetime at which the risk is "negligible" (see section 4.2).

6.1.4 Policy development

The processes of hazard identification, hazard characterisation, exposure assessment and risk characterisation may be readily viewed as rational, scientific activities. In contrast, policy development takes account of both rational assessment and societal values. It therefore requires the valuation of a specific health outcome (such as skin rash, gastro-enteritis, cancer or death). Most definitions of risk assessment therefore combine a frequency estimation with some valuation of the seriousness of the consequences.

Whilst several approaches have been proposed for the rational comparison of the "value" of different adverse health outcomes it should be recalled that such valuation is principally driven by public perception and societal concerns, and rational analysis may be of very limited relevance. Experience with the enforcement of recreational water quality standards leading to restrictions on bathing has shown that public reaction may vary between the extremes of proclaiming that "there is nothing wrong with the water, we have been swimming in it for years without any illness, the authorities do not know what they are talking about" to "this water has killed a pet dog and must be doing me harm too - something must be done about it immediately".

Knowledge of the characteristics of a hazard, the local occurrence of the hazardous conditions and an assessment of the seriousness of the outcomes of exposure, provides the basis for development of policy. However, other factors should be considered. These may include: the seriousness of other hazards and associated health outcomes that might compete for limited resources; the cost and effectiveness of remedial and preventative actions; and the availability of technical solutions and of experience in their application in the country or region. For effective policy development the above-mentioned factors must be brought together. In most circumstances this is best done in a forum which enables participation of all concerned parties including, for example, water utilities, professional associations, public representatives and experts in the topic under consideration (see section 7.1).

6.1.5 Policy implementation

One of the most frequent failures of policy development is the formulation of legislation in the absence of consideration of its sound and sustainable implementation. In the field of water supply, there has been an increasing recognition by governments of the general need to ensure availability of water supplies, rather than costly treatment only for favoured localities.

A number of actions are available to governments with which to support policy implementation, the most obvious being regulatory enforcement. This implies a capacity to monitor the implementation of the regulations and a will to enforce compliance when the regulations are not met. Other mechanisms for implementation include voluntary codes, conflict resolution, economic instruments and public information and participation (see Chapter 7). Most frequently, multiple actions will be used and will interact with one another to encourage safer behaviours and practices.

6.1.6 Evaluation of management plans and actions

The types of actions described above should, ideally, be combined in appropriate preventative, remedial and contingency plans according to local circumstances and should constitute part of a declared policy for control of adverse human health impacts from cyanobacteria. Thus, they would be integrated into water sector policy more generally, established at national level and operated at local levels supported by legislative frameworks, trained staff and effective institutions with proactive strategies for awareness raising and information dissemination (see Chapter 7).

These plans must be periodically reviewed. In particular, after an incident, it is useful to reflect on the parts of the contingency plan that worked well and those parts that did not

function effectively. With long-term, preventative or remedial management actions it is crucial to put in place a system of monitoring that will enable the efficacy of the actions to be evaluated. There may be long delays before any benefit of a management action is perceived in lakes and reservoirs - in some cases 5-10 years or longer.

It is important to consider the tenets and principles of Adaptive Environmental Management (AEM), particularly with long-term management actions. Decisions and actions are often made on the best available advice, recognising that with any complex ecological problem, such as with a toxic cyanobacterial bloom, the information available to guide a specific management action will always be limited and inadequate. It is important, therefore, to monitor the outcome of the management action, and then to modify or revise the action depending upon the response, or as new scientific information and techniques become available.

6.2 Situation assessment

Assessing the risk posed by toxic cyanobacteria, or the potential for development of cyanobacterial blooms, and linking this to effective measures for the protection of public health within available resources, is complex. Situation assessment may be proactive, for example to determine whether contingency planning is required or to inform long-term action, such as pollution control to minimise bloom formation; or it may be reactive, for example to assist in interpretation of specific local events or conditions to inform emergency or incident response.

An important factor in situation assessment is understanding the adequacy of available information with which to make the assessment. In many, if not all, cases epidemiological evidence of cyanobacteria-related health effects would not be available because of poor or non-standardised, or poorly differentiated, diagnoses; lack of awareness of cyanotoxins as potential causes of symptoms; and inadequate reporting systems, research programmes, or information analysis. In most situations a limited range of information is available to assist in identifying whether a problem or potential problem exists. The types of information possibly available to aid in assessment are summarised in Table 6.2.

6.2.1 Drinking water supply information

The monitoring of water bodies and supply systems for cyanobacteria and cyanotoxins is not yet common practice in most countries in the world. There are a number of critical control points in the potable water supply system where testing for cyanotoxins and intact cyanobacterial cells should be carried out if significant cyanobacterial populations occur in the source water. These may include the water storage reservoir or river; the treatment plant raw water intake; key points in the treatment process; and in the final treated drinking water, depending on local circumstances. Details of such critical points are given in Table 5.1 with more detailed monitoring information being provided in Chapters 10-13.

One requirement is to be aware of which members of the community receive drinking water from which water supply. This information is usually readily available in cities and large towns but this may not be the case in rural areas. In addition, the existence of water treatment systems and their effectiveness in cyanotoxin removal (see Chapter 9)

should be ascertained. For small community supplies there may be little or no water treatment, and this must be taken into account by health authorities when assessing any potential risk situation.

To aid in making a rapid situation assessment based on available water supply information, and critical control points as outlined in Table 5.1, the protection categories outlined in Figure 6.2 should be consulted. This schematic flow diagram may be particularly useful in those countries and regions where little or no consideration has been given previously to the potential risk posed by toxic cyanobacteria in drinking water supplies. The flow diagram focuses on the raw water supply and treatment stream, with attention being paid to the likelihood of toxin release from intact cells (either in the storage reservoir or during the transport network to the treatment plant), removal of intact cells (and their toxins), and the capacity for removal or destruction of dissolved cyanotoxins.

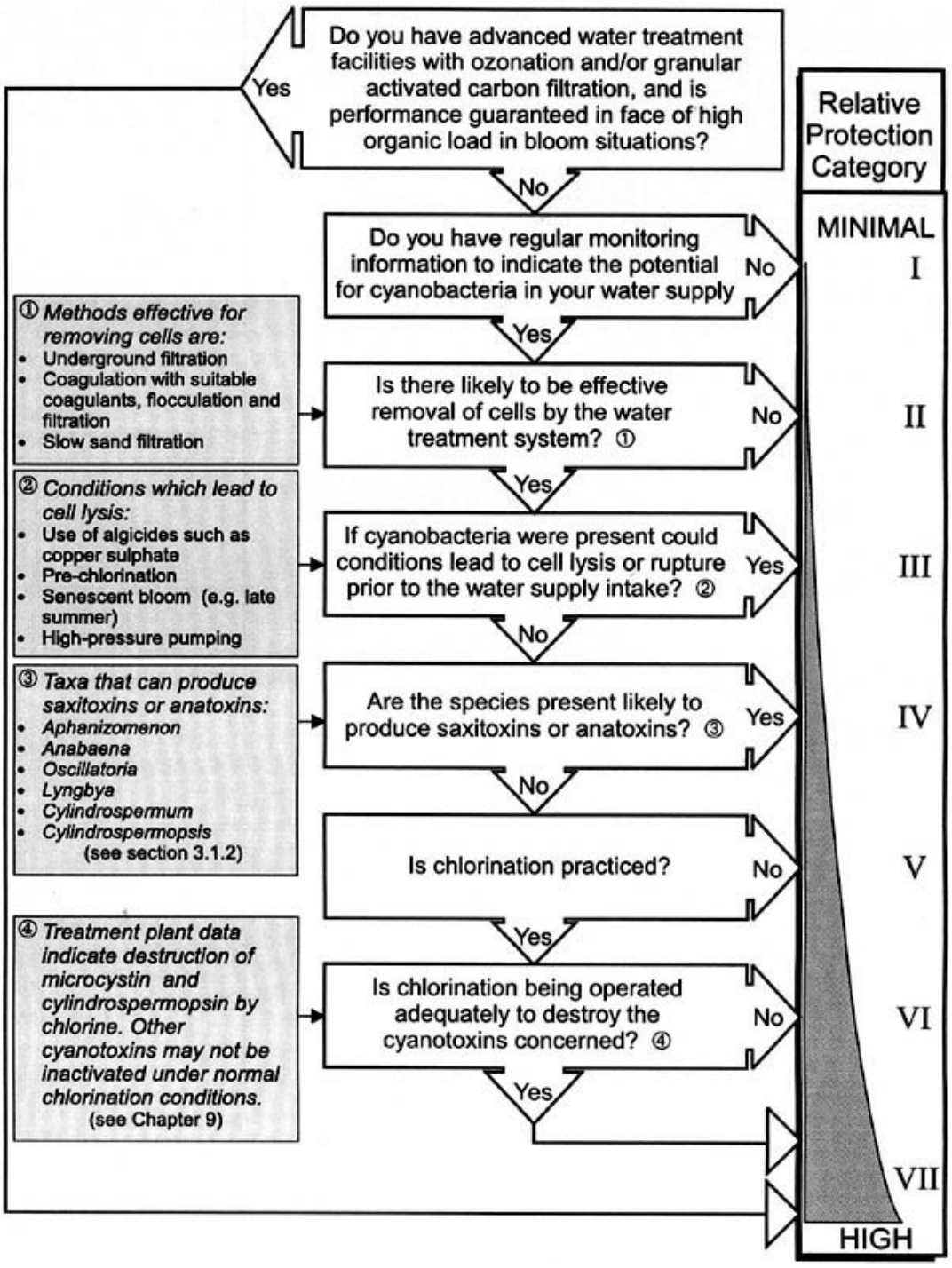
Table 6.2 Types of information of assistance to assess whether a health problem from a cyanobacterial bloom exists or is likely in a particular water body

Observation	Sources of information	Management options
Potential for bloom formation	Water quality monitoring data (nutrients, temperature, etc.)	Basis for proactive management
History of bloom formation	Cyanobacterial blooms may follow marked seasonal and annual patterns	Can inform proactive management
Monitoring of cyanobacteria and/or cyanotoxins	Turbidity, discolouration, cell microscopic identification, cell counts and toxin analysis provide increasingly reliable information	Possible only during event and enables only reactive management
"Scum scouting"	In areas of high public interest the general public and untrained agency staff may play a role in identifying and reporting obvious hazards such as scums	Possible only during event and enables only reactive management
Reporting of animal deaths and human illness	Requires both volition and a mechanism for data collection which may not exist	Possible only during event and informs only reactive management
Epidemiological detection of disease patterns in the human population	Requires both effective reporting and large-scale effects before detection likely	Normally well after an event; can inform future management strategies

6.2.2 Recreational water information

Assessing the potential hazard at recreational water bodies is complicated by the numerous points at which people may enter or move around the water and by the heterogeneous and often rapidly changing distributions of cyanobacterial populations, particularly scums which may be blown around by the wind. As noted in section 5.2.2, concentrated scums pose the greatest risk to bathers. However, monitoring of their potentially rapid formation and dispersal, as well the monitoring of all water bodies used for recreational purposes, is rarely likely to be achievable, nor is it appropriate unless conditions indicate otherwise. Therefore, additional approaches to assessing cyanobacterial risks at recreational sites are needed.

Figure 6.2 Simple and rapid assessment of the degree of protection afforded by drinking water supply systems. Note that this figure should be used with, rather than instead of, more detailed health investigations



Visual monitoring for scums around bathing areas considered to be at-risk is crucial for protecting human health and is quite easy to perform (see Chapters 11 and 12). Operators of recreational sites can be involved in assessing changes of situation.

Furthermore, participation of the public in monitoring for cyanobacteria, and particularly for scums, is a very effective approach (see Table 6.2 under "scum scouting"). This involves education on appearance and toxicity of scums, as well as on recognition of cyanobacteria at high cell densities when they do not form scums (see Chapter 7). An example of a message to convey this may be:

"If you walk into the water up to your knees, carefully, without stirring up sediment, and cannot see your feet because of a greenish discoloration, don't swim and inform the local authority using the following telephone number".
In many countries and regions, the current situation is that public awareness is negligible and knowledge of the risk posed by bathing in cyanobacterial scums is lacking. Eye witness accounts in Australia, Germany and Japan report swimmers deliberately covering themselves in cyanobacterial scum for the sake of an "exciting" photograph, and one well-documented poisoning case in the UK resulted from army cadets undertaking kayak "Eskimo rolls" in cyanobacterial scum. If public education not only addresses personal health risk but also stimulates a sense of responsibility in reporting scums, valuable support for risk assessment at recreational sites can be gained (see Chapter 7).

6.2.3 Environmental information

Environmental information, including physical, chemical and biological data can aid in the prediction and assessment of the likelihood, or presence, of cyanobacterial mass developments, including blooms, scums and mats. It can also help predict and assess types of cyanobacterial toxins and their location.

Historical records and local knowledge

Consultation of historical records, if available, can indicate whether a water body has been prone to cyanobacterial bloom development. Useful information is sometimes available from the local community, including descriptions of the water body and examples of human health incidents, livestock mortalities and fish-kills associated with blooms and scums. However, a lack of historical and local evidence of blooms cannot be taken as assurance that cyanobacterial blooms have not occurred, or will not occur, because data may be lacking and recognition of cyanobacterial blooms and associated problems may have been inadequate (Skulberg *et al.,* 1984) and because increases in cultural eutrophication may be ongoing.

Physical data

Environments in which various species of cyanobacteria can flourish, together with the physical conditions that promote such developments are outlined in Chapter 2. In the case of the many species of planktonic cyanobacteria whose growth is favoured in warm, thermally-stratified environments, the onset of favourable growth conditions is indicated by a rise in surface water temperature above about 18 °C and the establishment of persistent thermal stratification (Reynolds, 1984; NRA, 1990). For example, the Queensland Department of Natural Resources in Australia undertook a two-year survey of thermal stratification in its major water supply reservoirs to aid in its assessment of reservoirs potentially at risk of developing cyanobacterial blooms. The study enabled reservoirs to be categorised as seasonally strongly stratified, weakly stratified or well

mixed, with the strongly stratified bodies being considered most at risk. In addition, the seasonal timing and persistence of stratification was used as a trigger to increase monitoring effort (i.e. as an indicator of when to switch from monthly to fortnightly or weekly sampling) (Chudek *et al.,* 1998).

Hydraulic mixing and transport processes

The ratio between the depth of the mixed layer and the depth to which sufficient light for photosynthesis penetrates, strongly influences cyanobacterial mass development and the extent to which the populations may be dominated by particular cyanobacterial types. Data on flushing rates in lakes as well as river flow rates are useful because planktonic cyanobacteria do not usually attain high population densities in highly flushed environments with retention times (i.e. the time it takes for the water volume to be exchanged once) of less than 5-10 days, or in the open channels of flowing rivers. If river flows are reduced due to drought and/or excessive abstraction of water, cyanobacterial bloom development can be anticipated provided nutrient concentrations and light penetration are adequate (e.g. Bowling and Baker, 1996). Section 2.3 on cyanobacterial "ecostrategists" provides further details of this, together with section 8.5 on the hydrophysical control of cyanobacteria.

Chemical data

The mass development of cyanobacteria is dependent on the nutrient concentrations (especially phosphorus and nitrogen) in a water body. The relationship between mean chlorophyll *a* concentrations (as a simple measure of cyanobacterial and planktonic algal biomass) and annual mean phosphorus concentrations provides a valuable (but easily misused) basis for assessing the likelihood of planktonic biomass development; this is discussed critically in Chapter 8 (Vollenweider, 1968; Vollenweider and Kerekes, 1980). Inputs and concentrations of nitrate and ammonia should also be considered because these can influence growth rates, maximum biomass and phytoplankton species composition. The ability of several toxigenic cyanobacterial genera to fix dissolved atmospheric nitrogen under aerobic conditions (e.g. *Anabaena, Aphanizomenon, Nodularia),* but not others (e.g. *Microcystis, Oscillatoria,* emphasises the need to take physical, chemical and biological factors into account when attempting to predict the likelihood of cyanobacterial mass development.

Biological data

Long-term and within-year monitoring records are useful in contributing to the assessment of the likelihood, onset and persistence of cyanobacterial mass developments. Such long-term data sets are not widely available, and often their value may not be apparent to managers who may see long-term monitoring as difficult to justify. This may be the case particularly in water bodies that have no history of cyanobacterial problems. Health authorities responsible for the quality of recreational waters and drinking water resources in many countries may not be sufficiently informed of data available in environmental authorities or local research institutions. Establishing such contacts is strongly recommended for assessing potential cyanobacterial risks.

In addition to monitoring for total phytoplankton biomass (measured by cell counts or as chlorophyll *a* concentration) and cyanobacterial genera or species distribution and

numbers (see Chapter 12), information on other biota in the reservoir, lake or river can be useful. This could include the types and abundance of phytoplankton grazers (zooplankton) and of zooplanktivorous fish (see section 8.5 for details).

6.2.4 Health information

Information on the health of the population is collected in nearly all countries for the purpose of providing assistance in the prevention and control of disease. Often included in this information are records of outbreaks of gastro-enteritis and, where possible, their causes. Most sources of gastro-enteritis are infectious organisms, although in most cases of gastro-enteritis a specific cause is not identified. When a substantial outbreak of gastro-enteritis occurs it will generally be investigated in order to determine the source and the causative organisms. Faecal, food and water samples may be screened for a variety of possible pathogens, and only if no pathogens have been identified will the possibility of toxicity in the water or food be investigated. As awareness of cyanobacterial toxicity increases, the likelihood of these toxins being considered as a possible cause for clinical illness increases.

Routine monitoring for the presence of cyanobacterial cells or cyanotoxins in drinking water is undertaken in only a few countries at present, and then only by some water supply companies or authorities. In those countries where there is an awareness of the problem, monitoring of reservoirs after the onset of a cyanobacterial bloom has been detected is more common. As a result of the absence of routine monitoring, reports of gastro-enteritis outbreaks that have been later attributed to cyanobacterial poisoning have been made in the absence of cell counts or toxin measurement at the time of the event (for examples see section 4.1).

It is unlikely that an outbreak of illness will be related to cyanobacteria in the drinking or bathing water unless a specific local investigation is conducted. A link between data gathered by health authorities and cyanobacterial data obtained from water monitoring will be required if acute gastro-enteritis caused by cyanotoxins is to be understood and avoided. This may be established by the reporting of monitoring data collected by water supply agencies to health authorities. In outbreaks of gastro-enteritis in which no pathogen has been detected, it may be useful to look at the geographical distribution of cases to see whether the drinking water distribution system is the likely source. Investigation of the presence of cyanotoxins can follow (if still present), or proactive investigation for a subsequent bloom can be introduced.

Until cyanobacterial monitoring and cyanotoxin analysis are more widely established, it will remain difficult to correlate clinical findings with the toxic effects of cyanobacteria.

6.2.5 Other data

In addition to the drinking water, recreational water environment and health information that may assist in developing a situation assessment, additional information may be gleaned from veterinary records of animal deaths and *post mortem* examinations (see Chapter 4 for likely symptoms and pathologies). While in many cases it may not be possible to attribute unequivocally animal deaths, or even poor water quality, to toxic cyanobacteria, consistent relationships between these observations and particular water

bodies at certain times of the year may be indicative of water bodies with potential cyanobacterial problems.

6.3 Management actions, the Alert Levels Framework

An Alert Levels Framework is a monitoring and management action sequence that water treatment plant operators and managers can use to provide a graduated response to the onset and progress of a cyanobacterial bloom. Circumstances and operational alternatives will vary depending upon the source of the water supply and the analytical and water treatment facilities available. The managerial response model, presented as a "decision tree" in Figure 6.3, is based upon the critical control points identified in Table 5.1, the drinking water supply protection categories defined in Figure 6.2, and on an alert levels framework developed earlier in Australia. The decision tree should be seen as a general framework, recognising that it may be appropriate to adapt specific alert levels and actions to suit local conditions in different countries.

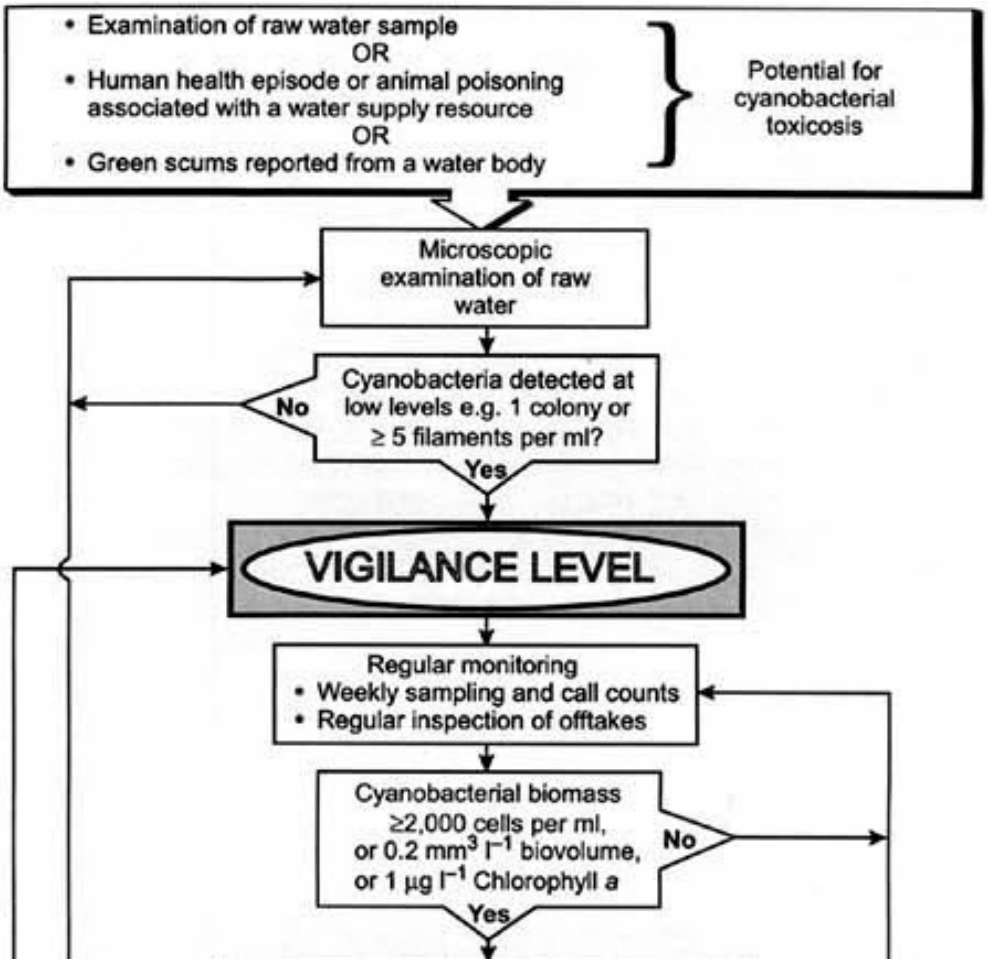
The decision tree provides for the assessment of a potentially toxic cyanobacterial bloom, with appropriate actions and responses, through three "threshold" stages. The sequence of response levels is based upon the initial detection of cyanobacteria at the Vigilance Level, progressing to moderate to high cyanobacterial numbers and possible detection of toxins above guideline concentrations at Alert Level 1. Alert Level 1 conditions require decisions to be made about the suitability of treated drinking water based on the efficacy of water treatment and the concentrations of toxins detected (if such measurements can be made). At very high cyanobacterial biomass levels in raw water, the potential health risks associated with treatment system failure, or the inability to implement effective treatment systems at all, are significantly increased. This justifies progression to a heightened risk situation denoted by Alert Level 2 conditions.

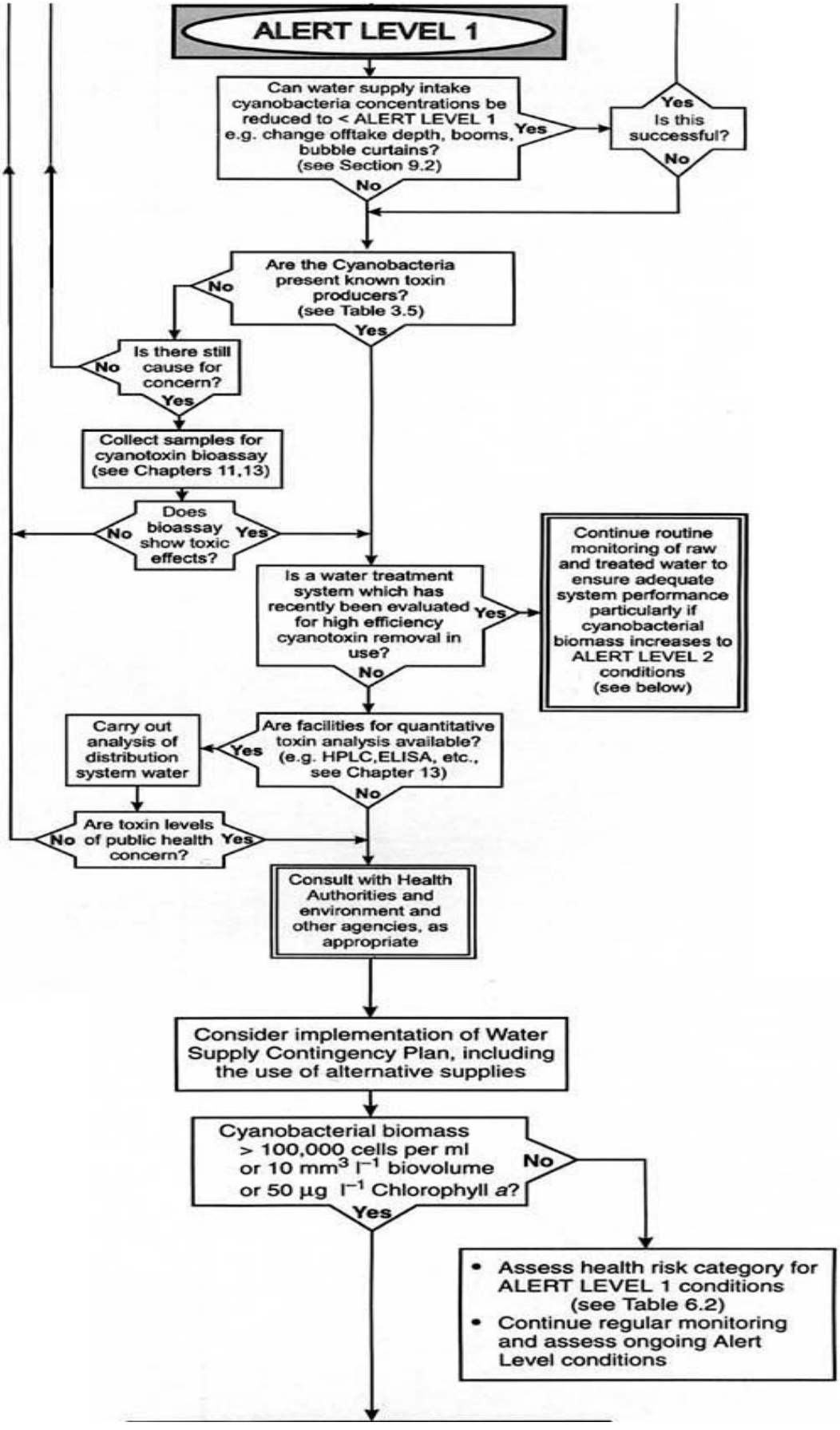
The framework has been developed largely from the perspective of the drinking water supply operator but is also important for the manager of the raw water supply. The actions accompanying each level cover categories such as additional sampling and testing, operational options, consultation with health authorities and media releases. An important part of the framework is consultation at various stages with other agencies, particularly health authorities that generally have responsibility to oversee the safety of water for potable supply.

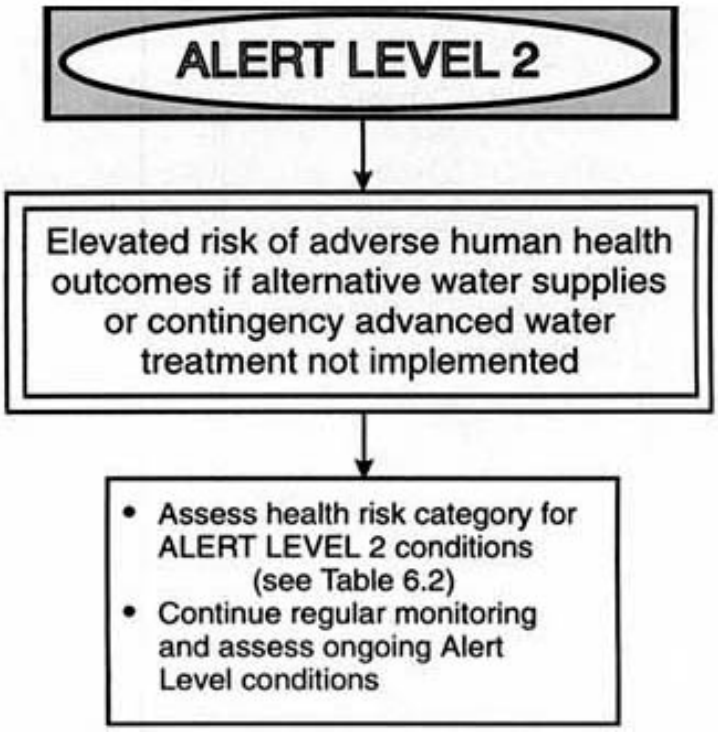
6.3.1 Vigilance Level

The Vigilance Level encompasses the possible early stages of bloom development, when cyanobacteria are first detected in unconcentrated raw water samples (see Chapter 12). The indicative value for the Vigilance Level is the detection of one colony, or five filaments, of a cyanobacterium in a 1 ml water sample, although this threshold may be adapted according to local knowledge and prior monitoring history. Taste and odours may become detectable in the supply as the population develops above the Vigilance Level, but their absence does not indicate absence of toxic cyanobacteria. For example, recognition of the highly odorous earthy/muddy smelling compound geosmin, produced by *Anabaena,* may occur at concentrations less than 1,000 cells per ml (Jones and Korth, 1995). In contrast, *Microcystis* produces weakly odorous compounds that will be detected only at cell concentrations 100-1,000 times higher than this, and are therefore unlikely to be noticed in raw water.

Figure 6.3 Decision tree incorporating a model Alert Levels Framework for monitoring and management of cyanobacteria in drinking water supplies. Note that this framework should be adapted according to local conditions




ALERT LEVEL 1
Can water supply intake cyanobacteria concentrations be reduced to < ALERT LEVEL 1 e.g. change offtake depth, booms, bubble curtains? (see Section 9.2)
Yes
No
Yes
Is this successful?
No
Are the Cyanobacteria present known toxin producers? (see Table 3.5)
No
Yes
Is there still cause for concern?
No
Yes
Collect samples for cyanotoxin bioassay (see Chapters 11,13)
Does bioassay show toxic effects?
No
Yes
Is a water treatment system which has recently been evaluated for high efficiency cyanotoxin removal in use?
Yes
No
Continue routine monitoring of raw and treated water to ensure adequate system performance particularly if cyanobacterial biomass increases to ALERT LEVEL 2 conditions (see below)
Are facilities for quantitative toxin analysis available? (e.g. HPLC,ELISA, etc., see Chapter 13)
Yes
No
Carry out analysis of distribution system water
Are toxin levels of public health concern?
No
Yes
Consult with Health Authorities and environment and other agencies, as appropriate
Consider implementation of Water Supply Contingency Plan, including the use of alternative supplies
Cyanobacterial biomass > 100,000 cells per ml or 10 mm3 l−1 biovolume or 50 µg l−1 Chlorophyll a?
No
Yes
• Assess health risk category for ALERT LEVEL 1 conditions (see Table 6.2)
• Continue regular monitoring and assess ongoing Alert Level conditions



The presence of cyanobacteria in low numbers (and sometimes detection of characteristic tastes and odours) constitutes an early warning for potential bloom formation; therefore, when the Vigilance Level is exceeded, it is generally appropriate to increase the sampling frequency to at least once a week, so that potentially rapid changes in cyanobacterial biomass can be monitored.

6.3.2 Alert Level 1

The Alert level 1 threshold (cyanobacterial biomass 2,000 cells per ml or 0.2 mm^3 l^{-1} biovolume or 1 µg l^{-1} chlorophyll *a*) is derived from the WHO guideline for microcystin-LR (see Chapter 5) and the highest recorded microcystin content for cyanobacterial cells (Chapter 3). In other words, threshold is the cyanobacterial biomass level, assuming the species is a potential producer of microcystin, where raw water microcystin concentration could exceed the WHO guideline. Consequently, Alert Level 1 conditions require an assessment to be made (preferably based on an analysis of total toxin concentration in the raw water by a quantitative technique such as high pressure liquid chromatography (HPLC) or enzyme linked immuno sorbent assay (ELISA), see Chapter 13) as to whether the concentration of potentially toxic cyanobacteria in the raw water supply can be reduced (e.g. by offtake management) or whether the water treatment system(s) available are effective in reducing toxin concentrations to acceptable levels (see Figure 6.2 and Chapter 9).

Alert Level 1 conditions require consultation with health authorities for ongoing assessment of the status of the bloom and of the suitability of treated water for human consumption. This consultation should be initiated as early as possible and continue after the results of toxin analysis on drinking water become available. Clearly, as the biomass of potentially toxic cyanobacteria increases in the raw water, so does the risk of

adverse human health effects in the absence of effective water treatment systems. Therefore, on-going monitoring for cyanobacterial biomass and toxin concentrations is essential. The monitoring programme, which should be at least weekly in frequency, may be extended throughout the source water body to establish the spatial variability of the cyanobacterial population and of toxin concentration. It may also be appropriate at this time to issue advisory notices to the public through the media or other means. Government departments and authorities with possible interests or legal responsibilities should also be contacted (see Table 7.1), as should organisations or facilities that treat or care for special "at risk" members of the public (such as kidney dialysis patients, see Chapter 4).

For toxic cyanobacteria other than those that produce microcystin (see Chapter 3, or as indicated by bioassay results) it will be necessary for local health authorities to undertake a detailed risk assessment based on the inherent hazard of the toxin detected (Chapter 4), its concentration in treated drinking water, and the exposure expected. If Alert Level 1 biomass levels are maintained, but toxins or toxicity are not detected in cyanobacterial or raw water samples, regular monitoring should continue to ensure that toxic strains or species do not develop over ensuing weeks or months (see Chapter 3).

6.3.3 Alert Level 2

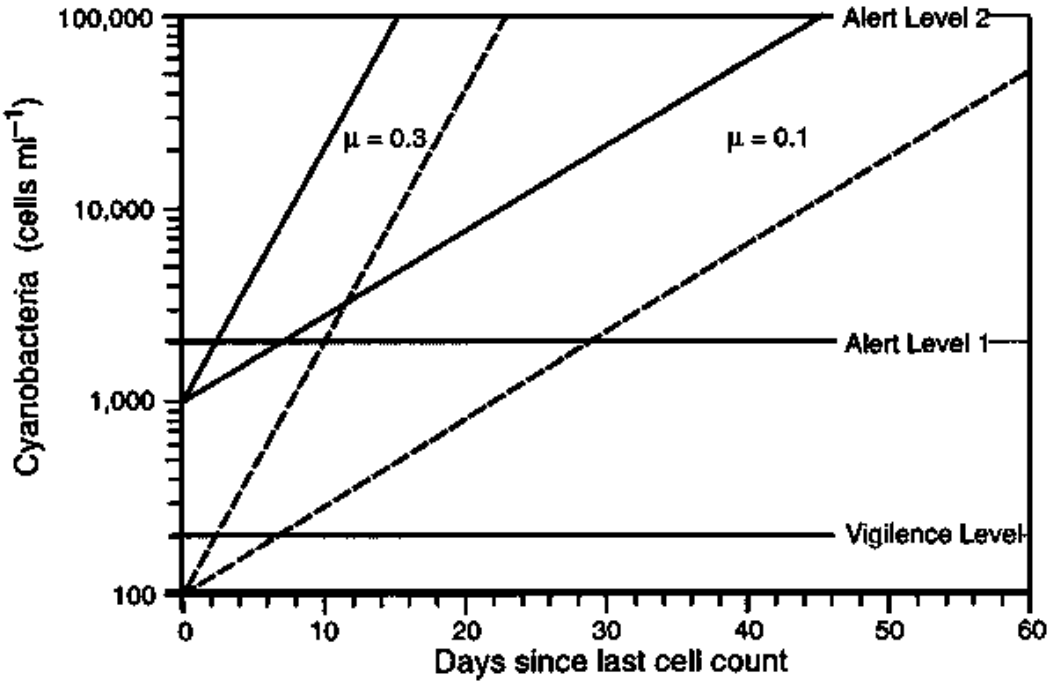
The threshold for Alert Level 2 (cyanobacterial biomass 100,000 cells per ml or 10 $mm^3 l^{-1}$ biovolume or 50 $\mu g\ l^{-1}$ chlorophyll *a,* with the presence of toxins confirmed by chemical or bioassay techniques) describes an established and toxic bloom with high biomass and possibly also localised scums (although scums may also form under Alert Level 1 conditions). The sampling programme will have indicated that the bloom is widespread with no indication of a cyanobacterial population decline in the short term. Conditions in Alert level 2 are indicative of a significant increase in the risk of adverse human health effects from the supply of water that is untreated, or treated by an ineffective system (see Chapter 9), even for short-term exposure. The need for effective water treatment systems and on-going assessment of the performance of the system thus becomes of heightened importance. Whereas hydrophysical measures to reduce cyanobacterial growth may still be attempted in this phase (Chapter 8), application of algicides can enhance the problem by causing high concentrations of dissolved toxins as a consequence of cell lysis. Whereas filtration systems (possibly combined with flocculation-coagulation) may have removed cell-bound toxins, dissolved toxin is more likely to break through treatment systems.

If effective activated carbon or other advanced treatment is not available, Alert Level 2 conditions should result in the activation of a contingency water supply plan which is appropriate for the operator and the user or community. This may involve switching to an alternative supply for human consumption, the implementation of contingent treatment systems, or in some circumstances the delivery of safe drinking water to consumers by tanker or in bottles. More extensive media releases, and even direct contact with consumers via letterbox delivery of leaflets with appropriate advice to householders, may be necessary (see Chapter 7). Where advice is provided to the public because of a cyanobacterial hazard to human health, it will generally be advisable to emphasise that the water would still be suitable for purposes such as washing, laundry, toilet flushing, etc. Withdrawal of a piped drinking water supply because of a cyanobacterial toxin hazard is usually not justifiable because the adverse health effects resulting from

disruption of supply (e.g. lack of toilet flushing, lack of fire fighting) are likely to outweigh substantially the cyanobacterial toxin risk itself.

Monitoring of the bloom should continue in order to determine when the bloom starts to decline so that normal supply can be resumed. The sequence at Level 2 may follow through to deactivation of alert level conditions with media releases, and advice to government departments and health authorities to confirm this. It is possible that the collapse of a bloom, or a management action such as flushing, could lead to a rapid decline from Level 2 back to Level 1 or beyond. Likewise the sequence might escalate rapidly, bypassing Level 1 to Level 2, if adequate monitoring and early warning information is not available. Cyanobacterial populations in natural water bodies, especially in hot climates, may double in size in less than two days (growth rate, μ, 0.35 d^{-1}). Therefore, monitoring frequency needs to take such potentially rapid population growth rates into account. Figure 6.4 gives an indication of the rate of change of an exponentially dividing population at two growth rates observed in field studies of cyanobacteria.

Figure 6.4 Predicted development of cyanobacterial population from initial concentration of 100 or 1,000 cells per ml and at growth rates (μ) of 0.1 and 0.3 d^{-1} (After Jones, 1997)



6.3.4 Overall risk assessment and summary of action-response thresholds

The information presented in Table 6.3 will enable water resource managers, treatment plant operators and health authorities to make a rapid assessment of the potential risks associated with increasing levels of potentially toxic cyanobacteria in water (based on the Alert Levels Framework presented above and in Figure 6.3) and the protection afforded by the monitoring and water treatment systems in place (based on the protection categories given in Figure 6.2). The risk category outcomes in Table 6.3 take

into account the possibility of toxin persistence after a bloom has collapsed and the possibility, therefore, that dissolved toxins are present in the absence of viable cyanobacterial cells (hence the different risk outcomes for long term compared with short term, low biomass), and the increased risk associated with treatment system failure at very high cyanobacterial biomass (Alert Level 2).

Table 6.3 Relative risk categories for adverse human health outcomes due to toxic cyanobacteria in drinking waters

Protection category[2]	**Cyanobacterial biomass category**[1]			
	Absent or low (long term)	**Absent or low (short term)**	**Moderate - high (Alert Level 1)**	**Very high (Alert Level 2)**
I	-	Low	High	Very high
II	Minimal	Low	High	Very high
III	Minimal	Low	Medium- high	High
IV	Minimal	Low	Low - medium	Medium
V	Minimal	Low	Low	Medium
VI	Minimal	Minimal	Minimal - low	Low
VII	Minimal	Minimal	Minimal	Minimal - low[3]

[1] The cyanobacterial biomass categories are defined as follows (see Figures 6.3 and 6.5):

Low: Cyanobacterial biomass < 2,000 cells per ml or 0.2 $mm^3\ l^{-1}$ biovolume or 1 $\mu g\ l^{-1}$ chlorophyll a; the category is "long term" when based on data compiled over at least a two-month period, and "short term" when based on data compiled over less than two months (including analysis of a single sample only and taking into account the risk of toxin persistence after a bloom collapses)

Moderate - high: Whether for a single measurement or for repeated measurements over several weeks, cyanobacterial biomass greater than in low category but, < 100,000 cells per ml or 10 $mm^3\ l^{-1}$ biovolume or 50 $\mu g\ l^{-1}$ chlorophyll *a*

Very high: Cyanobacterial biomass > 100,000 cells per ml or 10 $mm^3\ l^{-1}$ biovolume or 50 $\mu g\ l^{-1}$ chlorophyll a, and presence of toxins confirmed by chemical analysis or bioassay

[2] Protection categories from Figure 6.2

[3] Risk category is greater than minimal because of the increased risk associated with treatment system breakthrough or failure at high biomass loads

Finally, Figure 6.5 provides an "at a glance" summary of the threshold managerial action levels for drinking water (Vigilance and Alert Levels) and the health guidance levels for recreational waters outlined in Chapter 5, Table 5.2.

6.4 Planning and response

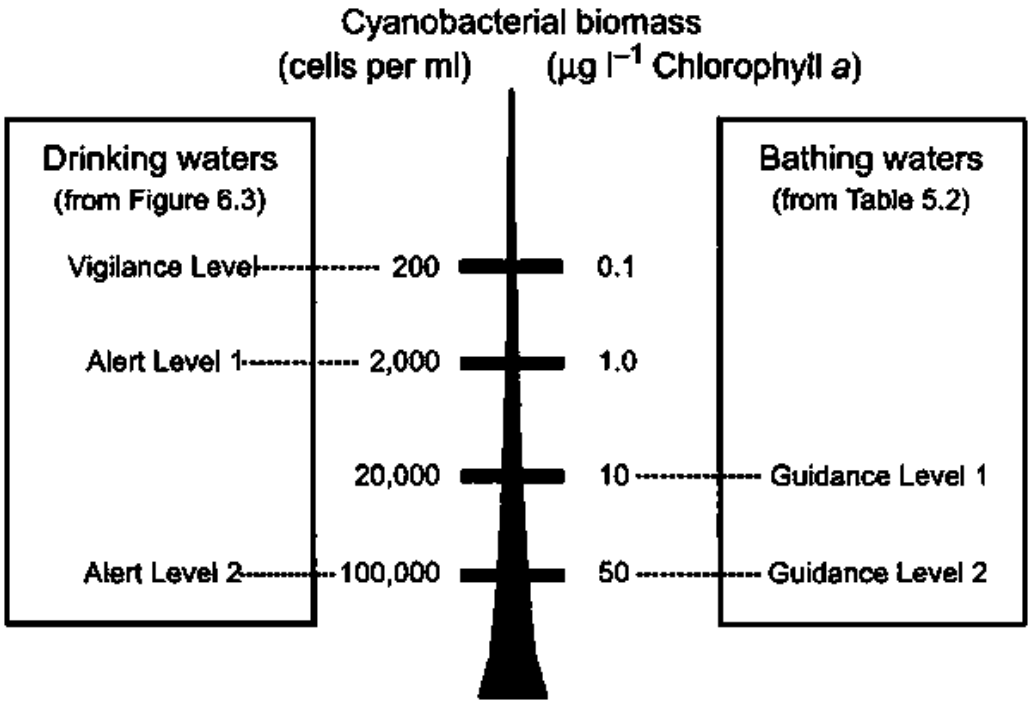
The intensity and scope of management actions to control human health hazards associated with cyanobacteria may vary widely. In the simplest case, a one-off study may indicate little or no cause for concern or it may lead to an information dissemination and public awareness and education campaign. An initial situation assessment that indicates significant risk calls for the establishment of a monitoring programme, formulation of contingency plans, changes in pollution control and water supply management, and a more substantial public information and participation strategy. Which type of response is appropriate in a given situation will depend on a number of complex and interconnected factors, of which rational assessment of human health risk is one part. Other important aspects include, for example, technical and institutional capacities, economic considerations and social values. The principal management actions that may be taken fall into three main groups:

- *Water resource and catchment management.* Most long-term actions are intended to minimise nutrient inputs to water bodies, both from external (watershed) and internal (sediment) sources, as well as altering the hydro-logic conditions in such a way as to prevent or reduce conditions favourable for cyanobacterial bloom formation (see Chapter 8 for guidance on long-term measures). Other actions may include manipulation of the aquatic food web to increase grazing pressure on cyanobacteria (see Chapter 8).

- Remedial measures in drinking water supply. Some of these actions may be seen to be applicable in short time frames and may therefore be deployed in response to a specific situation; other are also medium- or long-term actions, such as installing safeguards (such as treatment steps) in drinking water supply that will assist in controlling risks while such safeguards remain installed and operated. Other actions are associated with contingency plans to be activated in case of need (see Chapter 9).

- *Public information and awareness-raising.* A well-informed public (see Chapter 10), aware of the potential and real risks of toxic cyanobacteria, is important for many reasons. These reasons include an improved surveillance capacity through public participation; for example swimmers and other users can contact local authorities if they see cyanobacterial scums, and householders can report unusual odours in their drinking water supply. Several Australian States have community-based "WaterWatch" or "Stream-Watch" monitoring programmes that are undertaken by high school students and community groups. Experience has shown that more aware communities, which are adequately informed by appropriate authorities, will be less likely to react inappropriately in the event of a bloom.

Figure 6.5 Summary of managerial action levels for drinking waters (see Alert Levels Framework decision tree in Figure 6.3) and for bathing waters (see Guidance Levels in Table 5.2). Note that for bathing waters, the special Guidance Level 3 (scum formation) can be achieved during calm weather conditions at open water biomass levels similar to Guidance Level 1



Few remedial measures specific to recreational water sites are available in addition to those for minimising bloom formation, increasing public awareness, and informing the public. In some instances, fencing-off or the placing of a boom to prevent accumulation of scums may be considered for densely used and highly impacted areas, in addition to public information and awareness-raising.

Proactive management, that is taking action before significant cyanobacterial proliferation has occurred, is generally preferable to reactive (or crisis) management, that is placing controls in place once significant cyanobacterial proliferation has begun. Few countries presently operate monitoring programmes capable of identifying potentially hazardous conditions and early warning systems have not as yet been developed (for example giving several weeks notice of impending toxic cyanobacterial hazards), even though previous monitoring data may indicate annual "high risk" months or periods. In practice, therefore, management of cyanobacterial hazards typically occurs in three ways, preventative management plans, contingency plans and planning in emergency situations.

6.4.1 Preventative management plans

Management to prevent human health effects will typically involve prevention and minimisation of cyanobacterial blooms, deployment of appropriate drinking water treatment where required, and controls on recreational water use in order that human

populations are not exposed to significant risks. It will also involve general contingency planning, which is considered in section 6.4.2.

History has shown that single-action management plans tend to have poor success rates; one example of this was the ban during the 1970s in several European countries on the use of detergents containing phosphate. Where only this action was applied, little success was seen in the abatement of cyanobacterial and algal blooms because other sources of phosphorus pollution were largely ignored (including the remaining 50-70 per cent of phosphorus in sewage that does not arise from detergents).

Reduction of nutrient pollution below threshold values which control cyanobacterial bloom formation is highly effective and sustainable. However, many water bodies require large reductions in nutrient loads, and the implementation of the necessary measures is likely to take a number of years. Furthermore, the high nutrient concentrations within such water bodies may decline only slowly in response to reduced external loading, particularly if water exchange rates are low or release from sediments is high. Thus, total prevention of cyanobacterial bloom development may require extended recovery time spans, and may even be unachievable in naturally eutrophic systems. In such situations, bloom minimisation will generally be accompanied by contingency planning and, if blooms are expected to occur, by application of appropriate drinking water treatment either continuously or at times of cyanobacterial occurrence in the source water.

Preventative management may effectively address the hydrophysical conditions of cyanobacterial growth through the use of hydraulic management (flushing, artificial mixing), or reduce drinking water intake concentrations by offtake management.

An effective approach to preventative management may be changing the drinking water source (where this is feasible). This approach was illustrated by the change from using water in shallow eutrophic ponds and ditches to using groundwater in China (see Box 5.3).

There is no single formula that can be followed to compile a good preventative management plan. However, key elements may include:

- The convening of a multi-agency and multidisciplinary committee to develop the plan and to co-ordinate its implementation.

- Development of comprehensive policy relating to point and diffuse source pollution control, and for the regulation of river flows and reservoir management for the prevention of cyanobacterial problems.

- Compilation or review of relevant technical information (such as for bloom prevention and management, drinking water treatment and recreational water management), and the involvement of key technical (e.g. scientific and engineering) personnel to provide expert advice.

- Development of procedures (including financial and institutional procedures) for implementing key actions arising from the plan.

• Development of monitoring systems to determine the effectiveness of management actions.

• Establishment of response mechanisms for modification of action in the light of feedback on management plans.

• Development of means for effective communication between agencies and with the public and media.

Two examples of how preventative plans may be co-ordinated are provided in Boxes 7.2 and 7.4.

6.4.2 Contingency plans

Plans and actions for prevention of health hazards arising from cyanobacterial blooms should aim to prevent and curtail blooms and ensure that plans are in place to respond to blooms when they do occur. Planning for such events is an important part of the overall strategy for managing health hazards associated with toxic cyanobacterial blooms.

Contingency plans are normally developed and managed at a local or regional level. National and regional authorities may, nevertheless, have important roles to play in supporting and facilitating plan formulation and in providing expertise, should an event occur. Key elements of a contingency plan overlap with those for preventative management and include:

• The convening of a multi-agency and multidisciplinary committee to develop, maintain and, if necessary, modify the plan and co-ordinate its implementation if required. The members of such a committee should be aware of their authority and responsibility as committee members in advance of an occurrence (see Chapter 7).

• Development of a comprehensive response plan including specific actions at different alert levels and the responsibilities of different agencies.

• Compilation of a manual or guide for incident response addressing the major areas of activity and including management, drinking water treatment and recreational water management, and communication with the public and media.

• Plans for effective communication between key government agencies, health authorities, water supply agencies, hospitals and the public need periodic testing.

• Ensuring the availability of technical capacity (especially analytical capacity and access to expertise) to deal with the demands of the contingency plan. The specified experts or institutions should be able to respond to specific questions in time horizons relevant to incident response, such as:

• What is the size, extent and toxicity of the bloom?

• If toxic, what types and concentrations of cyanotoxins are present in the drinking water supply and how are they partitioned between cell-bound and dissolved phases?

• Is an adequate water treatment system in place (see Chapter 9 and Table 6.2 for details), and if not will the general public be exposed to "unsafe" concentrations of cyanotoxins?

• Special precautions (e.g. portable water treatment systems, or transported safe water supplies) may be advisable for "at risk" groups especially susceptible to cyanotoxins, such as patients with previous acute liver damage.

• Special precautions (usually additional treatment facilities with careful monitoring of performance) are of crucial importance for hospitals treating patients with kidney dialysis or intravenous therapy.

• Identification of potential alternative water supplies, preferably from uncontaminated groundwater (there is the possibility that other local surface water storage facilities may be suffering simultaneously from cyanobacterial problems) to be exploited in the case of severe health hazards. This may include plans for transporting clean, treated water from other areas or deploying portable water treatment systems.

• Establishment of awareness amongst local health practitioners where significant hazards are believed to exist and development of systems for communicating with them in the event of an outbreak, including for example advice regarding the possible symptoms of cyanobacterial intoxication and what treatments are advised.

• Prior agreements regarding standardised press releases and the conditions under which their release would be justified. Release of information to the media should be co-ordinated through the main organising committee or task force.

6.4.3 Emergency responses and incident investigation

The actions to be taken in responding to an incident are similar to the elements listed under "contingency planning" (above). However, time constraints will be greater and, because of lack of prior preparation, resources may be less available. The risk of contradictory "messages" from concerned authorities is proportionately greater. Experience has shown that initiating interagency co-operation, especially between the drinking water supplier and the health authority, securing an expert opinion on the real risk to human health and initiating communication with the media and public, are crucial elements in the earliest stages of responding to an incident. It should be recalled that whilst true emergencies can arise from cyanobacterial blooms, as was the case with the Caruaru dialysis tragedy in Brazil (see Box 4.4), an event may be perceived as an emergency or "environmental crisis" by the public and the media even if this is not the case from a health viewpoint.

If the incident is deemed to be severe (as was the case in the Palm Island Mystery Disease, see Box 4.3), a health investigation should be instigated without delay. A follow-up investigation of an incident will often provide valuable information for both preventative and contingency planning. It may also lead to substantial improvement of

the regional assessment of hazard due to cyanotoxin exposure, as in the case of one such investigation from Canada (Box 6.1).

Box 6.1 The Manitoba incident

Deacon Reservoir is the City of Winnipeg's main storage facility for water from Shoal Lake. The lake is generally considered to be of high quality and its water is only disinfected with chlorine prior to distribution for drinking. In late August 1993, a large cyanobacterial bloom developed in Deacon Reservoir. In an attempt to control both cyanobacterial density and taste and odour problems, municipal officials isolated the reservoir and treated it with copper sulphate. This action raised concerns that if the bloom contained toxin-producing cyanobacteria, significant quantities of the toxins may have been released into the reservoir.

Sampling determined that toxin-producing cyanobacteria were not present in the Deacon Reservoir, but they were present in Shoal lake, the dominant species being *Microcystis aeruginosa.* Analysis of water samples indicated that microcystin-LR produced by *M. aeruginosa* was present in samples collected from Shoal Lake and from the distribution system, but it was not present at detectable levels (< 0.05 µg l^{-1}) in samples from Deacon Reservoir. Maximum microcystin-LR concentrations measured in the raw water of Shoal Lake and in treated tap water were 0.45 µg l^{-1} and 0.55 µg l^{-1}, respectively. Subsequent monitoring showed a steep decline in concentrations, suggesting that higher microcystin-LR levels may have been present earlier in August 1993.

As the weather during the summer of 1993 was characterised by below-normal temperatures and above-normal precipitation (conditions that are usually not supportive of cyanobacterial bloom formation) there was concern that higher levels of microcystin-LR could develop in Shoal Lake during the more usual relatively hot, dry summers. As a result, Manitoba Environment, in co-operation with the City of Winnipeg, continued to monitor for microcystin-LR in Winnipeg's water supply. On six occasions between 1994 and the end of 1996, microcystin-LR was detected at concentrations ranging from 0.1 to 0.5 µg l^{-1}.

Because Shoal Lake (a relatively nutrient-poor water body) had supported a toxic cyanobacterial bloom, Manitoba Environment became concerned that toxic blooms might also occur in rural surface water supplies in southern Manitoba, which are generally more nutrient-rich. A comprehensive two-year study was conducted on water quality in rural south-western Manitoba surface water supplies in 1995 and 1996. In the first year of the study, microcystin-LR was found to be widely distributed in all water supply categories. Rural municipal water supplies had a higher detection frequency (93 per cent) than on-farm domestic/livestock dugouts (57 per cent), suggesting that conventional treatment methods were only partially successful in removing the toxin. Mean concentrations ranged from 0.23 µg l^{-1} in recreational sites to 0.35 µg l^{-1} in dugouts used exclusively for livestock. In the second year of the study, seven rural surface water supplies were intensively sampled for microcystin-LR. The hepatotoxin was found throughout the entire sampling period (June to December 1996), sometimes at levels greater than 0.5 µg l^{-1}, which was the "Emergency Health Advisory Guideline" formulated by Health Canada in response to the 1993 incident.

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Chapter 7. IMPLEMENTATION OF MANAGEMENT PLANS

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Control of hazards to human health from cyanobacteria is part of a wider picture of water quality and quantity management, environmental protection, policy formulation and general development concerns. Implementation of programmes to address cyanobacteria-related health hazards therefore takes place within the larger framework of other local, national, and international plans and activities relating to water. That framework is the subject of this chapter, which examines the principal elements required to respond effectively to cyanobacteria-related health risks. First the organisations, agencies and groups that may become involved where there is a cyanobacterial bloom are identified, then the tools governments can use to implement policy are examined, together with legislation as an implementation tool. The remainder of the chapter outlines how professionals and the public may be educated, informed and mobilised.

7.1 Organisations, agencies and groups

7.1.1 Vested interests

Every water body has associated with it a set of persons and organisations with a vested interest and an involvement. Vested interests can be associated with the level of water (flooding, hydroelectricity, transportation, recreation); with the contents of the water (fishing, recreation); with the quality of the water (drinking water/domestic use, recreation); with the quantity of water (irrigation, industry, transportation); with its motility (wastewater, transport); with the preservation of the water body itself (ecosystem maintenance, wildlife conservation); or with some combination of these.

Few rivers, lakes, estuaries and seas can support all vested interests equally and so compromises amongst conflicting activities are inevitable. Diverting too much water for irrigation lowers water levels for transportation and may increase the salinity and mineral content of the water, reducing the numbers offish and causing stagnation. It may also increase the likelihood of algal and cyanobacterial bloom formation in river reaches downstream of irrigation reaches, because many cyanobacteria species prefer slow-flowing river conditions for bloom development. Allowing unrestricted transportation of dangerous cargoes may damage the ecosystem and wildlife of the surrounding area.

Limiting the construction of dams to preserve a riverine environment may mean insufficient energy production and water inputs for industry, domestic needs and mining. Activities particularly likely to increase cyanobacterial hazards in water resources include:

- Land-use, land-use changes and abstraction practices, which may have an impact through diffuse and point sources of nutrients (such as agriculture) and through changing run-off regimes (e.g. caused by urbanisation and forest cutting).

- Pollutant discharges and especially nutrient-rich municipal wastewaters and sewage discharges, which are likely to increase eutrophication.

- Flow control and river flow reduction through, for example, damming, which may increase opportunities for cyanobacterial proliferation by increasing hydraulic retention times and the propensity for water column stratification.

- Abstraction, whether constant (such as for drinking water supply), seasonal (such as for irrigation) or intermediate (such as for power generation), which may tend to decrease flow rates and also increase opportunities for cyanobacterial proliferation by increasing retention times.

As with any other activity in relation to water resources, taking action in response to a cyanobacteria risk requires the recognition and balancing of vested interests. For example, increasing stream flows to decrease hydraulic residence time will have beneficial effects on drinking water and animal health, but may have detrimental effects on recreational use and agricultural (irrigation) interests.

Responding to an environmentally-related health issue, such as a toxic cyanobacterial blooms, implicates and involves many with vested interests who can be defined broadly as representing the public, the media, the government (or its agents) and the private or corporate sector. The largest and most vociferous vested interest group will often be the general public, who may demand that the quality of their drinking or recreational water be returned to a level that they deem acceptable, as soon as possible. Sometimes the water quality standard that the public expects may seem unrealistic (e.g. *"The water used to be crystal clear when I was a child, so why isn't it now?").* Farmers may also represent a vociferous lobby who may insist that the water necessary for farms and livestock be again made safe so that productive activity may resume. However, they may also be a strong lobby against reduction of diffuse pollution input by arguing that changes in agricultural practices, e.g. fertiliser application, would inevitably reduce crop yields. Water suppliers often have a strong vested interest in a better resource quality because that increases the safety of their product and reduces treatment costs, but agencies may tend to avoid publicity on this issue in order to avoid a negative image and adverse public comment. Thus, the greatest driving force for action will nearly always come from the user sector.

The media can play a key role in acting as vehicles for public awareness, thereby triggering public outcry. They can also be vital for advising the public of potential health risks (or their absence), for informing the public whether appropriate actions have been taken and of the efficacy of these actions.

Notwithstanding governments' primary public safety role, where they exist, private companies involved in water supply and wastewater treatment processes will also have a key role in any response. Collaboration with such companies will be necessary whether planning is of an emergency or long-term strategic nature. However, private companies may view the monitoring data they generate as proprietary or commercial and their active participation needs to be encouraged, whereas governments may view information they hold as being in the public domain.

Other important sectors with a vested interest in many countries are tourism and recreation. Hotels, holiday resorts, water skiing and fishing-based tourism operations, for example, can be affected profoundly by adverse publicity and recreational restrictions brought about by toxic cyanobacterial blooms. Involvement of these sectors will be extremely important if local acceptance of management plans (short- and long-term) is to be obtained. The fact that private companies operate on a profit motive does not necessarily place them at odds with governments' public safety goals, because safe water is also a prerequisite for most, if not all, kinds of tourism and recreation activities, and is seen increasingly as a priority amongst tourists and other recreational water users.

Where a cyanobacterial bloom occurs, private companies, governments, the media, the public and others with a vested interest will express and seek support for their particular interests, some of which may conflict with one another. As a result, multi-sectoral co-operation is essential.

7.1.2 Multi-sectoral involvement, agencies and their roles

Governments have ultimate responsibility for safeguarding public health and their role will be to minimise the damage and to assess the circumstances that led to the cyanobacterial problem (s) in the first place. They can also assist with public awareness activities, as outlined in section 7.4. Although governments usually take the leading role in co-ordinating policy amongst vested interests, they may later turn over leadership to a multi-sectoral committee or group to continue developing longer-term plans or strategies (see Box 7.1).

Human health issues affect many different aspects of environmental policy and management and therefore programme co-ordination is both desirable and necessary for effective action in environment and health. Yet in many countries, environmental health programmes are assigned to different specialised agencies, making co-ordination difficult to achieve. Chapter 18 of Agenda 21, the plan of action arising from the United Nations Conference on Environment and Development outlines the problem as follows: *"The fragmentation of responsibilities for water resources development among sectoral agencies is proving, however, to be an even greater impediment [to effective action]... than has been anticipated. Effective implementation and co-ordination mechanisms are required*" (Anon, 1992).

Box 7.1 Development of the New South Wales Blue-Green Algal Task Force and New South Wales Algal Coordinating Committee

In 1991, an extensive toxic cyanobacterial bloom along the Barwon/Darling River system in Australia triggered the implementation of policy for reactive and planned management. The Barwon arises in the Great Dividing Range of Eastern Australia and flows southwest for 2,735 km until it joins the Murray River. The Barwon/Darling River runs through an arid and seasonally very hot environment where temperatures of 40-45 °C occur during the summer; flow is regulated for irrigation and the supply of drinking water.

Flow in the Barwon/Darling River is controlled by the release of water from large reservoirs in the mountain catchment area, by numerous weirs along the length of the river, and by an extensive off-river storage towards the downstream end of the system. The release is determined largely by irrigation requirements. The river is subject to floods at irregular intervals, usually through subtropical storms. Rainfall in the catchment was low during 1991, leading to a high demand for water for irrigation and very low river flows. In late spring (October/November 1991), a massive bloom of *Anabaena circinalis* occurred in over 1,000 km of the river. As an example, 245,000 cells per ml were recorded in the weir pool from which drinking water is supplied to the town of Bourke, New South Wales (NSW). The drinking water in this town was, at the time, chlorinated river water with no additional treatment. Such supplies are common world-wide. Widespread scums occurred along the riverbanks where livestock drank.

The bloom came to public attention when the local newspaper printed a picture of several dead cows in a weir pool, with comments on the water quality. Water samples were collected and sent to the laboratory for toxicity testing. Intraperitoneal injection of lysed extracts of the *Anabaena* cells showed considerable neurotoxicity, with evidence of some neurotoxicity in drinking water samples. The toxins concerned have subsequently been identified as saxitoxin-type neurotoxins.

The NSW State Government declared a State of Emergency, which enabled rapid action including the deployment by the Army of portable water treatment plants capable of using highly contaminated water to produce a safe supply. These units provided dissolved air flotation with flocculation for cyanobacterial cell removal, followed by filtration and granular activated carbon adsorption of any toxic organic materials. The water supplied to the affected towns from these plants had no detectable toxin content. A major public information campaign was rapidly put in place with local meetings, radio and press coverage. Health officers recommended that swimming and water sports were discontinued, with variable success. Farms drawing their own water supply directly from the river were particularly at risk. Livestock deaths along the river were difficult to quantify, but about 2,000 sheep and cattle deaths were reported. The State of Emergency ended when heavy rain in the catchment flushed the river and increased turbidity greatly. No further blooms occurred in the river that summer.

As a consequence of the Barwon/Darling River bloom the State Government established the NSW Blue-Green Algal Task Force (BGATF). The task force was chaired by the State Water Resources Department and made up of representatives from key state government departments (environment protection authority, departments of agriculture, conservation and land management, health and public works, state emergency services board and state "total catchment management committee" (itself a co-ordinated multi-sector public group)), local water boards and corporations, research scientists and the Murray Darling Basin Commission, a multi-state water management agency. Later, when the state of Emergency passed, the BGATF became the NSW State Algal Coordinating Committee (SACC). Members were added to the committee from the adjoining states of Queensland, Victoria and South Australia (these states had, in the interim, set up their own algal task forces). Their membership *"helped speed*

information transfer, and reflected the principle that management measures must be implemented as part of an integrated resource management approach which cuts across established agency and geographical boundaries" (*NSWBGATF, 1993*).

During the emergency phase of the bloom, the BGATF co-ordinated cyano-bacterial monitoring throughout the river, the provision of alternative water supplies, the drilling of new boreholes (for groundwater), the installation of water boom curtains to minimise cyanobacterial access to pump inlet valves, the installation of emergency water treatment systems that could remove (or were believed to remove) cyanobacterial toxins from contaminated drinking water supplies and, most importantly, the transfer of information on a regular basis to the media. After the emergency phase, the BGATF went on to develop the State's comprehensive integrated "Algal Management Strategy" which aimed to minimise the future occurrence and impact of algal (mostly cyanobacterial) blooms. The strategy included contingency planning, improving water system management, reducing nutrient levels in waterways, education and awareness raising (including media interactions) and research. Specific activities of SACC under the Algal Management Strategy were: development of a nutrient control strategy (which included establishment of nutrient management plans with actions such as plans for the upgrade of sewage treatment and disposal systems); reduction in the phosphorus content of washing powders and detergents; upgrades to septic tank systems in "high risk" catchments; reduction of soil erosion; fertiliser and stormwater control strategies; and a public and local government "Phosphorus Awareness and Reduction Campaign". The Committee also subsequently co-ordinated or implemented water allocation and water system management plans, some of which were governed by multi-state agreements (Box 7.3).

To improve the transfer of information to and from the State's many regions (some of which are over 1,000 km from its headquarters in Sydney), regional algal co-ordinating committees (RACCs) were also established. The RACCs were charged with the responsibility for developing local contingency and management plans (based largely on guidelines put forward by SACC), co-ordinating local media and public education, co-ordinating local monitoring and training in cyanobacterial and algal sampling and identification, and identifying when cyanobacterial alerts should be issued for regional waterways.

Table 7.1 outlines some of the functions necessary for cyanobacterial bloom management and indicates the different government departments or other organisations that may have responsibility for each function.

In addition to single-issue government or quasi-governmental agencies in a particular country, there may be national or regional co-ordinating groups which have as their mandate to bring together, or to attempt to bring together, the various sectors, user groups and agencies involved in water management. There may also be water users associations or co-operative societies with interests in water. Any one or several of these agencies and co-ordinating groups may be involved when a cyanobacteria problem arises.

The co-ordination necessary for effective action on environment and health issues is difficult to achieve, not only because it takes place amongst those with vested interests, agencies and groups representing (at times) divergent agendas, but also because the co-ordination itself is complex. That is, it does not simply consist of linking different institutions towards a common goal. It also requires combining inputs from different areas of expertise (inter-disciplinarity); facilitating collaboration between policy-orientated institutions and regulatory institutions; and fostering co-operation among sectors that

positively and negatively influence environmental health quality, including industry, agriculture, local government and transport. Experience in the UK and Australia has shown that an effective strategy to achieve this complex co-ordination is the formation of interdepartmental task forces, with representatives from all relevant government agencies and other key groups with a vested interest (Box 7.1). Such task forces have been crucial to the success of emergency and long-term management of toxic cyanobacterial blooms.

7.2 Policy tools

A number of tools may be deployed by governments in the pursuit of water management and health protection policy. Although sometimes discussed in isolation, these tools are generally inter-related, and policy implementation is most effective when a range of tools is employed (see Box 7.2).

The principal interventions and tools relevant to the control of cyanobacterial hazards include:

• *Consensus through conflict resolution.* Conflict resolution involves negotiation, through formal and informal processes, guided or unguided by an expert, which may be binding or non-binding, depending on the agreement of the parties before entering into the negotiation. Each party's interests are aired, then jointly evaluated, ranked and then balanced. Ideally, this process precludes, or at least places in abeyance, any subsequent conflict, because it is through consensus that a plan of action (balancing all relevant interests) is developed and agreed upon.

• *Guidelines and recommendations.* An enhanced role for experts informs the development of guidelines and recommendations. Such guidelines and recommendations suggest methods of complying with desired policies. They are generally not binding, but rather serve an educational and advisory purpose, in that those studying them learn what actions to take. To be effective (because adherence is voluntary), each guideline or recommendation should be explained and justified.

• *Non-binding agreements, including industry codes.* Non-binding agreements, such as industry codes, are similar to guidelines and recommendations, except that they may be more formal. Although not binding, such codes have a high success rate, especially where industry members are involved in their formulation. Such involvement is desirable because industry members know their capacities and, furthermore, once they have signed up to a code there is "peer pressure" by other members to adhere to it. In addition, there might have been reluctance to follow codes developed by third parties, such as environmental and advocacy groups. Such concerns are assuaged where the codes have been developed following wide consultation. As with consensus building through negotiation, non-binding agreements involve all parties in creating acceptable compromises, thus ensuring greater success.

• *Economic measures.* A set of measures, both supportive and penalising, can be implemented to encourage the reduction of water pollution. Generally, such economic incentives and disincentives are contained in legislation, although they may also appear in non-binding agreements such as industry codes. Supportive measures can be direct

subsidies for water protection technologies or practices (such as setting aside agricultural areas), or various forms of tax reductions (such as for introduction of treatment steps). Penalties involve fees for discharge, which can be calculated according to the load of individual hazardous substances, with each substance tagged with a sum per unit of loading.

• *National legislation.* Because of its formality and inflexibility, law may not necessarily be the best avenue for implementing policy. Nevertheless, it should be recalled that in situations where there are competing interests, legislation offers certain benefits. It provides stability in a complex and changing environment and those with vested interests know where to go to seek the rules that apply to them. In addition, at least at the national level, legislation provides a coercive effect (the force of the State) upon those that have neglected to take action despite having agreed to do so (this has been known to occur particularly where there are financial interests at stake).

Table 7.1 Involvement of government agencies and other organisations in short-term (including emergency) and long-term management of cyanobacteria-related health hazards

Function	Agency
Water pollution control, water resources monitoring, enforcing water quality standards	Ministry of Environment, Ministry of Water Resources, Environmental Protection Agency
Protection of habitats and fisheries	Ministry of Environment, Environmental Protection Agency, Ministry of Wildlife, Ministry of Fisheries
Independent surveillance of drinking water supplies and recreational water quality, enforcement of corresponding standards	Ministry of Health, Ministry of Public Health, local government
Drinking water supply	Local government, private companies
Provision of recreational facilities, promotion of recreational water use	Ministry of Tourism, local and state government, private companies
Allocation of water rights	Government agencies, parastatals, water users' associations, courts adjudicating disputes
Water storage and river management, monitoring, water allocation policy	Ministry of Water Resources, Ministry of Environment, Ministry of Water, Ministry of Public Works, Environmental Protection Agency
Catchment (watershed) management, agricultural policy	Ministry of Natural Resources, Ministry of Agriculture
Upgrades to public infrastructure, e.g. drinking water and sewage treatment plants	Ministry of Public Works, private and semiprivate sector utilities and service organisations
Shipment of emergency water supplies	Local government, private companies
Public education, research into causes and control of toxic blooms	Ministry of Education, Ministry of Health, local government, universities and research institutions, recreational or other users' groups
Implementation of emergency response plans	Local defence organisations, local government, emergency preparedness agencies, Environmental Protection Agency, health ministries/departments

Box 7.2 A combination of tools successfully reduced pollutant loads in Germany

To reduce hazardous substances and nutrients in wastewater, the federal government of the former Federal Republic of Germany ("West Germany") introduced pollution fees per unit loading in the 1970s. The combination of continuously rising fees with options of exemption from their payment if new investments were made in wastewater treatment, resulted in great improvements in treatment technology and water quality. At the time, many critics expected the fees to have little effect because the fine per unit of loading appeared to be substantially lower than the costs for improving treatment. However, the imposition of fees was only one of several tools used by the government and society to achieve the goal of reducing loads. At the same time a strong "green movement" produced public pressure against industrial pollution, industry developed new production procedures which either avoided producing wastewater or included treatment, an international treaty in 1987 targeted the reduction of pollution loads to the North Sea by 50 per cent, and new legislation was discussed and implemented (such as European Union and national legislation to introduce nutrient stripping in all treatment plants handling more than 10,000 population equivalents). In conjunction, these measures caused a very clear downward trend for many pollutants, largely because industrial effluents have become substantially "cleaner", and many sewage treatment facilities have introduced steps for nutrient removal. The major source of nutrient loading into surface waters now is agriculture, and measures to reduce this source of input require implementation.

All legislation must be underpinned with a firm policy background so as to ensure that it will be effective once enacted. As one expert explains:

"Simply to promulgate laws and designate responsible officers will not suffice. It is first necessary to build an effective political base of opinion and power and then to design adequate systems, assign authority, provide resources and translate plans into action; finally the development of the systems must be monitored, so that they can be adapted as required. In most countries sectoral bureaucracies have to be brought under a considerable degree of control and given clear, consistent and persistent policy direction, if co-ordination schemes are not to flounder." (Schaefer, 1981, pp 42-43)

In most countries, before new legislation is introduced by a sector ministry, policy approval must be sought and obtained from the Cabinet or Council of Ministers. This ensures that the process of formulation of legislation is not an academic exercise, undertaken with no thought for the realistic chances for enactment and implementation. The existence of laws which are either unachievable or unenforceable means that financial or human resources have been diverted from other, perhaps more important (e.g. health), priorities. Moreover, the existence of such legislation reflects negatively on the entire legal system.

- *International and interstate plans and agreements.* As noted in section 7.1.1, actions taken to further one interest may have effects on other vested interests. Where a body of water crosses international or state lines, such actions have international and, at times, diplomatic implications. Few rivers and lakes remain within national or state boundaries, adding a diplomatic or more complex legislative dimension to many water resources management issues. There is a clear trend towards development of international plans and agreements which organise water management around particular water bodies. Box 7.3 gives one example of an inter-state response to cyanobacterial bloom management in Australia.

Box 7.3 The Murray-Darling Basin Algal Management Strategy

The Murray-Darling Basin (MDB) is the largest riverine catchment in Australia, crossing four states, Queensland, New South Wales, Victoria and South Australia, and the Australian Capital Territory. The MDB covers an area similar in size to Western Europe. The Murray-Darling River system is actually a network of more than a dozen major rivers that ultimately join with the Murray River and discharge to the sea at Lake Alexandrina in South Australia (the site of the world's first scientifically documented toxic cyanobacterial bloom in 1878, see Chapter 3). The catchment is the agricultural "bread basket" of Australia, with a heavy reliance on water abstraction from the major rivers and headwater storages for irrigation.

Australia is a Commonwealth of states and legislative responsibility for the control and management of water resources (indeed, all natural resources) is vested in the individual states. In 1990, it was recognised that the four states covering the MDB needed to co-ordinate their actions in controlling cyanobacterial blooms because actions, or lack of action, by one state could have a deleterious effect on downstream states. Thus the MDB Commission (a non-government, multi-state organisation that manages the water resources of the MDB) set up an Algal Management Working Group. The working group was made up of members from the four states, the commonwealth government and CSIRO (the national scientific research organisation). Its charter was to *"reduce the frequency and intensity of algal (actually cyanobacterial) blooms and other water quality problems associated with nutrient pollution in the MDB through a framework of coordinated planning and management actions".* Later the cyanobacterial management strategy was also to focus on the impact of the altered river flow regime on the occurrence of cyanobacterial blooms, as well as on problems associated directly with nutrient inputs.

The first project commissioned by the working group was the production of a major report identifying and quantifying (by modelling estimates only) all sources of nitrogen and phosphorus inputs in the MDB. Soon after that report was released in 1992, the MDB Commission convened 14 Technical Advisory Groups (TAGs) to provide expert advice on issues ranging from nutrient input control to cyanobacterial physiology and toxicology to water treatment. The 14 TAG reports were released in one major report (MDBC, 1993). Following the release of the TAG Report, the working group released a draft algal management strategy that was circulated widely among government departments, community organisations and interested members of the public. Public meetings and workshops were held to discuss key issues. The importance of gaining broad community support and "ownership" of the draft report were seen to be crucial to the success of the project. Comments obtained during this consultation phase were summarised and incorporated in the final report (Murray Darling Basin Ministerial Council, 1994).

Unlike the state task forces that were set up during a "crisis" period (see Box 7.1), there was little focus on short-term or emergency management. Rather, the MDB algal management strategy focused on longer-term, strategic initiatives to reduce the frequency and severity of cyanobacterial blooms. Its key elements were nutrient reduction, river flow management, education and awareness, research and monitoring, and funding. The published strategy also contains algal management case studies (on nutrient and flow management), techniques for determining nutrient targets for individual catchments (including the use of catchment-nutrient export models), and modelling studies on the impact of river regulation on natural flows in the MDB.

In addition to plans and strategies, many water bodies are governed by bi- or multilateral agreements which embody the customary rules and policy priorities of all the countries with an interest in the river, lake or sea. Such agreements may address issues such as reducing pollution, exchanging information on the level and volume of water, carrying out flood management, early warning systems, monitoring changes in the path of watercourses, sharing costs and managing dispute resolution mechanisms (Nanni,

1996). Few of these are of direct relevance to the control of cyanobacterial hazards beyond policies relevant to the control of eutrophication in general. Nevertheless, some international agreements specifically include provisions for notification of downstream countries of pollution events in order to enable them to take corrective action (Box 7.4). Including bloom formation as an issue requiring notification of downstream countries might assist in control of cyanobacterial hazards.

Box 7.4 The UN ECE Convention on the Protection and Use of Transboundary Waters and International Lakes

The Convention was drawn up under the auspices of the United Nations Economic Commission for Europe (UN ECE) and adopted at Helsinki on 17 March 1992 (UN ECE, 1992). It entered into force on 6 October 1996. The convention is intended to strengthen local, national and regional measures to protect and use transboundary surface waters and groundwaters in an ecologically sound way. The parties will prevent, control and reduce the pollution of transboundary waters by hazardous substances, nutrients, bacteria and viruses. The precautionary principle and the polluter-pays principle have been recognised as guiding principles in the implementation of such measures, together with the requirement that water management should meet the needs of the present generation without compromising the ability of future generations to meet their own needs. This will protect and conserve not only water resources but also soil, flora, fauna, air, climate, landscape and cultural heritage.

In order to comply with the Convention, emission limits for discharges from point sources shall be based on the best available technology. The Parties will also issue authorisations for wastewater discharges and monitor compliance therewith, adopt water quality objectives, apply at least biological or equivalent processes to treat municipal wastewater, and develop and implement best environmental practices to reduce the input of nutrients and hazardous substances from agriculture and other diffuse sources. Parties bordering the same transboundary waters will conclude specific bilateral or multilateral agreements which will provide for the establishment of joint bodies (e.g. river or lake commissions). They will consult each other on any measures to be carried out under the Convention, jointly elaborate water quality objectives, develop concerted action programmes, jointly monitor and assess transboundary waters, set up joint warning and alarm systems, and provide mutual assistance in critical situations.

Governments will also undertake any additional action that may be required to protect human health and safety. One of these measures is the preparation, under the auspices of UN ECE and the European Regional Office of WHO, of a protocol on water and health expected to be adopted at the 1999 London Ministerial Conference on Environment and Health. Its objective is to promote the protection of human health and well being and sustainable development through improving water management and preventing, controlling and reducing water-related disease.

At the international level there is no "supranational" enforcement mechanism for nations that decline to follow even the agreements they have signed. Nonetheless, international agreements do offer certain benefits, namely that they are formal, written, and embody two or more countries' policy compromises (Box 7.5). They are also usually vetted and approved by a majority of the national legislature before being signed.

Box 7.5 The Baltic Sea

The Baltic Sea, the largest body of brackish water on Earth, has notorious phenomena of blooms of cyanobacteria. The understanding of the biology of the species of cyanobacteria involved and their production of cyanotoxins is necessary for an effective water management of the Baltic Sea. The problems related to such mass development of cyanobacteria illustrate the need to combine efforts at the local level with national and international policies in order to improve the environmental situation in this geographical area.

The Baltic Sea has a surface area of 374,000 km^2 and a mean depth of approximately 60 m and is subdivided into a number of areas: the Gulf of Bothnia in the northernmost part; the Finnish Gulf, bordered by Finland, Russia and Estonia; the Gulf of Riga; the Baltic proper; the Gotland Sea and the Bornholm Sea. The Baltic Sea is connected by narrow channels to the Kattegat and Skagerrak that lead to the North Sea. The salinity gradient varies from 1-4 ‰ in the most eastern and northern parts, to 7-8 ‰ in the south.

The Helsinki Commission has estimated the pollution loads entering the Baltic Sea (Helcom, 1993, 1997). Nutrient input from the atmosphere (through rainfall and particulate deposition) and from the surrounding land by rivers and run-off is high. Elevated levels of nutrients in the water, especially of phosphate and nitrogen compounds, stimulate the growth of cyanobacteria and algae. In the Baltic Sea several cyanobacteria, such as the genera *Nodularia* and *Aphanizomennon, are* capable of biological nitrogen fixation.

The cyanobacteria blooms that are typical of the Baltic Sea may cause a number of serious problems: they discolour the water and may produce bad smells; the decomposition of their organic matter may result in depletion of oxygen, which leads to the death of fish and other animals; and cyanotoxins are produced with consequences to other living organisms, including people.

Co-operation between the countries in the Baltic Sea region occupies a central position in the field of environmental protection. A priority action plan that also addresses problems of blooms has been made and is being operated by the governments in order to determine the major problems and the main measures to reduce them (Ministry of the Environment of Finland, 1991).

7.3 Legislation, regulations, and standards

Making generalised observations about legislative frameworks is difficult, not least because of the diverse forms they may take (common law, civil law, Islamic law) but also because of the varied environments in which they operate. Nonetheless, it is possible to identify certain principal characteristics of the (national) legislative framework within which actions related to the control of cyanobacterial health risks may be effected.

7.3.1 Forms of legislation

The term "legislation" or "laws" refers broadly to all legal texts which are promulgated by the legislative arm of the state exercising its legislative powers under the Constitution, or by the executive arm of the state exercising either its own executive powers under the Constitution or powers delegated to it from the legislative branch. A basic law is usually

introduced by a sector ministry and enacted by Parliament or other equivalent national lawmaking body.

The term "subordinate legislation" (most commonly known as regulations but in some jurisdictions denominated orders, notices, rules, schedules, bye-laws, ordinances, instruments, directives, or proclamations) refers to all legal instruments promulgated by the executive branch exercising delegated legislative power. Subsidiary regulations to a particular law enacted by the national legislature are elaborated and issued by the Minister with responsibility for the subject matter (with or without the approval of all other Ministers, depending upon the country). There may also be schedules or annexes to the regulations, consisting of even more detailed subject matter.

Under the principle of supremacy, laws adopted by the national legislature nearly always take precedence over subsidiary regulations as well as over legal instruments adopted by the legislative branches of the country's political subdivisions. In some countries, however, a variant of this provides that legislation promulgated by the national legislature has primacy for certain subject matters, whereas legislation by the local legislatures takes precedence for others. In other constitutional systems, such as the USA or Australia, any powers not expressly granted to the federal government (or Commonwealth) in the Constitution are the responsibility of the states.

In addition to laws and regulations, there may be other administrative legal texts such as rulings, circulars, guidelines, standards, administrative notes and decisions. Such legal instruments, although non-binding, assist the governments in achieving the aims of the umbrella legislation while at the same time providing guidance for private groups as to how government authorities will go about enforcing and implementing the law (Chiaradia-Bousquet, 1995).

7.3.2 Water laws and standards

Enactment of a basic water law is important in countries where there are laws covering different subject matters and enacted at different times containing conflicting provisions relating to water. This might occur, for example, amongst a country's environment protection law, electricity law and public health regulations. However, during the process of updating or replacing legislation on water, it should be kept in mind that the trend in many countries is towards considering water issues in conjunction with other issues, such as the conservation and use of land, rather than viewing them in isolation (Burchi, 1991).

In order to be effective in addressing cyanobacterial hazards, the law should refer to the jurisdictions, responsibilities, and authority of specified competent agencies in relation to water and to their relationships to one another. Thus it becomes the instrument which brings together the diverse parts of the water medium from which problems arise, as well as the diverse groups associated with their control. These groups include water users, those constructing and administering water supply systems, those regulating different aspects of water and having responsibilities in related domains, and agencies involved in data and information management and sector planning (Table 7.1).

It is important for the water law to reflect the policy priorities and political realities of the particular country. For example, in the case of drinking water supply, legislation in the

established market economies is orientated principally towards the regulation of established water supplies administered by recognisable entities where extensive water supply infrastructure is in place. This may have little relevance to countries where the goal is principally to support the best use of available resources in the provision of incrementally improved service standards to the population as a whole. Such differences argue strongly for developing the policy for the particular country in light of its particular legislative and policy framework, rather than relying on some sort of "model" legislation which would not reflect the realities at hand.

Water laws generally follow the basic principle of national legislation, i.e., that a basic law is indeed basic, whereas any details, which may have to be changed in response to scientific advancements or other exigencies, are contained in the regulations or other subsidiary legislation. The rationale is that regulations are more easily changed because they are elaborated by the responsible Ministry and can be easily revoked, amended or replaced, rather than having to go through the Parliamentary process.

Among the details which will normally form part of the subsidiary legislation of a water law are standards of various kinds. For example, there may be standards on minimum treatment requirements linked to source type and quality. Chapter 5 describes the levels of safety provided by treatment regimes in relation to the nature and degree of cyanobacteria-related hazards in the source water. However, standard setting should not be restricted to water quality, but should also extend to water resource protection and water supply service quality more generally. It should also extend beyond the simple assessment of quality and include the adequacy of structures and systems, such as the definition of safe facilities and practices, minimum standard specifications, and minimum standard operating systems.

In some countries, service quality standards are contained in a "sanitary code" or "code of good practice". As mentioned above in the discussion of industry codes, such standards are most likely to receive support in their implementation if consultation with the affected entities (e.g. supply agencies and professional bodies) has occurred. Other standards consist of quality targets, or what are known as water quality standards. Comprehensive guidance on the setting of drinking water quality standards is available in the *Guidelines for Drinking-water Quality* (WHO, 1993). The *Guidelines for the Safe Use of Wastewater and Excreta in Agriculture and Aquaculture* (Mara and Cairncross, 1989) are also available and further guidelines for safe recreational-water environments are in preparation.

Table 7.2 outlines some of the many issues that may be addressed through water laws, regulations and standards. Not all of these should be seen as essential components of water legislation for all countries, because the contents of a particular country's laws, regulations and standards depend on the policy priorities of that country. This table does not attempt to indicate which matters "belong" in the basic law and which should be contained in subsidiary legislation. Although the general principle (that the more detailed material and the matters that may need to be changed should not be contained in the basic law) applies, the precise dividing line depends to a large extent on the particular legislative scheme. Other water management issues, which are not directly tied to a particular water use, but which might also be addressed through legislation, are given in Table 7.3.

The above discussion should make clear that legislation may play a positive, supportive role in target setting, particularly by virtue of the inclusion of details (such as standards) in subsidiary regulations. Nevertheless, penalties and other sanctions to assist in enforcement are also included in most laws and will occasionally be used. In order to moderate their use, it is appropriate that explicit schemes are provided for enforcement implementation and that they are pursued (Jensen, 1967). The imposition of other more innovative solutions, such as mandatory participation in working groups or monitoring programmes, may obviate the need for some of the harsher penalties. Alternatively, any of these may be used in combination (Box 7.6).

7.4 Awareness raising, communication and public participation

The understanding that cyanobacterial blooms and toxins present hazards to human and animal health is a prerequisite for anticipating, avoiding or reducing their adverse effects. Raising awareness on the causes and effects of cyanobacterial blooms serves two goals: protection from the health hazards presented by the toxins, and long-term reduction of toxic bloom development through public participation. Early recognition of blooms and scums facilitates better management of the blooms and associated problems and helps to reduce their impact on the community as a whole (NRA, 1990; NSWBGATF, 1992).

Table 7.2 Types of water use and features of laws, regulations and standards

Water use type	Area of regulation	Main features of laws, regulations and standards
Drinking water supply	Drinking water quality	Place obligation to supply continuously safe water upon the supply agency Require supply agency to exercise due care Define what is "safe" in terms of acute or long-term exposure (for cyanobacteria this would normally relate to concentrations of some specific toxins (see Chapter 4) as well as toxins not recognised at the time of standard formulation)
	Monitoring requirements	Divide monitoring obligations amongst a number of agencies or sectors (public sector oversight of water resource management, public health authorities, drinking water suppliers, and recreational facility providers including local government) Require information sharing amongst these agencies Define the extent of monitoring required of each agency and define the conditions under which monitoring should be undertaken Define the technical requirements against which monitoring results would be compared
	Treatment requirements	Require supply agency to treat water to achieve the required standards (may be implicit in the requirement of the basic law to supply safe water) Require newly constructed or rehabilitated supply systems to reach minimum treatment capabilities (these may be formulated in terms of performance criteria or processes required, generally in relation to the characteristics of both the source water and the supplied population) Require the upgrading of existing systems to reach the standards required of newly constructed or rehabilitated systems

		Define technical details of construction or operating practice
	Contingency plans	Oblige government agencies to establish contingency plans Define the conditions under which contingency planning is required, and the type of contingency to be planned for (for Alert Levels Framework, see Chapter 6) Describe the basic components of an "adequate" plan (this may include, for example, the need to have alternative supply capacity in very high risk areas)
Recreational waters	Recreational water quality	Impose a "duty of care" (the standard of reasonableness) on those promoting or offering the use of facilities for recreational water use (both private facility owners or operators and local governments may be bound by these duties) Relate use types and water types or qualities to alert levels, and associate alert levels with actions Define a methodology for defining alert levels with consideration for local conditions

Table 7.3 Water resources management: features of laws, regulations and standards

Management types	Main features of laws, regulations and standards
Pollution control	Require companies to control pollution (this could be interpreted as imposing a duty to control eutrophication and, by implication, cyanobacteria)
	Impose duty on industry to address nutrient discharge (especially sewage) and run-off
	Require agencies to manage river flows to prevent cyanobacterial blooms
Integrated management	Define alert levels and associate specific actions with each level (see Chapter 6)
	Include methodology for defining alert levels according to local conditions
Information and the public	Oblige government agencies to disclose information relating to public health risks
	Define the levels of information to be disseminated according to the alert level
	Impose a duty on public health authorities to inform the public and other government agencies of relevant risks
	Require public education and define its contents (this is different from disclosure)
Intersectoral co-ordination	Define roles of agencies and place responsibilities upon them
	Require minimum frequency of co-ordination at national/local level and wider consultation
	Establish local procedures for co-ordination and consultation

Case histories (e.g. Bell and Codd, 1994; Ressom *et al.,* 1994; Falconer, 1996; Codd *et al.,* 1997) and risk assessments (Chapter 4) point to the need for greater awareness of cyanobacterial bloom and toxin hazards amongst not only water users (such as the

general public, water recreational groups and water resource managers), but also professional groups. Awareness raising can serve several functions:

- To alert professionals and water users to the presence of hazardous accumulations of cyanobacteria and toxins and to the risks involved in drinking and using water containing blooms and toxins.

- To inform health-care professionals of the circumstances and exposure routes that lead to intoxications.

- To inform water users of the causes of cyanobacterial bloom development and of ways in which they can assist in reducing bloom formation.

- To inform environmental regulators, water user and water supply organisations about cyanobacterial toxin hazards, and to help identify and implement appropriate policies on water access, use, consumption and treatment.

Box 7.6 Imposition of a monitoring programme after prosecution of a local council for serious environmental harm

In May 1996, the waste management officers responsible for the safe operation of sewage treatment works in Tasmania, Australia, noted that a discharge was occurring from an almost empty sewage treatment pond into a nearby dry watercourse. This was not allowed under the terms of the operating licence. There was a dense bloom of *Microcystis aeruginosa* in the next pond, and the officers observed pools containing *Microcystis* in the watercourse and scums of blue-green slime along the banks and down into a recreational lake called Wrinklers Lagoon.

Water samples contained 511,000 cells per ml of *Microcystis* in the sewage pond and 144,000 cells per ml in a pool in the watercourse. Microcystin content in the pond was measured at 27 µg ml^{-1}, and the scum in the lake at 120 µg g^{-1} dry weight. Total nitrogen in the pond was 25.9 mg l^{-1}, and total phosphorus was 7.3 mg l^{-1}. About 14×10^6 litres had been discharged from the sewage pond into the lake, which had a volume of about 200×10^6 litres. The reason for the discharge was a subsidence in the sewage pond retaining wall, which caused the operators to employ repeated discharges from the pond to avoid collapse of the wall. Because the lake is a well-used swimming and fishing location, the discharge caused an immediate health hazard and a long-term risk of repeated blooms of toxic *Microcystis.* On receipt of the officers' report, the local Council excavated the retaining sand bank of the lake and established a channel to the sea, partly draining the lake and allowing sea water flushing.

Environment Tasmania, the state environmental protection agency, prosecuted the Council for serious environmental harm on eight counts of discharge of sewage pond contents containing toxic *Microcystis* into a recreational lake.

In discussions between the parties and an expert advisor, the Council proposed a monitoring programme for the lake and a reduction of the severity of the charges. The magistrate hearing the case found the Council guilty on a lesser charge of "material environmental harm", imposed a fine of A$ 30,000 (approximately US$ 20,000) and directed that a monitoring programme be carried out. The proposed monitoring programme was intended to make the lagoon safer with respect to blooms of *Microcystis* (or *Nodularia* which can be handled in a similar way to blooms of *Microcystis);* to improve the amenity value of the lagoon; and to minimise any risks to people, pets, livestock, or fishermen eating fish from the lagoon. The monitoring programme was to be

reviewed after three years.

Source: FCR Cattell v. Break O'Day Council; Court of Petty Sessions, Launceston, Tasmania, Australia. Complaint No. 9745521.

7.4.1 Professional group awareness

Professional groups are often no more cognisant of the circumstances leading to cyanobacteria-associated health problems than are the general public (Skulberg *et al.,* 1984). The kinds of professional groups that require up-to-date information on cyanobacterial blooms and toxins in order to manage effectively the associated problems include water treatment and supply authorities and companies, medical and veterinary practitioners, public and environmental health authorities, and national/state environmental agencies. Initiatives to increase the awareness of these groups and organisations have centred around conferences, workshops, correspondence in widely-read professional journals, review-type papers, and the large-scale publication of handbooks and reports. Such educational materials have been developed for the water supply, treatment and environmental sectors (NRA, 1990; Lawton and Codd, 1991; Carmichael, 1992; NSWBGATF, 1992; Yoo *et al.,* 1995); the human health-care sector (Codd and Roberts, 1991; Elder *et al.,* 1993; Ressom *et al.,* 1994), and the veterinary sector (Codd, 1983; Beasley *et al.,* 1989). In countries intending to address cyanobacterial risks, these communication routes should be established and developed further.

7.4.2 Public awareness

In some parts of the world, such as Australia, the UK and Scandinavia, cyanobacterial awareness programmes have operated for several years. However, in other countries with water resources and supplies containing toxic cyanobacterial blooms, awareness and the availability of information are more limited. These deficiencies can be combated in several ways. When cyanobacterial populations in water bodies exceed threshold levels, media warnings should generally be issued to the public. Such warnings and notifications may be issued through the newspapers, radio and television, and may require media targeting and press conferences. The content of any announcement varies according to individual bloom situations and the purposes for which the affected water body is used. For example, if a cyanobacterial bloom occurs in a municipal water supply source, media releases may be appropriate at lower cell populations than if the waters are used only for recreational purposes.

The information supplied in the warnings should be prompt, concise and should include details of expected changes in the quality of supply, such as tastes, odours, discolouration and the actions being taken to alleviate the problems. Where recreational activities, livestock watering, and further water uses other than human supply, are involved, media warnings may also contain a brief description of the bloom and how it can be recognised (such as a noticeable discolouration of the water or the presence of scums). Other information which may be supplied in the warnings and notices, if appropriate to the bloom situation, includes:

- Possible health problems including gastrointestinal upsets, skin rashes, and eye irritations.

- Warnings not to use the water for swimming, bathing, or showering.

- Advice on finding alternate sources of water for animals and preventing pets from swimming (and subsequently grooming scum material out of their fur) in affected areas.

- Notification that boiling the water will not destroy the toxins.

The community often has several concerns when a cyanobacterial bloom occurs in its drinking water supply or recreational amenity. Commonly-asked questions concern the use of water in evaporative air coolers; its use for drinking, laundry, dish washing, washing fruits and vegetables and cooking; whether fish, shellfish and crustaceans harvested from cyanobacterial bloom-containing water are suitable for human consumption; and where to obtain alternative water supplies. Information or fact sheets prepared in advance can be circulated upon request (see below).

It may be appropriate to display warning signs and notices at major points of public access to affected water bodies. For eutrophic waters with occasional bloom problems, provision of basic information to visitors is particularly likely to contribute to protecting them from health hazards, because the rapid appearance and disappearance of scums cannot always be detected in time for the responsible authorities to implement specific measures (such as temporarily restricting use). In such situations, health protection must, to some extent, rely on the users' own informed judgement.

Warning signs should be prominent and simple, with symbols or minimum text to indicate no swimming, paddling, drinking, livestock watering, pets in the water, or other activities deemed by the responsible agencies to present unacceptable exposure risks. The signs may be removed when the blooms decrease to acceptable levels, or they may be kept in place throughout the year in areas with year-round problems. If the latter option is taken, the gradual decrease in visual impact of warning signs and notices needs to be borne in mind.

Brochures and fact sheets are other tools which are increasingly used to convey to the general public and specialist water user groups information on the appearance and hazards presented by cyanobacterial blooms and scums. Examples are available from several countries which have experienced bloom-related problems, such as the UK, Australia, Denmark, Finland, South Africa, and the USA (NRA, 1990; NSWBGATF, 1992; Yoo *et al.,* 1995).

Brochures and fact sheets intended for the public should be simple and free of scientific jargon, and should include brief descriptions with the following kinds of information:

- The nature and occurrence of cyanobacteria.
- The health hazards presented by cyanobacteria and their toxins.
- The recognition of cyanobacterial blooms and scums.
- The propensity of cyanobacteria to produce toxins.
- Precautions and steps to be taken to avoid potential health problems.
- Whom to contact if the presence of a cyanobacterial bloom or scum is suspected.

Special fact sheets for farmers, regarding protection of farm water supplies for animals, and also for water sports organisations, have also been used. Professional associations

and user-group networks, such as national water sports bodies, provide a useful means of spreading information. Posters and displays in public places and association premises are additional tools for conveying information to the public on cyanobacteria and for increasing community awareness. Videos are another useful method of educating the public about cyanobacterial problems. They can be used to present the causes and consequences of cyanobacterial blooms, as well as to provide good visual examples of waters containing blooms and scums. They may also outline the strategies proposed and used by authorities to manage the problems, and to inform the public about what, as individuals, they can do to help. For example, to inform the public and to enable them to initiate action at a community level, Australia's Murray-Darling Basin Commission (Box 7.3) initiated and supported the development of a "study circle" kit for adults entitled "Blooming Blue-Green Algae". The kit contains a video, an audiotape, posters and printed information on cyanobacteria which enables people to discuss and find out more about cyanobacteria.

Education on the causes of blooms can begin through programmes run in schools. Students may be encouraged to participate in simple water quality investigations, such as in the Australian "Streamwatch" programme. Easy-to-use kits are employed to enable the early detection of impending cyanobacterial blooms.

The effectiveness of the tools discussed above is only as good as the efforts made to publicise and to distribute them. The need for contact points may be especially important in remote locations where monitoring waters by government agencies or water body owners is not possible and thus the assistance of the community is vital.

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Chapter 8. PREVENTATIVE MEASURES

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Cyanobacterial bloom formation can be avoided by measures which address their growth requirements, i.e. plant nutrients and light. The basis for excessive growth of cyanobacteria and other phytoplankton organisms (planktonic algae) is enrichment of aquatic ecosystems with plant nutrients. This process is termed eutrophication. The key nutrient in many cases is phosphate. In some systems, not all of the phosphate available is actually used for phytoplankton growth because other resources limit the maximum possible biomass. These may be light intensity or availability of nitrogen. Furthermore, other biota can affect the growth of cyanobacteria and phytoplankton organisms: submerged aquatic plants may compete for nutrients, and grazing by zooplankton may reduce the stock of many phytoplankton organisms and (to a lesser extent) also of some cyanobacteria.

The key management action for abatement of Cyanobacterial blooms is to address the source of the problem by control and reduction of external nutrient loading to the water body, and thus of the concentrations within it. Measures addressing light availability directly (e.g. artificial mixing) or targeting the community structure of the biocoenosis (e.g. biomanipulation) have been successful chiefly in less eutrophic situations. For highly eutrophic water bodies under restoration by reduction of nutrient loading, such measures may accelerate and enhance success.

Control and reduction of nutrient loading usually focuses on phosphorus (for the reasons discussed in section 8.1), but measures addressing phosphorus may be designed to reduce nitrogen input simultaneously. Targets for nutrient concentrations can be achieved by following basic principles of good catchment management with respect to agriculture and sewage treatment. This chapter aims to assist decision making by giving information on:

- Target values for phosphorus concentrations likely to control cyanobacterial blooms in a given water body.

- The potential impact of hydrophysical and biological methods for control of specific Cyanobacterial ecostrategists (see section 2.3) in a given water body.

• The likelihood of success of poorly substantiated methods, sometimes propagated with remarkably effective marketing strategies.

A decision tree for application of different management approaches in order to control phytoplankton growth is given in Reynolds (1997). It leads from restriction of all sources of phosphorus enrichment to enhancing flushing or sediment removal, biomanipulation and artificial destratification. Reynolds (1997) points out that decision points for application of measures are still being quantified by current research.

8.1 Carrying capacity

The concept of the carrying capacity of the resources in a given ecosystem to sustain a population has proved very helpful in planning measures to control the size of that population; Applied to cyanobacteria, this means asking questions such as:

• How much biomass can be sustained on the basis of the amount of nitrogen available?
• How much biomass can be sustained on the basis of the amount of phosphorus available?
• How much biomass can be sustained with the amount of light that penetrates into the water?
At any one point in time, it is likely that one of these three resources will limit the possible amount of biomass at a lower biomass level than the others. However, the limiting resource may change seasonally, for example at higher latitudes it changes in relation to the angular height of the sun and day length, and in tropical climates it frequently changes in relation to turbidity changes caused by seasonality of the flow regime. During winter (even in clear water) or in turbid situations, light is usually the limiting factor, whereas the available nitrogen and phosphorus could have allowed a higher level of biomass. As light intensity increases in spring or as water becomes clearer, phytoplankton organisms begin to multiply and incorporate available nitrogen and phosphorus into their biomass, often up to the point where either of these resources is depleted in the environment and further growth is not possible. If nutrient concentrations are excessively high, phytoplankton may reach a density that causes such a high level of turbidity that light availability limits any further growth, and in these situations populations will be light- rather than nutrient-limited.

For planning and management, it is important to be able to estimate which of the key resources (light, nitrogen or phosphorus) is likely to control phytoplankton biomass in any given system. In other words, the questions are:

• Which resource determines the carrying capacity for phytoplankton?
• How high is the carrying capacity?

For a first step in answering this, it is not important to differentiate between cyanobacteria and other phytoplankton, because the maximum amount of phytoplankton possible can be equal to the maximum amount of cyanobacteria possible, once cyanobacteria have become dominant. One approach at estimating the carrying capacity in relation to nitrogen and phosphorus is to look at the relative amounts of these nutrients in phytoplankton biomass, known as Redfield Ratio by mass (Round, 1965). These ratios are:

C H O N P
42 8.5 57 7 1

Among these components, hydrogen and oxygen are never limiting in aquatic environments. Carbon is available as carbon dioxide (CO_2) and is consumed by photosynthesis. Consumption of dissolved CO_2 enhances diffusion of atmospheric CO_2 into the water, a process which takes time. Carbon limitation has been extensively investigated and has been summarised by Reynolds (1997). He concluded that CO_2 limitation can occasionally have an impact, particularly in soft-water lakes with low bicarbonate alkalinity, but that these situations are generally brief and do not substantially limit the maximal amount of biomass possible.

8.1.1 Nitrogen

Nitrogen may enter water bodies as leachate from soils, as run-off from animal feedlots, and from untreated or biologically-treated sewage, unless treatment includes nitrification and denitrification. Phytoplankton can take up inorganic dissolved nitrogen in the form of nitrate, nitrite and ammonia. In some arid continental regions, nitrogen is found to be the chief factor limiting phytoplankton growth (Reynolds, 1997). The relevance of nitrogen to limitation of cyanobacterial biomass is under debate, because a number of cyanobacterial taxa can compensate for its lack by fixing atmospheric nitrogen at rates of up to 175 kg $ha^{-1}a^{-1}$ (Rönicke, 1986). Thus, lack of dissolved inorganic nitrogen may actually support the dominance of such species as *Anabaena* and *Aphanizomenon.* However, these taxa also occur under conditions of surplus inorganic nitrogen. More importantly, nitrogen fixation is a process requiring high amounts of light energy and will not be effective in very turbid waters (as is the case during dense algal blooms). Thus, in a given water body the maximum amount of biomass that can grow, in addition to the biomass already present, can be estimated from the Redfield Ratio (see above) on the basis of the concentrations of dissolved inorganic nitrogen.

8.1.2 Phosphorus

Phosphorus, like nitrogen, enters water bodies from untreated and from biologically treated sewage, and further treatment steps are required to eliminate it. Phosphorus is biologically available as phosphate, which binds to soil particles more effectively than nitrate. Thus, the main entry route into water bodies from land areas is as surface run-off and with erosion. Although biomass needs only about one seventh of the amount of phosphorus as it needs of nitrogen, phosphorus is the resource which most frequently limits phytoplankton growth in aquatic environments. Cyanobacteria and many other phytoplankton organisms have developed storage mechanisms for phosphate (known as luxury uptake). These enable them to store enough phosphate for 3-4 cell divisions. As a consequence, one cell can multiply into 8-16 cells without requiring any further phosphate uptake, and biomass can increase by a factor of 10 or more even when dissolved phosphate is entirely depleted. For this reason, the amount of biomass that can grow in addition to the biomass present cannot be predicted from the concentrations of dissolved phosphate (see Box 8.1).

Phosphorus is naturally abundant only in very few aquatic ecosystems (such as some lowland estuaries, some volcanic lakes and some ground-water-fed lakes). Furthermore, its inputs to aquatic environments are often easier to control than nitrogen inputs.

Methods for elimination of phosphorus from domestic sewage are well developed and currently more cost-effective than nitrification and denitrification (although current developments may provide better approaches to combined elimination of both nutrients). Measures to protect soils from erosion can also be very effective against loss of phosphorus, whereas control of nitrate leachate from over-fertilised soils may be more difficult. Nitrogen limitation may be to some extent compensated by fixation of atmospheric nitrogen by cyanobacteria, whereas there is no comparable compensation mechanism for phosphorus.

8.1.3 Available light energy

Light energy is a critical resource indirectly affected by nutrient concentrations. Light arriving at a water surface is partly reflected, and the remainder is very quickly absorbed by the water itself as well as by the dissolved substances and by the suspended particles in the water. An upper limit of phytoplankton cell density is reached when the cells shade each other to such an extent that further growth is no longer possible because the individual cells do not receive enough light. This level can be estimated following Lambert-Beer's basic law of exponential extinction with increasing thickness of the water layer. This law can be expressed as:

$$I_z = I_o' e^{-z\varepsilon}$$

where I_Z is the intensity at depth Z

I'_o is the surface intensity

ε is the vertical extinction coefficient, which in turn is the sum of extinction by the water itself and the substances dissolved in it such as humic acids ε_w, the algae suspended in the water ε_a, and other particles suspended in the water ε_p.
The average amount of light *I** available to a phytoplankton organism entrained in vertical mixing of the entire water body or (under conditions of thermal stratification) within the upper, warm water layer (the epilimnion) is the square root of the intensity at the surface I_o and at the bottom of the mixed layer or of the water body I_m (Reynolds, 1997). This relationship illustrates the decisive influence of depth on light availability and enables estimation of the carrying capacity for phytoplankton biomass. For otherwise clear water with the sum of ε_w and ε_p being only 0.2, Reynolds (1997) uses chlorophyll as a measure of phytoplankton biomass and demonstrates that at 1 m depth and a daily insulation of 10^{-3} mol photons m^{-1} s^{-1} a maximum of 670 µg l^{-1} of chlorophyll may be sustained, whereas at a mixed depth of 10 m, only 49 µg l^{-1} are possible, and if mixing occurs down to 30 m, carrying capacity declines to only 3 µg l^{-1} chlorophyll. At these phytoplankton biomass levels, turbidity has also increased (expressed as increase of the term εa), and not enough light can penetrate to enable further growth.

Nutrient availability often influences light limitation. If nutrients are limiting, phytoplankton cannot grow to density levels that reach the light-determined carrying capacity. If nutrient concentrations are excessive, phytoplankton will reach the biomass limit determined by light (unless other factors such as hydrological flushing prevent growth). Further increase of nutrient concentrations will then have no further effect on phytoplankton biomass. This is often the case in hypertrophic water bodies. Turbid situations where the light-determined carrying capacity has been reached are often dominated by

cyanobacteria, because at low light intensity these have a higher growth rate than many other phytoplankton organisms (see section 2.2).

8.2 Target values for total phosphorus within water bodies

In determining target values of phosphorus within water bodies to control cyanobacterial blooms, two questions are important:

- What phytoplankton biomass density can be expected at a given concentration of total phosphorus?

- At what threshold concentration of total phosphorus does phytoplankton density create a turbidity level high enough to reach the light-determined carrying capacity, and thus switch a water body from total phosphorus control of biomass to control by light limitation?

Finding answers to these questions requires a clear definition of total phosphorus. In the past, the soluble phosphate fractions have frequently been addressed when dealing with eutrophication issues. This has some predictive value if it can be measured in seasons where very little phytoplankton is present to consume dissolved phosphate (e.g. during severe light limitation in winter), and if inputs are fairly constant throughout the year. However, the carrying capacity for phytoplankton biomass is more reliably analysed in terms of the total amount of phosphate, i.e. the sum of phosphate bound in biomass and phosphate dissolved in the water, known as total phosphorus (Box 8.1).

Box 8.1 Monitoring total phosphorus as opposed to soluble phosphate fractions

Considerable confusion prevails in the use of the term "phosphate". Historically, soluble reactive phosphate (SRP) or orthophosphate has been measured and addressed when dealing with phytoplankton growth, because this is the fraction of total phosphate which is directly available for uptake by cyanobacteria and algae. However, recycling of phosphate molecules within the plankton communities has proved to be extremely rapid (within 5-100 minutes) (Wetzel, 1983), and phosphate liberated by degradation of organic material will be taken up by bacteria and algae faster than scientists can sample and measure it. Furthermore, cyanobacteria and algae can store enough phosphate for up to four cell divisions and increase 16-fold, even if no soluble reactive phosphate could be measured. If SRP is found above detection limits, this means that it is surplus to the requirements of the cyanobacteria and algae. The only informational value of such a finding is that growth is limited by some factor other than phosphate. The upper limit of the biomass of cyanobacteria and/or algae that can develop in a given water body is, therefore, often largely determined by the amount of phosphate bound within the cells, and total phosphate phosphorus is the variable that should be studied for biomass management. This variable is not equivalent to total phosphorus, which includes the mineral form (such as apatite) unavailable for biological uptake. However, mineral forms are of quantitative importance only in some water bodies (e.g. with high silt loading) and, for the sake of simplification, total phosphorus has become widely used to represent total phosphate phosphorus.

The term total phosphorus is preferable to the term total phosphate, because results are reported in terms of phosphorus rather than phosphate. This is important because the weight of the PO_4 molecule is about three times that of its central P atom, and lack of specification in reporting results as to whether they refer to µg PO_4 or µg P has caused considerable confusion in the literature.

For predicting phytoplankton density from total phosphorus concentrations several models have been developed. The most comprehensive statistical model was established through an international (largely European and North American) co-operative study organised by the Organisation for Economic Co-operation and Development (OECD) (Vollenweider and Kerekes, 1982). The concentration of chlorophyll *a* was used as an easy-to-analyse measure for phytoplankton density. Data for annual mean values of chlorophyll *a* and for maxima of chlorophyll *a* were compiled from a wide variety of phosphorus-limited lakes (77 for annual means and 50 for maxima) and related to annual mean concentrations of total phosphorus. The resulting regressions were almost linear and highly significant (Figure 8.1).

The result roughly means that per microgram of total phosphorus, an annual mean phytoplankton biomass corresponding to 0.25 µg of chlorophyll *a,* and a maximum of up to 1 µg of chlorophyll *a,* may be expected. These results, together with results on the occurrence of cyanotoxins given in Chapter 3, can be used as a basis for rough guide values for estimation of maximum cyanobacterial bloom biomass and toxin concentrations. In natural ecosystems, 1 µg of total phosphorus can support a biomass up to 100 µg of organic substance (corresponding to approximately 1 µg of chlorophyll *a),* which in turn may contain up to 1 µg microcystin. Substantially higher biomass and higher microcystin content are possible, but occur only through accumulation of cells in surface scums, or in some laboratory cultures.

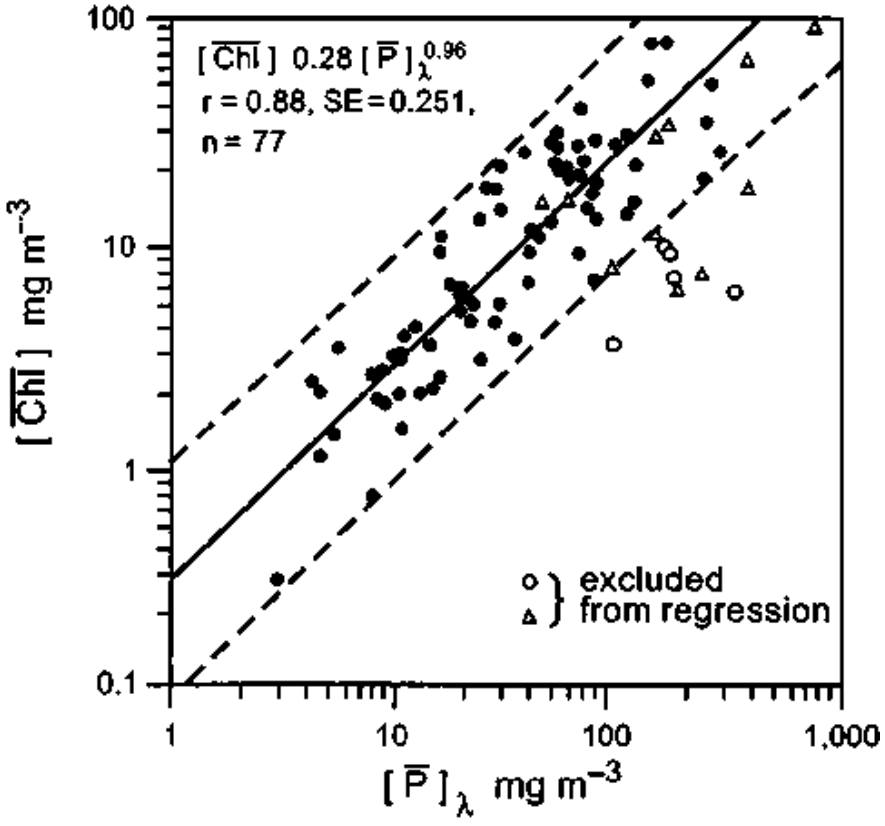
Regressions, such as the OECD model illustrated in Figure 8.1, can be applied as management tools to predict the average and the maximum phytoplankton biomass range likely at a given concentration of total phosphorus. However, this approach has been criticised because these models integrate the behaviour of a number of lakes rather than the response of any one lake to changes in phosphorus concentrations. It must be emphasised that regression B is useful to estimate the maximum phytoplankton biomass at a given phosphorus concentration. However, the estimate given by the double logarithmic regression is only rough, the scatter of points within the 95 per cent confidence limits covers a factor of 10. Maximum ratios of chlorophyll to total phosphorus of 2 are still within this limit. This scatter reflects the effects of other environmental factors controlling phytoplankton biomass, particularly depth and mixing conditions, and losses due to grazing of algae and some cyanobacteria by zooplankton. The carrying capacity for phosphorus will not have been reached in all of the lakes among the wide variety used for regression B. Further management actions in addition to phosphorus control may be useful to avoid this carrying capacity being reached, i.e. to move the vertical position of a water body downwards in Figure 8.1.

Predictability of maximum biomass levels is enhanced by combining this model of phosphorus-determined carrying capacity with a measure for light-determined carrying capacity by introducing the threshold concentrations where phosphorus limitation switches to light limitation. This requires knowledge of the depth of the water body and, if it is thermally stratified, knowledge of the depth of the warm upper mixed layer (epilimnion), also termed "mixing depth" Z_m. If data on light extinction are available as outlined above, average light intensity through the mixing depth I^* can be calculated. In absence of such data, light availability can be estimated by regarding Z_m in relation to the depth of light penetration (euphotic depth, Z_{eu}). If mixing is deep in relation to light penetration, cell or colony densities cannot become very high, because the deeply

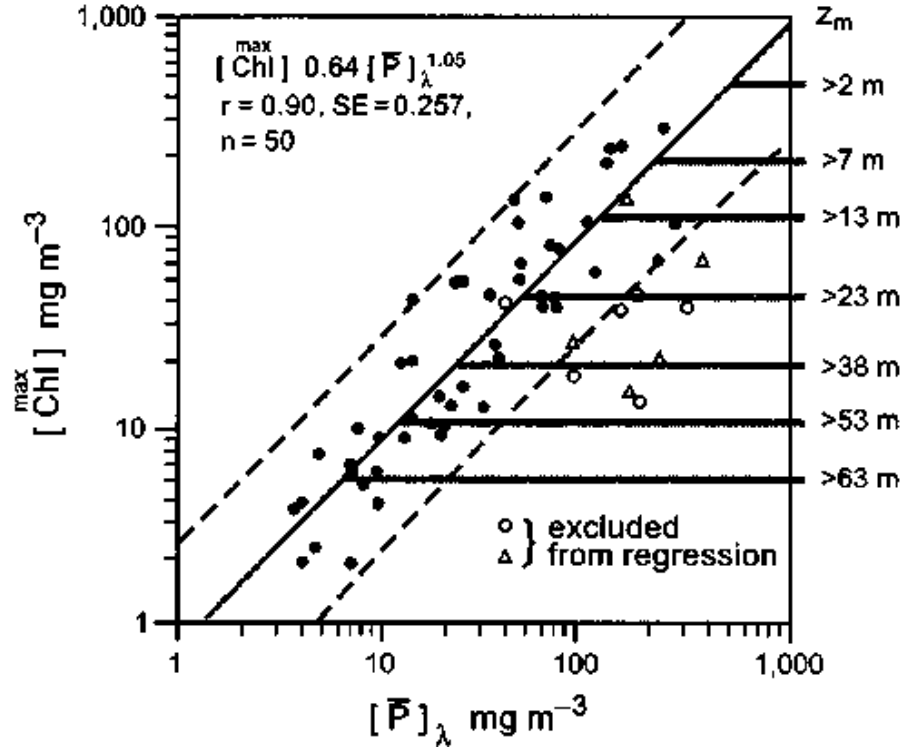
entrained cells would be spending too much time in the dark. If mixing is shallow, cells are frequently moved near the surface, where enough light can penetrate, even through a dense suspension, to enable extensive proliferation.

Figure 8.1 Vollenweider/OECD regressions for phytoplankton biomass (as chlorophyll *a*).

A. Annual means;



B. Maxima in relation to total phosphorus, amended with threshold levels for different mixing depths (Z_m) taken from Reynolds (1997) at which carrying capacity is limited by light ($\varepsilon_w + \varepsilon_p$ m^{-1} = 0.2). Dotted lines represent 95% confidence intervals of the regressions



The maximum biomass density which can be reached is higher in lakes with shallow mixing depths than in deeply mixed lakes, because the latter reach their light-determined carrying capacity at a much lower level of biomass. Figure 8.1B gives the chlorophyll levels at which light limitation truncates phosphorus limitation for different mixed depths in otherwise clear water ($\varepsilon w + \varepsilon p = 0.2$ m^{-1}), and further phosphorus input will not result in a further biomass increase. In turbid waters (e.g. due to silt loads), this level will be reached at much lower phosphorus concentrations (see Reynolds (1997) for chlorophyll capacities at higher light extinction coefficients).

When designing programmes to reduce cyanobacterial proliferation in highly eutrophic lakes, total phosphorus concentrations must be reduced below the threshold value for phosphate limitation of biomass in a given lake in order to have an effect; this threshold value will depend upon the depth of the lake. However, once phosphorus levels have been brought below the threshold value the OECD model can be applied. For example, if a shallow drinking water resource has total phosphorus levels of 600 µg l^{-1} and cyanobacterial densities corresponding to 200 µg l^{-1} chlorophyll *a,* and if restoration measures are applied that achieve total phosphorus levels of 200 µg l^{-1}, the annual mean chlorophyll *a* concentration is likely to decline to 70 µg l^{-1}. Although this is a step in the right direction, cyanobacterial biomass is still very high and problems of bloom formation are not yet resolved.

Experience collected during the past two decades with restoration of water ecosystems shows that phosphorus control for abatement of cyanobacterial blooms should target concentrations at least as low as 30-50 µg l^{-1} total phosphorus (Cooke *et al.,* 1993). In many water bodies, substantial reduction of cyanobacterial and algal population density can be expected at these concentrations when compared with higher concentrations. However, significantly lower total phosphorus concentrations (less than 10 µg l^{-1}) may be required, particularly in deep lakes, in order to prevent blooms of some stratifying ecostrategists in the long term. Information on the prevalent ecostrategists within the cyanobacterial population will help to predict the success of management measures (see section 2.3 and Box 8.2).

Box 8.2 Thresholds for phosphorus control of different cyanobacterial ecostrategists

Knowledge of the prevalent ecotypes in a given water body leads to the following consequences for total phosphorus management:

- *If scum-forming ecostrategists prevail* (such as *Microcystis* spp. or *Anabaena* spp.) cell numbers and biomass are likely to decline if total phosphorus concentrations can be brought well below 50 µg l^{-1} P. This will also reduce scum formation, because less cells and colonies will be available to concentrate into scums. Nonetheless, some scums will probably continue to occur until phosphorus limitation becomes so severe that cell density (and therefore turbidity) decreases to the point where the depth of light penetration is as deep as the depth of large areas of the water body ($Z_{eu} = Z_m$). Under these conditions, vertical migration of these taxa is less effective because their buoyancy regulating mechanism requires some time in the dark (see section 2.2). Therefore, they lose their competitive advantage over other phytoplankton.

- *If dispersed ecostrategists prevail* (such as the filamentous species *Planktothrix agardhii,* formerly named *Oscillatoria agardhii)* very pronounced "switches" may be expected. As phosphorus limitation reduces filament density, and thus turbidity, to the point where the relationship of the depth of light penetration to the depth of the mixed layer (Z_{eu}/Z_m) is greater than 0.4, these species are likely to disappear quite abruptly, and turbidity will increase even further, thus stabilising the result.

- *If metalimnetic ecostrategists prevail* (such as *Planktothrix rubescens*), the water layer above these cells is usually quite clear. Very low concentrations of total phosphorus (often below 10 µg l^{-1} P) are necessary to decrease turbidity further and thus increase light intensity down to the depth inhabited by these species. If this can be achieved, metalimnetic ecotypes may disappear. If not, hydrophysical measures may be more successful in controlling their density.

- *If nitrogen fixing ecotypes prevail* (such as *Anabaena* spp.), reduction of total phosphorus down to concentrations effectively limiting biomass will cause dissolved nitrogen concentrations in excess of uptake by phytoplankton. Nitrogen fixation is then no longer an advantage in competition over other cyanobacteria and algae. This may induce disappearance of the nitrogen fixing species.

8.3 Target values for total phosphorus inputs to water bodies

The OECD study, which provided the regression shown in Figure 8.1, evaluated data from 87 lakes with respect to the relationship between total phosphorus concentrations

in the lake (annual means) and the external load (input) (Vollenweider and Kerekes, 1982). The regression showed a close correlation between annual means of in-lake concentrations and the annual means of inlet concentrations in relation to the residence time of the water:

$$TP = 1.55[P_{inlet} /(1+\sqrt{\text{residence time}})]^{0.82}; r = 0.93, n = 87$$

Table 8.1 Permissible and dangerous inputs for phosphorus and nitrogen for different depths and for a renewal time of 2 $m^3\ m^{-2}\ a^{-1}$

Mean depth (m)	Permissible inputs[1]		Dangerous inputs	
	P (g m^{-2} a^{-1})	N (g m^{-2} a^{-1})	P (g m^{-2} a^{-1})	N (g m^{-2} a^{-1})
<5	<0.07	<1.0	>0.13	>2.0
<10	<0.1	<1.5	>0.2	>3.0
<50	<0.25	<4.0	>0.5	>8.0
<100	<0.4	<6.0	>0.8	>12.0
<150	<0.5	<7.5	>1.0	>15.0
<200	<0.6	<9.0	>1.2	>18.0

[1] Permissible inputs increases with residence time; a doubling of the residence time increases the permissible inputs by a factor of 1.6

Source: Harper, 1992

The "safe loadings" given in Table 8.1 were derived from this model. The model may serve for preliminary scaling of measures to reduce inputs of phosphorus. For prediction of the total phosphorus concentrations in a given water body, estimates of inputs from inlets, from surface run-off (especially from agricultural areas with tillage, fertilisation and erosion), from urban storm-water outfalls and from atmospheric precipitation are needed. Acquiring all of the necessary data may be difficult, and approaches to estimates are discussed in the context of lake and reservoir diagnosis by Cooke *et al.* (1993).

Currently, very little information on relationships between inputs and in-lake concentrations is available from tropical and subtropical aquatic ecosystems. Future research must investigate whether relationships established for water bodies in temperate climates apply, or whether changes are necessary. Differences may be expected, especially with respect to sediment-water interactions and mineralisation rates, because these depend strongly upon temperature and upon thermal stratification.

If inputs exceed critical values for a given system, increase of the concentrations of total phosphorus within that system are likely. In turn, cyanobacterial proliferation is likely, and management measures are then needed to reduce phosphorous inputs.

8.4 Sources and reduction of external nutrient inputs

In most cases, eutrophication is enhanced by anthropogenic activities. The three major sources of external nutrient inputs are run-off and erosion from fertilised agricultural areas, erosion resulting from deforestation, and sewage. Exceptions may occur and are illustrated by the example of Lake Victoria. This large lake has an area of 68, 000 km^2. Tributaries supply about 15 per cent of its water and 85 per cent originates from

precipitation. Burning of field stubble is widely practised and leads to substantial air pollution. Thus, 60 per cent of the phosphorus load is estimated to enter Lake Victoria through precipitation. Only 40 per cent originates from sewage and run-off into the tributaries (Lindenschmidt *et al.,* 1998). Replacement of the practice of burning stubble would substantially reduce this load.

Sustainable approaches aim at reducing nutrient loads at their source or as close to the source as possible (sections 8.4.1 and 8.4.2). If this is not feasible, approaches to reducing inputs from a main tributary (i.e. treating a main tributary as if it were a sewage channel) may be considered (sections 8.4.3). Reduction of external inputs beneath the threshold expected to be effective is an important basis for the success of further in-lake restoration measures (which may address internal nutrient loads or ecosystem structure, see section 8.5).

The first questions to ask in designing programmes for abatement of eutrophication by improving agricultural practices and/or by introducing or improving sewage treatment are:

- How high is the phosphorus input from wastewater (sewerage outfalls or diverse small sewage inlets) to a given water body (excretion of 2-4 g P per person per day may be assumed) (Siegrist and Boiler, 1996)?

- How high is the input from agriculture and run-off from other surfaces?

- Down to what concentrations must phosphorus input be reduced in order to reduce concentrations in the receiving water body beneath a total P threshold likely to be effective in the given water body (see section 8.2)?

Answering these questions requires specific evaluation of the resources to be protected, of their catchment land use, and of the water and effluent drainage network. Ideally, nutrient inputs and the relative share of different nutrient sources should be estimated and catchment characteristics, such as soil type, run-off potential and vegetation cover should be considered. In a region with nutrient-rich, erodible soils and reduced vegetation cover or natural eutrophication (e.g. river deltas or some tropical areas), reductions in inputs will not be possible to the same extent as in a region with sandy soils, flat relief and dense tree cover (Cooke *et al.,* 1993). Thus, the same measures and similar investments into reducing inputs are likely to be more successful in reducing eutrophication in a potentially oligotrophic ecosystem than in a naturally eutrophic one.

In many cases, quantitative assessments of inputs will not readily be available because this requires detailed analysis of hydrological conditions (e.g. assessment of stream flow rates and water retention times), as well as nutrient concentrations and their variations over time in all of the main tributaries. Such investigations require time and resources. Whereas inputs from point sources like sewage effluents are relatively easy to assess, diffuse inputs from agriculture are often very difficult to quantify. Managers are likely to be confronted with water bodies for which almost no limnological data are available, and perhaps not even the depth contours of the water body are known. Planning and implementation of resource protection measures may be delayed for several years before reliable data become available. The dilemma for managers is whether to begin with measures to reduce obviously substantial inputs, without having the data basis to predict whether the measures taken will reduce nutrient concentrations below the

threshold effective for controlling cyanobacteria, or whether to delay planning and decision-making until data become available.

In some countries, user friendly "decision support" software programs (see Box 8.3) have been compiled for use by managers and community groups in identifying the main sources of nutrients from a catchment, and for identifying possible actions e.g. off-river disposal of wastewaters or (re) construction of riparian buffer strips to protect against inputs from erosion. There is an array of computer models that may be used to simulate the hydrodynamic and transport conditions in a catchment system. Trudgill (1995) gives examples of available models and profound discussions of various processes that may be included in the compilation. Since numerous parameters are required for calibration of these models, the complexity of the model should be tailored to the extent of the data base available. For applying these models, the study should include the entire watershed (and, in some cases, the airshed as well) and not just the lake water body.

No general recommendation can be given to resolve the dilemma between the necessity of adequate planning data, and the need to implement obvious measures without delay. Although numerous restoration measures around the world have proved to be ineffective due to insufficient diagnosis and evaluation by scientists and managers, most of these were measures addressing ecological balances within the water body (see section 8.5). In contrast, measures addressing reduction of external nutrient inputs are not likely to be applied mistakenly. In the worst case they may prove to be insufficient and require further action - either further reduction of inputs, or in-lake action.

An effective alternative to quantitative assessment of loading, is a "common-sense" or qualitative approach. This begins with studying maps and geographical information to identify main tributaries, slopes critical for erosion, precipitation patterns and land use. Detailed and critical inspection of the catchment area may provide an excellent basis for recognising priority actions, some of which may be implemented at low cost. Such inspection is generally of underestimated value. Qualitative assessments should include identification of sewage outfalls (possibly illegal or unregistered), land use, vegetation cover, agricultural practices (e.g. soil tilling supportive of erosion, lack of protective riparian buffer strips with dense vegetation cover as a barrier between surface runoff and water body, and stubble burning).

Box 8.3 Testing the Catchment Management Support System (CMSS) in the Murrumbidgee River Catchment, New South Wales, Australia

The Catchment Management Support System (CMSS) is a simple computerised decision support system developed by CSIRO Land and Water, Australia. It combines land use, nutrient generation and land management information into a single model which can predict the impacts and costs of different land management practices in a catchment. As the set-up and use of CMSS does not require a technical or computing background, it is highly suited to use by catchment management committees and other community or management groups (Davis and Farley, 1997).

In a recent application (Cuddy *et al.,* 1997), CMSS was developed for the Murrumbidgee River Catchment, New South Wales (NSW) with the intention that it should be provided to local catchment committees as a tool in the development of nutrient management plans. This pilot study was used to demonstrate the application of CMSS to a specific area, as an initial phase of its application to major catchments in NSW. In applying CMSS to the Murrumbidgee Catchment, significant inputs were required from relevant management organisations and individuals. The

necessary data, ranging from soil types and rainfall distribution to the initial and ongoing costs of changed sewage treatment practices, were available from these organisations. The Murrumbidgee CMSS was developed within the timeframe of the pilot study and handed over to the local agencies. Although the pilot study successfully met its objectives and the program has now been widely adopted, CMSS has yet to be influential in the production of nutrient management plans. This reflects both the prolonged nature of developing plans through community consultation and the lack of involvement by local catchment committee members in the pilot study.

While investments in internal or "in-lake" control measures can be wasted without sufficient pre-restoration evaluation, reduction of external nutrient inputs will be at least a first step in the right direction. Managers are encouraged to implement nutrient control measures, even if the data base is not sufficient to predict the quantitative impact on concentrations within the water body.

8.4.1 Domestic wastewater

Wastewater emissions have frequently been managed by a philosophy of using water as a medium for transporting wastes out of the locality without considering the impairment of the function of the water as a resource further downstream, or of the possible enrichment of lakes with pollutants and nutrients.

Many developing and least developed countries are extending their coverage of drinking water supplies in order to improve human health. Evaluation of experience shows that this usually leads to a several-fold per capita increase in water consumption and thus also in the amount of waste-water generated. Improvement of human health therefore also requires development of wastewater collection and treatment, as highlighted by the World Bank:

"For urban water supply, experience indicates that the collection and proper treatment of sewage must be an integral part of water supply projects. Bringing water into a city without taking sewage out exposes the population - and particularly the poor -to increased pollution." (World Bank, 1993)
Possible exposure to pathogens is a major reason why collection and treatment of wastewater is important. Another reason is that cyanobacteria proliferate in eutrophic lakes and rivers fertilised by wastewaters.

Replacement of phosphorus in laundry detergents can typically reduce phosphate loads in sewage by 50 per cent at best, the remaining 50 per cent being inevitable because it originates from human excreta. Wherever sewage outfalls are considered to be a significant input of phosphorus to a water resource, phosphate elimination, alternative treatment approaches (see Box 8.4), or sewage diversion is necessary. Criteria for adequate technology largely depend upon population structure and on geographic conditions.

If population density is low, and the flushing rates of water bodies or phosphorus absorption capacity of the soils is high, nutrient elimination from sewage may not be necessary - high nutrient concentrations in water resources in such situations are more likely to originate from agriculture. Treatment methods adequate for protection from

infectious agents may also be sufficient in these circumstances (on-site treatment such as properly constructed latrines, septic tanks and sewage lagoons). If domestic wastewater is used in agriculture, health risks should be avoided by following the WHO guidelines for the use of wastewater in agriculture and aquaculture (Mara and Cairncross, 1989). A special aspect of such situations is tourism. Low population areas affected by tourism may need special consideration because the population may increase temporarily several-fold and overload sewage treatment capacities. In temperate regions, the tourism season may coincide with the cyanobacterial growth season.

Box 8.4 Nutrient retention using low- and medium-technology approaches

Alternatives to "high-tech" nutrient stripping methods in sewage treatment (involving an array of methods using lagoons or land treatment) have been in use for more than a century, and their advantages as well as their drawbacks are well established. Many land treatment systems require large areas (up to 10 m^2 per population equivalent), and infiltration of wastewater into the ground below has often occurred without control for hazardous substances or pathogens. Modern artificial wetland systems are being developed to overcome these shortcomings. These systems combine mineralisation processes in the water body with filtration through the soil substrate; they are sealed towards the bottom and they have controlled effluents. As with "high-tech" treatment plants, artificial wetland systems require careful maintenance operation, and control. They are by no means a solution for "letting nature do the job alone". Poor maintenance and overloading rapidly lead to malfunctioning and to poor hygienic conditions in the system. In temperate regions, performance during the cold season may be less effective, but as a means for handling additional sewage loads caused by tourism during the warm season, such systems may be excellent supplements to year-round treatment technologies. Other approaches comprise hygienic methods of collection of excreta, re-use of waste in agriculture and regular emptying of septic tanks. Care must be taken particularly with respect to occupational hazards, and with the designing and siting of alternative treatment methods in order to avoid relocation of the wastewater problem to another site (e.g. causing groundwater contamination).

Sparsely populated regions with water bodies highly susceptible to eutrophication, e.g. because of long water retention times (such as many lakes in Sweden), will require more carefully designed sanitation systems to protect these resources. Supplementary treatment techniques may be necessary, particularly during the tourist season (see Box 8.5).

In many densely populated areas, municipal sewage is the source of at least half of the total phosphorus inputs to rivers and lakes. In large urban areas, treatment of wastewater collected in sewerage systems requires industrial-scale plants for the protection of surface waters. Untreated municipal sewage contains more than 10 mg 1^{-1} of phosphorus. Biological (secondary) treatment oxidises organic matter, but does not substantially reduce phosphorus content. Where municipal sewage constitutes a significant source of phosphorus pollution, removal of phosphorus at treatment plants is necessary (see Box 8.5). Heavy seasonal tourism may also be a problem in such situations because it increases the demand on the capacity of treatment plants and sewerage and also causes substantial fluctuations in the sewage load. Sewerage and treatment for fluctuating amounts of sewage present specific technical difficulties. Lake Balaton is an example of such a situation, where the number of tourists during July and August is twice that of the local population (Somlyody and van Straten, 1986).

Box 8.5 Two well-established and widely used technologies for phosphorus removal in treatment plants

Chemical precipitation with ferric or aluminium salts, often performed simultaneously with biological treatment, can reduce phosphorus concentrations by an order of magnitude to values around 1 mg l^{-1}.

Biological phosphorus removal ("bio-P") can be achieved by alternating aerobic and anaerobic steps in biological treatment and thus substantially enhancing P-uptake by bacteria. This method saves flocculation chemicals and produces less sludge, but requires an adequate design of basins and careful operation of the process. In large treatment plants (> 100, 000 population equivalents) it is more economic than chemical flocculation, because operation costs are lower (Gleisberg *et al.,* 1995). It can reduce treatment plant effluent concentrations down to 0.2-0.5 mg l^{-1} P. Although the method has been known for several decades, experience has only recently accumulated to a level of understanding which allows stable and reliable performance, and it is advised to maintain chemical precipitation facilities as a back-up (Harremoes, 1997), especially for treatment plants discharging into water bodies with critical phosphorus concentrations.

In lowland regions, discharge of domestic and industrial wastewater may amount to 50 per cent and in dry seasons to almost 100 per cent of the total flow of the recipient river, e.g. River Thames in London (Gray, 1994) and Havel River in Berlin (Köhler and Klein, 1997). Such slow flowing rivers are suitable habitats for cyanobacterial growth but the river water may also be needed for production of drinking water. In such situations, phosphorus elimination by simultaneous chemical precipitation or "bio-P" is not adequate, because concentrations in the recipient water body will almost equal the outfall concentrations. Treatment objectives therefore are 0.03-0.05 mg l^{-1} P and this can be attained by adding a further treatment step (filtration) for removal of phosphorus in small, slowly settling floes. The removal of pathogens is usually also a further important objective of filtration treatment in regions with such intensive water use. Various methods of filtration over sand or gravel and pumice beds are available, and new methods of membrane filtration are also being developed.

The costs of such treatment technology may appear intimidating at first glance because of the necessary investments. However, even sophisticated procedures involving filtration need not cost more than US$ 0.15-0.30 per m^3 of treated water (Heinzmann and Chorus, 1994), and this is usually only a small fraction of the costs of drinking water. Effective resource protection in such densely populated areas will save the cost of drinking water treatment, for example by saving the necessity of activated carbon filtration.

Eutrophication due to sewage outfalls has been recognised as a widespread problem since the 1970s. Abatement was begun in that decade with several isolated projects, such as diversion of sewage around lakes with specific touristic value (e.g. at Lake Constance in the 1970s and at major parts of Lake Balaton in the 1980s) and the introduction of phosphorus precipitation in a few treatment plants. Comprehensive programmes began in the mid 1980s. In Europe, an international convention for the protection of the North Sea triggered introduction of phosphorus and nitrogen elimination in larger sewage treatment plants (those treating more than 10,000 population equivalents). Recently, the implementation of measures to eliminate nutrients in sewage has started to show substantial success:

• Denmark achieved a 79 per cent reduction of phosphorus inputs from sewage between 1985 and 1995 and further reduction is expected in 1997 when two plants in Copenhagen go into full operation (Harremoes, 1997).

• Switzerland achieved a 60 per cent decline in total phosphorus inputs from municipal wastewater (Siegrist and Boiler, 1996).

• The USA and Canada together achieved a 50 per cent reduction in phosphorus concentrations in Lake Ontario and the west basin of Lake Erie, with significant impact upon "algal blooms" and accumulations of filamentous cyanobacteria on shorelines, by the construction and upgrading of sewage treatment plants along the Great Lakes (Charlton, 1997). However, in order to maintain the quality now achieved despite the expected increase of population density by the year 2011, and in order to meet the quality targets for Hamilton Harbour, tertiary sewage treatment (effluent filtration) is considered necessary.

Charlton (1997) emphasises the importance of reliable performance of sewage treatment plants: *"Part of the difficulty in understanding sewage problems is the mistaken belief that sewage treatment plants, once built, will perform as planned, with no operational problems"* (Charlton, 1997). Steady degradation of performance during critical summer months has led to considerable phosphorus inputs, the highest being 2.7 times the target. Internal assessment, optimisation of performance, and identification of staff with the job and the achievement of effluent target concentrations, are crucial for reducing effluent loads.

8.4.2 Agriculture and erosion

Losses of phosphorus and nitrogen from deforested, agriculturally-used areas into surface waters are the other major factor enhancing eutrophication. As with phosphorus pollution from wastewater, this problem has increased exponentially in some parts of the world since the 1950s. The causes are structural changes in agriculture involving intensification by tillage of larger plots, extensive application of mineral fertilisers, and the establishment of large-scale animal husbandry. Simultaneously, other major parts of the world suffer substantial lack of phosphorus in topsoils. Zehnder (1996) points out a striking global imbalance between surplus phosphorus in most of the industrialised regions and a lack of phosphorus in most developing regions. On a global scale, phosphorus must be perceived as a limited resource. In regions with phosphorus deficiency, controlled fertilisation in combination with adequate protection from erosion, would help maintain fertility of tropical and subtropical soils, so that further deforestation would no longer be necessary in order to gain new (only transiently fertile) farmland. Further, development of sewage treatment methods that allow reclamation of phosphorus for reuse as fertiliser should be a long-term target for sustainable handling of this resource.

Excessive use of fertilisers and manure has created eutrophication problems in lakes and rivers. In regions with intensive agriculture and slowly flowing rivers with little discharge, e.g. in much of north-western Europe, water bodies without cyanobacterial problems have become scarce. Heavy surplus fertilisation has been enhanced by the widespread trust in phosphorus retention in soils (soils as "savings banks" for phosphorus), but soil erosion and surface runoff have proved to be major pathways into

surface waters, particularly through storms and intensive rainfall shortly after application of fertiliser or manure. The extent of these losses is site-specific and largely depends upon geographic and hydrological conditions: "It *has been suggested that up to 90 per cent of the annual phosphorus losses occur from only 5 per cent of the land during only one or two storms, especially in areas where surface runoff and erosion are the dominant routes for phosphorus losses"* (Oenema and Roest, 1997). The importance of leaching, first recognised as a pathway for nitrate inputs, is being recognised increasingly for phosphate in some types of soils, e.g. sandy, acidic soils with a high degree of saturation of their phosphorus adsorption capacity (Oenema and Roest, 1997). For the Netherlands, Oenema and Roest (1977) estimate 300,000-400,000 ha of phosphorus-leaking sandy soils to be pollution "hot spots" requiring high priority in identification and remediation.

In tropical and subtropical regions, eutrophication of lakes and reservoirs due to inputs associated with erosion is greatly enhanced.

Closing cycles by reuse of manure as a nutrient resource in agriculture, especially if combined with changing practices of land tillage and deforestation, can contribute to reduction of nutrient pollution. Such approaches are sustainable alternatives to considering animal slurry as waste, and reduce the costs of purchase of fertilisers. In this context, performance of large-scale livestock farming in industrial dimensions requires regulation just as for industry:

"The output of waste from many cattle and pig units measures up to that of a large town and needs to be managed with at least the same care as that accorded to human waste. At one time the only environmental requirement placed on farmers was that they should observe good agricultural practice. This is no longer a satisfactory basis for environmental protection. Agriculture must be put on the same basis as other major industries with strict controls on the quality and quantity of effluent discharges." (Packham, 1994)

Box 8.6 Good agricultural practices - best management practices

For fertilisation

- Planning land use, choice of crops and crop rotation to minimise erosion losses.

- Structuring the farmland to minimise erosion by measures such as terracing, interruption of large areas with shrub hedges, and buffer strips planted with shrubs along river banks and lake shores.

- Planning nutrient management in order to avoid losses from the farm by closing nutrient cycles, using manure as fertiliser and avoiding phosphorus import.

- Measuring current fertiliser content of soils and dosing according to the demand calculated for the crop.

- Timing application of fertilisers according to the growth of the crop.

- Cover crops to reduce erosion from bare soil.

- Managing irrigation and groundwater levels.

- Using animal slurry as fertiliser according to the demand of the crop, rather than misusing crops or grasslands as a deposit site for animal slurry.

For animal husbandry
- Reduction of livestock density to 1.5-2 cattle units per hectare.

- Closing nutrient cycles by limiting stock numbers to the fertilisation requirements of the area used for growing crops.

- Placing feedlots and watering sites away from surface waters.

- Protecting river banks and lake shores with fences to keep out livestock in order to reduce both direct pollution by excreta and increased erosion by treading.

The alternative to Packham's request is to redefine "good agricultural practice" to include sustainable resource use. Criteria for "best management practices" or "good agricultural practices" are listed in Box 8.6.

Apart from closing nutrient cycles, sustainable biological methods of production have been developed in many pilot projects and are becoming increasingly popular. Model projects have demonstrated that productivity of biological or "organic" farming methods is not substantially lower than that of conventional farming, provided methods are adapted adequately to the given geographic conditions. The economic balance of "biological" or "organic" farms is frequently equally good because of reduced expenditure for agrochemicals and, in some cases, better prices for the product. Co-operation between water supply agencies and farmers has supported this development in Germany (see Box 8.7) and has shown success in improving resource quality.

In drinking water catchments, it is particularly important that agriculture follows "best agricultural practices". This can be encouraged by designating protection zones around the drinking water source and regulating practices allowed or prohibited within these protection zones. Sophisticated models distinguish two to three degrees of protection, depending upon the relative impact of the respective part of the catchment upon the water quality. In addition to agriculture, other activities which impact water quality, such as forestry, fisheries and tourism, may be regulated in drinking water protection zones.

Countries with a traditional rural society may have options for reclaiming historical agricultural experience and combining it with modern approaches to sustainable "best agricultural practice" in order to attain high outputs of high quality products at low environmental and health impact. Such an integrated approach requires continuous development, evaluation of experience and training.

Countries with large-scale industrialised agriculture may have problems in implementing change. In contrast to the success achieved in phosphorus elimination by wastewater treatment, progress in abatement of agricultural phosphorus pollution has at best been modest. For Switzerland, Wehrli *et al.* (1996) estimate that, while phosphorus emissions

from sewage have been reduced by 60 per cent during the past 15 years, losses from agricultural areas into water bodies have rather increased and the need for a new agricultural policy is just beginning to be widely perceived. Some of the reasons for this delay in awareness and action are:

- Losses of phosphorus from farmland are rarely perceived as economic losses.

- Effective measures will differ regionally or even from farm to farm, and management practices must be optimised locally rather than administered generally. This requires shifts of attitudes.

- Even where programmes and regulations exist, their implementation and control may be difficult.

A basic change in attitude is required. In some countries this appears to be developing slowly as a new generation of farmers with better training (including education on sustainable farm management and ecological impacts) takes over, and as consumer awareness for quality criteria and the ecological impact of products is growing. A wide array of measures can be used by government authorities to support such developments. Examples are training and advice to farmers, eco-audits on products, subsidies for setting land aside from use, subsidies or tax redemptions during periods of transition to organic farming methods, pollution taxes and legislation to enforce water protection.

Box 8.7 Co-operation between water suppliers and agriculture for sustainable provision of healthy drinking water

Legislation should include the principle that use of land and water must occur in such a sustainable way that subsequent use by others Is not hampered. This provides a legal basis for requiring co-operation of agriculture with water supply agencies. Furthermore, protection zones above aquifers or around reservoirs must be staked out so that they cover the actual "intake" of the respective resource. Where protection zones already exist, new hydrological understanding often shows that in many cases these areas are much larger than previously presumed, and that protection zones must be expanded.

Such areas or zones are especially suitable for developing models of co-operation. One such model is the foundation of a voluntary association of those concerned, namely farmers, water and health administrations, representatives of agricultural associations and, amongst others, the water supply organisation. A steering committee or executive board should be elected, in which the water supply organisation should not take the lead. The guiding principle is "co-operation instead of confrontation". An alternative may be to have direct contracts between the farmers and the water supply agency. Such contracts bind the farmers to certain methods of production in return for some financial support, especially during the years of transition from intensive farming to sustainable methods. Tasks for such associations and their leadership are:

- Issuing regular advice, e.g. for suitable situations for applying manure, fertiliser or well-targeted pesticides, and issuing prohibited periods (e.g. "no liquid manure on frozen ground"), or computer-supported fertilisation schedules.

- Regulating the maximum density of livestock tolerable without risking pollution of the aquifer.

- Organising advice and training for farmers, e.g. for measuring soil content of fertilisers.

- Establishing time schedules for changes in land use (e.g. four years transition time for extensivation, eight years of use as pasture, but then use only for forestry). Often, lenient time schedules will be fulfilled much more quickly than required.

- Provide seeds for intercropping or keeping the ground covered to protect against erosion.

- Purchase suitable machines for demonstration or for communal use.

The general emphasis is on advice; on using, developing, publishing and making available local experience and expertise; and on expressing concern and requesting responsible co-operation rather than on issuing prohibitions. A very successful tool has been the installation of counsellors for sustainable farming whose salaries are paid by the water supply agency, but who work within the agricultural authorities and organise courses as well as giving individual advice and training.

The economic aim is to market products which have been audited in relation to health and sustainable land use. Farmers can join an organisation for organic production methods and sell their products under the name of the association; this should be encouraged by the association. Such organisations then take over the responsibility for checking that members comply with the rules and the methods of production; this helps to enforce sustainable production methods. Such organisations can be encouraged to advertise for membership in the region.

The costs of such models of co-operation vary considerably, depending on whether farmers must be supported during phases of transition and on the services provided. Nevertheless, experience shows that these measures increase the price of water only by a few cents per cubic metre (Such, 1996).

Establishing such co-operations is easiest if the land in the protection zone belongs to the water supply company or agency and is only leased to the farmers. Furthermore, employing an expert on agriculture may help a water supply agency considerably in negotiating with farmers. Success with this approach has been reported by Such (1996), Höllein (1996) and Fleischer (1996).

8.4.3 Treatment of drinking water reservoir inlets

Where drinking water reservoirs with one major inflow have a large share of diffuse, non-point source inputs and a strong need for rapid remediation, reduction of nutrients in the inflow may be the most effective option. Pre-reservoirs with retention times of at least several days can reduce total phosphorus inputs by 50-65 per cent (Klapper, 1992). Retention times should allow incorporation of phosphorus into algal biomass and sedimentation of that biomass, but should not be large enough for slow growing taxa, such as cyanobacteria, to establish dominance. Sediment dredging may be necessary at intervals of several years in order to counteract the re-release of phosphorus.

The Kis-Balaton reservoir in Hungary is an example of a special wetland and shallow reservoir system of 60 km^2 designed to retain phosphorus and reduce inputs to the tenfold larger Lake Balaton. Water is retained for one month (mean value) in an intricate system between coffer dams and reed zones. Phosphorus retention has been successful, but flooding with stormwater from the Zala River has caused pulsed phosphorus inputs to the lake (Padisák, Pers. Comm.).

If the largest nutrient share originates from a single major inflow, phosphorus stripping facilities can be very effective in reducing inputs. Successful examples, with different degrees of technological sophistication, are the Wahnbach Reservoir, Lake Schlachtensee, Lake Tegel (Sas, 1989), and the Haltern Reservoir (Paetsch and Kötter, 1980).

8.5 Internal measures for nutrient and cyanobacterial control

In planning restoration measures for lakes and reservoirs, it is important to realise that substantial time lags may occur between measures to reduce external inputs and the results achieved in the water body. Feedback mechanisms within the ecosystem (e.g. sediment-water interactions or the establishment of new dominant species) require time to reach a new equilibrium. Hypertrophic aquatic ecosystems have specific positive feedback mechanisms which stabilise trophic state and cyanobacterial dominance and therefore resilience effects are not uncommon, even after substantial reduction of inputs below thresholds calculated to be effective. Sas (1989) pointed out that resilience patterns occur on two levels:

- Delayed response of in-lake total phosphorus concentrations to a reduction of input, due to the time required for flushing phosphorus out of the water body, and the time required for establishment of new sediment-water equilibria.

- Delayed response of phytoplankton biomass and species composition to reductions of in-lake total phosphorus concentrations, due to stability of prevailing biocoenosis structures and/or biotic enhancement of internal load.

Experience shows that several years (up to 10) may be necessary between the implementation of a restoration measure that substantially decreases inputs, and visible success in terms of reduction of phytoplankton biomass and cyanobacterial blooms. Monitoring of phosphorus inputs and phosphorus concentrations in the recipient water body during this time is recommended. Usually, a declining trend in total phosphorus concentration will be the first detectable response to a reduction in inputs and will indicate whether a particular measure can be expected to be successful, but it may take years for phosphorus concentrations to decline below the threshold effective for controlling phytoplankton biomass. Often, such time lags are due to the (sometimes substantial) phosphate storage capacity of anoxic sediments typical in hypertrophic waters. Flushing rates (i.e. the inverse of retention times) strongly influence the time necessary to reach a new equilibrium. In some cases, particularly in water bodies with low water exchange rates, supplementary "internal" measures may be advisable in order to accelerate a response. The following sections briefly introduce and evaluate a number of such measures for which experience is available.

With very few exceptions, internal measures are appropriate only after, or in combination with, an effective reduction of external inputs. In principle, reduction of inputs should be the actual restoration or resource protection measure, and internal measures should serve as a further boost to switch the ecosystem out of resilience and into a new balance. If possible, a few years of patience and observation of nutrient concentration trends within the lake will show whether internal measures are necessary. Only rarely are internal measures without adequate reductions of inputs justified as an emergency approach; usually such measures require continuous operation (such as aeration, see

section 8.5.2) or repeated application (such as in-lake phosphate precipitation, see section 8.5.1). Medium- to long-term success of restoration investments is at stake if this principle is not considered.

8.5.1 In-lake phosphorus precipitation

In lakes and reservoirs with high water retention times, decline of phosphorus concentrations may be very slow, even after external inputs have been reduced to levels which should ensure a mesotrophic or oligotrophic state. If the water body has a high phosphorus content that is flushed out only slowly, only some of the phosphorus within the biomass will settle to the sediments. Much of it is released from decaying organic material, entrained back into the water body by water circulation, taken up by cyanobacteria or algae and, in part, passed on to higher levels of the food web. Degradation of organic material at the sediment surface has often led to anoxic conditions which may accelerate phosphorus release rates dramatically. Thus, phosphorus within a lake can be recycled many times, and no decline of cyanobacterial biomass can be achieved without reducing this in-lake phosphorus pool. Sometimes, this situation also applies to lakes which are naturally eutrophic, such as lakes in western Canada situated on phosphorus-rich glacial till (Prepas *et al.,* 1997). Precipitation of phosphorus from the water body to the sediment can be a successful measure, if it is undertaken so that phosphorus remains permanently bound in the sediment.

Prerequisites for lasting success are low external loading, sufficient depth to prevent sediment resuspension due to wind events, and adequate choice of flocculants. Experiments with precipitation of phosphorus have been undertaken with aluminium sulphate, ferric salts (chlorides, sulphates), ferric aluminium sulphate, clay particles and lime (as $Ca(OH)_2$ and as $CaCO_3$).

Ferric salts are effective in precipitating phosphorus, but difficult to handle because of their aggressive acidity. Furthermore, the iron-phosphorus complex is stable only under oxic conditions. Thus application of ferric salts usually requires subsequent continuous aeration to avoid re-dissolution of phosphorus under anoxic conditions. Due to the high mobility of iron ions, addition of iron frequently often has to be repeated at regular intervals. In addition, Prepas *et al.* (1997) point out that iron may be a limiting micro-nutrient in some systems and, in such situations, treatment with ferric salts may actually stimulate growth of cyanobacteria and algae.

Aluminium sulphate is poorly soluble under neutral and high pH conditions, but may decrease pH in waters with low buffering capacity, which leads to solubilisation and problems of alum toxicity.

Lime (both $Ca(OH)_2$ and $CaCO_3$) has been used as an algicide to coagulate and precipitate phytoplankton cells out of the water column (Murphy *et al.,* 1990; Zhang and Prepas, 1996). It is non-toxic, usually fairly inexpensive, and the pH-shock for the aquatic biota can be minimised by careful dosing over an extended time span. Unlike treatment with copper sulphate, the precipitation of cyanobacterial cells with $Ca(OH)_2$ does not appear to cause cell lysis and toxin release into the water (Kenefick *et al.,* 1993; Lam *et al.,* 1995). Lime also functions, to some extent, as a longer-term algal inhibitor, reducing eutrophication by precipitating phosphorus from the water (Murphy *et al.,* 1990). It appears that $Ca(OH)_2$ is more effective than $CaCO_3$ in precipitating phosphorus

(Murphy *et al.,* 1990). Many of the studies of both the mechanism and effects of liming for algal control have been carried out in eutrophic, hard water lakes or farm dugouts (dams) in Alberta, Canada (*Murphy et* al., 1990; Zhang and Prepas, 1996). It is possible that the technique may be more effective in these conditions than in soft water. The dose rates used are also quite high (e.g. 50-250 mg l^{-1} $Ca(OH)_2$) (Zhang and Prepas, 1996) which would make the technique prohibitive for large lakes. Techniques for the application of lime, which involve pumping or spraying of a slurry, are described by Prepas *et al.* (1990b).

Experience with in-lake precipitation of phosphorus is increasingly being compiled. A number of documented case studies show success either in terms of reducing phytoplankton biomass or in terms of shifting species dominance away from cyanobacteria. Nevertheless, numerous unsuccessful cases have also been documented, and further development of these techniques is ongoing (see compilation in Klapper (1992) and in Cooke *et al.* (1981, 1993)). Furthermore, in some water bodies, the concentrations of iron or calcium compounds in the inflow are naturally high and regularly provide sufficient binding sites for phosphate to induce natural phosphorus precipitation. Measures in the catchment area or changes in inflow regime may have considerable impact in either increasing or decreasing this input, and thus may have a significant impact on the trophic state of the water body.

8.5.2 Sediment dredging and phosphorus binding

Release from sediments may be a substantial source of phosphorus (sometimes referred to as internal loading) for many years after external inputs have been minimised. Water exchange rates, sediment chemistry, temperatures, mixing conditions, and bioturbation govern phosphorus release rates. Iron-bound phosphorus is highly sensitive to redox conditions; when sediment surfaces turn anoxic during summer stratification, phosphorus concentrations may increase dramatically, fertilising cyanobacteria in their optimum growing season. Under oxic conditions in shallow, unstratified systems, high pH (> 9.8) may strongly enhance oxic phosphorus release (Ryding, 1979). Because high pH values are a result of intensive photosynthetic activity, this phosphorus release pathway is a positive feedback mechanism in favour of cyanobacterial blooms. Other aerobic phosphorus release mechanisms may also be significant, especially bioturbation by feeding fish and invertebrates (Gardner *et al.,* 1981).

Options for measures to counteract sediment release are removal of sediment (dredging) or treatment to bind phosphorus. Dredging is costly and will reduce release rates only if:

- It is carried out down to sediment layers with a lower or less mobile phosphorus content.

- Phosphorus-rich interstitial water is handled in such a fashion that it does not reach the water body and cause additional inputs.

- Dredged sludge can be deposited where it does not create a new external input with erosion and stormwater runoff into the lake.

In some urban and industrial regions, dredging is precluded or complicated by high concentrations of heavy metals and organic contaminants in the sediments which would

then require disposal as hazardous waste. Dredging is particularly recommended for smaller water bodies where the trophic state can be further improved by gaining depth, or which also need to be cleared of dumped rubbish.

Sediment treatments aim at trapping phosphorus in the sediment, either by oxidation to insoluble iron compounds, or by adsorption onto calcium carbonate or clay particles. During the past two decades, broad experience collected with numerous failures (see Box 8.8) and a few successful cases has shown that effective treatment requires careful design on the basis of profound understanding of the sediment chemistry and hydrology of the water body to be treated. Oxidisation may be achieved by aeration, artificial mixing (see also section 8.5.5), or the introduction of pure oxygen. It appears to be most effective if achieved with nitrate, which transports more oxygen and penetrates more readily into sediments. Well-treated sewage effluent (not contaminated with harmful substances, fully nitrified and after phosphorus removal) may be suitable for this purpose, if the process is controlled so that nitrate concentrations are not elevated in drinking water.

Box 8.8 Is aeration effective in binding phosphate in sediments?

The frequent failures in the use of aeration to meet the objective of reducing phosphorus efflux from sediments require critical highlighting. Many aeration projects had several objectives, often not carefully distinguished and planned, such as (i) providing sufficiently high oxygen concentrations for survival offish and fish eggs in deep waters and on the sediment surface, (ii) destratification in order to entrain buoyant cyanobacteria, and (iii) oxidising sediment surfaces. Some of these objectives may be conflicting, e.g. destratification will increase sediment surface temperatures, thus potentially enhancing phosphorus release, and it will transport nutrient-rich, near-surface water into upper strata where these nutrients can be used for growth of cyanobacteria or other phytoplankton. Often, aeration has proved to be insufficient for achieving the aim of reducing phosphorus release. Even the prominent, carefully designed, experiment at the Swiss Baldegger See did not succeed in increasing phosphorus retention of the sediments after 10 years of operation (Wuest and Wehrli, 1996). Energy costs of aeration may be considerable. At the present state of the art, it can be recommended only for increasing the oxygen content of the water (e.g. as a fish habitat), or if artificial mixing is desired - success in increasing phosphorus retention in lake sediments appears doubtful. Injection of pure oxygen appears to be more successful in some cases (Gemza, 1997; Prepas *et al.,* 1997).

8.5.3 Withdrawal of bottom water from the hypolimnion

In thermally stratified eutrophic lakes, phosphorus accumulates in the hypolimnion (cold bottom water layer) during summer stagnation, partly from settled organic material originating in the upper water layers and, in many lakes, largely from the release of sediment-bound phosphorus under anoxic conditions. Although most natural outflows drain surface water, it is often possible to dam the natural outflow and to abstract hypolimnetic water instead (Olzewski, 1961). This is especially easy to apply to reservoirs and can reduce in-lake concentrations significantly. In the Swiss Mauensee the biomass of *Planktothrix rubescens* was reduced from 152 g m^{-3} to 42 g m^{-3} using this approach (Gächter, 1976).

Nürnberg (1997) compiled the advantages of hypolimnetic withdrawal during summer stratification as a method based solely on selective output of total-P rich water. The advantages of the method are:

- It addresses the cause of eutrophication.
- It does not add chemicals.
- It does not necessarily change the water budget.
- It can break the cycle of enhanced sediment accumulation of total phosphorus.
- It can flush more phosphorus out of the system than the sediments accumulate each year.

Hypolimnetic withdrawal is effective only if enough water flows into the lake. Furthermore, some lowering of the water level may be tolerable, but complete destratification by removal of most of the hypolimnion should be avoided, because increasing the contact area between warm surface water and sediments will enhance phosphorus release due to elevated temperatures. In addition, impairment of water quality downstream will require attention if the amount of phosphorus released is high in relation to the total flow. Downstream phosphorus pollution may be avoided by treatment of the hypolimnion outlet with chemical phosphorus precipitation. Nevertheless, the low temperatures of the hypolimnion water may have a substantial impact on downstream biological processes, such as fish breeding.

8.5.4 Reduction by flushing

Flushing with water of low phosphorus concentrations can greatly reduce external inputs and will also accelerate recovery from internal loading by removing in-lake phosphorus which would otherwise be recycled for a number of growing seasons. If suitable water is available in sufficient quantity, flushing can be a very effective tool for reduction of cyanobacterial proliferation. Successful examples are Veluwemeer in the Netherlands (Sas, 1989) and Moses Lake in the USA (Welch *et al.,* 1972). However, this measure also implies a relocation of the phosphorus to another water body, and this impact must also be evaluated.

8.5.5 Hydrophysical measures

Cyanobacteria show different "strategies" of survival in competition against other phytoplankton organisms (section 2.3). Many of these strategies are adapted to specific hydrophysical conditions. Changing these conditions may therefore substantially reduce the success of these cyanobacterial "ecostrategists" and allow other phytoplankton species to become dominant. This approach can be an effective temporary, supplementary measure alongside reduction of external inputs of nutrients, particularly if in-lake nutrient concentrations have declined to values around the threshold where success may be expected. In some cases, where eutrophication levels cannot be decreased, permanent installation of hydrophysical measures can be a solution (see Visser *et al.* (1996) for the example of Nieuwe Meer in Amsterdam).

The mass development of scum-forming species is highly dependant on the stability of the water column. In water without vertical mixing, the colonies of *Microcystis* or other colony-forming taxa can migrate up and down by changing their specific weight (see section 2.3). Interrupting this vertical migration of the colonies by artificial mixing of an otherwise stably stratified water body, can prevent rapid development of surface scums.

Furthermore, disrupting the possibility for these organisms to move into strata with optimum light conditions is likely to reduce their growth rate and thus their efficiency in competing against other phytoplankton. In contrast, mixing improves growth conditions for taxa such as diatoms, which depend on mixing to remain in suspension. Thus, increased mixing may shift species composition from cyanobacteria to, for example, diatoms.

Thermally stratified water bodies naturally have an upper mixed layer known as the epilimnion. If artificial mixing substantially increases the depth of this layer, it reduces the light-determined carrying capacity, or the concentration of phytoplankton biomass possible (see also section 8.1). To be successful, artificial mixing measures must satisfy three conditions (see Visser *et al*., 1996):

- At least 80 per cent of the water volume should be mixed.

- The artificial mixing rate must be higher than the rate of vertical movement of the colonies of cyanobacteria. Rates of colony movement depend on colony size and thus are somewhat variable (see section 2.3), but as a general rule, a mixing rate of 1 m h^{-1} is sufficient to prevent cyanobacterial blooms.

- A large part of the water body must be sufficiently deep. In most cases artificial mixing has been caused by installing aeration tubes which are connected to a compressor on the shore. The aeration tubes are situated in the deeper regions of the water body. Waters with extensive shallow areas have a low circulation rate which can negatively influence the results of artificial mixing. Furthermore, if the water body is too shallow, mixing cannot reduce the light-determined carrying capacity strongly enough to prevent cyanobacterial growth. The example of Nieuwe Meer (Visser *et al.,* 1996) shows that more than 20 m depth may be required.

A number of mixing projects have been unsuccessful because these principles were neglected. Many systems are now on the market, provided by different engineering companies. Engineering expertise is sufficiently developed to design systems that can meet the hydrophysical requirements. Care must be taken, however, to select competent companies, and to plan the measure to meet the ecological targets set in combating cyanobacterial blooms. Furthermore, in tropical and subtropical countries with high and prolonged insulation, the costs of systems are enough for mixing to become prohibitive.

8.5.6 Biomanipulation

Biomanipulation includes a range of techniques that influence algal growth by manipulation of parts of the food web of a lake. Examples are removal of planktivorous and benthivorous fish populations, providing refuges for zooplankton and introducing predatory fish such as pike (*Esox lucius*) in order to decimate planktivorous fish populations, and introducing submerged aquatic plants to compete with phytoplankton in consuming nutrients (Kitchell, 1992). These techniques aim at stimulating the growth or presence of phytoplankton-grazing organisms or of phytoplankton competitors.

Increasing grazing pressure

In shallow lakes, the removal of a large proportion of benthic and planktivorous fish can be helpful to diminish algal growth. Without this predation pressure, zooplankton and benthic fauna can develop and feed on algae and some species of cyanobacteria (e.g. early stages of *Microcystis* population growth when colonies are still very small). Selective removal of benthic fish reduces resuspension of sediments and thus mobilisation rates of sediment phosphorus (in deep lakes this is difficult). To stimulate these effects, predatory fish fingerlings can be introduced to diminish the population growth of the planktonic and benthic fish. Artificial refuges can be placed to provide habitats for zooplankton and pike. The artificial refuges are important when the development of submerged aquatic plants (macrophytes) is insufficient to serve this purpose.

The introduction of predatory fish can be effective. Interventions into established hypertrophic ecosystem structures by fish stock management techniques have proved successful in smaller ponds and lakes over shorter periods of time (Hrbácek *et al.,* 1978). If successful the water may become clearer due to a reduction of algal and/or cyanobacterial turbidity, the zooplankton populations increase, and fields of macrophytes may develop which compete for phosphate with the phytoplankton (thus reducing their capacity for growth) (see below). However, the breeding success of the remaining planktivorous fish stock in the lake will be high if insufficient predatory fish are present. Continued control of the development of the fish stocks is required, and the removal of planktivorous fish must be repeated regularly. Depending upon local salary levels, this may be expensive in terms of personnel. Biomanipulation is by no means a cheap method because of the continuous monitoring and management requirements. It is also unlikely that the technique will work naturally and unaided once the change in biological structure has been introduced.

A disadvantage of biomanipulation is that not all phytoplankton species are eaten efficiently by zooplankton. Stimulating the zooplankton without reducing concentrations of phytoplankton nutrients may stimulate dominance of inedible phytoplankton species, such as colony-forming (*Microcystis, Aphanizomenon*) or filamentous cyanobacteria *(Planktothrix agardhii*), or the green alga, *Enteromorpha.* High nutrient levels may also stimulate the growth of epiphytic algal species which grow on the surfaces of macrophytes and suppress their development.

Box 8.9 Ecosystem theory to explain how biomanipulation works

As indicated in section 2.2, eutrophic systems with blooms of *Planktothrix* (formerly *Oscillatoria*) *can* show enormous stability, with uninterrupted dominance over years. May (1977) indicated that multispecies assemblages of plants and animals can have several different equilibrium states. Scheffer (1990) highlighted this for shallow eutrophic lakes. He concluded that during the process of lake restoration, two different equilibria are possible at a state of moderate eutrophication - one with large populations of phytoplankton and planktivorous and benthic fish, and another in which nutrients are incorporated more evenly distributed among zooplankton, fish and macrophytes. The first system is turbid, the second system is clear. Resilience of the ecosystem during restoration maintains the turbid state over long periods, particularly if phosphate concentrations oscillate around the threshold effective for reducing phytoplankton biomass, but do not substantially decline below this level. In such situations, biomanipulation can help to switch the ecosystem from a turbid phytoplankton community to a clear macrophyte community.

Enhancing competition by introducing macrophytes

The introduction of macrophytes has the best chance of success in water bodies with a relatively large shallow littoral area (< 2-3 m deep) and at moderate concentrations of total phosphorus. Reynolds (1997) points out that if the areal nutrient input rate leads to phosphorus concentrations above 0.15 mg 1^{-1}, phytoplankton density can readily reach 0.15 mg 1^{-1} of chlorophyll *a,* and then submerged macrophytes will simply be "shaded out". However, at lower concentrations of phosphorus, particularly in spring, macrophytes have the chance to begin to grow and to incorporate enough of the available phosphorus to achieve substantial phosphorus limitation of phytoplankton biomass. Particularly if combined with the management of fish stocks, measures to support macrophytes may switch an aquatic ecosystem into a different, sometimes rather stable, biological structure resulting in clear water and low cyanobacterial biomass (Box 8.9).

General assessment

Reynolds (1997) summarises current knowledge on mechanisms of biomanipulation under the heading "bottom-up or top-down control"; "bottom up" implies control by nutrients and "top-down" implies control by the top end of the food chain, i.e. by consumers. Reynolds (1997) lists arguments against biomanipulation, such as self-starvation of consumers by outstripping the availability of phytoplankton or consequences for other components of the food web, and points out that the conceptual flaw is thinking in terms of "steady states". He comments:

"the state that generally attains is a lurching alteration between responses to plenty and responses to over-consumption.... When tropically-related organisms, with lives measured in hours to years, inhabit environments fluctuating with periods of days to months, their interactions are certainly likely sometimes to result in strong top-down pressures on producer biomass. At other times, however, the trophic cascade slows down to barely a trickle" (Reynolds, 1997).
These dynamic fluctuations are most likely to occur, such that they affect phytoplankton populations, in situations which are not extremely biased by total lack of nutrient limitation. Biomanipulation as a management tool to reduce algal or cyanobacterial growth is most likely to be successful in situations of moderate nutrient concentrations and in combination with reductions in inputs. Experience shows that, as long as the trophic level of the systems remains high, the risk that the ecosystem switches back into its original composition is also higher. For reviews of biomanipulation see Demerol *et al.* (1992), Carpenter and Kitchell (1992) and Moss *et al.* (1994).

8.5.7 Algicides

Algicides, especially copper sulphate, have been used rather widely in some regions to kill prevailing cyanobacterial blooms. As a result of the Palm Island catastrophe (see section 4.1) it was established that lysine a bloom may exacerbate problems because toxins previously contained within the cells are liberated and pass through drinking water filters far more readily than toxins within intact cells (see also Lam *et al.,* 1995). Nevertheless, preventative treatment at the beginning of bloom development has been widely used (Cameron, 1989) and may be necessary (see section 9.2).

Algicide treatment of water bodies is best considered as an emergency measure and may involve ecological risks. Toxic copper deposits may accumulate in the sediments (Prepas and Murphy, 1988). Repeated treatment may induce shifts in species composition towards more copper-resistant, but not necessarily more pleasant, species. This was the case in Lake Matthews, a drinking water reservoir for California, where taste-and-odour problems caused by *Oscillatoria* spp. were handled by copper sulphate application. Within very few years, the dosage needed to combat these species had to be increased from 27 to 400 t. The treatment led to the replacement *of Oscillatoria* by a more copper-resistant cyanobacterium, *Phormidium* sp., which prevailed for longer time spans and caused almost all year-round off-flavour problems (Izaguirre, 1992). Other undesirable ecosystem impacts of algicide treatment cannot be excluded. Wherever possible, it is preferable to choose abatement measures which address the source of the problem (i.e. growth conditions for cyanobacteria) because such solutions may be effective in the long term and actually safeguard human health by improving environmental quality.

8.5.8 Barley straw

The use of decomposing barley straw for the control of cyanobacteria and microalgae has been investigated recently (Welch *et al.,* 1990; Jelbart, 1993; Newman and Barrett, 1993; Everall and Lees, 1996). The effect of rotting barley straw in reducing filamentous green algal growth was reported by Welch *et al.* (1990) and algistatic effects were shown in laboratory cultures of the cyanobacterium *Microcystis aeruginosa* by Newman and Barrett (1993). The inhibitory effects were suggested to be due to antibiotic production by the fungal flora or to the release of phenolic compounds such as ferulic acid and f-coumaric acid from the decomposition of the straw cell walls.

A reduction in cyanobacterial populations has also been reported in reservoir trials after applying barley straw (Everall and Lees, 1996). These authors suggested that phytotoxic compounds released from decomposing straw inhibited the cyanobacterial populations, but that further chemical identification, and risk and environmental assessment were required prior to use in water supply reservoirs. However, a recent full-scale field trial has been carried out in a potable supply reservoir and was credited with reducing regular summer cyanobacterial populations (Barrett *et al.,* 1996). Although these trials gave apparently favourable algistatic results, they were carried out without replication or control trials and, as such, the study design cannot account for the influence of other factors (such as impact of weather conditions) on phytoplankton development and succession. An earlier trial which did include the dosing with barley straw of one of a pair of closely adjacent lakes (with similar hydrology and biology) resulted in a decrease in the cyanobacterial population in the straw-dosed lake as compared with the non-dosed lake, throughout the two years of post-dose monitoring (Harriman *et al.,* 1997).

There are conflicting data from Australia on the effects of barley straw. Jelbart (1993) failed to find any inhibitory effects with extracts of rotting straw on *Microcystis aeruginosa* isolates. Cheng *et al.* (1995) also found no algicidal or algistatic effects from barley straw over a six month period in a comprehensive field trial in six experimental ponds. The ponds were fertilised to encourage cyanobacterial growth and there were no differences in species composition or final standing crop between control and straw-dosed ponds.

These contradictory findings and the unknown identity of the inhibitory factors in rotting barley straw indicate that straw-dosing is still too poorly understood to recommend for reliable use as a cyanobacterial control measure, particularly in potable water supply reservoirs. Whether barley-straw dosing influences the composition and size of toxin pools in cyanobacterial populations still needs to be determined. Dosing with barley straw has gained unwarranted popularity and notoriety because it is an apparently simple procedure which is relatively inexpensive and highly visible. It is being used in freshwaters for cyanobacterial control in some countries even though the benefits are dubious (e.g. the introduction of rotting, oxygen-consuming organic matter needs consideration).

8.5.9 Other approaches

A market for rapid and cheap water resource protection and restoration methods is evolving. In Europe, poorly validated methods for reduction of cyanobacterial and phytoplankton growth are being advertised, some of which are questionable. In some cases, transient success was actually due to natural seasonal "clear water" phenomena. Although new approaches require field testing as part of development, independent verification of their success can reasonably be requested of their promoters prior to marketing. Public health officers and other public authorities are trained in other fields than environmental sciences and rarely have the expertise to judge restoration proposals. Review by environmental authorities or experts is therefore desirable prior to investment.

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Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management
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Chapter 9. REMEDIAL MEASURES

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The management and control of cyanobacteria in water supply storage facilities and of cyanotoxins in drinking water may be approached at a number of points and levels in the hierarchy of the total supply system. A detailed assessment of water supply systems with respect to the potential impact of blooms and cyanotoxins on water quality and public health has been presented in Chapter 6. The first preference for control is the prevention of eutrophication, which is discussed in Chapter 8. The next level of management response is reservoir and water body management which can include some engineering techniques to alter hydrophysical conditions in the water body in order to reduce cyanobacterial growth (section 8.5). The more immediate and short-term control techniques which can be used in the management of raw water abstraction include the avoidance of contamination by positioning of offtakes, selection of intake depth, offtake by bank filtration, and the use of barriers to restrict scum movement. Another intervention technique is chemical treatment with algicides. Algaecides have been, and will continue to be, used as emergency measures for the control of cyanobacteria, and their role in management strategies needs to be assessed from practical and environmental viewpoints. The final option for management of cyanobacterial problems and cyanotoxins in water supplies is within the treatment system. Research on removal of algal and cyanobacterial cells has been widely published (see review by Mouchet and Bonnélye, 1998) and recent work has generated quite detailed knowledge on cyanotoxin removal during drinking water treatment.

Much of the work on cyanotoxin removal has focused on single treatment steps, and a few studies have investigated the common combinations of coagulation, clarification and filtration. As a research approach, this is useful because assessments of the performance of individual treatment steps may be generalised more readily than observations gained in complete, but individual, supply systems with their respective site-specific characteristics. However, management approaches aimed at providing safe drinking water from cyanobacteria-infested surface waters require considering the system as a whole, and using different combinations of resource management tailored to the specific locality and different treatment steps. In such an overall approach, steps that individually may be unsatisfactory can have their place in combination with others and thus contribute to a multi-barrier approach. Furthermore, the individual aspects of each drinking water supply necessitate local assessment of performance as well as local optimisation of resource management and treatment strategies.

9.1 Management of abstraction

Management of raw water abstraction is effective in reducing the amount of cyanobacteria in the raw water, often by orders of magnitude. This can be achieved by choosing an optimum position for the offtake, or by abstracting surface water through bank filtration.

9.1.1 Direct abstraction from surface water bodies

General resource management, including hydraulic intervention measures, are described in Chapter 8. The horizontal and vertical distribution of cyanobacterial populations can vary enormously throughout a water body, whether in a lake, reservoir or river. This has obvious implications for both the siting of offtakes and the choice of offtake depth. Considerable contamination of raw water can be avoided by locating offtakes away from sheltered bays where scums may accumulate (usually downwind of the prevailing winds during the critical summer growth period). If this is not practical, it may be possible to employ temporary extensions to pipe intake points.

Selection of offtake depth can also be important in reducing contamination by avoiding surface or subsurface maxima of cell numbers. Many modern reservoir offtake structures (towers) have the provision for multiple offtake depths. If multiple offtakes are not available it may be possible to install siphon offtakes, at least as a temporary measure in small systems. In relation to cyanobacterial contamination, the choice of intake depth must take into account the time of daily maximum surface accumulation of cells and the amplitude of passive diurnal vertical sinking and rising of cells due to light-and photosynthetic-driven changes in cell buoyancy. In thermally stratified, mesotrophic reservoirs, attention must be given to the possibility of meta-limnetic maxima (i.e. maxima between warm upper and cold, lower water layers) of *Planktothrix rubescens* (syn. *Oscillatoria rubescens*).

Operators need to become familiar with the amplitude of vertical movement of cyanobacterial populations, and also with the potential for the formation of metalimnetic peaks, in order to avoid high cell densities as much as possible. This requires multiple depth sampling to determine vertical profiles of cyanobacterial cell density. Collecting information and building up knowledge and understanding of local ecology and conditions can increase flexibility in the management of blooms (see Chapter 10).

Another option to avoid contamination is to employ physical barriers or booms at the surface to prevent surface scums accumulating near the offtake site. Surface booms or curtains, similar to oil-spill containment booms, have been used successfully in Australia, the UK and North America to keep surface scums away from offtake structures (see Figure 9.1 in the colour plate section). These physical barriers often only extend to a depth of 0.5-1.0 m, and do not affect bulk horizontal flow significantly. This technique is a worthwhile emergency measure for transient blooms and its use will depend upon the practical aspects of installation.

9.1.2 Bank filtration and groundwater recharge

An abstraction method that has proved to be very effective in removing particles and many dissolved compounds in localities with suitable underground conditions is bank

filtration or abstraction of groundwater artificially recharged with surface water. The process uses bore holes or infiltration galleries which are located near to the banks of a surface water supply (river or reservoir). These wells fill with water which has infiltrated through the intervening porous soil materials. Depending upon the underground characteristics, water may travel for several hours, or even for weeks before it reaches the well. Longer retention times may enhance purification, but even retention times of between a few hours and days have substantially improved water quality. Many types of soils may be suitable, provided they allow water flow, are not too coarsely structured to achieve a filtering effect, or are not in contact with saline or otherwise unsuitable groundwater. Planning bank filtration requires local assessment of sites for their suitability.

Evaluation of bank filtration with respect to cyanotoxin removal is currently only beginning, and no published results are available. A study of elimination of algal and cyanobacterial taste and odour compounds has shown very effective removal by bank filtration at three study sites (Chorus *et al.,* 1993). Because of the generally positive experience with respect to removal of suspended materials, micro-organisms and a variety of chemical contaminants (Laszlo, 1984; UNDP/WHO, 1992) it may be expected that bank filtration will be a highly promising abstraction method to avoid contamination with cyanobacterial cells as well as dissolved toxins. This expectation is supported by the favourable results of a laboratory study from Finland which demonstrated good performance of experimental soil and sediment columns for both cell and toxin removal (Lahti *et al.,* 1996). In this case, lake water was inoculated with both toxic and non-toxic cultures of cyanobacterial cells and pure microcystin-LR and filtered through soil and lake sediment columns. It was found that during the experimental period of one week, both cells and dissolved toxins were removed very efficiently, although there was some breakthrough in sediment columns at high loadings. The mean rates of removal for cells were 93.7-99.7 per cent and 97.5-99.5 per cent for extracellular toxins for both soil and sediment columns. It was suggested that the removal of microcystins in this filtration process was the result of both adsorption and biodegradation (Lahti *et al.,* 1996). However, the relative performance of the two processes would be very site specific and dependent upon local soil characteristics and microbial activity.

The performance of bank filtration in relation to adsorption capacity, overloading and the potential for release over time of toxins from trapped cells would require monitoring (see Chapter 13 for methods).

9.2 Use of algicides

Algaecides are used in reservoirs to control cyanobacterial growth and to prevent or reduce to some extent the problems of toxins in the associated drinking water supply. Their role in the management scheme may be to provide effective short-term control of growth of cyanobacteria, at one point in time, particularly in circumstances where alternative drinking water sources are not available and preventive measures (as outlined in Chapter 8) are not feasible or not yet effective. Algicide treatment has been proposed as being more cost-effective than toxin removal in drinking water treatment plants, as has been suggested for the control of off-flavour problems (McGuire and Gaston, 1988), because an extended period of persistent blooms greatly enhances the need for additional treatment for toxin removal. However, experience with abatement of off-flavours caused by cyanobacteria through algicide treatment has also demonstrated

that this treatment may actually enhance the problem by supporting the development of species resistant to the treatment (Izaguirre, 1992).

Environmental concerns have been raised because the most commonly used algicide, copper sulphate, has broad ecological impact. It should be used only in dedicated water supply reservoirs in special circumstances, but is nevertheless an unsatisfactory long-term solution. In many countries, national or local environmental regulations prohibit or limit the use of algicides due to their adverse environmental impact. This needs to be established prior to considering the use of algicides.

Algicides, like all management techniques, must be applied correctly to work effectively. If algicides are used they must be applied at the early stages of bloom development when cell densities are low, in order to reduce the potential for liberation of the high concentrations of intracellular toxin that may be associated with dense blooms. Early application will further enhance the effectiveness of treatment because cyanobacterial cells can form a major part of the "copper demand" along with other organic matter in natural water.

A major limitation of any agent which disrupts cyanobacterial cells is the release of toxins and of taste and odour compounds from the cells. A range of studies have indicated that cyanotoxins are predominantly intracellular in healthy cells, and are only released into the water at an advanced stage of bloom senescence, or following treatment with chemicals such as algicides (Lahti *et al.,* 1996). This release can be quite rapid and has been shown to occur within 3-24 hours in different studies (Jones and Orr, 1994; Kenefick *et al.,* 1993). These dissolved toxins will then disperse and be diluted throughout the water body, but will not be removed by conventional flocculation and filtration procedures. Installation of additional treatment for removing cyanotoxins may be costly. The dangers of treating dense blooms with algicides was demonstrated in an incident which occurred on tropical Palm Island, Australia, where members of the community became ill with hepato-enteritis following treatment of the water supply reservoir with copper sulphate for a cyanobacterial bloom problem (Bourke *et al.,* 1983) (see Box 4.3).

If algicides are used to control toxic cyanobacteria, the reservoir should be isolated for a period to allow the toxins and odours to degrade (see section 3.4). Unfortunately, very little data exist on the withholding period in relation to toxin loss, but it could be in excess of 14 days (Jones and Orr, 1994).

In some cases algicide treatment may be unsuccessful or only partially successful. This can be due to inadequate dispersal and contact with the target organisms, variable sensitivity of cyanobacteria, and reduced toxicity due to complexation of the copper (Burch *et al.,* 1998). The form of copper most toxic to aquatic organisms is the free cupric ion (Cu^{2+}) and this can be reduced by complexation with both inorganic ligands under alkaline conditions, and organic ligands present in natural waters (McKnight *et al.,* 1983).

9.2.1 Copper sulphate

Chemical control of algae in water supply storage has been a widespread water quality management practice for over 100 years. Records of the use of copper sulphate date

from 1890 in Europe (Sawyer, 1962), from 1904 in the USA (Moore and Kellerman, 1905), and at least since the mid 1940s in Australia (Burch *et al.,* 1998). Copper sulphate has been regarded as the algicide of choice because it is economical, effective, relatively safe and easy to apply. It is also considered to be of limited significance to human health at the doses commonly used (WHO, 1996) and has been considered not to cause extensive environmental damage (McKnight *et al.,* 1983; Elder and Home, 1978). The latter point has been an issue of debate for some time (see Mackenthun and Cooley, 1952) because copper tends to accumulate in lake sediments (Sanchez and Lee, 1978; Hanson and Stefan, 1984). In some cases it appears not to be remobilised and is bound permanently to the bottom sediments (Elder and Home, 1978; Sanchez and Lee, 1978). However, in a study of 10 drinking water dugouts (small reservoirs) in Canada, sediment copper (previously accumulated from copper sulphate treatments) was released back into the open water under low dissolved oxygen conditions in the hypolimnion in summer (Prepas and Murphy, 1988). It has also been suggested that sediment-bound copper could have an impact on the benthic macroinvertebrate community (Hanson and Stefan, 1984). It is important to remember that copper and other heavy metals differ from some other toxic contaminants in that they are not biodegradable, and once they have entered the environment their potential toxicity is controlled largely by their speciation or physicochemical form (Florence, 1982). Copper sulphate treatment has been shown to cause short-term changes in phytoplankton abundance and species succession (Effler *et al.,* 1980; McKnight, 1981). Fish kills may also occur following copper sulphate treatment, although it is not clear whether this is as a result of copper toxicity or oxygen depletion (Hanson and Stefan, 1984).

A recent extensive survey of water utilities in the USA and Canada indicated that copper sulphate is by far the most widely used algicide, although other alternatives are used under some circumstances (Casitas Municipal Water District, 1987). Some of the compounds that have been used and evaluated for potential as algicides over the years are summarised in Table 9.1.

McKnight *et al.* (1983) give an assessment of the use of copper sulphate for the control of nuisance algae and cyanobacteria. They also indicate that there are wide differences in copper sensitivity among species. The relative growth inhibiting concentrations for a range of phytoplankton are given in terms of cupric ion activity (i.e. $[Cu^{2+}]$), derived from laboratory toxicity studies. The toxic cupric ion activities range from greater than 10^{-6}-10^{-11} M ($0.063\text{-}6.3 \times 10^{-7}$ mg l^{-1} Cu^{2+}) for species of diatoms, dinoflagellates, green algae and cyanobacteria - a difference of over four orders or magnitude (McKnight *et al.,* 1983). These toxic Cu^{2+} concentrations are very much less than the usual doses applied as total copper in copper sulphate treatments. The relative toxicity is given in terms of ionic copper because it is believed that phytoplankton react principally to the concentration of Cu^{2+} or loosely complexed copper rather than the total dissolved metal in the water.

McKnight *et al.* (1983) have used these findings to develop an experimental procedure to determine the required dose rates for target species in individual reservoirs, taking account of the particular water chemistry. This experimental procedure to determine dose rates is suggested as more effective than simple empirical formulae, based on pH and alkalinity, which were not very useful (McKnight *et al.,* 1983). The experimental approach requires access by the water supply operators to a good level of biological and chemical expertise and analytical capacity. The approach is based on first determining the cupric ion activity as a function of added copper, and thereby the complexing

capacity of the reservoir water by a copper ion selective electrode. This is followed by a culture assay to determine the sensitivity of the particular nuisance algae to copper. The local nuisance species preferably need to have been isolated into laboratory culture. The required copper sulphate dose rates can be derived from a simple formula relating growth inhibition, in terms of cupric ion concentration, to the Cu^{2+} concentrations in the reservoir after complexation (McKnight *et al.,* 1983).

Table 9.1 Compounds that have been used as algicides, their formulation and key references

Compound	Formulation	Reference(s)
Copper sulphate	$CuSO_4.5H_2O$	McKnight *et al*., 1983; Holden, 1970; Palmer, 1962; Casitas Municipal Water District, 1987
Cutrine® - plus	Cu alkanolamine. $3H_2O^{++1}$	Humburg *et al.,* 1989
Copper - triethanolamine complex	Cu $N(CH_2CH_2OH)_3.H_2O$	Humburg *et al.,* 1989
Copper citrate	Cu_3 $[(COOCH_2)_2C(OH)COO]_2$	Casitas Municipal Water District, 1987; Raman, 1988; McKnight *et al*., 1983; Fitzgerald and Faust, 1963
Potassium permanganate	$KMnO_4$	Fitzgerald, 1966; Holden, 1970
Chlorine	Cl_2	Holden, 1970

[1] Copper II alkanolamine complex

9.2.2 Copper chelates

The problem of the reduced effectiveness of copper sulphate treatment in hard alkaline water has long been recognised (Palmer, 1962; Casitas Municipal Water District, 1987). Chelated copper algicides were developed to overcome the problems of the complexation and precipitation loss of toxic copper under these circumstances. Examples of copper chelate algicides include copper ethanolamine complexes and copper citrate (Table 9.1). Copper citrate has been used as an algicide in the USA (Casitas Municipal Water District, 1987; Raman, 1988). It is available either as a commercial preparation (Hoffman *et al.,* 1982) or by simultaneously dosing copper sulphate and citric acid (Raman, 1988). It is claimed that the use of citric acid as a chelating agent enhances the solubility of copper allowing it to remain in solution longer under alkaline conditions (Raman, 1985; 1988). Raman (1988) recommends applying copper sulphate: citric acid in the weight ratio 2:1 in high-alkalinity waters (> 40 mg l^{-1} $CaCO_3$). A study which examined equilibrium speciation of copper in water to determine the changes in distribution of Cu(II) in relation to pH, dissolved organic carbon (DOC) and citrate was carried out by Casitas Municipal Water District (1987). This study demonstrated that citrate greatly enhances the solubility of copper even in the presence of appreciable alkalinity (100 mg 1^{-1}). McKnight *et al.* (1983) suggested that the advantage of using synthetic copper chelating agents in hard, alkaline waters probably results from decreasing the supersaturation of malachite ($Cu(OH)_2CO_3$) and tenorite (CuO) and thereby the rate at which equilibrium with these insoluble forms (precipitates) is approached. It is possible that a longer time taken to reach equilibrium would result in the maintenance of toxic ionic Cu^{2+} activities and the inhibition of algal growth for longer

periods after dosing (McKnight *et al.,* 1983). It is acknowledged that, despite their relatively widespread use in the USA, the efficacy of chelated copper algicides in relation to water chemistry is poorly understood (Casitas Municipal Water District, 1987).

9.2.3 Use of oxidants

Potassium permanganate has been used as an algicide from as early as 1935 (Holden, 1970). A survey of North American utilities indicated that a small number use potassium permanganate relative to those who use copper sulphate (Casitas Municipal Water District, 1987). Commercial formulations of potassium permanganate marketed specifically as algicides are available in the USA (Casitas Municipal Water District, 1987). Fitzgerald (1966) investigated the relative toxicity of potassium permanganate to eight species of algae and cyanobacteria and found the algicidal dose was in the range 1-5 mg l^{-1}, except for one green algae where up to 8 mg l^{-1} was required.

Chlorine is used mainly for control of algae in water treatment works but is also known to have been employed in reservoir situations (Holden, 1970). The effective dose rates are dependent on the chlorine demand of the water, but most algae are reported to be controlled by residues of free chlorine between 0.25 and 2.0 mg 1^{-1} (Holden, 1970).

9.2.4 When to use algicides

Because cyanobacterial toxins are primarily intracellular, algicides must be used with particular caution to avoid release of intracellular toxins. Algaecides should be used when cell numbers are low to avoid excessive toxins or taints following rupture of the cells. This should be checked by post-dosing monitoring. Algaecides may be used at higher cell numbers only if the reservoir can be taken out of supply until the toxins and taints degrade, or if treatment for removal of the toxins and taints is available. In the latter cases the use of algicide should be assessed against the capability for whole cell removal offered by treatment processes, because cell removal may be safer. It is important to know how effective the chosen algicide is in the specific waters. For example, copper may be less effective in waters with high dissolved carbonate or at alkaline pH.

Table 9.2 Distribution of microcystins during laboratory culture of *Microcystis aeruginosa*

Age of culture	Distribution of toxins (%)	
	Cells	Water
Young		
Slowly-growing cells	100	0
Rapidly-growing cells	75-90	10-25
Old		
Slowly-growing intact cells	70-80	20-30
Decaying cells (leaking cell contents)	30-40	60-70

Source: National Rivers Authority, 1990

Algaecides should only be used in waters where the environmental impacts are acceptable and this should be checked with the local environmental agency.

9.3 Efficiency of drinking water treatment in cyanotoxin removal

Cyanobacterial toxins represent a challenge to drinking water treatment which involves removal of organic substances in both soluble and insoluble form. Water treatment processes may remove target substances by either separation or conversion. Separation processes are those which remove the target substance from the treated water, usually to a treatment residual which becomes a waste stream for disposal. Conversion processes involve transforming the target substance into a different chemical form, thereby reducing the water quality problem. Although conversion processes are sometime characterised as though they achieve destruction, there will always be reaction products and thus transformation is a more accurate description than destruction. Ideal conversion processes are those which yield innocuous reaction products.

A major factor in assessing water treatment for cyanobacterial toxin removal involves consideration of soluble and suspended substance removal. The primary toxins which have been studied, microcystins, nodularins and anatoxins, are all water soluble. However, laboratory observations for microcystins have shown that these toxins are produced within the cyanobacterial cells and are expected to be predominantly found within slow growing, healthy cells (Table 9.2).

Recent work (Mole *et al.,* 1997) has shown that microcystin release from cultured *Microcystis aeruginosa* began to occur late in the exponential growth phase and increased significantly during the stationary phase. This release was linked to a decrease in the integrity of the cells as determined by staining with fluorescein diacetate. The amount of toxin release was influenced by the culture medium and reached as much as 50 per cent in most commonly used media late in the stationary phase of population growth.

Until a bloom collapses or is otherwise affected by some treatment practice, the majority of toxins will be retained within the cells, making removal of intact cells a high treatment priority. However, under bloom conditions, a substantial proportion of toxin would also be expected to be released to the water column, making removal of soluble toxin an unavoidable concern.

Physicochemical treatment has been shown to cause cell lysis and toxin release (James and Fawell, 1991). Operational investigations in Africa demonstrated significant cell lysis during extended transport in pipelines (Dickens and Graham, 1995). However, other experiments conducted with cultured *Microcystis* showed that the flow and mixing conditions associated with water treatment did not cause cell lysis or toxin release. In addition, changes in pH from 5 to 9, which can occur in the treatment of some waters, did not cause any release of the intracellular toxins (WRc, 1996). Effects of physical and chemical stress on toxin release from cyanobacterial cells should therefore be assessed in treatment and conveyance systems.

The following sections review the capacity of established and novel treatment processes for the removal of cells and dissolved toxins.

9.3.1 Screening and prefiltration

Water treatment facilities usually employ coarse screens to remove debris from the water intake. These screens have no effect on the removal of either cyanobacterial cells or soluble toxins. However, microstrainers or fine screens may be used to remove larger algae, cyanobacterial cells and aggregated cells. Mouchet and Bonnélye (1998) reported removal rates of 40-70 per cent for two cyanobacterial species but pointed out that smaller species (e.g. single cells and small colonies of *Microcystis)* are poorly retained (to sometimes less than 10 per cent). Concerns regarding possible cell rupture, lysis and toxin release resulting from pressure on the filter screen have not been sufficiently addressed.

9.3.2 Aeration and air stripping

There are a number of methods for contacting air with water in drinking water treatment that may be required for various purposes, such as to oxidise iron and manganese from soluble to insoluble forms, to prevent reducing conditions which may yield odorous compounds, and to remove dissolved gases such as carbon dioxide, hydrogen sulphide, other reduced sulphur compounds and other volatile organic compounds (Hamann *et al.,* 1990).

Neither aeration nor air stripping will be effective for removing soluble toxins because they are non-volatile compounds. Nor would they be effective for removal of cyanobacterial cells (for aeration techniques applied in reservoirs to reduce growth of cyanobacteria see section 8.5.5).

9.3.3 Coagulation and clarification

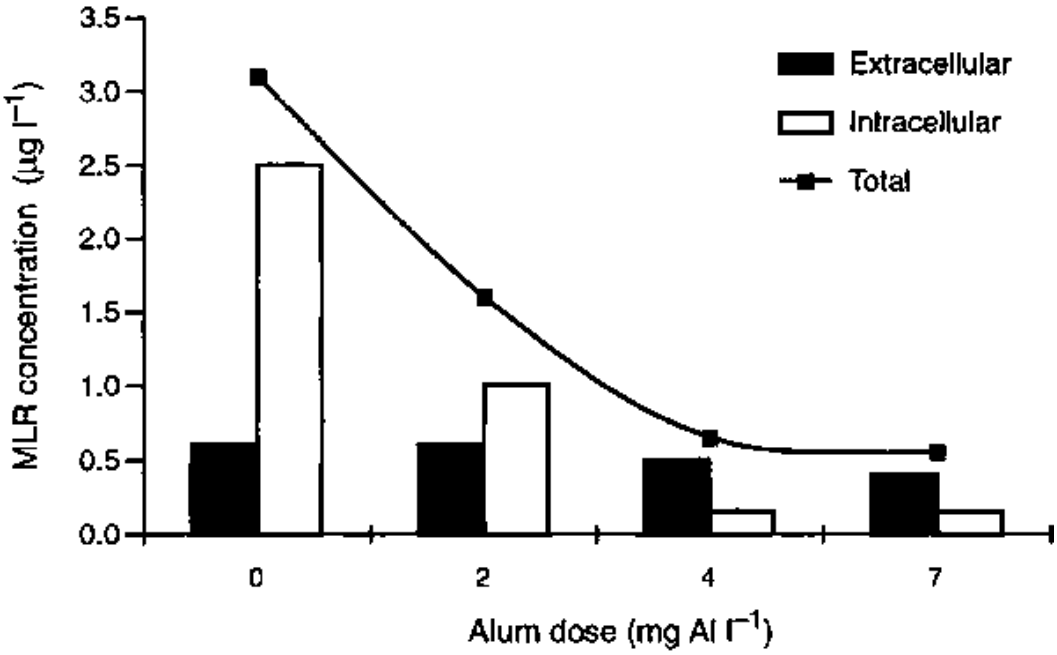
Coagulation promotes the aggregation of small, dispersed particles into larger particles which can be separated by sedimentation, filtration or flotation (Grohman *et al.,* 1985; Hamann *et al.,* 1990). Coagulation differs from precipitation because the latter involves converting soluble substances into insoluble particles, whereas coagulation deals with pre-existing dispersed particles such as mineral turbidity (clay, silt), larger molecular weight natural organic matter, micro-organisms including cyanobacteria, and oxidised, insoluble forms of iron and manganese.

Common chemicals used for drinking water coagulation include various aluminium and ferric iron salts. More recently synthetic organic polymers have gained some acceptance. Coagulation with multivalent metal salts can also be aided by adding various organic polymers to promote floe growth. Leuschner (1984) reported substantially improved flocculation of *Planktothrix agardhii* after addition of a cationic polymer. Efficient removal of algae is dependent on optimisation of chemical doses and coagulation pH. Mouchet and Bonnélye (1998) have shown that the coagulant dose necessary for algal removal is proportional to the sum of alkalinity and the logarithm of cell number. They emphasise that minimising turbidity in ajar test is not a sufficient criterion for adjusting treatment to remove algae and cyanobacteria, and recommend measuring the electrophoretic mobility of the cells (zeta potential) for optimising dosage (particularly because at insufficient coagulant dose, cyanobacteria will be the last phytoplankton cells to be removed). Bernhardt and Clasen (1991) have reported that coagulation of algal cells that are smooth and more or less spherical occurs largely by charge neutralisation. In

contrast, filamentous algae, large algae or species with bristles on their cell surface can be dealt with effectively only by sweep coagulation, by encountering the algae with large amounts of metal hydroxide floe.

Coagulation, by its nature, offers some promise for removal of intact cyanobacterial cells. For neurotoxins, Falconer (1989) reported that alum dosed at 120 mg l^{-1} alone and in combination with a number of polyelectrolytes removed about 20 per cent of the toxicity from a neurotoxic bloom of *Anabaena circinalis.* For microcystins, a number of published studies have shown that coagulation has a negligible capability for removal of any soluble toxins present in water. This has been demonstrated with aluminium sulphate coagulation jar tests in which total toxin concentration was reduced as a result of the removal of algal cells rather than the extracellular toxin (Figure 9.2) (WRc, 1996). Rositano and Nicholson (1994) also demonstrated this expectation by evaluating removal of purified, soluble microcystins by three coagulants: ferric sulphate, alum and polyaluminium chloride. In all cases they found essentially no toxin removal. Lambert *et al.* (1996) found inconsistent and low levels of microcystin removal (0-39 per cent) across the coagulation-sedimentation stage of a small, full-scale water treatment plant using an alum dose of over 60 mg l^{-1}.

Figure 9.2 The effect of coagulation with alum on the concentration of intra- and extracellular microcystin-LR (After Hart *et al.,* 1997. Reproduced courtesy of Blackwell Science)



By contrast, it must be emphasised that a study on raw water treatment with high doses of alum (200 mg l^{-1}) found over 23 per cent of the cell-bound microcystin-LR was released, mostly within two days of treatment (Lam *et al.,* 1995). However, at concentrations and conditions that would occur in water treatment plants, Velzeboer *et al.* (1995) found that aluminium sulphate did not appear to cause lysis of cells of cultured *Anabaena circinalis* or *Microcystis aeruginosa.* Flocculation under laboratory conditions, which simulated operating water treatment plants, resulted in removal of cells in a

healthy state, with no additional release of geosmin or microcystin-LR. Further work by Chow *et al.* (1997a) using ferric chloride as the coagulant showed similar results with some stimulation of growth of both algal species. There was no increase in concentration of microcystin in the water following treatment of *Microcystis aeruginosa,* although it appeared that *Anabaena circinalis* may be more susceptible to damage from chemicals. Later work using alum in a pilot plant with cultured *Microcystis aeruginosa* harvested at the late exponential phase of growth confirmed that the cells were not damaged through the treatment process and that no additional toxin was released during treatment (Drikas *et al.,* 1997). However, this study also confirmed that the low concentrations of extracellular microcystin present in the feed water (2-6 µg l^{-1}) are not removed during the treatment process. It was further found that the total cell number in sludge collected from the pilot plant decreased to half its initial value after two days, and that toxin release began virtually immediately, reaching almost 100 per cent after two days. After five days the toxin concentration began to decrease and was reduced by approximately 80 per cent after eight days and completely removed after 13 days. This corresponds to findings of Jones and Orr (1994) who observed that bacterial degradation of microcystin-LR occurred after nine days in a lake after chemical treatment of a *Microcystis aeruginosa* bloom. The importance of toxin release from sludge depends on the time that sludge is retained in sedimentation tanks and it could have implications for sludge management, particularly if supernatant is returned from sludge treatment processes to the head of the plant.

Selection of clarifier type will also affect cell removal rates. Mouchet and Bonnélye (1998) have summarised experience largely from warm climates and have shown that sludge blanket-type clarifiers are substantially more effective than static settlers (largely because of longer flocculation time), particularly if upflow pulsed systems are used. This achieved consistent reduction of total phytoplankton by 95-99 per cent at a plant treating Seine River water, 95-98 per cent elimination of cyanobacteria at a Philippine plant (as compared with 90-95 per cent removal by static settling), and 96.7-99.5 per cent removal of *Anabaena* and *Microcystis* at an industrial-scale plant in Harare (Zimbabwe). In Cairo and Alexandria, Egypt, older settling tanks were successfully upgraded to upflow pulsed sludge blanket clarifiers, thus not only improving performance for algal and cyanobacterial removal, but also efficiency per unit area and a reduction in coagulant consumption by 15-45 per cent and chlorine consumption by 15-35 per cent.

9.3.4 Dissolved air flotation

Although coagulation is normally followed by a sedimentation step, in some waters where the content of the suspended matter is low it is often easier to float the floe rather than attempting to settle a light floe. Recycled water saturated with air under pressure is introduced following the flocculation stage. Following the release of pressure the air comes out of solution and forms tiny bubbles which attach to the floe and cause it to float to the surface. The floated sludge is then collected and removed. This process is called dissolved air flotation (DAF) and is more effective than sedimentation, particularly for water with low turbidity and high colour, because the resultant floe is lighter and floats easily.

Dissolved air flotation is also generally more effective than sedimentation processes for treating algal-rich waters; for example floe blanket clarification has been shown to remove 76.5 per cent of *Microcystis* cells whilst DAF removed 98 per cent in the

presence of other algae (Gregory and Zabel, 1990). A Belgian DAF plant achieved 40-80 per cent removal *of Microcystis,* 90-100 per cent removal of Anabaena but only 30 per cent removal *of Planktothrix* (syn *Oscillatoria)* (Steffensen and Nicholson, 1994). Markham *et al.,* 1997) have reported on the efficiency of algae removal at eight DAF plants. Like Bernhardt and Clasen (1991), they observed that the characteristics of algae influence their removal by any clarification process. They found that most of the treatment plants produced more than 80 per cent removal and they expected this would be improved by optimisation. Vlaski *et al.* (1997) found that, in a pilot plant, DAF achieved high particle (algae) removal during a cyanobacteria bloom (mainly *Microcystis aeruginosa).*

Dissolved air flotation is unlikely to be more effective than conventional sedimentation processes for removing extracellular toxins. It may, however, remove more intact cells because the floating sludge tends to be removed more frequently than settled sludge in horizontal flow tanks, where the algae may die and then lyse. This assumption needs to be evaluated further.

Periods of high turbidity often cause problems for DAF, and any interruption in the process leads to an interruption in the treatment process. Thus a stock of spare parts and regular maintenance by qualified personnel are critical issues when using this approach (Mouchet and Bonnélye, 1998).

9.3.5 Precipitation for hardness reduction

Conversion of soluble compounds into insoluble particulates for separation by sedimentation or filtration is commonly used for water softening (calcium and magnesium removal) and for iron and manganese removal (Hamann *et al.,* 1990). Some concurrent removal of soluble metals and dissolved natural organic matter may also be achieved. Lime is commonly used for adjusting hardness or for precipitation of soluble metals. This process typically uses rapid mixing followed by flocculation and sedimentation.

No studies evaluating lime precipitation as a separate process in a water treatment plant sequence are available. However, some insight into the expected removal of intracellular toxins has been provided by two studies looking at treatment of raw water blooms with lime. Kenefick *et al.* (1993) found that lime doses from 100 mg l^{-1} as $Ca(OH)_2$ precipitated the cells in cyanobacterial bloom material containing microcystin-LR without releasing toxin compared with control batches over 14 days, while Lam *et al.* (1995) found only 4 per cent release of microcystin-LR for the same lime dosage. These studies suggest that lime softening would be effective at removing intracellular toxin by removing the cyanobacterial cells without causing cell lysis, but that there is no evidence to suggest that lime softening can reduce extracellular toxins.

9.3.6 Direct rapid filtration

Filtration is a process for the removal of suspended particulate matter, typically including clay, silt, natural organic matter, coagulated floes, lime softening precipitates, iron and manganese precipitates, and microorganisms (Hamann *et al.,* 1990). Filters most commonly use granular media such as coarse sand, crushed anthracite coal, garnet and granular activated carbon (GAC). Direct filtration is applied for low turbidity waters by

filtering directly after coagulation/destabilisation without an intervening clarification stage to remove the bulk of the floe. Conventional water treatment uses rapid filtration rates which require regular backwashing to maintain performance.

Mouchet and Bonnélye (1998) reported poor removal rates of 10-75 per cent, depending upon phytoplankton species, by direct rapid filtration without prior chemical treatment. Drikas *et al.* (1997) found that removal of *Microcystis aeruginosa* cells in the filtration stage of a pilot plant varied between 14 and 30 per cent following alum coagulation/sedimentation. Lepisto *et al.* (1996) evaluated full scale water treatment plants for their ability to remove cyanobacterial cells and found rapid sand filtration achieved only a 14 per cent reduction in cells. Rapid sand filtration, including GAC was somewhat better achieving 42 per cent reduction of cyanobacterial cells. These researchers expressed concern over the possible fate of intracellular toxins which may be released from degrading cells trapped in the filtration stage. Lambert *et al.* (1996) found inconsistent incremental removal of microcystins from 14-60 per cent across a dual media sand-anthracite filtration stage, following an alum coagulation-sedimentation stage, at a small, full-scale water treatment plant.

As an overall assessment of direct rapid filtration for elimination of algae and cyanobacteria, Mouchet and Bonnélye (1998) have indicated that direct filtration is generally not satisfactory, unless more sophisticated multimedia filters and adequate initial treatment are applied. They particularly emphasised the excellent results in algal removal after pre-ozonation (explicitly with the aim of enhancing cell removal through further steps, rather than for oxidation of cyanotoxins, see section 9.4.1).

A potential issue of concern, which currently has been inadequately investigated, is the effect of long filter runs between backwashing. Death and lysis of cyanobacteria retained on filters could lead to substantial toxin release.

9.3.7 Combined coagulation, sedimentation and rapid filtration

Conventional water treatment commonly involves the combination of coagulation, clarification (sedimentation or dissolved air flotation) and filtration. Consequently, much of the limited research that has been published on water treatment performance for the removal of cyanotoxins has looked at overall removal across the common combinations of coagulation-filtration and coagulation-clarification-filtration, rather than looking at each stage individually.

Himberg *et al.* (1989) evaluated hepatotoxic fractions from *Microcystis wesenbergii, M. viridis* and *Planktothrix agardhii* (syn. *Oscillatoria agardhii)* in bench-scale treatment processes consisting of alum or ferric chloride coagulation combined with sand filtration and chlorination. Alum coagulation, at doses from 36 to 71 mg l^{-1}, with filtration achieved toxin removals from 11 to 32 per cent, while ferric chloride at 55 mg l^{-1} achieved from 9 to 16 per cent. The removal contribution of the low chlorination dosage in this case was apparently negligible. They also studied a similar conventional process at pilot scale using freeze dried *Microcystis* bloom material and found negligible toxin removal (Keijola *et al.,* 1988). Nonetheless, Lambert *et al.* (1996) found combined microcystin removal was 50-60 per cent across coagulation, sedimentation and dual media filtration in a small full scale plant.

Similar studies at bench scale with anatoxin-a have indicated no removal for either alum or ferric chloride process combinations at a toxin concentration of 20 µg l^{-1}, but at 10 times higher toxin concentrations, the alum process achieved a 14 per cent anatoxin-a removal and the ferric chloride process achieved a 49 per cent anatoxin-a removal (Keijola *et al.,* 1988).

Leuschner (1984) studied phytoplankton retention by flocculation, sedimentation and rapid filtration in a plant treating highly eutrophic river water. Whereas *Microcystis* spp. (occurring as large colonies) were rarely observed in the finished water, *Planktothrix agardhii* was poorly retained, showing an average breakthrough of 27 per cent of the filaments. As also reported by Mouchet and Bonnélye (1998), addition of a cationic polymer during flocculation substantially improved retention.

The removal of whole, intact cells presents the best opportunity to remove toxins in separation processes, whereas the literature indicates removal efficiencies are low with extracellular toxins. Some unsatisfactory results reported with lysis of entire cells may have been due to an excessive time delay between flocculation and analysis. In summary, currently available results indicate that conventional coagulation and rapid filtration processes assist in toxin removal, particularly if cyanobacterial cells are kept intact, but cannot be generally relied upon as the main removal process. Mouchet and Bonnélye (1998) have emphasised the need for:

"... *pilot scale investigation in order to estimate the technical and economical advantages of this choice in each case. Generally, a conventional treatment line, including coagulation, flocculation, settling or flotation, and filtration, is preferred to treat algae-rich waters. However, algae removal is somewhat more delicate than turbidity removal and, consequently, greater attention is required when selecting technology and adjusting the chemical treatment.*" (Mouchet and Bonnélye, 1998)

9.3.8 Slow sand filtration

In contrast to rapid filtration, slow sand filters operate at lower rates and develop a surface filter cake which performs most of the filtration together with (often high) biological treatment activity. These biofilms establish after some time of operation and contribute significantly to degradation of dissolved substances. Mouchet and Bonnélye (1998) reported a likely removal of 99 per cent of algal cells by slow sand filtration. Operation of these filters in the dark can prevent intensive algal growth on the filter. However, overloading of filters with algae or cyanobacteria from the raw water may lead to rapid blocking, requiring removal of the bioactive surface layer, thus temporarily reducing the efficiency for retention of dissolved substances. For removal of toxic cyanobacteria, this constitutes a dilemma because bloom-containing waters are likely to lead to rapid blocking and thus undermine the practicability of slow sand filtration. However, experiments have shown that before blocking, slow sand filters may be quite effective in the removal of toxic cyanobacteria and dissolved toxins.

Keijola *et al.* (1988) evaluated laboratory-scale slow sand filters and reported over 80 per cent removal of toxins from *Microcystis,* 30-65 per cent removal of toxins from *Planktothrix* (syn. *Oscillatoria)* and about 70 per cent removal of anatoxin-a. Because filtration itself would not be expected to achieve any removal of extracellular toxin, these results suggest that the mechanisms were at least biosorption, and perhaps some

biotransformation. Australian studies (Sherman *et al.,* 1995) with roughing filters followed by slow sand filters showed that *M. aeruginosa* and some *Planktothrix* (syn. *Oscillatoria)* cells from toxic bloom material could be removed by physical means and biological processes. Superior microcystin removal, in one of two river water sources being treated with GAC filters, was attributed to biological activity (Drikas, 1994). Freeze dried bloom material was used in this study.

Work on microcystin-LR degradation using an isolated bacterium for use in water treatment has been undertaken by Bourne *et al.* (1996). A pseudo-monad has been isolated which possesses an enzyme system capable of degrading microcystin, but the work is currently only at the laboratory scale. Pilot plant studies using a solid phase support for this bacterium to investigate this process are to be undertaken.

Developments have occurred in the exploitation of slow sand filters at large treatment works, notably in the UK and Netherlands. These have included use of various pretreatments, such as conventional treatment by coagulation and filtration and pre-ozonation to control the rate of blocking by algae and cyanobacteria. Whilst these processes will assist with removal of cells containing toxins, they have not been adequately assessed for their reliability in degradation of extracellular toxins. A notable development has been the sandwiching of a layer of GAC within the bed of sand in slow sand filters in order to assist in removal of dissolved toxins.

General experience with slow sand filters suggests that they are potentially very useful for removal of particles and dissolved substances, particularly if further developed or combined with other treatment steps to avoid blocking when loaded with waters rich in algae and cyanobacteria (or other particles). New approaches to slow sand filtration are experimenting with horizontal rather than vertical water flow (as used in cross-flow membrane techniques). This requires larger amounts of water but will remove most of the potentially filter-blocking particles and, in particular, would keep cyanobacteria suspended. Such systems may be developed locally, particularly to serve small communities. For large treatment facilities, bulk cell removal by coagulation and clarification before slow sand filtration may be an effective approach for obtaining the benefits while avoiding rapid blocking.

9.3.9 Activated carbon adsorption

The use of activated carbon adsorption has expanded greatly in Europe and North America during the past two to three decades because most other water treatment processes are ineffective in removing soluble organic matter. This approach uses either powdered activated carbon (PAC) which can be added intermittently whenever the need arises or GAC adsorbers which are used continuously. Accordingly, GAC may be more expensive than PAC when used only intermittently, but it is also generally more effective and more reliable for consistent removal of soluble organic compounds (Hamann *et al.,* 1990). Given the nature of cyanobacterial toxins, activated carbon adsorption would be expected to offer some promise for toxin removal.

Powdered activated carbon

Keijola *et al.* (1988) found that 20 mg l^{-1} of PAC was able to achieve a 90 per cent removal of hepatotoxins following conventional treatment combined with pre-ozonation.

Hart and Stott (1993) and Croll and Hart (1996) have reported the evaluation of several PACs for the removal of microcystin-LR at an initial concentration of 40 µg l^{-1}. With the most effective PAC tested (wood based), doses greater than 20 mg l^{-1} were required to achieve toxin removal of greater than 85 per cent.

Donati *et al.* (1993) also evaluated several different PACs for the removal of dissolved microcystin-LR at an initial concentration of 50 µg l^{-1}. For the best PAC they studied, a dose of 25 mg l^{-1} with 30 minutes contact time was able to achieve 98 per cent removal, while for the poorest a dose of 50 mg l^{-1} only achieved a 60 per cent removal. They suggested that the mesopore volume of the various carbons was the best predictor of carbon performance (Donati *et al.,* 1994a). Nodularin was also removed with PAC (Donati *et al.,* 1994b). Likewise, Bernazeau (1994) found that 12 mg l^{-1} of PAC could achieve a 95 per cent reduction of dissolved microcystin-LR from an initial concentration of 50 µg l^{-1}. Monitoring of a full scale conventional water treatment plant which was using a PAC dose of 30 mg l^{-1} showed the combined treatment processes removed an average of 82 per cent when microcystin levels in raw water were above 0.5 µg l^{-1} (Lambert *et al.,* 1996).

There is general agreement that to achieve high removal efficiencies, very high doses of PAC are required for toxin removal and that contact time is very important. Lower doses of PAC are required with pure water compared with natural water containing organic matter and when using actual plant mixing conditions and contact times. Alum coagulation in conjunction with PAC was also found to affect adversely toxin removal (Jones *et al.,* 1993).

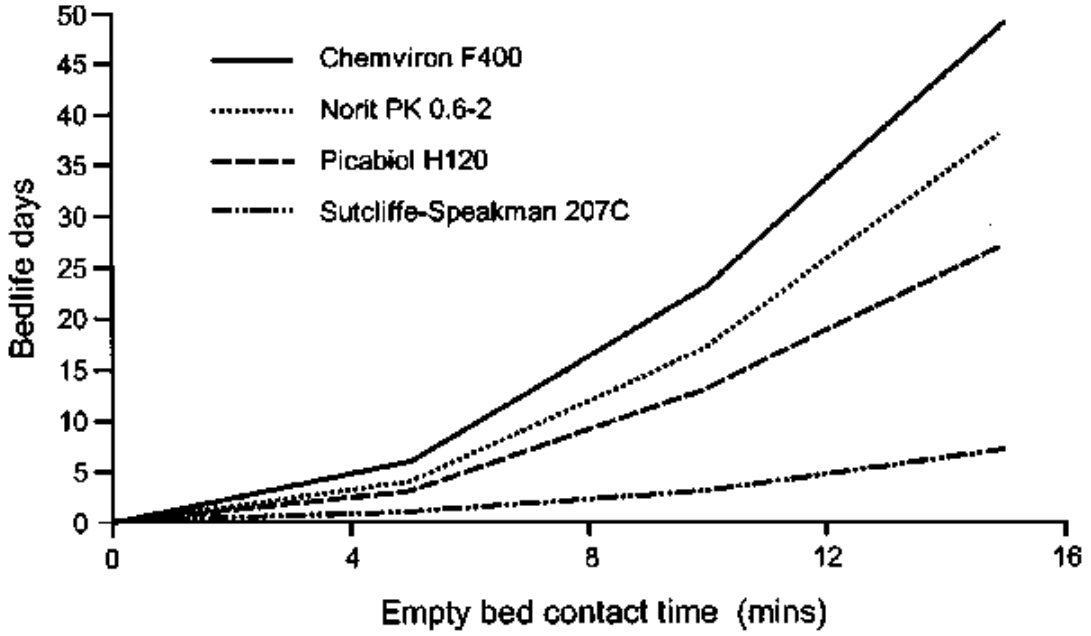
Granular activated carbon

As might be expected, research into the performance of GAC has shown effective removal of toxins, provided the adsorption capacity of the GAC has not been compromised. Pilot scale tests treating microcystins at 30-50 µg l^{-1} showed greater than 90 per cent toxin removal for water treatment volumes up to 7,000-10,000 activated carbon bed volumes before efficiency dropped to less than 63 per cent (probably because of saturation of the GAC with dissolved organic carbon (DOC)) (Bernezeau, 1994). In these trials, the raw water had DOC levels at 5-6.5 mg l^{-1}, more than 100-fold greater concentration than the microcystins. The DOC: toxin ratio would be at least this high under any realistic bloom conditions.

Studies by Hart and Stott (1993), using rapid column tests to simulate the performance of GAC under dynamic conditions predicted bedlives to be fairly short for continuous exposure to microcystin concentrations of 5-20 µg l^{-1}. For example, Figure 9.3 shows predicted bedlife for four different carbons, based on rapid column tests. The bedlife is the time taken to reach 1 µg l^{-1} in the treated water with a constant concentration of 10 µg l^{-1} in the feed water, for a range of empty bed contact times (EBCTs). For EBCTs typically used in water treatment of 10-15 minutes, the best performing carbon for this water gave a bedlife of only 30-45 days. These results were confirmed in Australian studies by Jones *et al.* (1993) and Craig and Bailey (1995) in both laboratory and pilot plant studies, using air dried bloom material. The results showed that while various GACs were effective for microcystin-LR removal, the life of the GAC was limited. Saturation conditions probably explain the observations that a full-scale GAC adsorber was achieving only between 40 and 60 per cent microcystin removal down to 0.6-1.2 µg

l^{-1} for raw water which typically had DOC levels of 20 mg l^{-1}, 2,000 fold greater than the toxin levels (Lambert *et al.,* 1996).

Figure 9.3 Predicted GAC bedlives for 10 µg l^{-1} microcystin-LR input and 1 µg l^{-1} limit in filtrate from rapid column test results for four different carbons (After Carlisle, 1994. Reproduced courtesy of the Foundation for Water Research, UK)

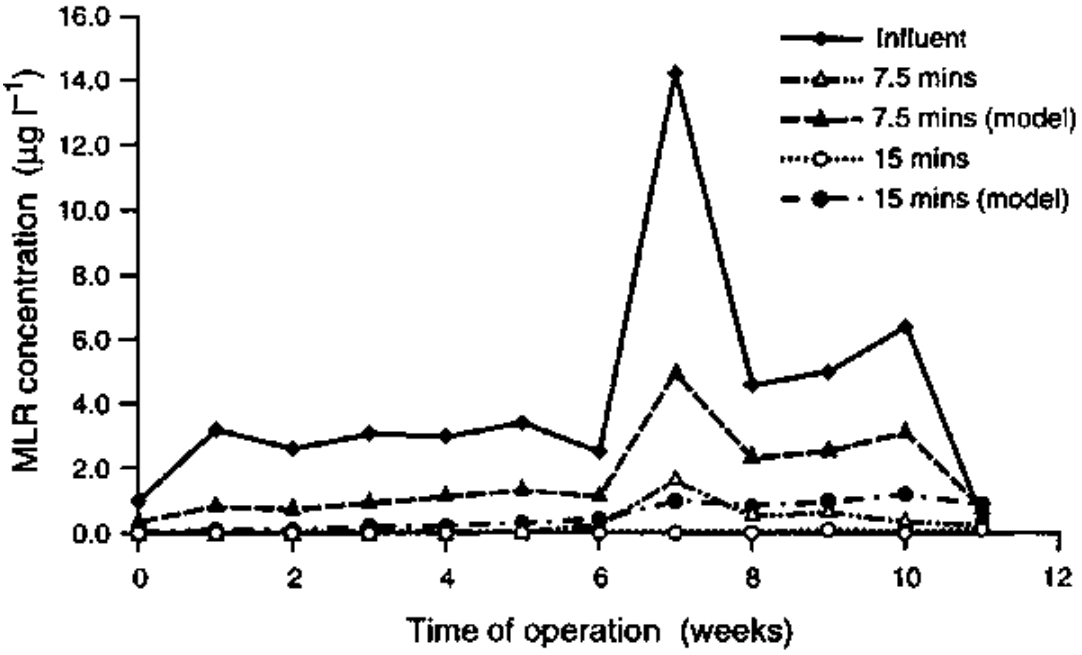


No published studies have been found using GAC specifically for removal of nodularin, cylindrospermopsin or PSP toxins. However, a report by Falconer *et al.* (1989) showed removal of *Anabaena* neurotoxicity on GAC. Given the later identification of the toxin of the population studied, their study almost certainly assessed PSP toxin removal. Carlile (1994) repeated the tests by Hart and Stott (1993), using the GAC they found to be most effective, and found anatoxin-a to be adsorbed better than microcystin-LR.

Biologically active carbon

Granular activated carbon is not only an effective adsorption process but it is also an effective medium for biological treatment. Because microcystin-LR has been shown to be biodegradable (Fawell *et al.,* 1993), it is therefore possible that the toxin could be degraded on a biologically active GAC. Carlile (1994) undertook pilot scale tests using two GACs, one that had been previously used on a pilot plant for total organic carbon (TOC) removal and an unused GAC. For the tests, for each GAC, two different contact times of 7.5 and 15 minutes were used. The pilot plant results showed that there was no significant difference between the performance of the unused GAC and the used GAC at both contact times. However, the comparison of pilot plant results with results of modelling assuming removal only by adsorption, shows poorer removal by adsorption only (without any biological activity) at both contact times (Figure 9.4). The implications from this are that the better removal on the pilot plant resulted from biological activity on the GAC, and that this biological activity developed very quickly also on the unused GAC.

Figure 9.4 Comparison of pilot scale test results for microcystin-LR removal by used GAC (probably biologically active) with performance predicted from models for adsorption only (After Hart *et al.,* 1997. Reproduced courtesy of Blackwell Science)



Pilot plant trials investigating anatoxin-a removal by GAC showed no breakthrough, whereas modelled results for the same operating conditions predicted breakthrough (UK WIR, 1995). This suggested that biological activity was also important for anatoxin-a removal by GAC.

In practice, it is difficult to exclude biological activity from GAC adsorbers and therefore better removal of both toxins than indicated by rapid column tests would be expected. The pilot-scale experiments discussed above suggest that when biological activity is established, GAC at 15 minutes effective bed contact time provides a high degree of security for both microcystin-LR and anatoxin-a removal. However, as these results currently are poorly confirmed in full scale application, careful surveillance of treatment performance is essential for treatment plants removing cyanotoxins in the raw water with GAC. This particularly pertains to monitoring of breakthrough when saturation with DOC is approached.

9.4 Chemical oxidation and disinfection

Drinking water is treated with chemical oxidants to fulfil a wide variety of objectives including: control of biofilm growth, colour removal, odour control, enhancement of coagulation and flocculation, and iron or manganese oxidation. The most critical application of chemical oxidants is for disinfection. The chemicals used most commonly in municipal water treatment are chlorine, chloramines, ozone, chlorine dioxide and potassium permanganate.

9.4.1 Oxidation combined with disinfection

Once cyanobacterial cells have been removed from water, dissolved cyanotoxins are potentially susceptible to oxidation by disinfectants. Several substances have been tested for this purpose in drinking water treatment.

Chlorine

Early work reported that substantial doses (5 mg l^{-1}) of chlorine were ineffective in destroying toxicity from algal extracts, as measured in mouse bioassays (Hoffman, 1976). Likewise, combined treatment processes which included chlorination at 0.5 mg l^{-1} were also found ineffective, suggesting little contribution from the chlorination stage (Keijola *et al.,* 1988; Himberg *et al.,* 1989). Similarly, Lambert *et al.* (1996) found that chlorination achieved negligible reduction in microcystin levels of 0.3-0.5 µg l^{-1} in treated water. In these studies, chlorine may have been consumed rapidly by the high concentrations of organic matter reported to be present, leaving insufficient available for removal of microcystins. However, Nicholson *et al.* (1994) showed that chlorination could be very effective at destroying microcystin-LR and nodularin under the correct treatment conditions, i.e. free chlorine residual of 0.5 mg l^{-1} after 30 minutes contact time with pH < 8. In contrast, they found that chloramination was completely ineffective at destroying microcystin-LR and nodularin, and this creates a problem for treating natural waters with any substantial nitrogenous chlorine demand.

Carlile (1994), Croll and Hart (1996) and Hart *et al.* (1997) have reported tests with a variety of oxidants using water spiked with dissolved microcystin-LR or anatoxin-a in the range 5-10 µg l^{-1}. The tests with chlorine used an applied dose of 1.7 mg l^{-1}, which was found to give a free residual of approximately 0.7 mg l^{-1} after 30 minutes. The effectiveness of the chlorine in reducing microcystin-LR concentration was very dependent on pH and time. At pH 5, removal was more than 93 per cent within 30 minutes whilst at pH 7 removal reached only 88 per cent after 22 hours. Tests with a water containing Microcystis cells indicated that chlorination could be similarly effective. Chlorination during treatment at a pH sufficiently low to show maximum effect might not be feasible in practice. However, in conjunction with extended contact times with a residual free chlorine concentration, microcystin is likely to be degraded. Monitoring of this effect is important.

Chlorination tests have also been undertaken with water containing dissolved anatoxin-a. Nicholson *et al.* (1994), as well as Carlile (1994), reported no discernible removal of anatoxin-a by chlorination. Rositano and Nicholson (1994) also showed that chlorination of anatoxin-a was ineffective with a dose of 15 mg l^{-1} at pH 7 for 30 minutes contact time, providing only a 16 per cent removal. Recent Australian studies (unpublished results) have shown that removal of cylindrospermopsin can be achieved with chlorine doses of 1-2 mg l^{-1} at pH levels between 6 and 7.5 and a chlorine residual of 0.5 mg l^{-1}.

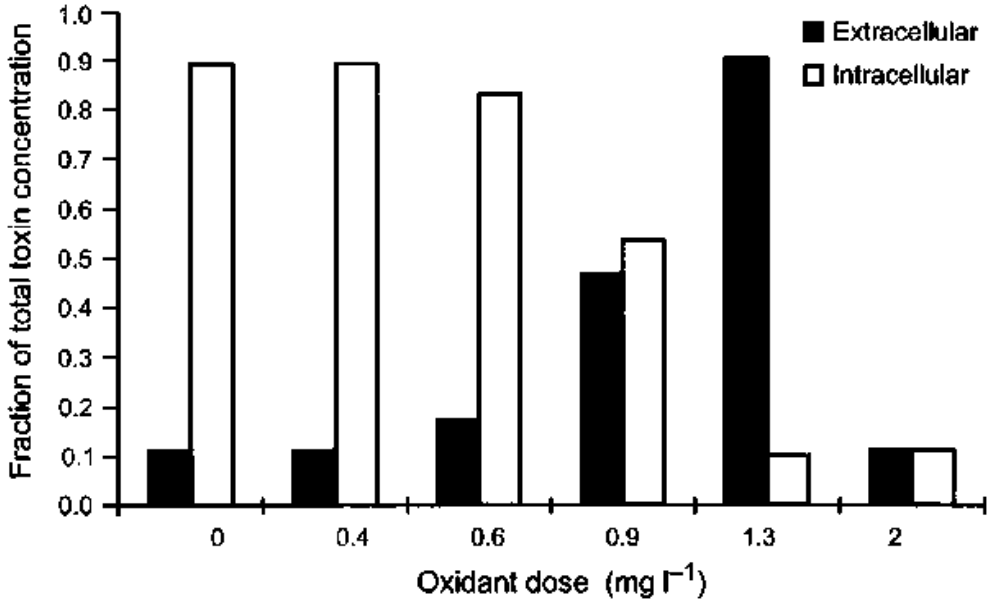
Care must be taken with chlorination procedures to avoid occupational exposure to toxic levels of chlorine in the air, or the formation of excess levels of trihalomethanes.

Ozone

The most consistently efficient process for destruction of both ultra- and extracellular microcystins appears to be ozonation, which can rapidly achieve essentially complete destruction of microcystins, nodularin and anatoxin-a (Keijola *et al.,* 1988; Himberg *et al.,* 1989; Rositano and Nicholson, 1994; Croll and Hart, 1996; Rositano *et al.,* 1996; Hart *et al.,* 1997). The major consideration in the application of ozonation is the ozone demanded by background DOC concentrations because, at a DOC level of 8.5 mg l^{-1}, ozone doses above 1 mg l^{-1} were necessary to achieve complete microcystin-LR destruction (Rositano and Nicholson, 1994). The results of Hart *et al.* (1997) demonstrate the importance of sufficiently high ozone doses (Figure 9.5). At low doses up to 0.6 mg l^{-1}, ozone degraded DOC and had little effect on microcystin-LR. Only after the DOC demand was satisfied, did the ozone show an effect on microcystin-LR. However, between 0.6 and 1.3 mg l^{-1}, this effect consisted almost entirely of cellular lysis, and only at 2 mg l^{-1} was extracellular toxin subsequently converted. These results highlight the crucial importance of sufficiently high ozone doses as well as of careful monitoring of performance, particularly with variable DOC concentrations in the water source as occur during cyanobacterial blooms. As discussed in section 9.4.2, the performance of ozone may be improved substantially if it is applied in several steps, e.g. before destabilisation/flocculation as well as after filtration.

Recent work in Australia (unpublished results) has shown that the ozone dose necessary to achieve removal of a range of PSP toxins in the concentration range 10-100 µg l^{-1} was less than the ozone demand of the water. Other recent studies in Australia (unpublished results) have shown that ozone is also effective for the removal of cylindrospermopsin.

Figure 9.5 Effect of ozonation on the distribution of both intra- and extracellular microcystin-LR from *Microcystis* dosed into a raw lowland water (After Hart *et al.,* 1997. Reproduced courtesy of Blackwell Science)



Care must be taken with ozone procedures to avoid occupational exposure to toxic levels in the air.

Potassium permanganate

Potassium permanganate at 1 mg l^{-1} was found to achieve 95 per cent removal of microcystin-LR in 30 minutes. However, in the presence of live intact cells removal was much poorer, suggesting that permanganate was unable to penetrate or lyse the cells effectively and was therefore unable to come into contact with the toxin (Rositano, 1996). Hart and Stott (1993), Carlile (1994), Croll and Hart (1996) and WRc (1996) have all reported similar observations for the removal of dissolved microcystin-LR and anatoxin-a and the same limitation in treating *Microcystis* cells. Lam *et al.* (1995) reported that potassium permanganate caused some cell lysis and liberation of microcystin-LR. This finding may be influenced by longer contact times than those used by Rositano (1996).

Hydrogen peroxide and UV radiation

Hydrogen peroxide was found ineffective in toxin removal, whereas either UV alone or UV with hydrogen peroxide achieved about a 50 per cent removal of microcystin-LR after 30 minutes (Rositano and Nicholson, 1994).

In contrast, Croll and Hart (1996) and WRc (1996) found UV radiation was capable of efficiently degrading both microcystin-LR and anatoxin-a, but only at very high doses of about 20,000 mWs/cm^2. A typical water disinfection dose is about 30 mWs/cm^2, and therefore UV on its own cannot be regarded as a practical method of toxin reduction. A recent finding has shown that very high concentrations of microcystin-LR (50-200 mg l^{-1}) were rapidly (10-40 minutes) destroyed using UV light in the presence of a titanium dioxide catalyst (Robertson *et al.,* 1997). The potential applications of this finding in water treatment remain to be explored.

Chlorine dioxide

Chlorine dioxide has strong oxidising ability, although only limited studies have been conducted with this oxidant. Hart and Stott (1993) found that whilst a dose of 6 mg l^{-1} was required to reduce 4.6 µg l^{-1} of dissolved microcystin-LR to less than 1 µg l^{-1}, a dose as great as 10 mg l^{-1} had no effect on about 4 µg l^{-1} of intracellular microcystin.

9.4.2 Pre-oxidation (before cell removal)

Pre-oxidation has been widely reported to assist coagulation, especially in the removal of some algae and cyanobacteria. Oxidants have been shown to breakdown some cyanotoxins effectively under certain conditions (see section 9.4.1) but may also lead to cell lysis and toxin release. Thus pre-oxidation of toxic cyanobacteria is a highly critical issue in treatment design.

Ozone has been most effective in oxidation of cell-bound microcystin, if applied at a sufficiently high dose and contact time (see section 9.4.1). Dissolved air flotation has been proposed in which the recycled water is saturated with ozone-rich air (Baron *et al.,* 1997). Ozone-rich air has also been proposed to be used in dispersed air flotation.

These approaches might result in reduction of extracellular toxin as well as enhanced removal of cells.

Chlorine has been applied to destroy cell-bound microcystins before further treatment. However, Lam *et al.* (1995) showed that chlorination of bloom material using a high dose of 44 mg l^{-1}, resulted in release of 64 per cent of the intracellular microcystin. Thus, pre-chlorination of raw waters containing cyanobacterial cells risks the release of toxin from otherwise intact cells.

Mouchet and Bonnélye (1998) have compared pre-ozonation and pre-chlorination with respect to their effect in elimination of algae and cyanobacteria, as well as toxin release and formation of by-products. They concluded that pre-chlorination is slightly more effective than pre-ozonation in enhancing coagulation (96.9 per cent removal as compared with 94.1 per cent in one treatment plant in France). However, this advantage is offset by the problems of cell damage resulting in release of DOC and metabolites which either may be toxic or may impart offensive taste and odour, as well as leading to formation of by-products (particularly highly unpleasant chlorophenols). In contrast, for pre-ozonation (usually dosed at 1 mg l^{-1}) these authors found little, if any, cell lysis at doses up to 3 mg l^{-1}. They recommend pre-ozonation as the better choice, especially in conjunction with a main ozonation step further in the treatment line, e.g. between clarification and filtration. It is however acknowledged that pre-chlorination is still very common, particularly in developing countries. While the advantages for improving clarification, keeping filters clean, eliminating ammonia and enhancing post-chlorination are well established, pre-chlorination in plants without subsequent adsorption onto activated carbon is not recommended.

Prior to cell removal, the total and dissolved organic carbon load of water with cyanobacterial blooms will vary by orders of magnitude, and consumption of the oxidant will therefore also vary widely. Continuous control of the oxidising step and very high doses may be necessary to ensure complete oxidation of cyanotoxins in one pre-treatment step. This is likely to be difficult in practice, and is associated with a risk of toxin liberation. Removing cyanobacterial cells before application of oxidant is safer. In contrast, pre-oxidation with a low ozone dose may be useful because it substantially enhances cell removal by subsequent steps. Safe and effective operation is possible if further cyanotoxin barriers (such as a further ozone step or GAC) are available. Consequently, pre-oxidation may be regarded as a step for enhancement of cell removal rather than cyanotoxin degradation, and requires either monitoring for breakthrough of dissolved toxins during cyanobacterial blooms or for the use of further multiple barriers in the treatment system.

9.5 Membrane processes and reverse osmosis

Membrane processes, particularly microfiltration (MF) and ultrafiltration (UF) are increasingly seen, under some circumstances, as economically viable treatment alternatives to conventional treatment for small and large communities. They should be effective in the removal of cyanobacteria and intracellular toxins.

Experimental studies at laboratory scale with flat-sheet UF and MF membranes, in both dead-end and crossflow modes, have shown high efficiency of removal (> 98 per cent) of whole cells of toxic *M. aeruginosa* (Chow *et al.,* 1997b). This study also examined the

effect of the filtration process on cell integrity by fluorescence microscopy and assessed cell damage by measuring the leakage of cell chlorophyll and toxin (microcystin-LR) into the permeate. There was evidence of damage to a small proportion of cells following filtration, but no significant increase in toxin in the permeate with all modes of filtration. In experiments with the ultrafiltration membrane, the amount of microcystin was significantly lower in the permeate than in the feed, which suggested that the particular UF membrane employed may have rejection properties or adsorption ability for microcystin. This would not be expected for UF membranes although removal of soluble toxin may be achieved with a very low molecular weight cut-off pore size, such as those offered by nanofiltration membranes. Hart and Stott (1993) evaluated the effect of nanofiltration for the removal of microcystin spiked into natural water at concentrations between 5 µg l^{-1} and 30 µg l^{-1} and found removal to below 1 µg l^{-1}. Australian studies with membranes (Muntisov and Trimboli, 1996) also showed that using nanofiltration microcystin-LR and nodularin at 8 µg l^{-1} were removed from water from the River Murray that had been spiked with toxin.

Neumann and Weckesser (1998) have tested three reverse osmosis membranes at 25-35 bar for elimination of microcystin-LR and microcystin-RR from tap and salt (3,000 mg l^{-1} NaCl) water. Initial toxin concentrations in the retentate were in the range 70-130 µg l^{-1}. With a detection limit of 0.2 µg l^{-1}, average retention levels were 96.7-99.6 per cent. There was no statistical difference in retention of the microcystins between the two waters.

9.6 Microcystins other than microcystin-LR

Most of the published research relates to microcystin-LR, even though its concentration can be exceeded by those of other variants, or by the sum of the concentrations of other variants (Codd and Bell, 1996).

Computer models are available which can be used to predict the properties of chemical compounds, based on their chemical structure, in order to provide information in relation to toxicology and environmental impact. Such models have been used to predict the properties of the microcystin variants which would be important in relation to removal by water treatment processes (WRc, 1997).

A physical measure of solubility and interaction with water molecules that gives an indication of the potential adsorption by activated carbon is the octanol-water partition coefficient, K_{ow}. This is defined as the ratio of the concentration in the octanol phase to the concentration in the water phase in a two-phase octanol-water system at equilibrium, and is usually expressed as a logarithm. Readily adsorbed, hydrophobic compounds have high values and poorly adsorbed hydrophilic compound have low values (often negative, indicating a higher concentration of the compound in the water phase). K_{ow} values have been estimated from molecular structure to provide an indication of the relative hydrophobicity compared with microcystin-LR (for which some information on activated carbon adsorption is available). The calculated K_{ow} values suggest that the majority of variants would be adsorbed by activated carbon similarly to, or better than, microcystin-LR. Hence any strategy for using activated carbon, based on the data available for microcystin-LR, would probably be suitable for the majority of the other microcystins.

Attempts to model the reactivity of microcystin variants with oxidants have been unsuccessful because of the complexity of the molecular structure. A principal mechanism of action of oxidants, particularly ozone and chlorine, on organic compounds is by the breakdown of double bonds. Any modifications to the basic microcystin structure which increases the degree of double bonding in the molecule would therefore be expected to enhance its reaction with ozone or chlorine. It has been concluded, from consideration of the amino acid functional groups in the variants, that some variants would be expected to be more reactive with oxidants than microcystin-LR, although the effect may not be important in practical terms because the basic molecular structure is not changed radically. For the same reason, the other variants would not be expected to be much less reactive with oxidants than microcystin-LR. Hence, any strategy for oxidant application based on microcystin-LR data would probably be just as effective for the other microcystins.

The modelling approach available for biodegradability can only class compounds as biodegradable or non-biodegradable, and cannot provide any further quantification to the degree of biodegradability. Modelling has classed microcystin-LR as biodegradable, and changes to the amino acids have not changed this classification. Hence all the variants would be expected to show similar biodegradability to microcystin-LR. This would be of significance in relation to the performance of biological GAC and slow sand filtration processes.

The lack of experimental data on the elimination of microcystins other than microcystin-LR emphasises the need to monitor performance of any treatment system that is applied for cyanotoxin removal.

9.7 Effective drinking water treatment at treatment works

There are a number of messages that arise from the published work with respect to good practice as well as effective design and operation of water treatment works. These include:

- Resources and abstraction should be managed to minimise the presence of algal concentrations in the raw water delivered for treatment.

- Chemical preparation and dosing facilities must be of adequate size, process control should ensure rapid dispersion and appropriate retention times, and chemical doses should be optimised at the appropriate pH.

- Some oxidants, e.g. ozone, can be dosed before coagulation and clarification but require particular care, not only to avoid lysis of cells but also to limit problems with disinfection by-product formation. Separation of steps into a low pre-oxidation dose to enhance flocculation and a higher dose after cell removal to oxidise dissolved toxins is a safer approach.

- Granular activated carbon plants with a high EBCT and ozone-GAC facilities may remove toxins effectively, especially if the GAC supports substantial biological activity.

- The effectiveness of treatment plants without ozone but with GAC will depend on the GAC EBCT value, on the degree of biological activity on the GAC, on the extent of exhaustion of the GAC and of the magnitude and duration of toxin occurrence.

- Conventional treatment plants without ozone and GAC might remove cyanobacterial cells and dissolved toxins satisfactorily if coagulation, clarification, filtration and superchlorination-dechlorination (with a contact time of>15 mg min l^{-1}) or ozonation are carried out effectively.

- Slow sand filter plants remove algal cells effectively, although pre-treatment steps are generally applied to maximise filter runs and efficiency. Because of the biological activity in slow sand filters and long contact times, some removal of dissolved toxin should be expected but this capability is unclear. Slow sand filter plants with pre-ozonation and/or sand-GAC sandwiching would be expected to be effective for dissolved toxins (although confirmation of this expectation is needed).

- Frequent monitoring of treatment performance is crucial to ensure safety, particularly with respect to cyanotoxin removal, because available information on the performance of different treatment steps is specific to the conditions of the experiments reported, and performance under other conditions is unclear. Variable and often high loads of DOC during cyanobacterial blooms may rapidly compromise treatment procedures that were initially successful.

- Most procedures have been studied for cyanotoxin removal as isolated treatment steps, rather than as a combination following the multi-barrier principle. Planning of treatment will lead to best results if combinations are considered, and if cell and dissolved toxin removal are separately evaluated (e.g. combinations of pre-oxidation to enhance cell removal with effective post-oxidation to ensure destruction of liberated toxin, or combinations of cell removal and slow sand filtration).

Perhaps because of the intermittent nature of cyanobacterial blooms, very little information has been reported from full-scale treatment plants treating water at naturally occurring toxin levels. Laboratory and pilot-scale investigations have shown that dissolved toxins can be removed effectively to less than 1 µg l^{-1} under conditions normally used in water treatment by biologically active GAC, ozone, potassium permanganate and chlorine (microcystin only). The information which has been reported to date is summarised in Table 9.3.

Table 9.3 Summary of water treatment performance on microcystins

Treatment technique	Expected removal[1]		Comments
	Cell bound	Extracellular	
Coagulation/sedimentation/dissolved air flotation	> 80%	<10%	Removal only achievable for toxins in cells, provided cells are not damaged
Precipitation/sedimentation	> 90%	<10%	Removal only achievable for toxins in cells, provided cells are not damaged
Rapid filtration	> 60%	<10%	Removal only achievable for toxins in cells, provided cells are not damaged
Slow sand filtration	~ 99%	Probably significant	Removal effective for toxins in cells; efficiency for dissolved microcystin is likely to depend on biofilm formation and thus on filter run length
Combined coagulation/sedimentation/filtration	> 90%	< 10%	Removal only achievable for toxins in cells, provided cells are not damaged
Dissolved air flotation	> 90%	Not assessed, probably low	Removal only achievable for toxins in cells, provided cells are not damaged
Adsorption - Powdered activated carbon (PAC)	Negligible	> 85%	For adequate PAC doses (> 20 mg l^{-1}) with a PAC shown to be effective, DOC competition will reduce capacity
Adsorption - Granular activated carbon (GAC)	See rapid filtration	> 80%	For practical EBCTs, DOC competition will reduce capacity and hasten breakthrough, filtration also removes algal cells
Biological granular activated carbon	See rapid filtration	> 90%	See GAC, biological activity enhances removal efficiency and bed life
Pre-ozonation	Very effective in enhancing coagulation	Potential increase	Useful in low doses to assist coagulation of cells; risk of toxin release requires careful monitoring and possibly subsequent treatment

			steps
Pre-chlorination	Very effective in enhancing coagulation	Causes lysis and release of dissolved metabolites	Useful to assist coagulation of cells but applicable for toxic cyanobacteria only if subsequent treatment steps will remove dissolved toxins and other released metabolites
Ozonation (post clarification)	-	> 98%	Rapid and efficient on soluble toxin provided that DOC demand is satisfied
Free chlorine (postfiltration)	-	> 80%	Effective when free chlorine is > 0.5 mg l^{-1} after > 30 minutes at pH < 8 and low DOC; effect negligible when dose low or pH > 8
Chloramine	-	Negligible	Ineffective. Free chlorine application will yield ineffective chloramines in waters enriched with nitrogenous organic matter
Chlorine dioxide	-	Negligible	Not effective with doses used in drinking water treatment
Potassium permanganate	-	95%	Effective on soluble toxin but only in absence of whole cells
Hydrogen peroxide	-	Negligible	Not effective on its own
UV radiation	-	Negligible	Capable of degrading microcystin-LR and anatoxin-a, but only at impractically high doses
Membrane processes	Likely to be very high (> 99%)	Uncertain	Depends on membrane type, further research required to characterise performance

DOC Dissolved organic carbon

Source: Adapted from Yoo *et al*., 1995

[1] Likely efficiency of removal when continuously applied at optimal doses and pH and under proper operating conditions

9.8 Drinking water treatment for households and small community supplies

Domestic upgrading of piped drinking water supplies has been a recent issue of concern in some countries. Many central supplies provide excellent quality drinking water and additional household treatment may actually cause deterioration rather than improvement. However, domestic treatment may have a role in regions supplied with poor quality drinking water, or for especially sensitive sub-populations. Furthermore, in many parts of the world, simple and easily maintained treatment for households and small communities may improve the quality of water otherwise used for drinking without any treatment. Boiling water will not remove or degrade cyanotoxins (Chen *et al.,* 1998).

Lawton *et al.* (1998) tested three different domestic jug filtration units for their capacity to remove extracellular microcystins (LR, LY, LW and LF) and one unit for removal of intact cells *(Microcystis aeruginosa* as single cells, spiral filaments of *Anabaena flos-aquae* and straight filaments of *Planktothrix* (syn. *Oscillatoria) agardhii).* Treatment in the jug units is based on activated carbon and ion exchange resins. Whereas approximately 60 per cent of the filamentous cyanobacteria were removed, 90 per cent of the single cells of *Microcystis* passed through the filter (removal of large *Microcystis* colonies was not tested but may be more effective). Cell morphology was thus considered crucial for elimination performance. Removal of microcystin variants ranged from 32 to 57 per cent (using new cartridges) and could be augmented to a cumulative removal of 88 per cent by three repeated passages of the same water through the filter. On filter cartridges which have reached the half-life recommended by the manufacturer, performance for extracellular microcystin-LR dropped for two of the three brands tested, in one case down to 15 per cent elimination. The study draws attention to the possibility of lysis of cells retained on the filter. It also highlights the need for further development of domestic jug filters if they are to be suitable for microcystin removal. In addition, the study emphasises the need for evaluation of performance of treatment processes in specific situations, particularly if scaled down for domestic use.

Other approaches for individual households and small communities involve methods of filtration, activated carbon and oxidation. As for large-scale plants, slow sand filtration will be effective in removal of cells, and will probably contribute to removal of dissolved cyanotoxins. Rapid blocking can be avoided by pre-treatment to control turbidity or by management of flow regime. Bankside filtration could also be effective and applicable to small community supplies. Addition of chlorine to filtered water at a dose high enough to oxidise microcystins has already been discussed with respect to the benefits for microcystin removal in relation to the problems of by-product production at high DOC levels.

Household treatment approaches have the problem of assessment of performance and quality control. Furthermore, they may enhance social differences, if they are available only to those who can afford them rather than providing "health for all".

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Chapter 10. DESIGN OF MONITORING PROGRAMMES

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Cyanobacterial blooms and cyanotoxins present a special challenge to monitoring programmes because the requirements are different from well-recognised monitoring designs both for pathogenic bacteria and for toxic chemicals. Pathogen concentrations are highest close to sewage outfalls or the inflow of agricultural runoff polluted by livestock faeces and they are diluted with increasing distance from such sources. In contrast, cyanobacteria multiply in the open water environment and scum-forming species are often dramatically concentrated by wind action. Furthermore, formation and dispersion of scums may change within days or even hours, making assessment of the associated hazard difficult. Toxic chemicals are dissolved in the water or bound to sediments. In contrast, at least while the producer cells remain intact, cyanotoxins are chiefly contained within the cells. Therefore, they may shift position in the water body with the cells and accumulate to hazardous concentrations. Consequently, monitoring strategies must encompass cell-bound toxins in addition to extracellular toxin pools.

Cyanobacterial distributions and their changes in space and time depend on the morphological, hydrological, meteorological and geographic characteristics of a given water body. Because the distribution of cyanobacteria is central to hazard assessment, the design of monitoring programmes should be specifically tailored for each water body to optimise the relation of information output to work input. Monitoring approaches also need to be more flexible than for many other parameters. Local knowledge of bloom history and a good understanding of the local growth conditions for cyanobacteria will greatly enhance the capacity to anticipate bloom formation. As knowledge and understanding of a given water body accumulate, regular patterns of Cyanobacterial growth may be noticed, so that in the long-term, monitoring may be focused upon critical periods and locations. To ensure efficiency of Cyanobacterial monitoring programmes, they should be reviewed regularly to provide the most cost effective use of resources and in order to continue to satisfy the primary needs for which they were established. Rapid evaluation and interpretation of results is important in order to achieve feedback into ongoing programmes and their adaptation to current needs.

Analytical quality assurance, as well as data analysis, interpretation and presentation are important aspects of monitoring programmes. These topics are covered in two of the companion volumes in this series: *Water Quality Assessments* (Chapman, 1996) and *Water Quality Monitoring* (Bartram and Ballance, 1996).

10.1 Approaches to monitoring programme development

10.1.1 Objectives of monitoring programmes

The objectives of a monitoring programme determine the approach, the design and the resources required. The aims and further applications of monitoring programmes focused on cyanobacterial populations and toxins may include, for example:

- Assessment of health hazards caused by cyanobacteria and their toxins.

- Identification of contaminated areas (e.g. in relation to drinking water intakes and recreational sites).

- Development of regulations concerning the development and use of recreational sites.

- Public education and information.

- Assessment of the causes of cyanobacterial problems (nutrient concentrations and other limnological data for understanding cyanobacterial growth).

- Development of a nutrient pollution control programme.

- Checking whether compliance with cyanobacterial cell (or biomass) and toxin level standards for the respective water use is being achieved.

- Prediction of levels and changes in cyanobacterial populations and toxins resulting from natural phenomena and human influence.

- Information of the effect of interventions, including lake and reservoir management and water treatment methods, on cyanobacterial cell and toxin levels.

- Wider contribution to the knowledge of cyanobacterial ecology, hydrobiology and the state of the environment.

The approach to monitoring programme development will differ for each of these aims. These examples would each require a programme with combinations of monitoring for cyanobacterial cells, cyanotoxins and growth conditions. Frequently, cyanobacterial monitoring will be connected to, or included in, other general purpose water quality monitoring programmes.

10.1.2 Monitoring strategies

Monitoring water bodies can be facilitated by using a structured approach which may significantly enhance efficiency of laboratory resource use, especially where resources are limited (see Figure 10.1). Because many commonly occurring cyanobacteria are more often toxic than non-toxic, the simplest approach is to assume toxicity and to monitor cyanobacteria rather than their toxins in the water body. Such an approach begins with simple visual inspection. If this indicates a possible cyanobacterial problem,

the approach moves on to assessing which level of cyanobacterial development can be sustained by the nutrient concentrations available (i.e. the carrying capacity, which is most frequently determined by total phosphorus, see section 8.1.) If nutrient concentrations are high enough for cyanobacterial proliferation to be likely, cyanobacteria must be monitored at time intervals adequate to identify hazards (see section 7.5 for time intervals of monitoring).

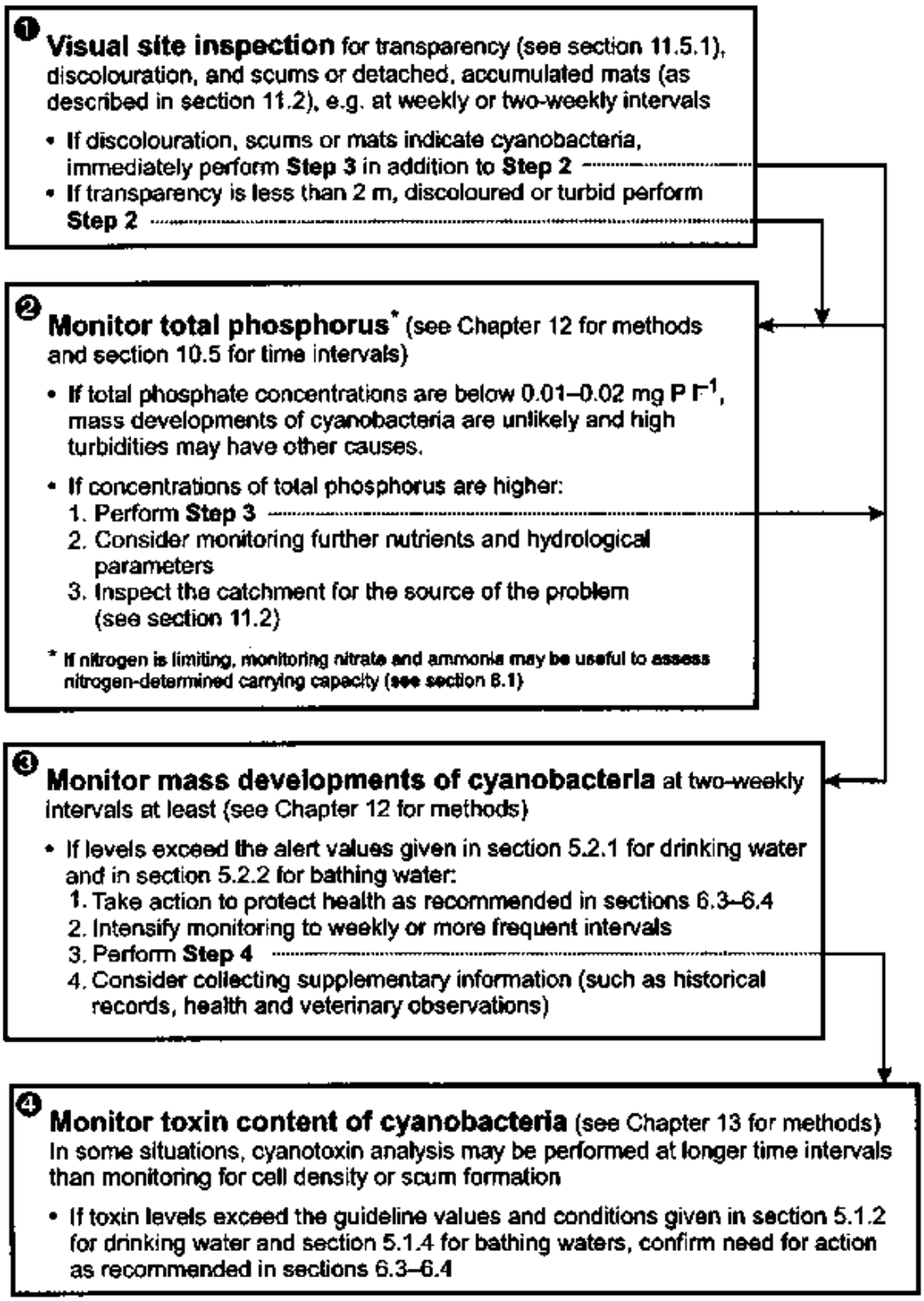
Monitoring may be supplemented with valuable information by collection of historical data on bloom occurrence and of health information, including veterinary records of animal poisonings (see Chapter 6).

10.1.3 Variable selection

Easy-to-assess visual indicators (Step 1 in Figure 10.1) may provide valuable information concerning cyanobacterial proliferation at low cost and often enable a high frequency of observation, especially if they are periodically supported by microscopy in order to ascertain that observed phenomena are due to cyanobacteria. If performed by local staff with alert and flexible observation skills as well as increasing experience, regular site inspection (including monitoring and recording of transparency, discolouration and scum formation) can provide much information in relation to the effort required. Including an assessment of land-based pollution in critical areas of the catchment may also provide information on nutrient pollution sources.

Monitoring of variables which enhance cyanobacterial growth and/or accumulation is valuable in recognising which water resources are at risk of bloom development and scum formation. In many regions, this may also assist substantially in ruling out resources unlikely to sustain major cyanobacterial populations. Monitoring of total phosphorus as a key factor for mass developments may be particularly relevant for setting priorities in monitoring recreational waters where short-lived minor surface scums are less of a problem. Total phosphorus data can further provide the basis for planning and assessing the success of measures to counter the causes of the problem (see Chapter 8). The collection of further environmental data on hydrological conditions (such as retention times and thermal stratification), light availability (as assessed by the relation of depth of light penetration to mixed depth, see section 2.2) as well as dissolved nitrogen (nitrate and ammonia) provides a basis for understanding why certain cyanobacterial genera or species dominate.

Figure 10.1 Example of structured, quantitative investigation approach to monitoring and surveillance for toxic cyanobacteria and their growth potential



Box 10.1 Limitations of assessing toxin risk from monitoring cyanobacterial populations

Although cyanobacterial identification and quantification provide a basis for estimating the toxic risk, the monitoring of cyanobacterial populations alone cannot be used as a surrogate for monitoring cyanobacterial toxins because:

- Toxin types and levels per unit cyanobacterial biomass can vary widely, ranging from undetectable to present at acutely toxic levels.

- Toxin levels per cell vary widely between individual strains and blooms of the same species.

- Individual cyanobacterial strains of the same species can contain more than one type of toxin.

- The toxins can persist in water bodies and water treatment plants in extracellular (soluble) form after release from the producer-cells upon cell lysis caused by biological, physical or chemical agents.

It thus follows that cyanobacterial toxins can be present in a waterbody or treatment facility in the absence of intact cyanobacterial cells or cell debris if the waterbody had recently contained cyanobacterial cells.

Monitoring cyanobacterial taxa and population densities (cell numbers or biomass) can provide an excellent basis for assessing risk, particularly if supported periodically with toxicity tests or toxin analysis. If data on toxin content of prevalent cyanobacteria are available for specific water bodies or regions, and have been found to remain fairly constant for the predominant taxa, a tentative prediction of toxin risks can be inferred from quantitative assessments of cyanobacterial taxa present using samples taken between the occasions of toxin analysis (see Box 10.1 for the limitations of this approach). Microscopy may also be used for monitoring finished drinking water prior to distribution, as well as different treatment steps, for breakthrough of potentially toxic cyanobacterial cells. The necessary laboratory equipment for monitoring cyanobacteria is limited to a microscope and some accessories (see Chapter 12). The training requirements for staff are lower than is frequently assumed (see Box 10.2).

In situations where the phytoplankton is largely dominated by cyanobacteria (e.g. they constitute more than half of the biomass seen through a microscope), measurement of the concentration of chlorophyll *a* can be used as an estimate of cyanobacteria present (see Chapter 12). A simple photometer is adequate for this approach.

Monitoring for cyanobacterial taxa, cell numbers and/or biomass may provide data which are of value for a variety of assessments, including:

- The occurrence, types, distribution and abundance of cyanobacteria, including potential toxin-forming types, in natural and controlled water bodies, water treatment and distribution systems and in potable water supplies.

• Spatial and temporal changes in cell populations, their composition, abundance and integrity.

• Relations between cyanobacterial populations and the types and levels of cyanobacterial toxins (if accompanied by toxin analysis), and associated water quality problems and health incidents.

• Warning systems to trigger contingency action plans in the event of cyanobacterial mass development in waters required for human or animal use.

• Responses of water bodies and water supplies to eutrophication control and strategies to destroy and/or remove cyanobacterial cells in water treatment.

Box 10.2 Adequate training for identifying important cyanobacterial taxa

A common misunderstanding is the assumption that the sophisticated taxonomic training, on a level adequate for ecological research, is necessary for practical monitoring of cyanobacterial hazards. This may be intimidating for beginners and practitioners. In practice, it may only be necessary to focus training on determining the taxa relevant in the region or waterbody to be monitored, frequently only to the level of genera (e.g. *Microcystis),* and down to the level of species only if these are easy to identify and if species differentiation is particularly important for indication of toxin content (e.g. *Planktothrix agardhii* and *Planktothrix rubescens).* Basic identification of cyanobacteria by local personnel should be supplemented by periodic quality control by experts (see the Alert Levels Framework Level 2 in section 6.3.3) to ensure adequate recognition of the important groups, especially after conditions in a water resource have been changed and other taxa may have proliferated.

Monitoring cyanotoxin concentrations and/or assessment of toxicity may be warranted to characterise the hazard presented by a given cyanobacterial population. The role of such monitoring is different in hazard assessment for drinking water and for recreational water use.

The increasing evidence of health outcomes due to unknown irritative cyanobacterial components may discourage the use of recreational sites with high cyanobacterial population densities for water contact-intensive activities, regardless of the concentrations of known toxins (e.g. microcystins, neurotoxins, cylindrospermopsin). Analysis of these toxins will not characterise the hazard of irritative effects (see section 5.2.2). However, monitoring of microcystins or other known toxins can assess whether specific blooms present hazards from the cyanotoxins which are of greater concern for public health. In particular, it may identify some blooms as not presenting high risk levels. This information may be particularly relevant for heavily used sites, such as those associated with holiday facilities and for which temporary closure might have a substantial economic and social impact.

Monitoring of recreational sites should emphasise bloom prevalence and the potential of bloom formation. Step 1 of the structured approach presented in Figure 10.1 is particularly important here. This approach can easily be communicated to the general public and to bathing site users, and can involve them in assisting with hazard assessment (in addition to providing a basis for their own decision-making on water

contact activities) by encouraging reporting of scums or strong discolouration and turbidity to authorities.

Monitoring toxin concentrations is especially important in drinking water supply systems in order to detect toxins released from the cells into the water during treatment and to determine the level of risk associated with a specific bloom in the water resource. Monitoring for cyanobacterial toxins is necessary to provide data:

- On the occurrence, types, abundance and distribution of cyanobacterial toxins in aquatic environments, water treatment and distribution systems, potable supplies and food products which contain, or have been exposed to, cyanobacteria and their toxins.

- On relations between environmental conditions, cyanobacterial populations and cyanobacterial toxins.

- For use in alert levels schemes and in the activation of contingency action plans.

- On relations between cyanobacterial toxins and water quality and health, with reference to human and animal exposure levels and health effects.

- For the derivation of standards for drinking water quality, to enable compliance with these values to be achieved and to determine, in the longer-term, whether such standards remain appropriate or need to be changed.

10.2 Laboratory capacities and staff training

Monitoring for cyanobacterial health hazards makes a range of demands upon analytical resources, some of which are different from those required by water quality monitoring for other types of variables. An overview of the requirements for the monitoring approaches discussed in section 10.1 and their respective information return is given in Table 10.1. Proper interpretation of information concerning cyanobacteria and their toxins requires expertise from the health and water resource sectors. However, these areas of expertise occur within a single authority in a few countries. Multisectoral co-operation is therefore important. Planning of monitoring programmes should generally involve co-operation between the environmental and the health sectors, bringing in further agencies or organisations where appropriate (e.g. drinking-water suppliers and authorities responsible for tourism, for public education or for water management).

Table 10.1 Approaches to monitoring for cyanobacteria and analysis for cyanotoxins: requirements and options for their organisation

Monitoring type	Parameters/variables	Demands on equipment and skills	Who	Where
Basic		Minimal		
Site inspection for indicators of toxic cyanobacteria in waterbody	Transparency, discolouration, scum formation, detached mat accumulation	Secchi disc, regular site inspection by trained staff; skill requirement basic, training easily provided	Environmental or health officers, trained health staff or supervised local	Local
Background		Low to moderate		
Potential for cyanotoxin problems in waterbody	Total phosphorus, nitrate and ammonia, flow regime, thermal stratification, transparency	Photometer, boat, depth sampler, Secchi disc, submersible temperature/oxygen probe; skills basic but require specific training and supervision	Environmental officers or experts with limnological expertise	Local, regional
Cyanobacteria		Low to moderate		
In waterbody and drinking water	Dominant taxa (quantity): often determination to genus level only is sufficiently precise; quantification only as precise as needed for management	Microscope, photometer is useful; specific training and supervision is required, but quite easily achieved	Environmental or health officers (with occasional quality control by experts); consultants with limnological expertise	Local, regional
Toxicity assessment		Moderate		
In waterbody and drinking water	Toxicity	Demands on equipment are low, but rather high on skills	Toxicologists	Central
Toxin concentration		Moderate to high		
In waterbody and drinking water	Toxin concentration	New methods with lower financial demands presently in development for some cyanotoxins (e.g. immuno-assay); skill requirements vary widely from moderate to very high	Skilled analysts	Central

Monitoring for visual indicators of cyanobacteria focuses on critical site inspection and requires almost no facilities. Training of staff is necessary, but not difficult and experience leads to improved performance. However, much time in the field is required and this can be reduced substantially by involving local people who have been given

specific training for visual inspection. Nevertheless, professional staff should exercise periodic quality control over their work.

Environmental monitoring of chemical and physical variables indicating bloom-forming potential, such as nutrient concentrations, hydrophysical conditions and transparency, make limited demands upon analytical resources (a submersible temperature and oxygen probe and a photometer with optical filters to provide the necessary wavelengths are the most complicated instruments required). Such analytical capacities may be readily decentralised. While laboratory analysis can be carried out by any capable chemical laboratory, some limnological expertise is necessary for the planning of field work, quality control of data and interpretation of results. The staff time required can be reduced once seasonal patterns of variation are known and sampling regimes can be adjusted to be most effective.

Health authority staff with experience in microscopy can learn to recognise the most important toxin-producing cyanobacteria in the water bodies under their responsibility, if training by experts can be provided (attention may need to be given to dampening the general taxonomic enthusiasm of some experts, in order to concentrate on the skills really needed for monitoring the cyanobacterial taxa relevant for the water resources in question, see Box 10.2). Cyanobacterial identification and quantification can be centralised or subcontracted because preserved samples can readily be transported (see Chapter 11). However, the development of local skills is recommended because this should enable more rapid identification of, and response to, current cyanobacterial problems.

Cyanotoxin analysis with customised immuno- or enzyme assays, or toxicity tests with simple bioassays (see Chapter 13), may make only moderate demands on equipment and can be performed potentially by local health or environmental authorities. However, these techniques require specific staff training and periodic quality control by comparing results with those of more elaborate methods. More advanced programmes addressing toxicity assessment and toxin analysis require developed analytical capacities and exacting quality control. Even in countries with extensive advanced analytical facilities it is unlikely that the demand for toxin analysis would justify establishment of widespread or local facilities and some form of co-operation on a broader scale of centralisation is therefore advisable. Methods for cyanobacterial quantification and toxin analysis should be standardised and a system of official accreditation for analytical laboratories should be implemented.

Several options may be available for conducting analyses of water samples. The agency responsible for the monitoring programme may have its own laboratory or laboratories, the facilities of another agency or of a government ministry may be available, or some or all of the analytical work may be done under contract by a private laboratory. Some analytical work may be done in the field using either field kits or a mobile laboratory. Regardless of the options chosen, the analytical services must be adequate for the volume of work expected. Furthermore, good communication between those planning and performing field work and the analytical laboratory is crucial for ensuring appropriate sample collection, preservation and transportation (see Chapter 11). Periodic quality control is highly recommended particularly with respect to the handling of samples from field to final analysis.

10.3 Reactive versus programmed monitoring strategies

Monitoring strategies can be regarded as either reactive or programmed, although these are not necessarily mutually exclusive. A reactive strategy is needed when an unexpected cyanobacterial bloom develops and affects, or has the potential to affect, water supplies, recreational water and human health. It can be triggered by an unanticipated bloom event, by health impairments reported to authorities and related to cyanobacterial proliferation, by results of routine visual site inspection, or if routine analysis in drinking water treatment facilities detects cyanobacteria or toxins in the raw water intake or in recreational areas. This response strategy can include a range of *ad hoc* assessments of cyanobacterial numbers, toxicity assessment and toxin analyses. Programmed monitoring strategies are being applied increasingly to the investigation of cyanobacterial population and toxin problems where cyanobacterial problems are ongoing, occur regularly, are anticipated or have occurred in the past. These structured programmes can provide additional preventative benefits by warning of necessary actions before a developing cyanobacterial population presents an operational, environmental, or health problem, or by triggering the implementation of preventive measures at the source of the problem (see Chapter 8).

The benefit and information obtained from reactive as well as from programmed monitoring strategies can be greatly enhanced by ensuring that samples of cyanobacteria or water from natural or controlled environments are supplemented by clinical observations and clinical samples in the event of associated human and animal health incidents. This can be assisted by heightening the awareness of medical practitioners, public health authorities and veterinarians through training and information programmes (see section 7.4).

The potential for community involvement in monitoring strategies is high, provided that adequate information is supplied using leaflets, publicity and educational campaigns (section 7.4.2). "Algae Watch" programmes, or "scum scouting" to report on the appearance of blooms and scums. Schools programmes and water sports associations in Australia, the USA and the UK have provided useful information to monitoring agencies, as well as having helped to promote community action and joint responsibility for the causes and cures of cyanobacterial bloom problems.

Programmed monitoring strategies have the potential to detect and anticipate changes in cyanobacterial populations and potential levels of toxins. Such strategies can thus provide information to trigger appropriate contingency plans. Alert Levels Framework (ALF) systems are finding application in some countries; these are systems of programmed monitoring which incorporate action sequences in the event of warning thresholds being exceeded. Alert Levels Frameworks may be used for the monitoring of cyanobacterial populations only, or cyanobacterial populations plus toxins, depending on monitoring objectives and resources (see section 6.3).

10.4 Sample site selection

The selection of sampling sites is a key factor in determining the value of the data to be sought from the subsequent sample examination and analysis procedures. Sample site selection should be tailored to meet the overall aims and objectives of the monitoring

programme (or even a single sampling visit). Thus site selection must consider and take account of the following:

- The uses made of the water body must be considered (e.g. potable supply, recreation, animal watering). For recreational use, sampling will include shoreline areas particularly frequented by visitors and may focus on public bathing sites. It may also include offshore sites where immersion sports take place. For drinking water resources, sites at or close to the raw water intake are important, and sampling within a treatment plant might include sampling at different treatment steps.

- If sampling aims at assessing the total population size of the cyanobacteria and their scum-forming potential, or the nutrient concentrations which influence the maximum possible population size, it should cover a central reference site in open, mixed water (experience may indicate if this can be used as a representative site for the main water mass). Selection of adequate depths must consider stratification of organisms and nutrients using depth-differentiating or depth-integrating sampling techniques as described in Chapter 11.

- Morphometric and hydrophysical characteristics of the water body (e.g. exposure to wind or thermal stratification) may help identify sites which are prone to scum accumulation. These factors are likely to influence the development and fate of cyanobacterial populations and their subsequent location in parts of the water body.

- Current weather conditions, particularly wind direction, which lead to scum accumulation along certain shorelines may require flexible choices of sites, particularly if the aim is assessing the highest cyanotoxin concentrations by sampling maximum scum densities.

- Specific incidents, such as animal deaths or human illness, if these are suspected to be associated with exposure to cyanobacteria and toxins at a specific location in the water body concerned.

- The history, if available, of cyanobacterial population development and occurrence of toxins in the water body, because this information may indicate sites particularly likely to harbour scums.

- Local logistical resources, accessibility and safety factors (e.g. Secchi transparency should be measured from a pier or boat, offshore sampling requires access to a boat and to a site for launching it, and sampling from steep shores or reservoir dams might be dangerous).

- Potential local sources of nutrient pollution (e.g. inlets or slopes affected by erosion).

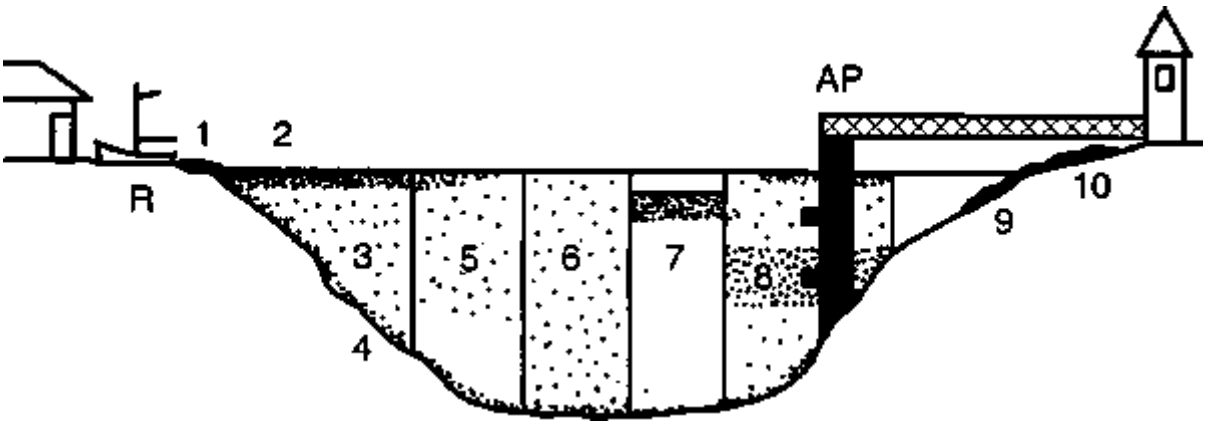
For adequate sampling site selection it is of critical importance to consider the location and potential concentration of cyanobacteria in a water body, as described in Chapter 2 and in section 11.3.2. This site selection should account for accumulations of cyanobacteria as scums (usually quite unevenly distributed over the area of a water body), as subsurface maxima at some metres depth, as homogeneous distributions throughout the mixed strata of the water body, or growing on the sediment surface (from

which they may become detached and driven onshore to present acutely toxic accumulations). Possible scenarios are shown in Figure 10.2.

The heterogeneous and dynamic nature of many cyanobacterial populations presents difficult problems for sample site selection. A flexible response to the current situation when choosing the sampling sites may, at times, be more appropriate than following a rigid programme. Alternatively, fixed sites always sampled within a broader monitoring programme may be supplemented with the sampling of sites currently harbouring cyanobacterial scums.

The horizontal and vertical heterogeneities in cell distribution are compounded by further variability in cyanobacterial toxin levels and distribution. Although the toxins are largely retained within the producer-cells during growth, they are released into the water during cell lysis due to natural agents, some algicides and pressure-induced disruption. Circumstances therefore arise where cyanobacterial toxins may be present in the absence of intact cyanobacterial cells. Sampling site selection for dissolved toxins may thus include locations such as the water close to decaying scums and in water treatment works and distribution systems, when it is suspected that cyanobacterial breakdown may have occurred.

Figure 10.2 Some locations of cyanobacteria in thermally stratified lakes or reservoirs. R, recreational area; AP, water abstraction point; 1, shoreline scum of planktonic cyanobacteria (often decaying); 2, planktonic cyanobacterial scum on open water during calm conditions; 3, dispersed cyanobacteria in epilimnion; 4, planktonic cyanobacteria on sediment; 5, upper mixed layer during autumn overturn; 6, spring and autumn conditions of complete mixing and in summer in shallow lakes in windy conditions; 7, scum under ice; 8, subsurface maximum of planktonic cyanobacteria (not apparent at surface); 9, mats of benthic cyanobacteria on sediment in shallow water; 10, shoreline accumulation of detached benthic cyanobacteria (Modified from Lindholm *et al.,* 1989 with additions)



10.5 Monitoring frequency

Cyanobacteria generally have fairly slow growth rates compared with many other micro-organisms. This helps to simplify monitoring frequency requirements. For those taxa which do not form scums but are dispersed in the water, weekly or even two-weekly

monitoring intervals are often sufficient, even during their growing season, in order to monitor population development and to assess the cyanotoxin hazard. However, the ability of scum-forming cyanobacteria to change their concentration and position in the water body within very short time spans of only a few hours poses a specific challenge to the design of monitoring programmes.

Monitoring frequencies for cyanobacteria and cyanotoxins are suggested in the Alert Levels Framework given in Chapter 6. For example, monitoring may begin on a fortnightly or weekly basis and be increased to twice-weekly, or to daily, whilst alert levels are exceeded, and then be reduced again after values decline below alert levels and guideline values for cyanobacterial cells and toxins. The same principles should be applied to the monitoring of recreational waters, whether they are used on a year-round or seasonal basis (section 5.2.2). If a water body prone to cyanobacterial mass developments is used for water-contact sports on a seasonal basis, or for a single event, monitoring should begin not less than two weeks before the start of the season or the event. As monitoring is continued, frequency may be adjusted to enable decisions to be made on access to the facility throughout the season, or on whether to proceed with a special event.

Structured approaches to monitoring (e.g. Figure 10.1) introduce nutrients as a further variable for analysis. This may improve the information return for effort expended, particularly if each step in the structure is monitored at an appropriate frequency. Patterns of investigation may begin with longer time intervals and may be intensified as cyanobacteria begin to proliferate. As knowledge and understanding of a given ecosystem and the behaviour of its cyanobacterial populations accumulates, monitoring frequencies can be optimised to meet the demands of the specific situation. For this reason involving limnological expertise is particularly important in the planning of monitoring programmes, in the evaluation of the data, and in periodic reassessment of the adequacy of ongoing programmes. The following monitoring frequencies are suggested for the structured approach given in Figure 10.1:

- Visual site inspection may begin at weekly or two-weekly intervals, which can be increased to weekly or even more frequent intervals once cyanobacteria begin to proliferate.

- Assessment of the carrying capacity for cyanobacteria in terms of nutrients (phosphate and in nitrogen-limited systems, possibly also dissolved inorganic nitrogen) may be undertaken less frequently in many situations. This depends on prevalent nutrient levels and their rate of change. For example, in some water bodies, total phosphorus concentrations may show little seasonal change, or they may always be far too high to limit cyanobacterial biomass. In either case, occasional monitoring (in temperate climates once in spring and once in summer) may be sufficient. On the contrary, if the hydrological regime shows pronounced fluctuations, or if the total phosphorus concentration oscillates around levels critical for limiting cyanobacterial biomass (0.03-0.05 mg l^{-1} P), monthly or biweekly measurements may be necessary in order to assess the carrying capacity for cyanobacteria. Often it will be advisable to begin a programme with monthly sampling for one or two years. Evaluation of patterns may then enable a reduction of sampling frequency to be justified.

• Assessment of cyanobacterial cell numbers or biomass can be affected by the rapid changes discussed above, particularly if scum-forming taxa prevail. Knowledge of a water body's carrying capacity for cyanobacteria, of the taxa typically occurring, and of seasonal time patterns of their occurrence will help anticipate critical situations which require increased monitoring frequency. If the aim of monitoring is to check compliance with standards for drinking water or recreational use, and toxin levels in a given water body are in the borderline range or above, sampling and analysis may be necessary several times a week. Time patterns of water body use, particularly for recreation, may be a useful further criterion for determining the time patterns for sampling.

• Toxin analysis may be necessary less frequently than assessment of cyanobacterial cell numbers or biomass. Although toxicity of populations is variable, it does not appear to change within a few days. Assessment of toxicity is particularly recommended when situations in the water body change, e.g. when other taxa appear or if bloom lysis occurs.

Ideally, "real-time" information on the state of the cyanobacterial population and their toxins is desirable for scum-forming taxa. Some approaches to meeting this demand are currently being developed. The simplest approach is semiquantitative assessment of cyanobacterial cell numbers through a microscope. This can be performed within an hour or less of sampling, provided a microscope is available locally, and can be repeated frequently during problem phases provided the laboratory is close to the water body (which is often the case at drinking water supply reservoirs). Continuous automatic provision of fluorescence data indicating pigment concentrations has become possible by means of submersible flow-cell fluorescence spectrophotometers or by continuous water flow through laboratory fluorimeters and cytometers. These procedures are currently beginning to differentiate successfully between cyanobacteria and other components of the phytoplankton. Installation of such devices may be especially attractive for drinking water supply abstraction points, in order to adapt offtake levels to the current location of cyanobacteria (if offtake depths are flexible), or in order to have an immediate indication of the need to apply further treatment steps. Another approach currently investigated for acquisition of real-time data for chlorophyll distribution and levels, and potentially for cyanobacterial phycobiliprotein pigments in freshwaters, is remote sensing of the optical properties of the water body by high resolution airborne scanners (Cracknell *et al.,* 1990; Jupp *et al.,* 1994). However, flight times may be infrequent and data collection depends on factors beyond human control, e.g. cloud cover. Nevertheless, the remote sensing of cyanobacterial populations as a contribution to water body management has excellent potential particularly for the monitoring of scum locations. Remote sensing may become cost-effective for areas that have to monitor many recreational sites.

Further aspects to consider in determining monitoring programme frequencies are, as with site selection (section 10.4), the monitoring programme objectives, water-use, specific incidents associated with exposure to cyanobacteria and their toxins, historical evidence of blooms in the water body, local water body and catchment characteristics, and the wider knowledge of cyanobacterial ecology. Available resources, sampling and analytical logistics also need to be taken into account, for example, the time needed for sample transportation, processing and analysis, and for the interpretation and reporting of results.

Monitoring intervals should be timed to provide information for the following situations:

- To give warnings of developing cyanobacterial populations and toxin levels.

- On the duration of cyanobacterial populations and toxin levels which exceed guideline values.

- On the decline of cyanobacterial populations and toxins due to natural processes or the persistence or reduction in cyanobacterial populations and toxin levels due to intervention, such as eutrophication control and water treatment.

In subtropical and tropical latitudes where appreciable cyanobacterial populations can occur all year round, it may not be feasible or necessary to maintain high frequency sampling programmes throughout the year, particularly if the population density is subject to less change because there is little seasonal change in growth conditions. In this event, sampling of the natural or untreated water may need to be at less frequent intervals, e.g. monthly, with laboratory resources being directed towards higher frequency monitoring of treated drinking water.

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Chapter 11. FIELDWORK: SITE INSPECTION AND SAMPLING

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Fieldwork, including site inspection, sample collection and, in most programmes, some on-site analysis, determines to a large extent the quality of information obtained and represents a significant proportion of the total cost of a cyanobacteria or cyanotoxin monitoring programme. A well-designed and implemented fieldwork programme enhances the quality of the data obtained and may assist greatly in containing overall costs. The importance of careful programme design has been emphasised in Chapter 10, together with the need for pilot testing to refine programmes and to assess logistics.

Properly trained field workers are the backbone of effective sampling and inspection programmes; aspects of their professional development and training are described in Chapter 7. Inclusion of members of the public as active participants in monitoring programmes is rapidly gaining acceptance and can contribute significantly to the quantity and quality of information obtained from a monitoring programme. Special interest groups (such as non-governmental organisations and user associations) and also concerned local persons in sensitive or affected areas can provide useful information. Proper orientation and training, for example in site inspection, in flexible responses to the results of inspections, and in conducting basic tests such as transparency, can assist in providing valuable additional data.

Further information concerning planning and performing fieldwork is presented in *Water Quality Monitoring* (Bartram and Ballance, 1996), a companion volume in this series.

11.1 Planning for fieldwork

The principal components of effective planning for fieldwork comprise:

- Timing and preparation (in the context of the monitoring programme plan and information needs for management).

- Prior liaison with the laboratory that will receive and process samples.

- Logistic preparation.

- Local co-ordination as required.

- Prior liaison with other information recipients (local or central).

Preparation of the monitoring programme principally concerns the timing of visits and careful cataloguing of sampling sites. The timing of sampling and inspection visits is described in Chapter 6.

Before routine field visits are performed, a period of pilot testing should be implemented. This will help ensure that time requirements for inspection and sampling are reconciled, and that activities are planned to make the best use of staff time and other resources (e.g. vehicles). Realistic estimation of travelling time is important to avoid exceeding allowable sample storage times prior to analysis. Pilot testing should lead to the development of a detailed inventory and description of sampling sites. If changes in water quality with time are to be interpreted with confidence, samples must be taken consistently from the same locations and/or from precisely identified locations. Pilot testing also provides an opportunity for training personnel and allows their familiarisation with particular aspects of the monitoring programme itself.

Co-ordination with the laboratory is an important aspect in determining the final value of the sampling expedition. In some cases, the laboratory will be responsible for preparation of sample containers and chemical additives for sample preservation, and may also be responsible for the provision and maintenance of equipment for on-site testing.

Laboratory capacity is an important area of concern which should be addressed in programme design and in pilot testing. It is essential that the workload generated by a sampling expedition is properly managed within the laboratory; it is therefore vital that analysts know how many samples will be arriving, the approximate time of arrival and the analyses that are to be carried out. Excessive delays before sample processing and analysis may render the sample results invalid (and thereby useless) for the management purposes for which they had been collected. Therefore, the timing of sample delivery to the laboratory and the workload management within the laboratory should be co-ordinated prior to fieldwork.

Good logistical preparation prior to fieldwork requires that equipment is checked to ensure that it is functioning properly (e.g. electrodes tested and calibrated, batteries charged). The correct number, size and type of sample containers must be prepared and transport must be arranged, ensuring permission and local access to any restricted sites. It is essential to prepare a sampling checklist which includes maps of sampling site locations, a list of equipment required and a detailed explanation of the methods for sample collection. The checklist should also include lists of the types and numbers of samples to be taken at each site, as well as of the required volumes. Good preparation for sampling involves previous labelling of sampling containers with at least the site, date and depth from which the sample is to be taken.

11.1.2 Frequency of field visits

The frequency of site inspection and sampling must be adapted to the local situation as described in Chapter 10. Key criteria are:

• Potable water supply reservoirs may need to be monitored regularly throughout the year if perennial persistence of cyanobacteria cannot be ruled out. This applies particularly to warm climates and in temperate zones to water bodies populated by certain taxa such as *Planktothrix.*

• Monitoring of recreational lakes in tropical countries may cover the whole year, while in temperate zones it can be focused on the warm season from early summer to autumn.

• The frequency of site inspection and sampling should be increased during development of cyanobacterial populations or when persistent blooms occur.

11.1.3 Safety

Caution and attention are appropriate while working with cyanobacteria, particularly when they are highly concentrated in scums. It is wise to treat all blooms as highly toxic. Contact with water should be minimised during sampling and gloves and rubber boots should be worn because cyanobacteria might contain toxins and could also have a high allergenic potential.

In some areas of the world other water-based hazards such as schistosomes (the cause of schistosomiasis or bilharzia) may also be present. In such circumstances water contact should be minimised and following contact the skin should be vigorously dried.

Caution during fieldwork should also apply during the use of boats or other vessels and whilst wading, especially in waters with low transparency where underwater hazards may not be readily visible.

11.2 Site inspection

Sites used for drinking water abstraction or recreation should be subject to programmed inspection by trained professional staff, and preferably in conjunction with sampling expeditions. Careful inspection can assist interpretation of results from sample analysis. Moreover the development of personal expertise in relation to specific water bodies can provide the best form of early warning system.

A protocol for site inspection should be established (see Box 11.1) that includes the ambient data of temperature, wind and other weather conditions as well as an estimate of the situation during the previous 24 hours.

High nutrient input from, for example sewage outlets or run-off from excessively fertilised areas favours the development of cyanobacteria (Chapter 8). Site inspection protocols should also address the identification of sources of nutrient input and significant land uses, as well as land use changes that may assist in the interpretation of findings. Such assessment is not necessary during every sampling tour but is particularly recommended during the pilot phase, and at moderate intervals, such as annually, when changes in the catchment area are suspected.

Box 11.1 Example protocol for site inspection and follow-up

1. Note ambient and weather conditions and, if possible, also those conditions during the past 24 hours, especially wind direction and velocity.

2. Assess the areas most likely to be affected by cyanobacterial blooms first, i.e. the downwind shores.

3. Determine if:

- The bottom of the lake is clearly visible at approximately 30 cm depth along the shore line.

- Note any distinct green or blue-green discolouration of the water; if a Secchi disc is available, note the transparency.

- Note if cyanobacteria can be seen as green or blue-green streaks on the surface, or as accumulations in bays and along shorelines.

- Note whether green or blue-green scums affect large parts of the water surface.

4. If cyanobacteria are present (according to Item 3 above):
- Initiate monitoring, if not already in place.

- Initiate an inspection of the catchment area for sources of nutrient inputs.

- Initiate temporary intensification of monitoring, if necessary, for safeguarding healthy use for drinking water or recreation (see Chapter 10).

5. If heavy blooms or scums are observed, immediately:
- Inform other parties concerned (water suppliers, health authorities, operators of recreational sites).

- Inform public and consider posting warning notices at bathing sites, and intervening against use for water contact sports (see sections 5.2.2 and 6.2.2).

6. If cyanobacteria or dense algal growth is a problem, check whether nutrient pollution sources are apparent, or whether a specific catchment inspection tour should be initiated (see Chapter 8).

When scums appear on the water surface, cyanobacteria may be present in densities hazardous to human health, and thus appropriate responses should be initiated quickly (see sections 11.3 and 6.4) and samples for further analysis should be taken. Sampling of scums outside designated or habitual bathing sites is also of great value for determining and predicting further risk, e.g. for when wind directions change during the following days.

11.3 Sampling

Sampling may address both cyanobacterial population development and hazardous accumulations. Informed on-site decision-making is necessary to refine sampling

programmes. Samples addressing population development as a basis for assessing the potential for hazardous concentrations are usually taken at one or several points in the water body. These points should be representative for the whole water body and are often where it is deepest. For assessing hazards, samples must also be taken in areas where accumulations of cyanobacteria can affect both humans and livestock, or they should be taken at the raw water intake of drinking water reservoirs. The potential for spatial heterogeneity demonstrated by some species (see section 2.2), i.e. horizontal and vertical variations in both cell numbers and toxin content, must also be considered when selecting the number and location of sampling sites.

Sample collection should always be accompanied by a site inspection (see section 11.2) because the data obtained during the inspection will be important in the interpretation of the results of sampling.

Sample collection and storage procedures differ depending on the type of analysis which will be carried out. The three principal categories of analysis usually performed are:

- Nutrient analyses (phosphorus and nitrogen).
- Cyanobacterial identification and quantification.
- Cyanotoxin analysis, e.g. toxicity testing and analysis of cell-bound and dissolved toxin.

11.3.1 Sample containers

Containers and bottles for the transport of samples should ideally be provided by the laboratory that will conduct the analyses. This helps to ensure that they are of a suitable volume, are properly prepared and that due consideration has been given to the need for pre-treatment and chemical addition. Field work is easier if bottles are pre-labelled and well-arranged in suitable containers (in climates where storage in insulated containers is not necessary, soft-drinks crates with subdivisions for each bottle are cheap and practical). For routine sampling of the same sites, it is advisable to always use the same bottle for each site and each parameter. This avoids cross-contamination, which is a particular concern for phosphorus analyses. For most samples, glass bottles are appropriate but often plastic containers can be used that are considerably lighter and unbreakable. It should be decided in advance whether it is more practical to subdivide a water sample into aliquots for each analysis prior to transportation, or whether a single sample can be divided on receipt in the laboratory. The following containers are recommended for the transport of cyanobacteria and related samples:

- *Phosphorus analysis.* Use 100 ml glass bottles pre-washed with and stored containing sulphuric acid (4.5 mol l^{-1}) or hydrochloric acid until usage. Phosphate is indicative of the potential for cyanobacterial growth even when it is at very low concentrations (µg l^{-1}) and, therefore, special care must be taken to avoid contamination of samples. Contamination may arise from phosphate-containing detergents or from previous storage of samples with very high phosphate concentrations. Phosphates are easily adsorbed to glass surfaces and may be released later when the bottle is filled with a new sample with low concentration (see Chapter 12).

- *Nitrate, ammonia and total nitrogen.* Use clean 100 ml glass or polyethylene bottles.

• *Microscopic identification of cyanobacteria.* Wide-mouth polyethylene bottles are appropriate for studying living material in a fresh grab or net sample (see below).

• *Microscopic identification and quantification of cyanobacteria.* Brown glass bottles (100 ml) are preferable. These should already have about 1 ml of Lugol's iodine or formaldehyde solution added, or the preservative may be added immediately after filling the bottles with the sample (see below). Clear glass bottles may be used but must be stored in the dark because sunlight destroys iodine, thereby allowing the sample to degrade.

• *Cell material for analysis of cell-bound toxin concentrations.* Use 1 litre (minimum volume) containers, preferably made of glass although good results have also been obtained with plastic. Containers should be pre-cleaned to prevent contamination.

• *Cell material in large amounts (usually enriched with a plankton net) for structural identification or some toxicity assays.* Use plastic containers with wide necks to facilitate filling. If samples are to be freeze-dried, the sample must be frozen in a layer not thicker than 1-2 cm. Specimen containers (100 ml) for urine samples (easily obtained from medical suppliers) are particularly suitable. Well-sealed, heavy-duty household plastic bags can also be used, but care must be taken to avoid puncture because highly concentrated cyanobacterial material can present a safety hazard. Bags should be placed in watertight ice boxes for cool storage and as a precaution against leakage.

• *Dissolved cyanotoxins.* Use 1 litre (minimum volume) containers, preferably made of glass although good results have also been obtained with plastic. Containers should be pre-cleaned to prevent contamination.

• *Chlorophyll* a *analysis.* Brown glass bottles of 1 litre capacity are recommended to avoid degradation of chlorophyll by sunlight. Clear bottles may be used if the samples can be stored in the dark.

Preparation of Lugol's iodine solution for preserving phytoplankton samples Dissolve 20 g of potassium iodide in 200 ml of distilled water, mix thoroughly and add 10 g of sublimated iodine. The solution must not be supersaturated with iodine because this can result in crystal formation which interferes with cell counting. Supersaturation can be tested by diluting 1 ml of stock solution to 100 ml with distilled water to give concentrations similar to those used for preserving samples. If iodine crystals appear after standing, more potassium iodide (approximately 5 g) should be added and the test repeated. If no crystals appear, 20 ml of glacial acetic acid is added. Use about 1 ml of Lugol's iodine to preserve 100 ml of phytoplankton sample. The resultant sample should be the colour of whisky).

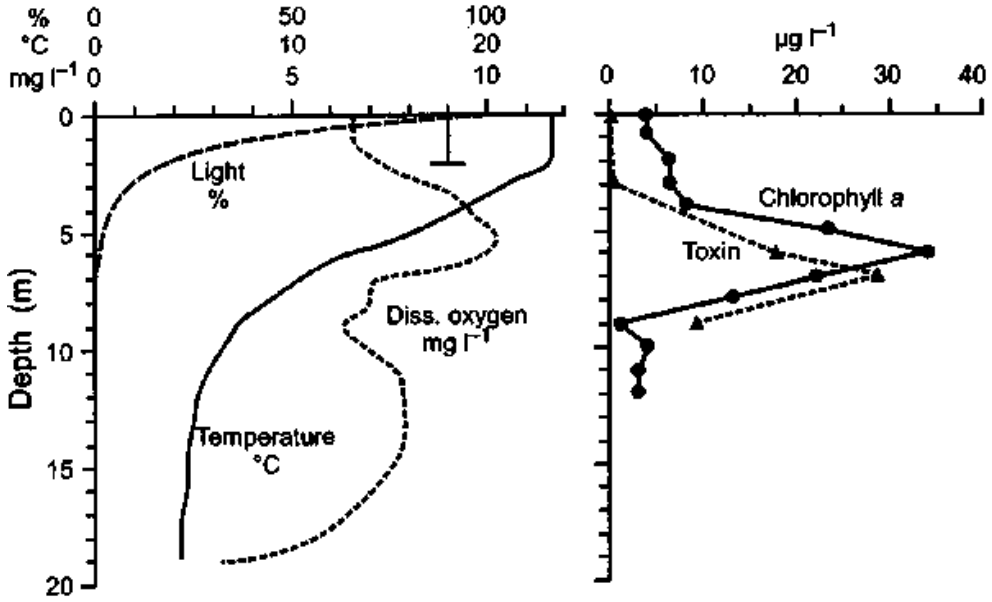
Dense scum samples may rapidly consume the iodine from the Lugol's solution. If samples lose the whisky colour, and particularly if they no longer smell of iodine, they are likely to decay. Samples stored for more than 10 weeks should be periodically checked for sufficient preservation; further drops of Lugol's solution may be added if necessary. If substantially more than 1 ml per 100 ml are required, the volume of Lugol's solution added to the sample should be determined because the sample volume will have changed appreciably and a corrective calculation is required for the cell counts.

11.3.2 Types of sample

Two different types of sample can be taken from lakes or similar surface waters. The simplest, a "grab" sample, is a discrete volume of water taken at a selected location, depth and time. Grab samples are also known as "spot" or "snap" samples (see Bartram and Ballance (1996) for different sampling devices and a description of available methods). In contrast, composite or integrated samples are made up of several sub-samples from different parts of the water body. These are aimed at representative sampling of a water body. Whereas grab samples are suitable for analysing situations at specific sites (e.g. maximum density of cyanobacteria or cyanotoxins at a bathing beach), composite samples are preferable for assessing the water body's total content of a substance (e.g. total phosphorus potentially available for phytoplankton growth) or the population of an organism (e.g. the size of a cyanobacterial population). Composite samples are particularly important if the variables to be assessed are unevenly distributed. If knowledge of the precise distribution is required, each sample can be evaluated individually. However, the integration of samples prior to analysis is often far more cost-effective.

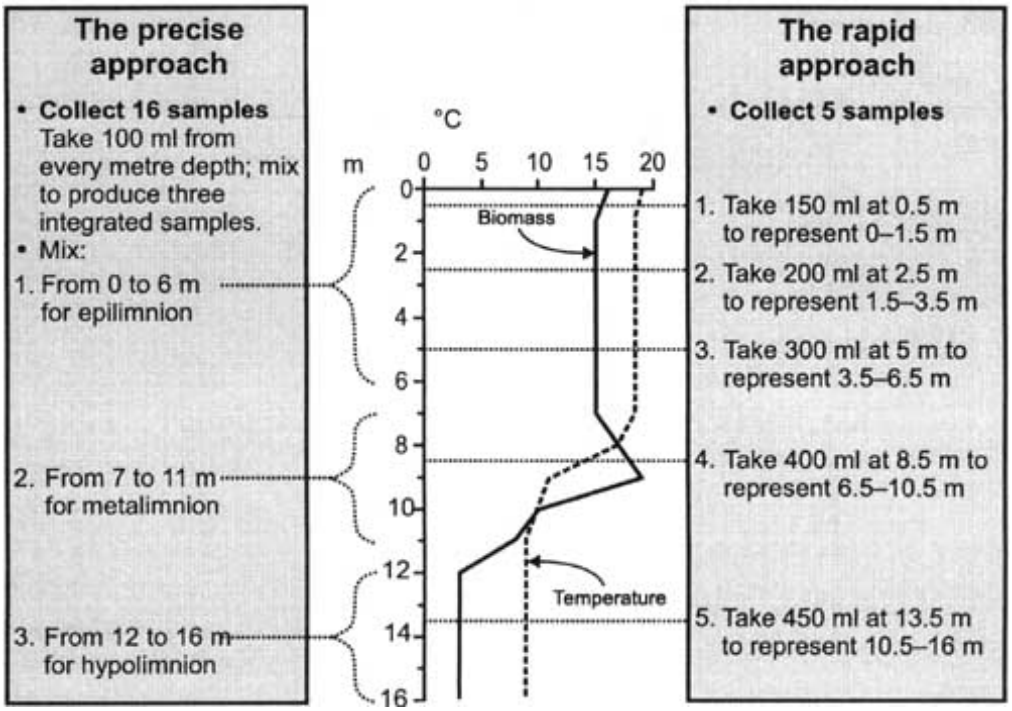
Depth-integrated samples may be obtained either by continuously sampling the total column of water from the surface to just above the sediment, or by discontinuously taking grab samples from representative depths and then mixing them together. The latter is particularly appropriate for deep lakes. However, in order to choose representative depths and to achieve meaningful integration, knowledge of thermal stratification of the water body is necessary.

Figure 11.1 Vertical distribution of chlorophyll a and a toxin (desmethyl-microcystin-RR) from the species *Planktothrix agardhii* in Lake Östra Kyrksundet in relation to hydrophysical conditions on 6 July 1988. Light intensity is given as a percentage of surface intensity (Reproduced from Lindholm and Meriluoto, 1991, *Can. J. Fish. Aquat. Sci.,* 48, with permission)



The thermal stratification of a water body is influenced by the morphology of lakes and reservoirs, the latitude, weather conditions and the physical nature of the water. It can be determined by measuring vertical profiles of temperature within the water body. Where thermal stratification occurs, it results in a water body functioning as two separate masses of water (the epilimnion and the hypolimnion) with different physicochemical characteristics and cyanobacterial populations, and with a transitional layer (metalimnion) sandwiched between (Figures 11.1 and 11.2). In temperate climates, thermal stratification generally occurs seasonally in water bodies of appropriate depth, whereas in tropical climates it often follows diurnal time patterns. Thermal stratification has important implications for the depth at which cyanobacteria are likely to be found (some species may accumulate on the surface, some in the metalimnion, see also section 2.2), as well as for concentrations of nutrients and interpretation of phosphorus and nitrogen data. Usually, shallow (2-3 m), wind-exposed lakes are unstratified, whereas in temperate climates deeper lakes usually exhibit a stable stratification from spring to autumn. Lakes of intermediate depth (e.g. 5-7 m) may develop transient thermal stratification for a few calm and sunny days, and which is then disrupted by the next event of rain or wind.

Figure 11.2 Derivation of representative depths for taking discontinuous samples and derivation of the volume of each sample

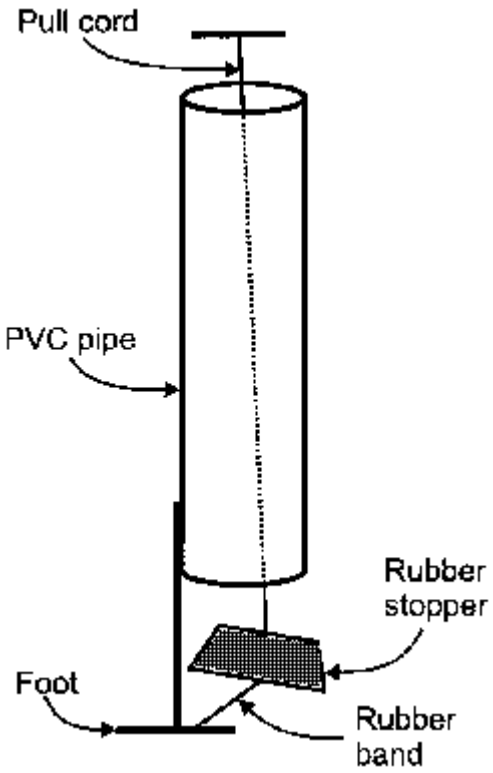


Thermal stratification usually results in inhomogeneous distributions of oxygen, nutrients, and populations of algae, cyanobacteria and other organisms. However, even when temperature is uniform throughout depth, stratification of organisms may develop on calm days. Depth gradients of oxygen concentration and pH are good indicators of this. Depth-integrated samples are more adequate than surface grab samples for the assessment of population size and nutrient concentration in such homothermous situations.

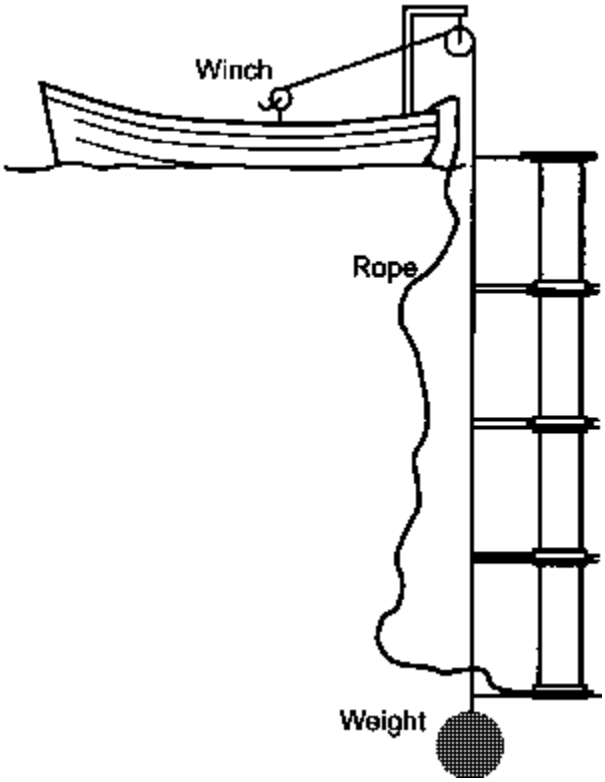
In deeper lakes or reservoirs with thermal stratification, depth-integrated sampling of representatively chosen depths is necessary - taking samples evenly spaced over depth may not yield fully representative results. When background information on the typical stratification characteristics of a given lake is available (e.g. from a temperature profile previously taken with a probe), sample numbers can be reduced by selecting adequate depths to represent specific strata. If depth intervals are unequal and samples are to be integrated, the volume of each sub-sample must be chosen to represent the actual fraction of the vertical gradient it represents (see Figure 11.2).

Figure 11.3 Simple tube devices for taking depth-integrated samples.

A. From shallow lakes or water layers (< 5 m);



B. From deeper layers (< 20 m)



Continuous depth-integrated samples are often quite adequate for shallow and moderately deep water bodies. They can be obtained using a water pump (submersible pumps are available which allow sampling at depth) attached to a garden hose which is operated at a steady pumping rate while the water inlet is drawn upwards between the desired depths at a uniform speed. The sample obtained is therefore representative of the water column. A very simple hose-pipe sampler may be used to depths of 30-35 m. This sampler is made of a piece of flexible plastic piping (a garden hose will do) of about 2 cm diameter and appropriate length; the pipe is weighted at one end and open at both. The weighted end, to which a cord is attached, is lowered slowly into the water so that when the tube is fully extended it encloses a columnar section of the water. Before hauling in the lower end with the attached line, the upper end should be closed in order to avoid loss of water once the lower end emerges from the surface. Hoses need to be stored clean and dry between sampling trips to avoid microbial growth inside the tube.

A simple depth-integrating pipe or tube sampler for shallow water columns (5 m deep) or for the surface layers of deeper water bodies is shown in Figure 11.3A. By combining tube segments as described by Sutherland *et al.* (1992) the sample may be extended to a total length of up to 20 m (Figure 11.3B).

Sampling scums

Scums of cyanobacteria often occur near shorelines at low water depths, and therefore working with a grab-sampler or a plankton-net may be difficult. Sampling scums is carried out more easily with a wide-necked plastic or glass container. When sampling scums, a suitable approach must be developed which allows for their observed heterogeneous density. Two samples can be useful for different purposes. One sample

can be aimed at assessment of the maximum density of cyanobacteria or highest toxin levels by taking a sample from where the scum is thickest (move the bottle mouth along the surface to collect the dense mats of buoyant cyanobacteria). The other sample can aim to simulate conditions where shallow waters are mixed by bathers and playing children (agitate the scum before submerging the bottle). Both types of sample can be used for comparison.

Plankton net sampling

Sampling of cyanobacteria with a plankton net is mainly carried out when large quantities of cell material are required (for example for toxicity testing or for extended chemical analysis) or when only a qualitative analysis of the phytoplankton is necessary. In contrast to the use of depth samplers which quantitatively trap all of the particles in a defined volume, the water volume filtered through a plankton net cannot be determined precisely. Calculations based on the area of the net opening and on the length and the distance it has been hauled are not recommended because they strongly overestimate the amount of water actually filtered (due to clogging of the pores in the net, only a fraction of the water volume actually passes through the net).

The depth at which the plankton net is deployed is dependent on the taxa of algae and/or cyanobacteria present. Floating cells (e.g. *Microcystis, Anabaena)* are harvested within the upper metres of the water column, while the sampling of well mixed or stratified water bodies with distinct depth distributions of cyanobacteria (e.g. *Planktothrix,* see Figure 11.1) may include deeper water layers.

11.4 Nutrients, cyanobacteria and toxins

11.4.1 Nutrient analysis (phosphorus and nitrogen)

Mass developments of cyanobacteria are associated with high nutrient concentrations. Phosphorus is usually the key nutrient controlling proliferation, although the availability of nitrogen may be an important variable to assess because it can influence whether or not nitrogen-fixing species dominate (see Chapters 2 and 8).

Total phosphorus (from unfiltered samples) determines the capacity of a water body to form cyanobacterial blooms. If dissolved phosphate (soluble reactive phosphate (SRP) determined from filtered samples) is detected at concentrations of only a few micrograms per litre, cyanobacterial growth and biomass are not limited by phosphate (see Chapter 8). Because such low concentrations are critical for interpreting the situation, sample contamination (e.g. through a few micrograms of dissolved phosphate leaching from contaminated sample bottles, or through the release of phosphorus by degradation of organic material during sample transport) may very easily lead to misinterpretation. In contrast, samples for total phosphate analysis are less sensitive. If SRP is important in a sampling programme, rapid filtration in the field or even on the boat may be desirable, particularly in warm climates.

Transformations between nitrate and ammonia may occur, if samples are not stored properly (cooled) and analysed rapidly (within 24 hours).

11.4.2 Identification and quantification of cyanobacteria

Cyanobacteria tend to accumulate in layers (at the surface or bottom) of sampling containers. When samples are integrated or when sub-samples are taken, the sample must be mixed immediately before sub-sampling.

In addition to the samples preserved with Lugol's solution, fresh unpreserved samples are useful to aid microscopic identification, because Lugol's solution masks the characteristic colours of the cyanobacteria, making it more difficult to recognise some species. Unpreserved samples can be qualitative and are easily obtained by hauling a plankton net (10 µm mesh) through the water column, or by taking a grab sample if the density of cyanobacteria is high. Unpreserved samples for identification may be stored for up to 24 hours, provided ambient temperature and light conditions are kept similar to those in the field. If longer storage is necessary, preservation with formaldehyde may be useful.

11.4.3 Samples for chlorophyll *a* analysis

When collecting samples for chlorophyll *a* determination it should be born in mind that this pigment will start to degrade rapidly after collection; therefore it is essential to keep storage times to a minimum. Filtration at the sampling site and storage of the filters in an ice box is recommended for warm climates. If samples are to be filtered and analysed in the laboratory, they should be transported as soon as practically possible (e.g. within 4 hours) and processed immediately on arrival.

Apparatus

- Measuring cylinder
- On-site filtration equipment, including vacuum hand pump and filter cup
- Glass fibre filters, GF/C, 47 mm
- Plastic Petri dishes and/or aluminium foil

Procedure

1. Thoroughly mix the sample and pour immediately into a measuring cylinder. Pass the measured sample through the glass fibre filter with the aid of a hand vacuum pump. If samples are dense, begin by filtering a small volume. If this is easily filtered, add more sample, mixing every time a sub-sample is poured from the bottle. Use as much sample as will easily pass through the filter leaving a distinctly visible greenish layer on the filter. Record the volume of water filtered.

2. Once filtration is complete, carefully remove the filter from the filtration apparatus and either place it in a Petri dish or wrap it in aluminium foil.

3. Fold the filter with the cells innermost because this both protects the pigment from light and also prevents the sample from being dislodged from the filter surface.

4. Keep filters at near freezing temperatures and in the dark prior to their delivery to the analytical laboratory. Deep-freeze filters if storage of several hours or more is necessary.

11.4.4 Bulk samples of cyanobacteria for cyanotoxin detection

For some purposes, it is important to collect a large amount of cell material using a plankton net as described in section 11.3; toxicity tests for example require large quantities because bioassays (e.g. mouse test) are less sensitive than chemical analysis. Large amounts of cell material may also be needed for some detailed chemical analyses (see Chapter 13). If the results are to be related to the dry weight of cellular material, a quantitative sample (i.e. sampling all particles of a defined water volume) is not necessary.

For later expert identification of the cyanobacterial taxa, and particularly for relating toxin concentrations to cell numbers or biomass of specific taxa, a subsample for cell counts may be taken directly from a well-mixed net sample before it is frozen. It is important to realise that plankton net hauls accumulate particles selectively (enriching large cells and colonies more than small or thin ones) and are therefore likely to contain a different quantitative distribution of taxa than the sample used for assessing the composition of cyanobacteria (as described in 11.3.2). Such subsamples for microscopy should be preserved in 10 per cent formaldehyde or Lugol's solution in a separate container; small volumes (e.g. 10 ml) are sufficient.

High biomass densities in such concentrated samples may rapidly consume the preservation capacity of Lugol's solution. If not analysed within several days, samples should be periodically checked for their preservation capacity; if they no longer show a brown colouration, more Lugol's solution should be added. Dilution of the sample may be useful to avoid rapid consumption of preservative (i.e. 1 ml net plankton sample, 8 ml water, 1 ml Lugol's iodine solution or formaldehyde).

11.4.5 Quantitative analysis of cell-bound and dissolved toxins

For recreational sites and for assessment of the total toxin concentration, cell-bound toxins are of primary importance. The concentration of dissolved toxin in the water is of special interest for drinking water reservoirs and where large amounts of cyanotoxin such as anatoxin-a are released into the water (Bumke-Vogt *et al.,* 1996). Large volumes may be required for dissolved toxin analysis (e.g. 1 litre) and this should be checked with the analytical laboratory. If the concentration of cell-bound and dissolved cyanotoxin is to be related to the water volume from which the cells were collected, a defined volume must be filtered. Several methods have been proposed (Coyle and Lawton, 1996; Gjølme and Utkilen, 1994) and are described in detail in section 13.1. Essentially samples are handled in a similar manner as those prepared for chlorophyll *a* analysis. The filtration step can be readily performed at the sampling site but with the following modifications:

- Glass fibre or membrane filters may be pre-weighed so that the amount of material retained (i.e. dry weight of cells) can be determined. Thus, toxin concentration can be related both to dry weight and to water volume.

- The filtrate can be collected, placed in a glass bottle and processed as described in Chapter 13 to enable the dissolved cyanotoxin concentration to be determined.

- Filters and filtrate should be kept cool.

Laboratory studies have shown that the microcystin content of material collected and dried on filters is unaltered for several months, when the filters are stored in the dark at room temperature and low humidity. So far this method has been shown to be useful for microcystin as well as for anatoxin-a.

11.5 On-site analysis

Analyses for many variables can be carried out in the field. A significant advantage of on-site testing is that tests are carried out on fresh samples that have not been contaminated or otherwise changed as a result of storage. Variables generally analysed on-site are:

- *Transparency* (with a Secchi disc). Cyanobacteria may occur as floating streaks or mats on the water surface (see Chapter 2) making it difficult to obtain representative transparency data (depending on the measuring site values can vary from 0.2 to 2 m). It may be useful to determine the transparency in areas without floating cells as well as within scums. The Secchi disc has to be lowered very carefully so as not to destroy the formation of accumulated cyanobacterial cells, and before taking the measurement the surface scums should be given time to return to their original position (i.e. their position prior to disturbance by the Secchi disc).
Bathers can easily perform an improvised transparency determination while standing knee-deep in the water and being careful to avoid suspending the sediment. If greenish turbidity obscures their view of their feet, a significant cyanobacterial bloom could be present corresponding to a level at which some authorities advise against bathing (see Chapter 5).
- *Temperature* (with a probe lowered into the water, or a thermometer reading taken immediately from the sampling device).

- *Dissolved oxygen* (electrometrically with a probe or chemically with the Winkler method).

Further details of the methods mentioned above are available in Bartram and Ballance (1996).

11.6 Field records

As with every sampling programme, careful field records are of critical importance for interpretation of results (see Bartram and Ballance, 1996). Field records involve pre-prepared lists and questionnaires, but which also leave plenty of space for additional comment and for the recording of observations. Such observation should include:

- Presence of scums.

- Weather conditions on the day of sampling and, if available, an indication of conditions over the previous 2-3 days, because this affects the accumulation of cyanobacteria.

- Any additional or unusual observations, such as scum distribution, estimation of numbers of people bathing despite the presence of scums, defect warning or prohibition signs regarding bathing in scum areas, suggestions for improvement of the sampling

programme, previously undetected inlets, changes in agricultural practices (such as previously unknown distribution of manure) or other use, etc.

11.7 Sample preservation and transport

As has been emphasised in several sections earlier in this chapter, samples must be clearly labelled with sampling site (station), date and time of sampling and depth of sampling. In general, samples should be stored cool and dark in a storage box, taking into consideration the following criteria:

• Cool storage is most important for all samples to be filtered because biological activity may enhance shifts between particulate and dissolved fractions of the variable to be analysed (biological activity is strongly enhanced by high temperatures). This is particularly important for nutrient samples, chlorophyll *a* samples and samples for cell-bound cyanotoxin analysis (whether collected on filters or with a plankton net). If storage time exceeds 2-3 hours, particularly in warm climates, cooling during sampling and transportation is recommended.

• Unpreserved samples for identification of cyanobacteria, fixed samples for determining cyanobacterial cell numbers and biomass, and samples fixed with Winkler's reagents for oxygen determination should be stored at ambient temperatures but protected from direct sunlight.

• Storage times should be kept as short as possible, generally in the range of a few hours. Storage times over 24 hours should be avoided. Specific descriptions of preservation and transportation of samples for toxicity testing or toxin analysis of cyanobacterial cells are given together with the sampling methods and in Table 11.1.

Table 11.1 Overview of sampling methods for the determination of cyanobacteria, toxicity testing and toxin analysis

Purpose	Equipment	Preservation	Transport	Advantages	Disadvantages
Determination of cyanobacteria					
Qualitative	Plankton net (10 µm); depth sampler[1]	Unpreserved, or formaldehyde solution	Cool and dark	Formaldehyde does not discolour the sample	Caution necessary when using formaldehyde
Quantitative	Depth sampler[1]	Lugol's solution	Cool and dark	Lugol's solution enhances settling in counting chambers	Colour of algae changed by Lugol's solution (identification difficulties for less experienced staff)
Toxicity tests and toxin analysis					
Qualitative, (large amounts)	Plankton net (25-50 µm mesh)	Must be frozen within 24 hours, freeze-drying	Cool up to 24 hours, otherwise frozen or freeze-dried	Provides large amount of cell material for toxicity testing and toxin analyses	No relation to water volume; sampling efficiency less than 100%; selective

Quantitative					
1. Cell-bound toxin Filter method	Depth sampler[1]; hand vacuum pump or filtering device; glass fibre or membrane filters	Directly frozen until analysis or air dried to send to laboratory	Cool up to 24 hours, or send dried filters	Cheap, quick, easy to send; direct relation to volume; biomass estimate possible with pre-weighed filters	No large amounts of cell material for further analysis; amount of toxin on filters may be below detection limit; caution required in humid climates with dried filters
Plankton net with defined water volume	Plankton net (10 µm mesh) graduated bucket	Must be frozen within 24 hours; freeze-drying	Cool up to 24 hours, otherwise frozen or freeze-dried	Provides large amount of cell material for toxin analysis and toxicity testing: relation to water volume	Selective sampling with a net
2. Dissolved toxin	Depth sampler[1]; hand vacuum pump or filtering device; glass fibre or membrane filters	Frozen	Cool up to 24 hours, otherwise frozen	Filters and filtrates can be obtained in one step	

[1] Depth samplers include Ruttner, Van Doom, hose sampler or water pump

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Chapter 12. DETERMINATION OF CYANOBACTERIA IN THE LABORATORY

This chapter was prepared by Linda Lawton, Blahoslav Marsalek, Judit Padisák, Ingrid Chorus

Identification and quantification of cyanobacteria in water resources is the principal component of cyanotoxin monitoring programmes and can provide an effective early warning system for the development of potentially toxic blooms. Data on concentrations of total phosphate, nitrate and ammonia are valuable for assessing the potential for cyanobacteria to develop and whether or not nitrogen-fixing species are likely to occur. Whereas methods for these nutrients have been extensively reviewed and internationally harmonised by the International Organization for Standardization (ISO), approaches to the species determination and quantification of cyanobacteria are very variable and can be undertaken at different levels of sophistication.

Rapid and simple methods can be employed to analyse the composition of a sample at the level of differentiation by genera (rather than species), which is often sufficient for a preliminary assessment of potential hazard and for initial management decisions. Further investigation may be necessary in order to address quantitative questions, such as whether cyanobacteria are present above a threshold density. Rapid quantitative counting methods can give useful estimates of cell numbers with a counting effort of less than one hour per sample (sometimes within minutes), and the bulk method of biomass estimation by chlorophyll *a* determination can be very time-effective with only moderate equipment demands. More detailed taxonomic resolution and biomass analysis is required if population development or toxin content needs to be predicted. Distinction between these approaches is important because management must decide how available staff hours are most effectively invested. In many cases, the priority will be evaluation of a larger number of samples at a lower level of precision.

The choice of methods further requires informed consideration of sources of variability and error at each stage of the monitoring process, particularly with respect to sampling (see Chapter 11). Water bodies with substantial temporal and spatial variation of cyanobacterial cell density may show several-fold deviation in cell numbers between samples taken within a few minutes or within 100 m, and precise determination of biomass in one sample per week therefore will not produce a basis for assessment of population size. Much better information can be gained by investing the same effort into a less precise evaluation of a larger number of samples (e.g. 10 samples taken at

intervals of 100 m, or every day). Information return on working time investment can further be optimised by regular intralaboratory calibrations of methods and their quality control by comparing results with the rapid methods to results of elaborate and precise methods.

This chapter describes methods for cyanobacterial determination and quantification at different levels of accuracy. For determination of the concentrations of key nutrients which control cyanobacterial biomass and species composition, the standard international methods developed by ISO are also reviewed.

12.1 Sample handling and storage

Consideration of the type of information required and decisions regarding the type of analysis required should be made prior to sample collection (see Chapters 10 and 11). However, this is not always possible, particularly when a routine monitoring programme is not in place. Samples may therefore require immediate evaluation on arrival in the laboratory to determine if pretreatment is needed prior to appropriate sample storage.

Samples that have been taken for microscopic enumeration should ideally be preserved with Lugol's iodine solution at the time of collection (Chapter 11). These samples will be relatively stable and no special storage is required, although they should be protected from extreme temperatures and strong light. However, samples should be examined and counted as soon as practically possible because some types of phytoplankton are sensitive to prolonged storage, and Lugol's iodine solution disintegrates over extended storage periods (usually in the range of months, but in a shorter period in very dense samples).

Unpreserved samples for quantitative microscopic analysis require immediate attention either by addition of preservative or by following alternative counting methods which do not use preserved cells. Where unpreserved samples cannot be analysed immediately they should be stored in the dark with the temperature kept close to ambient field temperatures. Unpreserved samples are preferable for species identification because some characteristics cannot be recognised in preserved samples. For example, colonies of *Aphanizomenon* have a characteristic bundle structure which facilitates identification, but preservatives tend to disintegrate the colonies, and the single filaments are more difficult to distinguish from other genera. While samples for quantification must be preserved immediately or counted, samples for identification may be analysed within 24 hours because changes in numbers are less important.

Samples for the analysis of chlorophyll *a,* total and dissolved phosphate as well as nitrate and ammonia, should be filtered as soon after sampling as possible. Storage for a few hours in the dark in glass bottles is usually acceptable if temperatures do not exceed 20 °C. Filtration at the sampling site is recommended, particularly in warm climates, or filtration should occur immediately upon arrival in the laboratory (see section 11.4.3). Filtered samples for nutrient analysis may be stored in the refrigerator for a few hours prior to analysis, or deep-frozen at -18 °C for several weeks. Although the suitability of storage of filters for chlorophyll *a* analysis at -18 °C is currently under debate, the method is employed by many laboratories if immediate extraction cannot be organised.

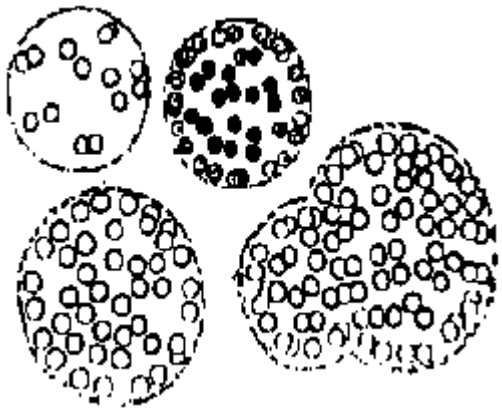
12.2 Cyanobacterial identification

Microscopic examination of a bloom sample is very useful even when accurate enumeration is not being carried out. The information obtained regarding the cyanobacteria detected can provide an instant alert that harmful cyanotoxins may be present. This information can determine the choice of bioassay or analytical technique appropriate for determining toxin levels (see Chapter 13).

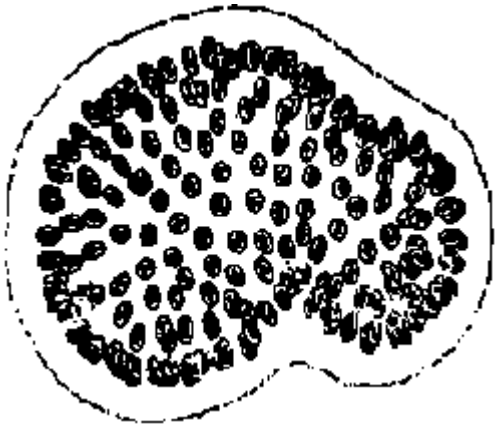
Most cyanobacteria can be readily distinguished from other phytoplankton and particles under the microscope by their morphological features at a magnification of 200-1,000 times. Figure 12.1 shows the most frequently occurring of the species known to produce toxins. Cyanobacterial taxonomy, following the established botanical code, differentiates by genera and species. However, this differentiation is subject to some uncertainty, and organisms classified as belonging to the same species may nonetheless have substantial genetic differences, e.g. with respect to microcystin production (see Chapter 3). Genetically identical cells, obtained by isolation of one colony and cultivation of its daughter cells, are termed strains or genotypes, and field populations of one species (or morphotype, i.e. identified as species on the grounds of morphological similarity) consist of a number of genotypes which cannot be differentiated microscopically. Current understanding of the regulation of cyanotoxin production indicates that distinction of genera is very important for assessing potential toxicity (see Chapter 3), but that microcystin content varies extremely at the level of genotypes or strains, rather than at the level of species. This is one reason why identification to the taxonomic level of genera (e.g. *Microcystis, Planktothrix, Aphanizomenon, Anabaena)* is frequently sufficient. It is preferable to give only the genus name, especially if differentiation between species by microscopy is uncertain on the basis of current general taxonomic knowledge, a lack of locally available expertise, or lack of characteristic features of the specimens to be identified. This must be emphasised because "good identification practice" has frequently been misunderstood to require determination down to the species level, and this has lead to numerous published misidentifications of species.

Figure 12.1 The most frequently occurring species of cyanobacteria known to produce toxins

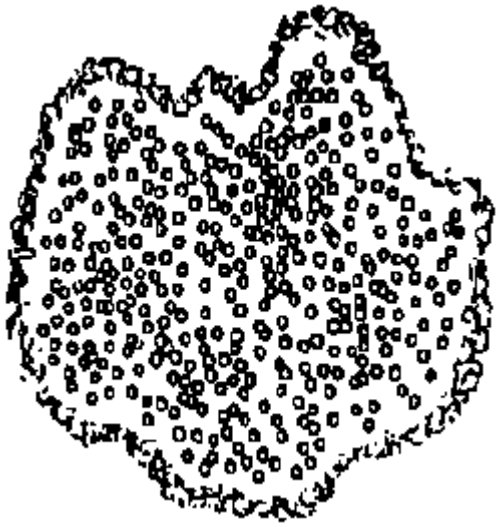
Coelosphaerium



Gomphosphaeria



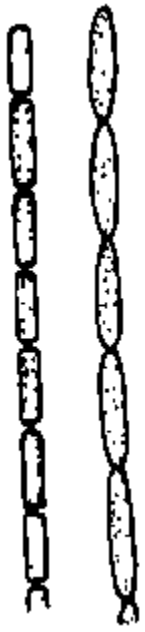
Microcystis



Synechococcus

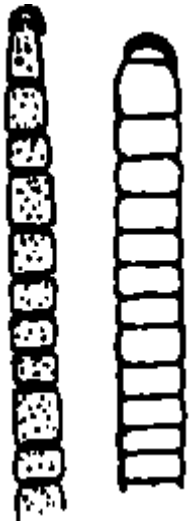
Synechocystis

Pseudanabaena



Oscillatoria

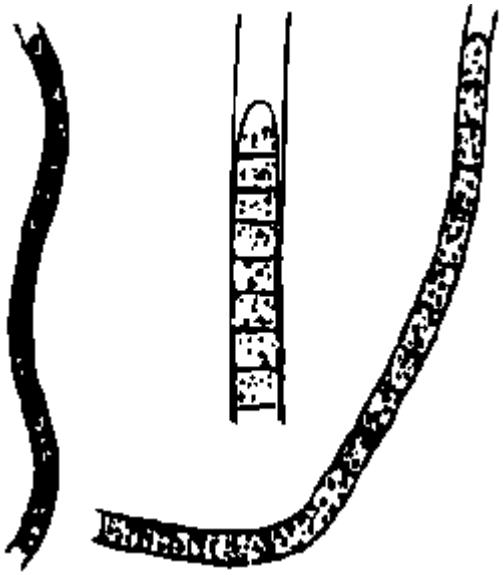
Trichodesmium



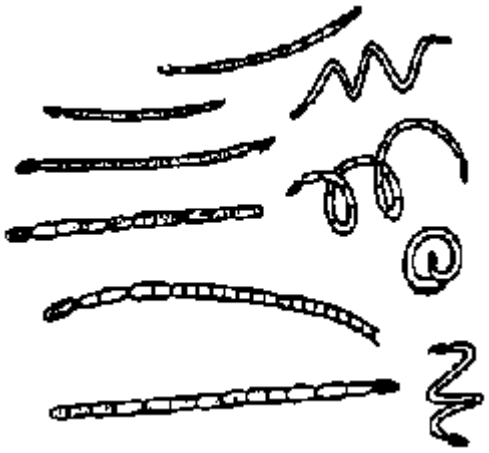
Schizothrix

Lyngbya

Phormidium

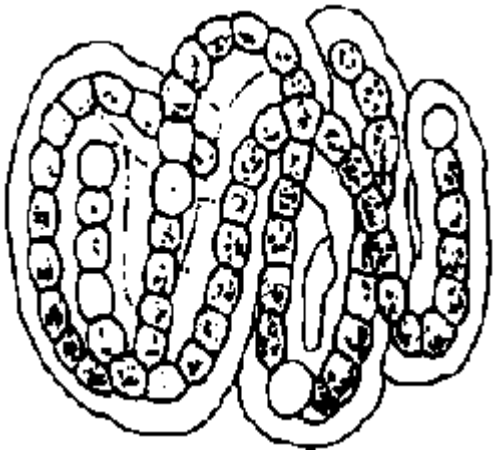


Cylindrospermopsis

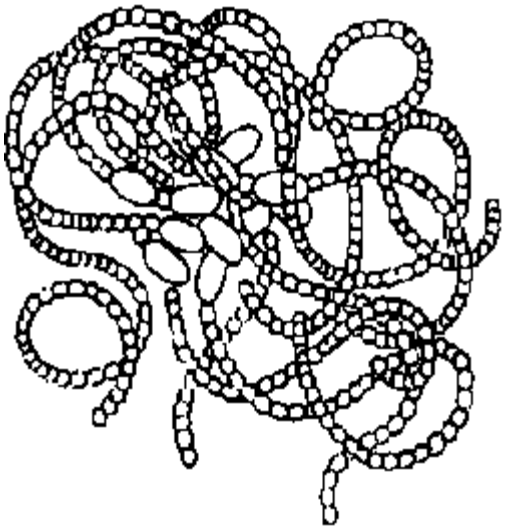


Aphanizomenon

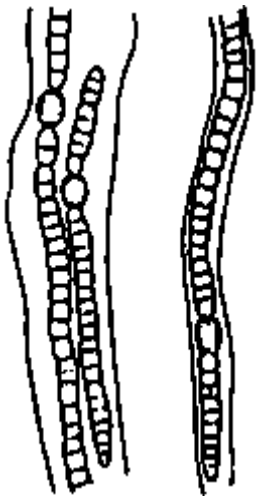
Nostoc



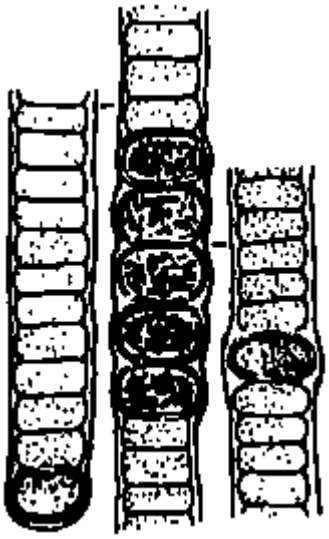
Anabaena



Hormothamnion



Nodularia



Gloeotrichia

Practitioners in health authorities with some experience in using a microscope can easily learn to recognise the major cyanobacterial genera and some prominent species which occur in the region they are monitoring. They should not be deterred by the pitfalls of current scientific work in cyanobacterial taxonomy which targets differentiation to the species level. Nevertheless, more precise identification of the dominant organisms down to species level may be useful for a more accurate estimate of toxin content. For example, *Planktothrix agardhii* and *Planktothrix rubescens* have both been shown to contain microcystins, but each species contains different analogues with different toxicity.

For establishing cyanobacterial identification in a laboratory, initial consultation and later occasional co-operation with experts on cyanobacterial identification (as outlined in Chapter 10) is helpful. Training courses for beginners should focus on the genera and species relevant in the region to be monitored. Experts can assist in initially deriving a

list of these taxa and the criteria for their identification. In the course of further monitoring, experienced experts should be consulted periodically for quality control and for updating such a list.

Publications assisting in the determination of cyanobacterial genera and species include Komárek and Anagnostidis (1986) and Anagnostidis and Komárek (1988).

12.3 Quantification

Rapid methods for frequent monitoring of large numbers of water bodies or sampling sites have been developed in some countries. These cannot be readily standardised and evaluated internationally, but require adaptation to regional or local conditions. Deciding on the appropriate classification of units to count depends upon variations such as whether prevalent taxa are filamentous or coccoid, whether they are colony-forming or occur as single filaments or cells, and whether populations are very diverse or largely mono-specific. One rapid method which is highly standardised and simplified involves counting a 0.5 ml sample of water for 2 minutes and determining the number of cyanobacterial units present. A table has been prepared which provides adjustment factors to take into account differences in the number of cells present in filaments or colonies.

Cyanobacterial biomass can also be determined using indirect methods, the most common being chlorophyll *a* quantification. This is a rapid and simple method but it is open to interference by chlorophyll *a* from other phytoplankton, hence it is best used when cyanobacteria are the main or dominant organisms present.

12.3.1 Enumeration

Microscopic enumeration of cyanobacterial cells, filaments or colonies has the advantage of directly assessing the presence of potentially toxic organisms. Little equipment in addition to a microscope is required. The method may be rather time consuming, ranging from a few minutes to several hours per sample, depending upon the accuracy required and the number of species to be differentiated. The remaining of this section begins by outlining precise and widely accepted counting procedures which are more time consuming and require a moderate level of expertise, but serve as a bench mark to assess the performance of simplified methods developed to suit the expertise and requirements of a sampling programme.

Sample concentration by sedimentation or centrifugation

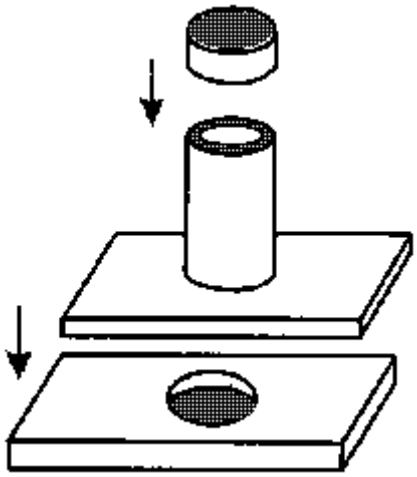
Direct counting of preserved cells is typically carried out by Utermöhl's counting techniques using a counting chamber and inverted microscope (Utermöhl, 1958). This method is well suited for assessment of a large variation in cell type and is widely accepted as one of the most reliable. Counting chambers and sedimentation tubes are commercially available or can be constructed by the investigator. The most commonly used chambers have a diameter of 2.5 cm and a height of about 0.5-2 cm and thus can contain 2-10 ml of sample. These chambers can fit easily on the stage of an inverted microscope. If larger volumes of water need to be analysed, as is the case when cell density is low (e.g. in drinking water or at the beginning of population development), then the height of the tube has to be increased. Such extended tubes, however, are too tall to

fit on the inverted microscope stage and the light would have to pass through a considerable thickness of liquid before reaching the sedimented specimens. This problem can be overcome by using a tube in two sections which allows the supernatant to be removed (after settling the sample) without disturbing the sedimented cells on the bottom glass (Figure 12.2). The amount of sedimented water required depends on the density of cells, on the counting technique (fields or transects, see later) and on the magnification. If concentrations are high (such as from bloom material), even 2 ml may contain too many cells for enumeration, and the sample will need to be diluted.

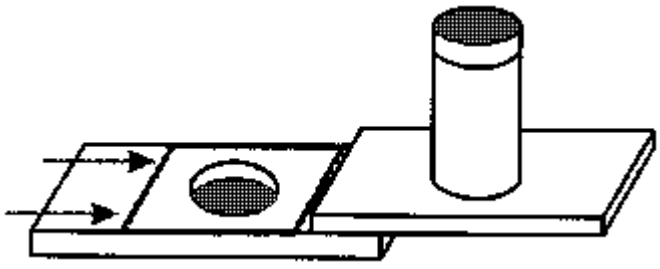
Apparatus

- Inverted microscope with 10x and 40x objectives
- Counting chamber with sedimentation tube
- Cyanobacterial identification key
- Sample preserved in Lugol's iodine solution (see section 11.3.1)

Figure 12.2 Counting chambers for use with inverted microscopes



Step 1
Cylinder is pressed firmly onto the counting chamber, sample is poured in, cylinder is sealed with heavy round glass slide. Sedimentation requires 4 hours per cm of cylinder height.



Step 2

Thin, square cover slide is used to slide cylinder and supernatant off the counting chamber.

Procedure

1. Allow the sample to equilibrate to room temperature. If cold samples are placed directly in the counting chamber, air-bubbles develop and prevent sedimentation.

2. Gently invert the bottle containing the sample several time to ensure even mixing of the phytoplankton.

3. Pour the sample into the sedimentation tube in place over the counting chamber.

4. Place the counting chamber on a horizontal surface where it will not be disturbed or exposed to direct sunlight.

5. Allow the sample to settle. Sedimentation times will vary depending on the height of the sedimentation tube. Allow at least 3-4 hours per cm height of liquid. Where neutralised formalin has been used as a preservative, double the time allowed for sedimentation. Note that buoyant cells (i.e. those with gas vesicles) may not settle and may require disruption of the gas vacuoles (see below). However, this problem is frequently overcome by several days of storage with Lugol's solution, because uptake of iodine increases the specific weight of the cells.

6. Phytoplankton density can now be determined by counting either the total number of organisms on the base of the chamber or by counting subsections (transects, fields).

If an inverted microscope is not available, and samples with low cyanobacterial density need to be counted, other techniques may be applied in order to concentrate samples sufficiently (e.g. sedimentation in a measuring cylinder, followed by careful removal of the supernatant).

Apparatus

- Glass measuring cylinder, 100 ml
- Glass pipette with pipette bulb or filler
- Standard laboratory microscope with 10x and 40x objectives
- Sample preserved in Lugol's iodine solution (section 11.3.1)

Procedure

1. Allow the sample to equilibrate to room temperature.

2. Gently invert the bottle containing the sample several times to ensure even mixing of the phytoplankton.

3. Pour 100 ml of the sample into the measuring cylinder.

4. Allow the sample to sediment (3-4 hours per cm height of liquid) in a location where it will be out of direct sunlight and it will not be disturbed.

5. Using the glass pipette with pipette bulb or filler attached, carefully remove the supernatant, leaving only the last 5 ml undisturbed.

6. The sample has now been concentrated by a factor of 20 and can be counted using a counting chamber (e.g. Sedgewick-Rafter or haemocytometer).

Where sedimentation is not possible, centrifugation can offer a rapid and convenient method of concentrating a sample (Ballantine, 1953). Fixation with Lugol's iodine solution enhances the susceptibility of cells to separation by centrifugation. However, buoyant cells (i.e. those with gas vesicles) may still be difficult to pellet and may require disruption of vacuoles prior to centrifugation by applying sudden hydrostatic pressure (see below) (Walsby, 1992). Once concentrated, a known volume can be quantified using a counting chamber or by counting a defined volume using a micropipette to place a drop on a microscope slide. Observation and counting can be done with a standard microscope.

Apparatus

- Centrifuge
- Centrifuge tube, 10-20 ml
- Syringe or bottle with cork, or plastic bottle with screw cap
- Standard laboratory microscope with 10x and 40x objectives

Reagents

- Aluminium potassium sulphate, 1.0 g $AIK(SO_4)_2.12H_2O$ in 100 ml distilled water

Procedure

1. Place 10-20 ml of sample in a centrifuge tube, seal with cap, and centrifuge at 360 × g for 15 minutes.

2. When pelleting needs to be enhanced, add 0.05 ml of aluminium potassium sulphate solution per 10 ml of sample. Mix and centrifuge as described.

3. Where problems occur with the pelleting of buoyant cells, try one of the following:

i) Place sample in a plastic syringe, ensure the end is tightly sealed, then apply pressure to the plunger.

ii) Place sample in a bottle with a tightly fitting cork then bang the cork suddenly.

iii) Place sample in a well sealed plastic bottle and bring it down sharply onto a hard surface.

These three approaches should be carried out with extreme care to avoid accidental exposure to toxic cyanobacteria. Once they have been subjected to this pressure shock, the gas vesicles should have been disrupted and cells should pellet when centrifuged.

4. Once cells have been centrifuged, carefully remove the supernatant and resuspend the pellet in a small known volume (e.g. 0.5 ml).

5. Samples concentrated by centrifugation can be counted using a counting grid or haemocytometer.

Counting cyanobacteria

Counting cyanobacteria involves defining the units to be counted. The majority of planktonic cyanobacteria are present as filamentous or colonial forms consisting of a large number of cells which are often difficult to distinguish. The accuracy of quantitative determination depends on the number of counted objects (e.g. cells or colonies); the relative error is approximately indirectly proportional to the square root of the number of objects counted. The number of colonies, not the number of cells, is decisive for accurate enumeration. However, the number of colonies is often not very high, even in water containing a dense bloom where only 20-40 colonies may be present in a 100 ml sample. Both filaments and colonies can differ greatly in the number of cells present, hence results given as number of colonies (e.g. stating that 1 ml of sample contains an average of 2.43 colonies of *Microcystis aeruginosa)* give little information on the quantity of cyanobacteria present).

Typically, unicellular species are counted as cells per ml and filamentous species can either be counted as number of filaments, and quoting an average number of cells per filament. The cells per filament in the first 30 filaments encountered are often counted and averaged. Alternatively, the total filament length per ml may be assessed as the sum of the extension of each filament within a counting grid placed in the ocular of the microscope. The latter approach is more precise when filament length is highly variable. For colonial species, disintegration of the colonies and subsequent counting of the individual cells is preferable to counting colonies and estimating colony size (Box, 1981). Disintegration of colonies sometimes occurs several days after fixation with Lugol's iodine solution. For more stable colonies, it can be achieved by alkaline hydrolysis (80-90 °C for 15 min, followed by intensive mixing) or gentle ultrasonication. These methods often separate cells very effectively, and even where colonies are not totally broken down into single cells the colony size may be reduced sufficiently to allow single cells to be counted. If this approach is impossible, the geometric volume of individual colonies may be estimated. If colonies are relatively uniform in size, the average number of cells per colony may be determined and then the colonies can be counted. Generally, the use of published values for numbers per colony is not recommended because the size of colonies varies greatly.

There are several systematic methods for counting cyanobacteria. Most approaches aim at counting only a defined part of the sample and then calculating back to the volume of the entire sample. The most common methods are:

- *Total surface counting* which requires the counting of all cells settled within the chamber and may be very time consuming. This method is usually only appropriate for counting very large units at low magnification.

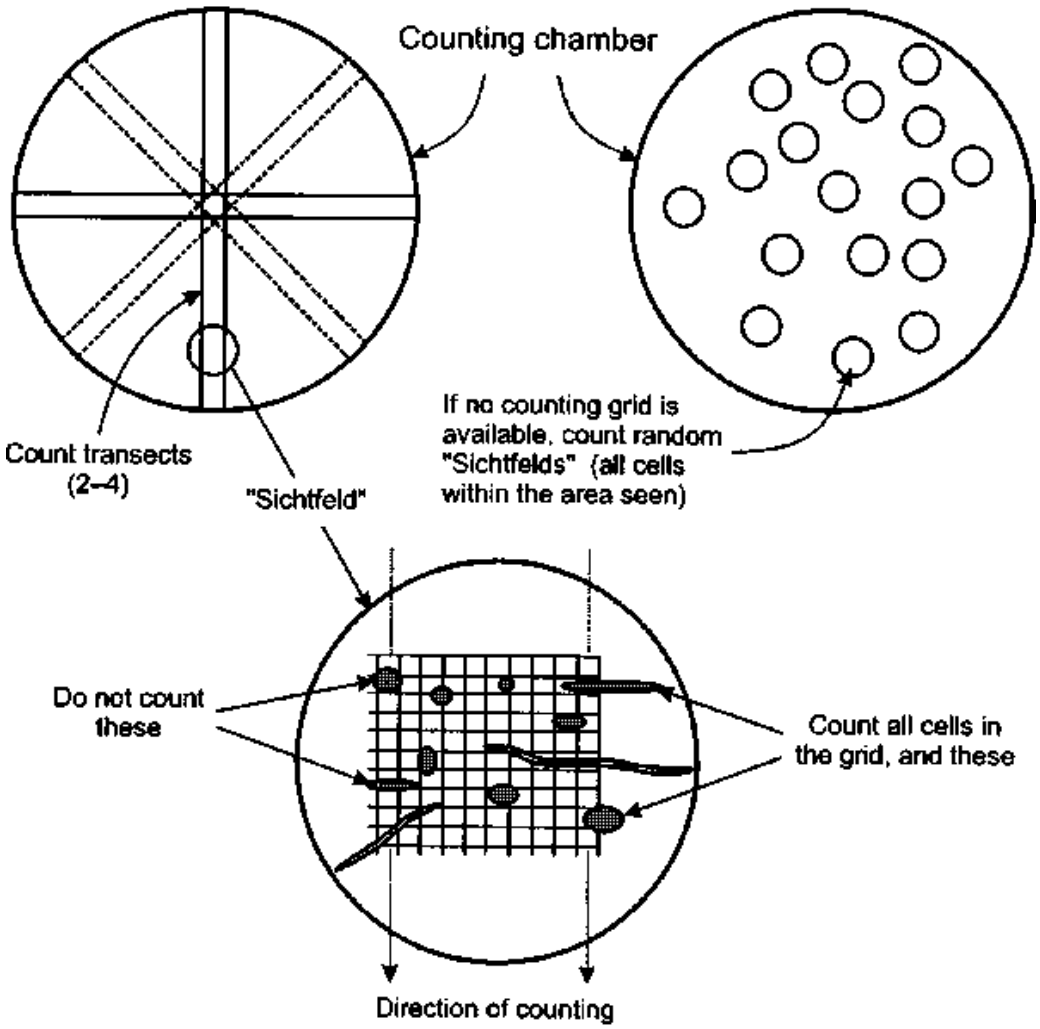
- *Counting cells in transects* from one edge of the chamber to the other (Figure 12.3). Transects should pass through the central point of the chamber. Some inverted

microscopes are equipped with special oculars so that the transect width can be adjusted as required. However, in many cases, the horizontal or vertical sides of a simple counting grid can be used to indicate the margin of the transect. Back-calculating to a millilitre of sample requires measuring the area of the transects and of the chamber bottom as well as the volume of the counting chamber.

• *Counting cyanobacteria occurring in randomly selected fields* ("Sichtfeld") (Figure 12.3). It is recommended that the position of the chamber to find the next field should be changed without looking through the microscope in order to prevent a bias in the selection of fields. The Sichtfeld area covered by a counting grid is usually considered as one field. However, if no counting grid is available the total spherical Sichtfeld can be considered as a single field. Back-calculating to 1 ml of sample requires registration of the number of Sichtfelds counted, measuring the area of the Sichtfeld and of the chamber bottom, as well as knowing the volume of the counting chamber.

The density of different species in one sample can vary and there can also be several orders of magnitude difference between the size of different species; hence it is necessary to select the counting method to suit the sample. Total chamber surface counting with low magnification (100x) is required for large species whereas transect or field counting with higher magnification (200x, 400x) is used for smaller or unicellular cyanobacteria. Accurate enumeration using transects or fields assumes on even distribution of cyanobacteria on the bottom of chamber surface after sedimentation. Due to inevitable convection currents, cells very rarely settle randomly on the surface of the bottom glass and are, almost always, more dense either in the middle or around the circumference of the chamber. Sometimes density also varies between opposite edges. The inaccurate estimate that arises from uneven distribution can be minimised by transect counting. Consequently, transect counting is the preferred method and counting four perpendicular diameters minimises the error. The relation of precision to counting time is very effective if about 100 counting units (cells, colonies, filaments) are settled in one transect (for simplification, see Box 12.1). Samples are best diluted or concentrated so that the number of units of the important taxa lies within this range.

Figure 12.3 Counting techniques used to enumerate cyanobacteria in water samples



A note on filaments:

• **Either** count them just as cells (inaccurate if length is highly variable). In that case, the one extending out of the grid to the right would be counted, but not the one extending out to the left.

• **Or** estimate the length of filament within the boundaries of the grid. In this case: 3 boxes (bottom left) + 5 boxes (mid-right) = 8 boxes

Specimens occurring exactly on the margin of the counting area (transect or field) present the common problem of whether to count them or not. When counting transects, those specimen that lie across the left margin are ignored while those that cross the right margin are included. When counting fields two predetermined sides of the grid are included and the other two are ignored (Figure 12.3).

Box 12.1 Simplification for biomass estimates

With some experience and a flexible approach, the time needed for enumeration and measurement of cell dimensions can be considerably reduced (down to 1 hour or less, if only one or two species require counting) without substantial loss of accuracy. The procedure is as follows:

- If the deviation of numbers of dominant species counted in two perpendicular transects is less than 20 per cent between both transects, do not count further transects.

- If the standard deviation of cell dimensions measured on 10 cells is less than 20 per cent, do not measure further cells.

- If a set of samples from the same water body and only slightly differing sites (e.g. vertical or horizontal profiles) is to be analysed, enumerate all samples, but measure cell dimensions only from one. Check others by visual estimate for deviations of cell dimensions and conduct measurements only if deviations are suspected.

There are different recommendations regarding the number of units per species that must be counted to obtain reliable data. It is particularly difficult to count each species with an acceptable error (20-30 per cent if 400 individual units are counted) in each sample. Mass developments of cyanobacteria are characterised by dominance of one to three species. Even if total phytoplankton is to be counted (for example in order to assess the relative share of cyanobacteria), it is rare for more than six to eight species to contribute to the majority of the biomass. Therefore, for total phytoplankton counts, it is suggested that 400-800 specimens in each sample are counted, giving a maximum error for the total count as 7-10 per cent. In this situation there will be a 10-20 per cent error for the few dominant species, 20-60 per cent for the subdominant species and the rest of the species can be considered as insufficiently counted. If only cyanobacteria are to be counted, and only one or two species are present, counting up to the precision level of 20 per cent, by counting 400 individual units per species, can be accomplished within less than one hour.

The use of mechanical or electronic counters for recording cell counts can shorten counting time considerably, especially if only a few species are counted. Computer keyboards can also be used together with suitable programmes for recording cell counts.

The use of an inverted microscope with counting chambers is generally the best approach for estimating cyanobacterial numbers. However, a standard microscope is sufficient for preconcentrated samples or for naturally dense samples from mass developments, provided the size of the water drop enumerated can be defined (e.g. by using a micropipette). Other counting chambers (e.g. Sedgewick-Rafter or haemocytometer) are available for use with a standard microscope. It can also be useful to monitor samples under high magnification with oil-immersion (1,000x) to check the sample for the presence of very small species which may be overlooked during normal counting.

An alternative counting method which has been found to be useful is syringe filtration. This method is considerably less time consuming because it does not depend on lengthy sedimentation times and uses a standard laboratory microscope.

Apparatus

- Syringe, 10 ml
- Membrane filters, 13 mm diameter with 0.45 µm pore
- Membrane filter holder
- Glass microscope slides plus coverslips
- Standard laboratory microscope with 10x and 40x objectives

Reagents

- Immersion oil

Procedure

1. Mix water sample by inverting several times.

2. Take up 10 ml of the sample into the syringe.

3. Place filter holder with filter in place, on the end of the syringe.

4. Gently filter the sample through the filter by applying pressure to the syringe piston.

5. Once all the sample has passed through the filter, remove the filter from the holder and place it on a glass microscope slide with the captured cells uppermost.

6. Allow the filter to dry at room temperature then carefully add one or two drops of immersion oil to the filter. The will make the filter appear transparent and permit observation of the cyanobacterial cells trapped on its surface.

7. Finally, cover the filter surface with a glass coverslip and examine under the microscope.

8. The density of cyanobacteria can be easily calculated by counting the number of cells on the filter and dividing this by the volume of water filtered (i.e. number of cells per ml).

12.3.2 Determination of cyanobacterial biomass microscopically

Cell size can vary considerably within and between species, and toxin concentration relates more closely to the amount of dry matter in a sample than to the number of cells. Hence, cell numbers are often not an ideal measure of population size or potential toxicity. This can be overcome by determining biomass. Two approaches are available, either estimation from cell counts and average cell volumes, or from chemical analysis of pigment content.

Cyanobacterial counts and cell volumes

Biovolume can be obtained from cell counts by determining the average cell volume for each species or unit counted and then multiplying this value by the cell number present in the sample. The result is the total volume of each species. Given a specific weight of almost 1 mg mm^{-3} for plankton cells, this biovolume corresponds quite closely to biomass. Average volumes are determined by assuming idealised geometric bodies for each species (e.g. spheres for *Microcystis* cells, cylinders for filaments), measuring the relevant geometric dimensions of 10 to 30 cells (depending upon variability) of each

species, and calculating the corresponding mean volume of the respective geometric body.

Example 1

By measuring 20 *Microcystis* cells, an average diameter of 5 µm was established. Assuming spherical-shaped cells the average cell volume is $4/3\ \pi r^3 = 65.4\ \mu m^3$. Enumeration resulted in 1 million cells per ml and thus the total biovolume is $65.4 \times 10^6\ \mu m^3\ ml^{-1}$.

Example 2

Measuring 30 *Planktothrix* filaments resulted in an average length of 225 µm and an average diameter of 6 µm. Assuming cylindrical shaped filaments, the average filament volume is $2\ \pi r^2 \times L = 6{,}359\ \mu m^3$. Enumeration resulted in 10,000 filaments per ml. Thus the biovolume of *Planktothrix was* $63.6 \times 10^6\ \mu m^3\ ml^{-1}$.

Thus, although the number of *Planktothrix* was 100-fold less than that of *Microcystis,* biovolumes were similar because the volume (and biomass) of a single *Planktothrix* filament is about 100 times as large as that of a single *Microcystis* cell. Both species often contain microcystins, and it is possible to compare the relative toxin content per biovolume or biomass whereas there is little point in comparing toxin content in relation to the respective cell numbers.

12.4 Determination of biomass using chlorophyll a analysis

The pigment chlorophyll *a* generally contributes 0.5-1 per cent of the ash-free dry weight of phytoplankton organisms. Although the pigment content may vary according to the physiological state of the organisms (e.g. it increases if light availability is low), chlorophyll a is a widely used and accepted measure of biomass. It is an especially useful measure during cyanobacterial blooms, when the phytoplankton chiefly consists of cyanobacteria, often of only one species. However, when chlorophyll *a* determination is used with mixed phytoplankton populations (cyanobacteria and other species), it gives an overestimation of cyanobacterial biomass. Rough microscopic estimations of the relative share of cyanobacterial cells among the total phytoplankton may be used to correct the overestimate.

Analysis of chlorophyll *a* requires relatively simple laboratory equipment, principally filtration apparatus, centrifuge and spectrophotometer. It is considerably less time-consuming than microscopic biomass determination (but also less specific and less precise). Standard protocols have been described (e.g. ISO, 1992) but preferred methods vary somewhat between laboratories. However, the main procedural steps in most methods are essentially the same: solvent extraction of chlorophyll *a,* determination of the concentration of the pigment by spectrophotometry, and adjustments to the result to reduce the interference by phaeophytin *a* which is a degradation product of chlorophyll *a.* A simple method following the ISO procedure for the determination of chlorophyll a in a lake water sample is outlined below.

Apparatus

- Spectrophotometer suitable for readings up to 750 nm, or photometer with discrete wavelengths at 665 and 750 nm

- Glass cuvettes, typically of 1 cm path length, or 5 cm for very low concentrations (e.g. from drinking water reservoirs at the beginning of population development)

- Centrifuge

- 15 ml centrifuge tubes, graduated and with screw caps

- Water bath at 75 °C or other heating device for boiling ethanol

- Glass fibre filters (GF/C), 47 mm diameter

- Filtration apparatus and vacuum pump

- Tissue grinder or ultrasonication device

- Pipette or similar for addition of acid

Reagents

- Ethanol (90 % aqueous)
- Hydrochloric acid, 1 mol l^{-1}

Procedure

Perform the following steps in low intensity of indirect light because light induces rapid degradation of chlorophyll.

1. After recording the initial volume of water, separate the cells from the water by filtration. Filter continuously and do not allow the filter to dry during filtration of a single sample. If extraction cannot be performed immediately, filters should be placed in individual, labelled bags (filters folded in half with cells innermost) or Petri dishes and stored at -20 °C in the dark (this may cause some pigment degradation and is not recommended by ISO). This step can be carried out at the sampling site and the samples are readily transported in this form. In preference to freezing, samples may be stored in the extraction medium (see below) for up to 4 days in the refrigerator.

2. Place the filter in a tissue grinder, add 2-3 ml of boiling ethanol, and grind until the filter fibres are separated. Ultrasonication can also be used. Pour the ethanol and ground filter into a centrifuge tube, rinse out the grinding tube with another 2 ml ethanol and add this to the centrifuge tube. Make up to a total of 10 ml in the centrifuge tube with ethanol. Place cap on the tube, label and store in darkness at approximately 20 °C for 24-48 hours.

3. Centrifuge for 15 minutes at 3,000-5,000 g to clarify samples. Decant the clear supernatant into a clean vessel and record the volume.

4. Blank Spectrophotometer with 90 per cent ethanol solution at each wavelength.

5. Place centrifuged sample in the cuvette and record absorbance at 750 nm and 665 nm (750a and 665a; absorbance at 750 is for turbidity correction and should be very low). Readings at 665 nm should range between 0.1 and 0.8 units.

6. Add 0.01 ml of 1 mol l^{-1} HCl to sample in cuvette (adjust volume to suit the volume of cuvette being used, calculating approximately 0.003 ml of 1 mol l^{-1} HCl per ml of ethanol solution) and agitate gently for 1 minute. Record absorbance at 750 nm and 665 nm (750b and 665b).

Calculation

1. Correct for turbidity by subtracting absorbance 665a-750a = corrected 665a absorbance
665b-750b = corrected 665b absorbance

2. Use the corrected 665a and 665b absorbance to calculate:

$$\text{Chlorophyll } a = \frac{29.62(665a - 665b) \times V_e}{V_s \times 1} \text{ mg m}^{-3}$$

$$\text{Phaeophytin a} = \frac{20.73(665b) \times V_e}{V_s \times 1} \text{ mg m}^{-3}$$

where: V_e = Volume of ethanol extract (ml)
V_s = Volume of water sample (litres)
l = Path length of cuvette (cm)

Note, the ratio of chlorophyll a to phaeophytin a should give an indication of the condition of the cyanobacterial (and algal) population, but may also reflect the effectiveness of sample handling and preservation, because high levels of phaeophytin a indicate degradation of chlorophyll a either in scenescent field populations or during analysis. When samples are concentrated by filtration for the purposes of analysis, the cells die. Consequently, the chlorophyll immediately starts to degrade to phaeopigments. If filters are not rapidly extracted or frozen, chlorophyll a concentrations are thus reduced. Occasionally, other factors affect this method, resulting in very low or even negative values for chlorophyll *a*. This can be checked by calculating:

$$\text{Chlorophyll } a + \text{Phaeophytin } a = \frac{12.2(663a) \times V_e}{V_s \times 1} \text{ mg m}^{-3}$$

The result of this calculation should give a similar value to the sum of the concentrations of both pigments determined separately, as above. Note also:

- If no centrifuge is available, filtration may be used instead.

- If no tissue grinder or ultrasonication device is available, proceed without this step. Slight underestimations may occur. For cyanobacteria, these are not likely to be too serious.

12.5 Determination of nutrient concentrations

The capacity for development of a cyanobacterial bloom depends on the available concentrations of elements that the cells are composed of (chiefly carbon, hydrogen, oxygen, phosphorus, nitrogen and sulphur). These elements are needed in the ratio in which they occur in living cells (in weight units: 42 C, 8.5 H, 57 O, 7 N, 1 P and 0.7 S). Hydrogen and oxygen are available in unlimited supply in an aqueous environment, and sulphur is usually also present in surplus concentrations. Carbon has been investigated as a potentially limiting factor, but has rarely been found to be relevant. Most often, phosphorus concentrations limit the amount of biomass that can form in a given water body but sometimes, nitrogen is limiting. The chief sources of nitrogen are nitrate and ammonia, but to some extent their lack can be compensated by some cyanobacteria through fixation of atmospheric nitrogen. Thus, even if phosphate is clearly the factor limiting carrying capacity, knowledge of nitrogen availability helps to predict whether nitrogen-fixing species are likely to grow.

Cyanobacterial cells appear to have little means of storing excess nitrogen, but can store enough phosphate for up to four cell divisions, which implies that one cell can grow into 16 without needing to take up dissolved phosphate. Information on dissolved phosphate concentrations, therefore, only demonstrates that if it can be detected, the phytoplankton population is not currently limited by phosphate. In order to assess the capacity of the water body to carry a cyanobacterial population, total phosphate must be determined, which can then be compared with the total concentration of nitrogen salts and organic nitrogen. However, in order to assess whether nitrogen may be limiting, analysis of dissolved components (chiefly nitrate and ammonia) is sufficient.

Among the methods available, the procedure of Koroleff (1983) for determining total phosphate has proved to be most reliable and is the basis of an ISO protocol. For nitrate and ammonia, several methods are available, but the ISO method with the least demands on equipment is described below. Details of ISO methods can be obtained directly from ISO at Case Postale 56, CH-1211, Geneva 20, or requested through the Internet on central@iso.ch.

12.5.1 Analysis of phosphorus according to ISO 6878

Phosphorus in various types of waters can be determined spectrometrically by digestion of organic phosphorus compounds to orthophosphate and reaction under acidic conditions to an antimony-phosphormolybdate complex which is then reduced to a strongly coloured blue molybdenum complex. The internationally harmonised method described by ISO/FDIS 6878 (ISO, 1998a) is applicable to many types of waters (surface-, ground-, sea- and wastewater) in a concentration range of 0.005 to 0.8 mg l^{-1} (or higher if samples are diluted). Differentiation by the following fractions is possible through filtration procedures:

Option	Fraction	Filtration/procedure
1	Soluble reactive phosphorus (SRP or orthophosphate)	Filtered sample
2	Dissolved organic phosphate	Digested filtered sample
3	Particulate phosphorus	Option 4 minus option 2

4	Total phosphorus	Digested unfiltered sample

Digestion or mineralisation of organophosphorus compounds to orthophosphate is performed in tightly sealed screw-cap vessels with persulphate, under pressure and heat in an autoclave (in the absence of which good results have also been obtained with household pressure cookers), or simply by gentle boiling. Polyphosphates and some organophosphorus compounds may also be hydrolysed with sulphuric acid to molybdate-reactive orthophosphate. The following gives an overview of the procedure, necessary equipment and chemicals, see ISO (1998) for details and specific problems.

Apparatus

- Photometer measuring absorbance in the visible and near infrared spectrum above 700 nm; sensitivity is optimal at 880 nm (and reduced by 30 per cent at 700 nm); sensitivity is increased if optical cells of 50 mm are used (if 100 mm cells are available, determination down to 0.001 mg l^{-1} may be possible)

- Filter assembly and membrane filters, 45 mm diameter with 0.45 µm pore

- For digestion of samples, an autoclave (or pressure cooker) suitable for 115-120 °C

- For digestion of samples, borosilicate vessels with heat-resistant caps that can be tightly sealed

- Bottles for samples as described in Chapter 11

- Pre-cleaned glass bottles for filtered samples

Reagents

All reagents should be of a recognised analytical grade and the distilled water used must have a negligible phosphorus concentration when compared with the samples

- Sulphuric acid (H_2SO_4): 9 mol l^{-1}

- Sulphuric acid (H_2SO_4): 4.5 mol l^{-1}

- Sulphuric acid (H_2SO_4): 2 mol l^{-1}

- Sodium hydroxide (NaOH): 2 mol l^{-1}

- Ascorbic acid ($C_6H_8O_6$): 100 g l^{-1} (stable for 2 weeks in amber glass bottle, refrigerated)

- Acid molybdate solution I: ammonium heptamolybdate tetrahydrate [$(NH_4)_6Mo_7O_{24}$. 4 H_2O] 13 g per 100 ml and antimony potassium tartrate hemihydrate [$K(SbO)C_4H_4O_6$. ½ H_2O] 0.35 g per 100 ml (stable for 2 months in amber glass bottle)

• Orthophosphate standard stock solution: sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) 1.2 g in 100 ml water, stabilised with 0.05 g of anhydrous sodium carbonate (Na_2CO_3) as preservative

• Potassium peroxodisulphate: ($K_2S_2O_8$) 5 g per 100 ml (stable for 2 weeks in amber glass borosilicate bottle)

Procedure

All glassware (including sampling bottles) must be washed with hydrochloric acid (1.12 g ml^{-1}) at 40-50 °C and thoroughly rinsed. Do not use detergents containing phosphates and preferably use the glassware only for the determination of phosphorus.

For measuring orthophosphate:

1. Filter samples with pre-washed filters; discard the first 10 ml of filtrate, collect 5-40 ml (depending on concentrations expected).

2. Carry out a blank test with distilled water, using all of the reagents and performing the same procedure as for the samples.

3. Prepare orthophosphate calibration solutions in the concentration range of the samples (e.g. from 0.05 to 0.5 mg l^{-1}) with a volumetric pipette in 50 ml volumetric flasks (filling them only up to about 40 ml).

4. Transfer samples into 50 ml volumetric flasks with volumetric pipettes. Depending on expected concentrations, use 5-40 ml of sample, fill up to about 40 ml with distilled water.

5. Add, while swirling, first 1 ml ascorbic acid solution and then 2 ml acid molybdate solution, fill flask up to the 50 ml mark with distilled water and mix well.

6. After 10-30 minutes, measure absorbance at 880 nm using distilled water in the reference cell.

7. Plot absorbance of calibration solutions against their concentration and determine slope; check for linearity. Run an independently-prepared calibration solution with each series of samples, but especially when new batches of reagents are used.

8. Occasionally dean the glassware used for developing the colour complex with sodium hydroxide solution to remove colour deposits.

For measuring total, particulate and dissolved organic phosphorus:
1. Clean digestion vessels with about 50 ml of water and 2 ml of sulphuric acid (1.84 g ml^{-1}) in autoclave for 30 minutes at 115-120 °C, cool and rinse, repeat procedure several times, store covered.

2. Carry out a blank test with distilled water, using all of the reagents and performing the same procedure as for the samples.

3. Add 1 ml of sulphuric acid (4.5 mol l^{-1}) to 100 ml of sample to adjust pH to about 1 (further adjustment with sulphuric acid or sodium hydroxide solution (2 mol l^{-1}).

4. Pipette 5-40 ml of sample into digestion vessel, add 4 ml of potassium peroxodisulphate solution, mineralise in autoclave (or pressure cooker), or boil gently for 30 minutes.

5. Cool, adjust pH to between 3 and 10 with sodium hydroxide solution or sulphuric acid (2 mol l^{-1}), transfer to 50 ml flask and proceed as above for orthophosphate.

If large quantities of organic matter are present, oxidation with nitric acid-sulphuric acid may be necessary. Furthermore, arsenate may cause interference (see ISO, 1998a).

The test report should contain complete sample identification, reference to the method used, the results obtained and any further details likely to have influence on the results.

12.5.2 Analysis of nitrate

Several methods for determination of nitrate have been provided by the ISO, the simplest being a spectrometric measurement of the yellow compound formed by reaction of sulphosalicylic acid with nitrate and subsequent treatment with alkali (ISO, 1988). The equipment required is a spectrometer operating at a wavelength of 415 nm and optical path length of 40-50 mm, evaporating dishes, a water bath capable of accepting six or more dishes, and a water bath capable of thermostatic regulation to 25 °C. This method is suitable for surface and potable water samples and has a detection limit of 0.003 to 0.013 mg l^{-1} (depending on optical equipment). Interference from a range of substances, particularly chloride, orthophosphate, magnesium and manganese (III) is possible. Interference problems can be avoided with other spectrometric methods ISO (1986a,b).

12.5.3 Analysis of ammonia

A manual spectrometric method is given by ISO (1984a) which analyses a blue compound formed by the reaction of ammonium with salicylate and hypochlorite ions in the presence of sodium nitrosopentacyanoferrate (III) at a limit of detection of 0.003-0.008 mg l^{-1}. An automated procedure is given by ISO (1986c). A distillation and titration method is given by ISO (1984b).

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Chapter 13. LABORATORY ANALYSIS OF CYANOTOXINS

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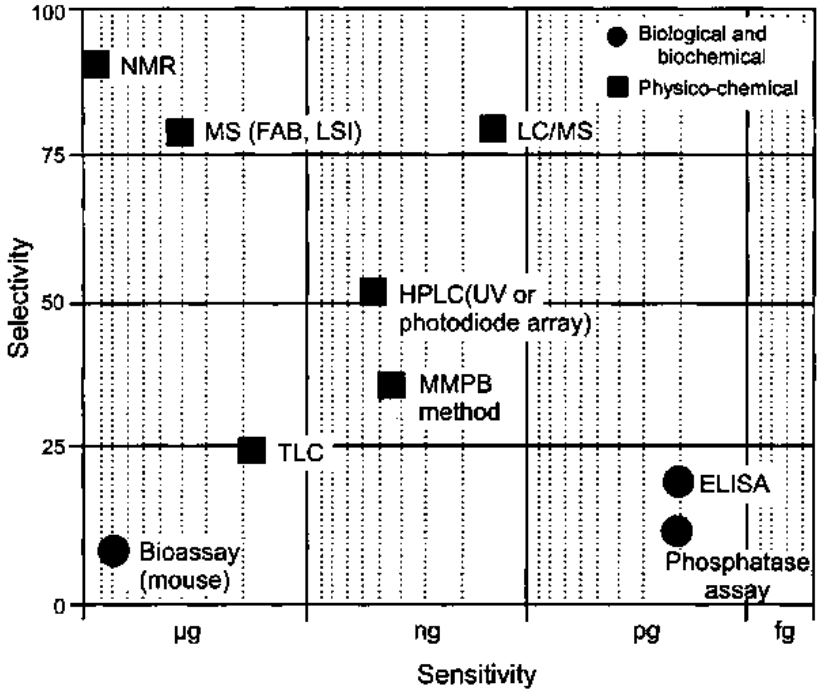
There is a diverse range of laboratory methods used to detect and identify cyanotoxins in water and cyanobacterial cells. These methods can vary greatly in their degree of sophistication and the information they provide. Relatively simple low cost methods can be employed which evaluate rapidly the potential hazard and allow management decisions to be taken. In contrast, highly sophisticated analytical techniques can be employed which determine precisely the identity and quantity of cyanotoxins. Techniques can be selected depending on the facilities and expertise available, coupled with the type of information required. For example, analysis of water for recreational purposes may only require detection of potentially harmful cyanobacteria, whereas ensuring potable water does not exceed guideline levels for cyanotoxins may require highly specialised equipment and expertise. Information obtained from simple, rapid screening methods such as microscopic examination can be used to make an informed decision on the type of bioassay or physicochemical technique which should be employed. It is important to remember that, currently, there is no single method which can be adopted which will provide adequate monitoring for all cyanotoxins in the increasing range of sample types which have to be evaluated. Selectivity and sensitivity are important criteria for the selection of methods. Figure 13.1 compares three biological and six chemical methods with respect to these criteria.

13.1 Sample handling and storage

When samples arrive in the laboratory, the type of analysis that will be carried out should have been anticipated previously. Full consideration should be given to the type of information required prior to sample collection (see Chapters 10 and 11). However, this is not always possible, particularly when a routine monitoring programme is not in place. Information obtained from the microscopic examination of the cyanobacterial taxa (Chapter 12) may also influence the choice of analysis but this information is not usually available until samples have reached the laboratory. Many samples will therefore require immediate evaluation on arrival in the laboratory to determine if any pre-treatment is needed prior to appropriate sample storage.

Figure 13.1 Relationship between sensitivity and selectivity of analytical methods for microcystins (see text for explanation of methods)



Samples for cyanotoxin analysis should be refrigerated in the dark to prevent toxin degradation but it is essential that storage be kept to a minimum (preferably less than 24 hours). Where prolonged storage is required, samples can be frozen, although this will release toxins from the cells and only the total amount of toxin in the sample can then be determined. It is often desirable, especially for water treatment purposes, to estimate the amount of toxin contained within cells as well as that which is dissolved in the water. Where this information is required, samples should be gently filtered as soon as practically possible and if necessary the filters and filtrate can be refrigerated or frozen.

A concentration step for cyanobacterial cells can be useful especially for less sensitive cyanotoxin assay methods. This can be achieved at the sampling location with the use of a plankton net or by using lakeside filtration apparatus (Chapter 12). In the laboratory, concentration can be achieved by allowing buoyant cells to accumulate in the upper part of a separating funnel which enables excess water to be removed. This method can concentrate cells by at least ten fold but is dependent on cells being vacuolate (i.e. floating). It may also lead to a bias if most of the cells do not accumulate at the surface because floating cells may have different toxin quotas than cells which do not. Centrifugation is useful but is often limited by the relatively small volume of laboratory centrifuge tubes and, furthermore, problems can be encountered when trying to pellet vacuolate cells. Filtration is increasingly popular because it can allow concentration of cells by several orders of magnitude and enables the weight of the cell mass to be determined. The following filtration method can be employed (Figure 13.2).

Apparatus

- Oven/incubator set at 45 °C or freeze-drier

- Vacuum dessicator

- Balance accurate to 0.0001 g

- Glass fibre filters 70 mm, GF/C, 1.2 µm will retain most cyanobacteria but the smaller pore size of GF/F (0.7 µm) is required for picoplankton cyanobacterial cells.

- Filtration cup and vacuum pump

- Measuring cylinder

- Plastic Petri dishes

Procedure

1. Place filters in dessicator under vacuum, then remove at intervals and weigh. Filters are ready to use once a constant weight is obtained. It is useful to place each weighed filter in a separate Petri dish with the weight recorded on the dish. This makes samples easy to handle and prevents confusion.

2. Mix the water sample by inverting the bottle several times, then measure a known volume using the measuring cylinder. This volume will differ depending on the concentration of cells present, because only small volumes of water with a high concentration of cells can be filtered before the filter becomes blocked.

3. Using one of the pre-weighed filters, filter the water sample and then return the filter to the labelled Petri dish.

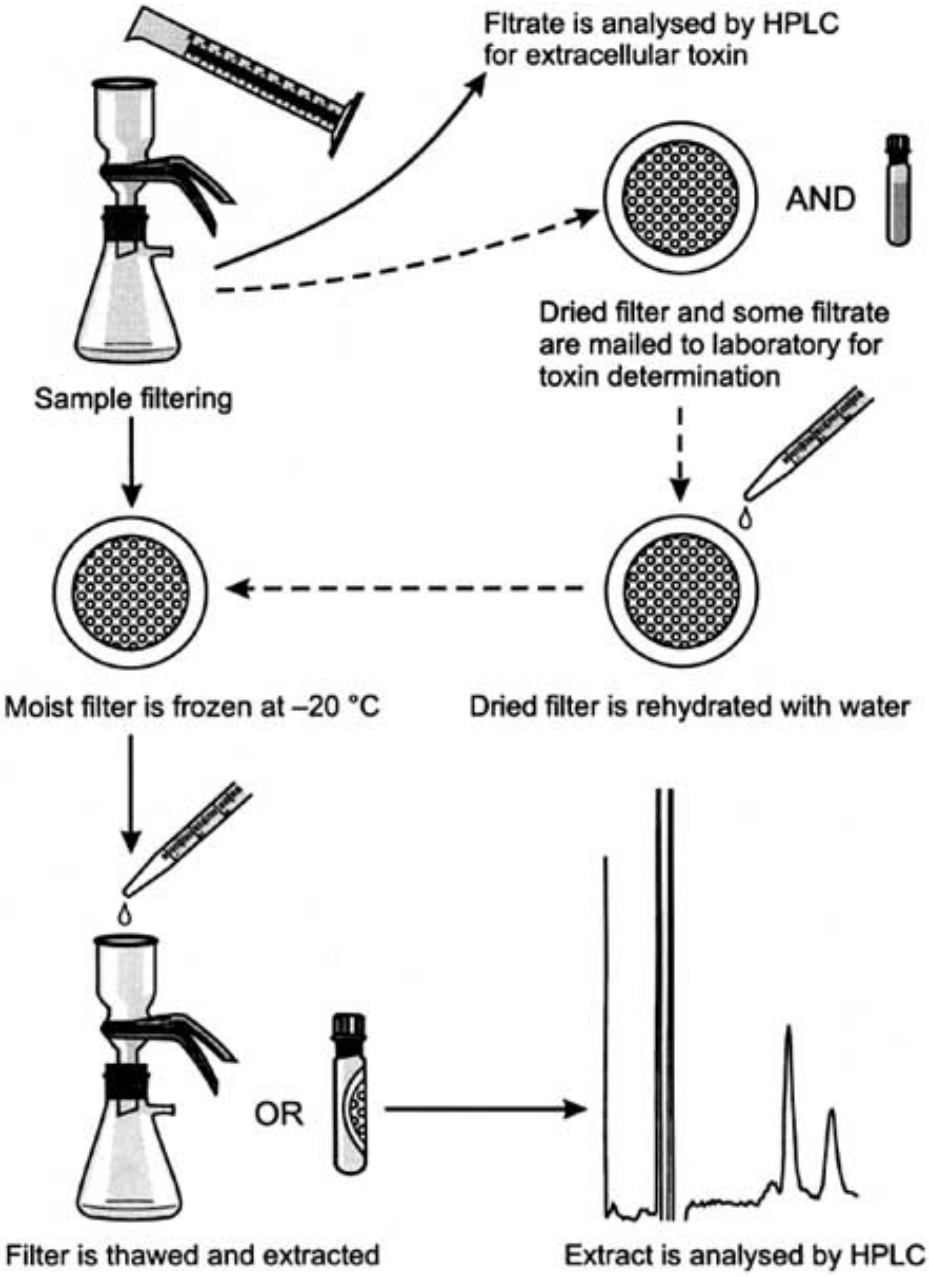
4. Keeping the filter in the Petri dish place it either in the drying oven or freeze-drier. If using on oven, the temperature must be kept below 50 °C.

5. Once dry, return filters to the dessicator and weigh to constant weight. Calculate the dry weight of cells collected by subtraction of the initial weight of the filter.

6. Cyanotoxins can either be extracted immediately, or filters may be stored until required, preferably in a freezer.

By following this procedure it is possible to relate cyanotoxin content to the dry weight of particulate matter extracted as well as to the volume of water filtered. Sometimes dry weights are affected by the presence of large particles in the sample (e.g. zooplankton or feathers from waterfowl). This can easily be overcome by carrying out pre-screening using a 1-2 mm sieve if necessary. Furthermore, particulate matter (i.e. seston) may consist of plankton organisms in addition to cyanobacteria (e.g. algae, rotifers, bacteria, detritus). A brief microscopic check of the fresh sample or a preserved sub-sample (see Chapter 11) will reveal whether or not the dry weight can be attributed mainly to cyanobacteria. In dense blooms and scums this is usually the case but, in samples from more homogenously dispersed situations, other components may dominate and the relating of the toxins to dry weight will therefore underestimate the toxin content of the cells. If an accurate balance is not available, the filtration method can be used but the cyanotoxin content can be related only to the volume of water filtered.

Figure 13.2 Toxin extraction by filtration



Dense cyanobacterial scums or samples concentrated using a plankton net are often freeze-dried. This provides a dry powder which can easily be weighed prior to extraction. However, great care must be taken with such dry powders because they can easily become airborne and may present a health hazard through inhalation.

Most sample handling and storage methods for cyanotoxin analysis have been evaluated primarily for microcystins, hence the stability of other cyano-toxins may not be fully understood. It is therefore important to evaluate the chosen method if other cyanotoxins are being monitored.

13.2 Sample preparation for cyanotoxin determination and bioassays

13.2.1 Extraction

For cyanotoxin detection, samples may be extracted from cells or biological matrices such as animal tissue, or water samples. Cells have successfully been extracted in a number of different liquid phases, and some of the most popular are 5 per cent acetic acid, methanol, acidified methanol (Trifluoroacetic acid (TFA) added), aqueous methanol, and butanol:methanol:water (1:4:15) (Harada, 1996). The efficiency of these methods depends on the sample and the cyanotoxins present. It has been found that the more polar extraction media, such as 5 per cent acetic acid, provide reasonable extraction efficiency of the more polar microcystins but give very poor recovery of hydrophobic microcystins (Lawton *et al.,* 1994a). Methanol has been advocated as the most suitable solvent because it gives good extraction efficiency and has the added advantage of allowing rapid sample concentration through evaporation. It has since been suggested that 100 per cent methanol may give poor recovery of more polar microcystins; but the addition of a small percentage of water overcomes this. Fastner *et al.* (1998) showed that, especially for lyophilised field samples dominated by *Microcystis* spp., extraction with 75 per cent methanol and 25 per cent water (by weight) was most effective. One approach which has been used routinely, with success, for microcystins is as follows:

Apparatus

- Rotary evaporation equipment
- Glass beakers, 50 ml
- Measuring cylinder, 20 ml
- Rotary evaporation flasks, 50 ml, pear-shaped
- Pipette, 0.25 ml
- Glass vials or microcentrifuge tubes, ~1 ml.

Reagents

- Methanol, high purity if possible.
- Aqueous methanol (75 per cent, v/v)

Procedure

1. Place each filter containing cells into a glass beaker with 20 ml of either pure methanol or 75 per cent aqueous methanol. Filters may be cut into small pieces with scissors but gloves must be worn and care must be taken not to cross contaminate samples or to lose material sticking to the scissors.

2. Allow filters to extract for 1 hour. If time is limiting, extract for 30 minutes because the shorter time has been found to reduce only slightly the recovery of microcystins.

3. Decant extract into rotary evaporation flask and dry *in vacuo* at 45 °C.

4. Add a further 20 ml of extraction solvent to the filter in the beaker and allow to extract as before.

5. Repeat this process a total of three times, each time decanting the extract into the same rotary evaporation flask.

6. Add 0.25 ml of methanol to the dry extract in the rotary evaporation flask, mix and remove the resuspended extract to a glass vial or microcentrifuge tube. Repeat this with a second 0.25 ml of methanol, placing both aliquots in the same vial or tube.

7. Consideration must be given to the type of analysis which is going to follow extraction because organic solvents are toxic to bioassay organisms. This can often be overcome by using a volatile medium, followed by evaporation and resuspension in a medium which is compatible with the assay method. With very toxic samples and/or sensitive bioassays, dilution with the assay medium (using a control containing the same per cent of solvent) is sufficient to obtain non-toxic solvent concentrations.

Two other rapid extraction procedures have also been successfully applied:

- After freezing and thawing to disrupt cells, toxins may be extracted in the filtration device by passing aqueous methanol or water, followed by methanol, through the filters by suction (use a total of 5-25 ml of solvent). Extracts can be used directly for analysis or further concentrated if enhanced sensitivity is needed (Figure 13.2) (Utkilen and Gjølme, 1994). This method requires little equipment other than a filtration device and deep-freezer.

- Membrane filters with approximately 20 mg of freeze-dried material may be extracted in 2 ml microcentrifuge tubes by adding 1.5 ml solvent (preferably 75 per cent aqueous methanol), sonicating, shaking for 30 minutes and centrifuging. This step extracts a large share of the microcystins, and extraction is more complete if the pellet is re-extracted twice and the supernatants are pooled (Fastner *et al*., 1998).

Saxitoxins are often extracted in acidified media including acetic acid, hydrochloric acid (HCl) and acidified methanol (Fernandez and Cembella, 1995). A recent study found methanol acidified with TFA was the most efficient solvent when extracting saxitoxin and neosaxitoxin from cyanobacterial cells (McElhiney *et al.,* 1998). Anatoxin-a has been successfully extracted with water, acidified water, acidified methanol (Edwards *et al.,* 1992), chloroform followed by hydrochloric acid (Harada *et al.,* 1989) or by dichloromethane after an acidification and neutralisation step (Bumke-Vogt *et al.,* 1996).

Box 13.1 When is sample cleanup needed?

Try analysis or bioassays without performing cleanup if:

- Samples are dominated by cyanobacteria; or

- Precise quantification of toxins and complete identification of minor toxin components is less important than rapid analysis of a larger number of samples.

Introduce cleanup if:

- Cyanobacteria are a minor component of the sample.

- Low concentrations of cyanotoxins are anticipated.

- Identification and quantification is important.

- Chromatograms obtained without cleanup show considerable baseline problems or poor peak separation.

- Bioassay results suggest influence from further substances.

To store extracts prior to analysis or bioassays, blow to dryness and deepfreeze (-18 °C). Samples may then be re-dissolved in the solvent at a concentration adequate for the subsequent chemical analysis or bioassay.

13.2.2 Sample cleanup

The purpose of cleanup is to eliminate impurities by a simple operation without loss of analyte and where the concentration of cyanotoxins is low, it also enables enrichment of the analyte. Whether or not cleanup is necessary depends largely on the precision of the toxin determination and quantification required. Without cleanup, small toxin peaks in chromatograms may be missed due to masking by other matrix substances eluting simultaneously, and toxin concentrations may be overestimated if peaks are not clearly separated from matrix substances. However, for many screening and monitoring purposes, particularly of bloom samples containing little material other than cyanobacteria, the carrying out of bioassays or toxin analysis without sample cleanup has given satisfactory results (see Box 13.1 for criteria when to perform cleanup).

The establishment of a versatile cleanup method would give an additional advantage to the analysis of microcystins in other matrixes such as biological samples, fish and shellfish. Furthermore, it would give useful information for preparative separation of microcystins and their degradation products. Octadecyl silanised (ODS) silica gel has been employed extensively to facilitate sample cleanup and trace enrichment of microcystins and nodularin because it retains the toxins and allows interference compounds to pass through (Lawton *et al*., 1994a; Harada, 1996).

Concentration and cleanup method for microcystins and nodularins in water samples

Apparatus

- Porcelain filter funnel, 110 mm diameter and Buchner flask, 1 litre or similar
- Filter disks, GP/C, 110 mm
- Measuring cylinder, 500 ml
- Pipettes, various
- Water vacuum pump
- Glass bottles, 500 ml
- Vacuum manifold
- Solid phase extraction cartridges, 1 g trifunctional, end-capped C18
- PTFE tubing and cartridge adapters
- Glass sample tubes
- Drying hot block (45 °C) with blow-down nitrogen
- Microcentrifuge tube (1.5 ml)

Reagents

- Sodium thiosulphate solution, 1 g $Na_2O_3.5H_2O$ made up to 100 ml with distilled water
- Aqueous methanol, solutions containing 10, 20 and 30 per cent methanol in water
- Trifluoroacetic acid solution, 10 per cent TFA v/v in water
- Trifluoroacetic acid solution, 0.1 per cent TFA v/v in methanol
- Methanol
- Water

Note: all reagents should be analytical quality

Procedure

1. Mix the water sample by inverting the container several times, then measure a 500 ml portion of the sample and filter it gently through a GF/C filter disc. The filter can be retained and extracted as described above to determine the particulate microcystin concentration.

2. Add 0.1 ml sodium thiosulphate solution to eliminate free residual chlorine. Shake the water sample vigorously and let it stand for a few minutes, then add 5 ml of the 10 per cent TFA and mix before passing the sample through a GF/C filter disc.

3. Place the sample in a 500 ml glass bottle, add 5 ml methanol and mix. The sample is now ready for solid phase extraction (SPE).

4. Solid phase extraction cartridges are prepared by attaching them to the vacuum manifold system and then conditioning them with 10 ml methanol followed by 10 ml water, ensuring that the cartridge does not become dry at any time. The methanol and water eluates are discarded.

5. Using PTFE tubing and adapter, attach a tube from the bottle containing the water sample to the top of the SPE cartridge. The vacuum draws the sample through the tubing and through the cartridge. The water is not collected but allowed to run to waste through the water pump.

6. Once all of the water sample has passed through it, the cartridge is washed with 10 ml of the 10 per cent methanol followed by 10 ml of the 20 per cent methanol and then

finally washed with 10 ml of the 30 per cent methanol. The eluate from the three washes is discarded.

7. The cartridge is eluted with 3 ml of 0.1 per cent TFA in methanol. This is collected in a sample tube and dried on a hot block (45 °C) under a gentle stream of nitrogen gas.

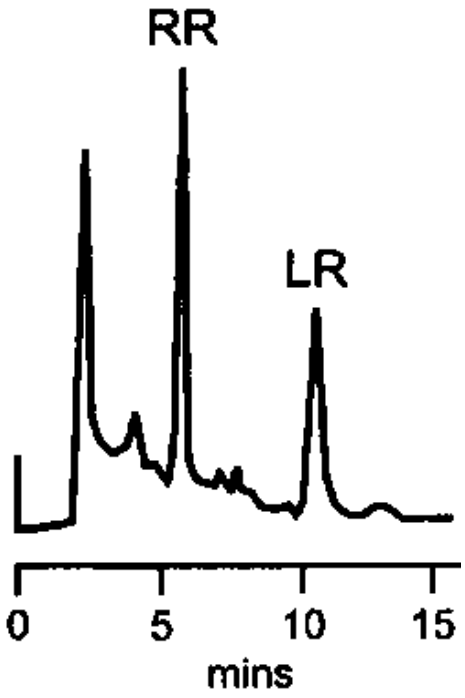
8. Samples are resuspended in 0.1 ml methanol and placed in a microcentrifuge tube. A further 0.1 ml of methanol is used to rinse the sample tube and this is combined with the first aliquot.

9. This sample can now be analysed or it can be dried and stored in the freezer until required.

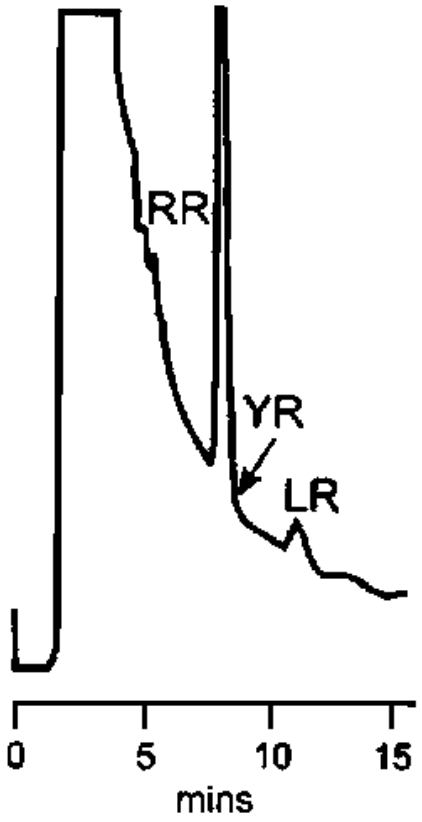
10. It is recommended that this procedure is carried out in duplicate for each water sample analysed.

Figure 13.3 HPLC profiles of water samples containing microcystins.

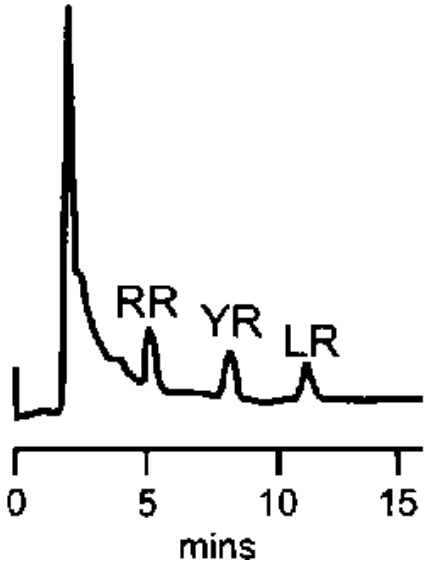
A. A toxic fraction from a bloom sample and of fractions from lake water (1 µg each of microcystin-RR, -YR, -LR added);



B. Before cleanup with silica gel cartridge;



C. After cleanup with silica gel cartridge



Notes

- Different systems can be used to pass the water sample through the SPE cartridge but a specially designed vacuum manifold apparatus is available for use with SPE cartridges. Most of these systems are designed so that a number of cartridges can be attached at one time (e.g. 10 or 20). Automated systems are also available, and peristaltic pumps have also been used successfully, but are not as practical.

- It is vitally important that all apparatus used is thoroughly cleaned to ensure no external contamination. Equipment used for trace enrichment should be dedicated to this procedure only, in order to reduce the opportunities for contamination.

Some modifications to the single SPE method have been proposed with promising results. Figure 13.3A shows the High Performance Liquid Chromatography (HPLC) chromatogram of a toxic fraction from a cyanobacterial bloom sample; the microcystins have been detected with limited interference. However, this method is not always effective for analysis of the toxins in raw water samples because of the occurrence of serious background peaks as shown in Figure 13.3B. To eliminate effectively the impurities, Tsuji *et al.* (1994) established a tandem cleanup method using ODS silica gel and silica gel cartridges as follows. The water sample was first applied to an ODS silica gel cartridge and the desired fraction was eluted with 90 per cent aqueous methanol. Next, the resultant eluate, in 100 per cent methanol, was applied to a silica gel cartridge. After washing with methanol, the toxin-containing fraction was obtained by elution with 10 per cent water-0.1 per cent TFA in methanol. Figure 13.3C shows the chromatogram after the silica gel cleanup, indicating that most of the impurities can be removed. Tsuji *et al.* (1996) used HPLC with ultra violet (UV) detection and Liquid Chromatography/Mass Spectrometry (LC/MS) combined with this tandem cleanup system to report the intracellular and extracellular microcystin levels between 1992 and 1995 for Japanese lakes. Microcystins-LR, -YR and -RR were detected at 0.02-2.64 μg l^{-1} in cell-free water and at 0.02-378 μg l^{-1} in the cells during this period.

Recently, an immunoaffinity purification method has been developed using an anti-microcystin-LR monoclonal antibody (Kondo *et al.,* 1996). This cleanup method was found to be remarkably effective in the removal of contaminants in the hepatic cytosol and enabled the analysis of microcystins and their metabolites, formed *in vivo* in mouse and rat livers, by HPLC and Frit-FAB LC/MS. Figure 13.4 shows the HPLC profiles of a cytosolic extract from mouse liver spiked with 5 μg each of microcystins-RR and -LR. Before the immunoaffinity purification, the spiked microcystins could not be accurately quantified due to the many impurities (Figure 13.4A), whereas after the immunoaffinity purification, the impurities were effectively eliminated and the peaks of the microcystins were clearly detected (Figure 13.4B). Although this immunoaffinity column is not commercially available, it has the potential to enable significant progress in a number of important areas of research; for example in metabolism studies and in elucidation of the fate of microcystins in the environment.

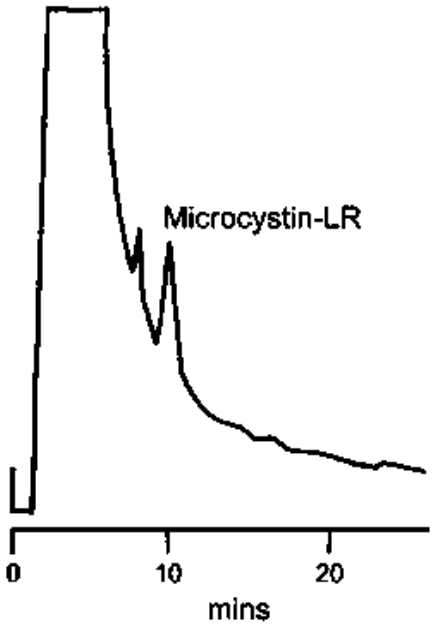
Research into appropriate cleanup methods for other cyanotoxins has still to be carried out, although a similar approach to that which has been developed for microcystins may prove useful.

13.3 Toxicity tests and bioassays

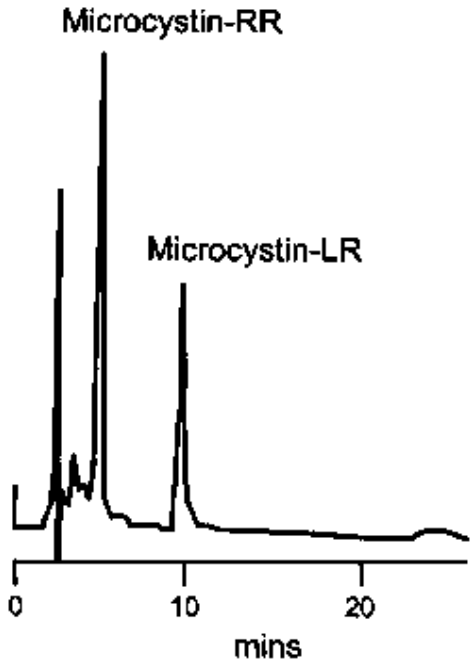
There have been many biological detection methods developed for cyanotoxins that use the bioactivity of the toxins (Table 13.1), such as potent hepatotoxicity, neurotoxicity, cytotoxicity, enzymatic activity and immunological interactions. However, for many years, the mouse bioassay alone has been used to determine bloom toxicity. Although this bioassay provides a measure of the total toxicity (response) within a few hours, it is generally not very sensitive or specific. Considerable research efforts have been made to find suitable alternative methods to the mouse bioassay as a routine monitoring assay for cyanotoxins and many novel and sensitive methods have become available in recent years. However, no single method is currently available to replace the mouse for the detection of all cyanotoxins using a single assay, and further validation and comparison of methods is needed before general recommendations on their application can be given (see also Box 13.2).

Figure 13.4 HPLC profiles of a cytosolic extract from mouse liver spiked with 5 µg each of microcystin-RR and -LR.

A. Extract after heat-denaturation, pronase digestion, and ODS silica gel cleanup;



B. Extract after further immunoaffinity purification



Different bioassays are described below. Users are advised to test their suitability for locally-prevalent cyanotoxins with other methods, such as chemical toxin analysis (see section 13.4). Sensitivity and selectivity are important criteria for the selection of methods. Figure 13.1 shows the sensitivity of three different biological methods in relation to six methods of chemical analysis.

13.3.1 Mouse bioassay

Male Swiss Albino mice are the most used animals for toxicity testing for cyanotoxins. Toxicity is tested by intraperitoneal injection (i.p.) of 0.1-1.0 ml of a lysate of cyanobacteria prepared either by sonication or by freeze-thawing of a cell suspension which has been sterilized by membrane ultra-filtration. Samples can be suspended in water or physiological saline solution which is preferred if the volume to be injected is 0.5 ml or greater.

Table 13.1 Bioassays for the detection of cyanotoxins

Method	Toxins[1]	Cost[2]			Comments	Reference(s)
		Cap.	Con.	Pers.		
Vertebrate						
Mouse	M,N,A, A(s),C,S				Requires licence, not permitted in some countries	Falconer, 1993
Invertebrate						
Brine shrimp	M,N	L	L(H)	M	Commercial kit available but increases cost	Kiviranta *et al*., 1991; Campbell *et al.,* 1994
Daphnia sp.	M,N	L	L	H	Culturing is labour intensive	Lawton *et al.*, 1994b
Thamnotox	M,A,C(?)	L	H	M	Commercial kit available but requires full evaluation for cyanotoxins	Kozma, 1997
Mosquito	M	L	L	M	Difficult to handle	Kiviranta *et al*., 1993
Fruitfly	M,N	L	L	L	Easy to culture	Swoboda *et al*., 1994
Locust	S	L	M	L	Easy to handle	McElhiney *et al*., 1998
House fly	S	L	L	L	Difficult to administer toxin	Ross *et al*., 1985
Bacterial						
Microtox	M,N	H	H	L	No correlation	Lawton *et al*., 1994b
Serratia sp.	M,S	L	L	M	Poor correlation	Lawton *et al*., 1994b
Biochemical						
PPase inhibition	M,N				Very sensitive	
Radioactive		M	M	L	Requires special facilities	Holmes, 1991
Colorimetric		L/M	M	L	Requires purified enzyme	An and Carmichael, 1994
AChE	A(s)	L/M	L	L	Only alternative bioassay for A(s) may react with OP[3] pesticides	Mahmood and Carmichael, 1987
ELISA					Very sensitive	
Polyclonal	M,N	M	H	L	Reactivity for variants may vary	Chu *et al*., 1989
Monoclonal	M,N	M	H	L	Reactivity for variants may vary	Ueno *et al*., 1996
Polyclonal	S	M	H	L	Variable cross-reactivity, does not detect C-toxins	Cembella *et al*., 1995
Mammalian cells						
Hepatocytes	M,N	M	M	M	Sensitive and rapid bioassay	Heinze, 1996

V79 fibroblasts	M	H	H	H	Some false negatives observed	Lawton *et al.,* 1994b
Neuroblastoma	S	H	H	H	Requires careful standardisation	Cembella *et al*., 1995; Gallacher and Birkbeck, 1992

ELISA Enzyme linked immuno sorbent assay

AChE Acetylcholinesterase

[1] M Microcystins; N Nodularins; A Anatoxin-a A(s) Anatoxin-a(S); C Cylindrospermosin; S Saxitoxin

[2] Cap. Capital; Con. Consumable; Pers. Personnel; L Low; M Medium; H High

[3] OP Organophosphorus

Box 13.2 When are bioassays needed?

Bioassays are recommended if any of these conditions are fulfilled:

- A laboratory can easily establish them, but has little or no access to adequate equipment and expertise for establishing physicochemical analysis, or little means of subcontracting analysis.
- There is indication of cyanotoxins other than (or in addition to) the known toxicants.
- Cyanobacterial taxa dominate, the toxins of which have not yet been well studied and which may therefore contain unknown toxic metabolites.
- Confirmation of results from physicochemical analysis is required, especially to confirm bioactivity.
- Validation of physicochemical methods by an alternative method is desired.

Mice should be observed for 24 h and then killed by an approved method (Falconer, 1993). The observation period must be extended to seven days where cylindrospermopsin is suspected and animals should be injected with sterile samples. This toxin demonstrates protracted symptoms which result from progressive organ failure, specifically liver and kidneys. At the end of the observation period post-mortem examination of tissue injury is performed. The observed symptoms and the results of the post-mortem are used to determine which cyanotoxin is present (see Chapter 3 and 4 for toxicology). However, where more than one type of cyanotoxin is present, the more rapid-acting toxin may mask other symptoms.

Toxicity is expressed as LD_{50} mg cell dry weight per kg mouse body weight. Values are classified as follows (Lawton *et al.* 1994b):

> 1,000	non toxic
500-1,000	low toxicity
100-500	medium toxicity
< 100	high toxicity

Note that in some countries non-toxic limits may be at least 2,000 mg cell dry weight per kg mouse body weight.

13.3.2 Invertebrate bioassays

A number of invertebrates have been investigated for use in routine bioassays for cyanotoxins. Of these, the brine shrimp (*Artemia salina*) has been the most popular, because no culture maintenance or specialist equipment is required. Brine shrimps have been exploited for many years for the detection of toxic secondary metabolites and are now commercially available as standardised test kits (although use of the kits increases the cost of performing the assay). Brine shrimp eggs are readily available from biological supply companies and can be stored for several years at -20 °C without loss of viability.

Brine shrimp bioassay

Apparatus

- Conical flasks, glass, 250 ml
- Microtitre plates, 96-well
- Incubator, 25 °C
- Desk lamp
- Pasteur pipette and bulb
- Pipette, 0.1 ml
- Universal bottle, 25 ml
- Dissecting microscope with low power objective

Reagents

- Brine shrimp eggs, stored in freezer
- Brine shrimp medium (BSM) stock solution, composed of:

Sodium chloride (NaCl) 300 g
Calcium chloride dihydrate ($CaCl.2H_2O$) 3 g
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) 15 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 5 g
Potassium chloride (KCl) 8 g
Glycine 60 g
Disodium glycerophosphate 30 g

- Methanol or formalin

The stock solution chemicals are dissolved in 1.25 litres of distilled water, dissolving each chemical separately in the order shown. It is important to add the disodium glycerophosphate last to prevent an insoluble precipitate occurring. The stock solution should be stored in a brown glass bottle in the refrigerator (~4 °C).

Procedure

1. Mix 20 ml of BSM stock solution with 140 ml distilled water in a 250 ml conical flask.

2. Add 100 mg of brine shrimp eggs and incubate at 25 °C until they hatch (usually 36-48 hours). Once a regular hatching time has been established try to keep it constant.

3. Separate the hatched larvae from unhatched eggs and egg cases by transferring them into a shallow dish, e.g. Petri dish or slightly deeper.

4. Position a desk lamp to one side of the dish and allow the larvae to concentrate towards the light.

5. Using a Pasteur pipette carefully, using slow steady suction on the pipette bulb, collect the hatched larvae.

6. Place larvae in a universal bottle and repeat the collection process until most of the hatched larvae have been collected.

7. If a substantial number of unhatched eggs have been collected in error, the separation can be repeated by placing the collected larvae in a clean dish and illuminating.

8. Using a mechanical pipette with plastic tips (first use a sharp blade to remove the first few mm of the tip) pipette 0.1 ml of the larvae suspension into a microtitre well. Make sure the larvae suspension is regularly mixed because they tend to cluster rapidly in the bottle. Mixing is easier if the bottle is only half full.

9. Examine the well containing larvae under the microscope to determine the approximate number of larvae in 0.1 ml. Ideally this should be between 15 and 25 larvae. Dilute the sample with fresh media if the larvae are too concentrated. Repeat the pipetting a few times to ensure reasonably reproducible numbers of larvae are being deposited in each well and that they are alive and look healthy.

10. Fill as many wells with larvae suspension as will be required to carry out the bioassay.

11. To determine the toxicity of cyanobacterial cells, the sample must first be extracted. This can be done as described above (section 13.2.1) from filter discs, but if the final extract is in methanol it must be diluted in BSM so that the final concentration of methanol does not exceed 5 per cent in the test well.

12. A dilution series of each sample should be prepared and 0.1 ml of the test solution added to the larvae in the wells in triplicate. The microtitre plate is then incubated for 18 hours at 25 °C after which the percentage mortality is calculated.

13. First, with the help of a microscope, count the number of dead or immobilised larvae in each well, then add a few drops of formalin or methanol and wait until all the larvae are dead. Now count the total number of larvae in each well and calculate the percentage mortality using a mean of the three replicates.

14. By plotting the concentration of cyanobacteria against the percentage mortality the LC_{50} value (i.e. the concentration of cell extract which caused 50 per cent mortality) for each sample can be determined.

Notes

- Care must be taken to exclude particulate material from the assay and therefore extracts must be filtered or centrifuged.

- High concentrations of cell extract cause false positives, probably due to oxygen depletion caused by the activity of bacteria, therefore sample cleanup (see section 13.2.2) is necessary to detect low concentrations of toxin successfully. Sample cleanup, e.g. the use of SPE, can also enhance the specificity of the assay because it selectively concentrates microcystins and nodularins, reducing interference by other compounds.

When 21 hepatotoxic bloom samples were assessed by this assay the results compared very favourably with both mouse bioassay and HPLC (Lawton *et al.,* 1994b). This assay has not been fully evaluated for toxins other than microcystins, although there does appear to be a correlation between anatoxin-a content and toxicity.

Daphnia bioassays can successfully detect microcystins, although standardised culturing is extremely labour intensive (Baird *et al.,* 1989). A commercially prepared test kit, similar to the brine shrimp assay in that it uses the resting stage of an aquatic invertebrate, *Thamnocephalus platyurus* has been found to be sensitive to a number of cyanotoxins (Kozma, 1997). These kits are relatively expensive and have a limited shelf-life (6 months), although the standardised format of this bioassay leads to highly reproducible results and low inter-laboratory variability (Kozma, 1997). The use of mosquito adults and larvae have both been investigated as potential bioassays (Turell and Middlebrook, 1988; Kiviranta *et al.,* 1993). Adults were injected and larvae immersed in aqueous extracts. Both methods were relatively sensitive but have not been widely adopted due to the difficulties of handling this organism. The other insect which has been shown to detect microcystins successfully in bloom samples is the fruit fly (*Drosophila melanogaster*) (Swoboda *et al.,* 1994). These organisms are easy to maintain in the laboratory, with no special equipment required. Toxin is administered orally by adding filter discs spotted with sample plus sucrose to tubes containing pre-starved (24 h) flies. The flies were not, however, sensitive to neurotoxic *Aphanizomenon* (Swoboda *et al.,* 1994).

Two invertebrate assays have been investigated for the detection of saxitoxins. Firstly, adult house flies injected with purified toxins and natural samples (shellfish extracts) gave results which compared well with toxicity determined by mouse. However, the flies are difficult to handle and require microinjection (1.5 µl) which is difficult to administer (Ross *et al.,* 1985). A locust bioassay has recently been found to detect saxitoxins successfully in a range of samples, namely cyanobacteria and shellfish (McElhiney *et al.,* 1998). Locusts are easy to handle because they can be readily immobilised by holding their rear legs. Samples are administered by injection (10 µl) and results are obtained within 90 minutes (McElhiney *et al.,* 1998). The LD_{50} for pure saxitoxin was 8 µg g^{-1} but the bioassay was not sensitive to microcystin-LR or anatoxin-a.

Locust bioassay for saxitoxins

Apparatus

- 10-25 pi syringe, e.g. type used for GC analysis
- Desert locust (*Schistocerca gregaria*), male early fifth instar
- Plastic container, 500 ml, foil covered with a number of small air holes

Reagents

- Simple saline, 7.5 g sodium chloride (NaCl) and 0.37 g potassium chloride (KCl) dissolved in 1 litre distilled water

Procedure

1. Prepare a dilution series in simple saline of the test sample. It has been found that saxitoxins can be extracted well from cyanobacterial cells with acidified methanol (TFA added). Samples can be dried then resuspended in saline for use in the bioassay.

2. Pick up locust by hind legs and inject 10 pi along the abdomen, (parallel to the body) between the second and third segment. Inject three locusts for each concentration and inject controls with saline only.

3. Place each locust in a clear plastic container and observe for 90 minutes.

4. Death or the inability to self-right when placed on their back is recorded as a positive result.

5. The time of death is an indication of saxitoxin concentration.

13.3.3 Bacterial bioassays

Bacterial bioassays have been investigated to determine if they can provide simple routine methods for cyanotoxin detection. The one that has received the most attention is the Microtox bioluminescence assay which indicates toxicity by a reduction in the light emitted by the test bacterium (*Photobacterium phosphoreum).* Initial investigations suggested that this system may be suitable for the rapid detection of microcystins in bloom samples (Lawton *et al.,* 1990) although more detailed analysis revealed that the assay responded to unknown components of cyanobacterial extracts other than microcystins (Campbell *et al.,* 1994). Several studies have now been published that clearly indicate there is no correlation between response in the Microtox assay and cellular content of the known cyanotoxins (Lawton *et al.,* 1994b; Vezie et al., 1996).

A second bacterial bioassay which used the inhibition of pigment (prodigiosin) formation in *Serratia marcescens* as an indication of toxicity has been proposed by Dierstein *et al.* (1989). This bioassay was thought to be useful for saxitoxins and microcystins. However, like the Microtox system, little correlation was found between actual content of known cyanotoxins and inhibition of pigment formation (Lawton *et al.,* 1994b).

13.3.4 Biochemical assays

The protein phosphatase inhibition assay is a sensitive screening method for microcystins and nodularins which uses the biochemical activity of these toxins. One

version is based on the quantitation of ^{32}P-phosphate released from a radiolabelled substrate (Holmes, 1991; Lambert *et al.,* 1994) by the activity of the protein phosphatase enzyme (PP1 and PP2A). It is sensitive to sub-nanogram levels of microcystin and is a rapid assay allowing the analysis of many samples in a few hours. The method has been applied to detect microcystin class compounds in the marine environment, to extracts of liver tissue taken from Atlantic salmon afflicted with netpen liver disease (Andersen *et al.,* 1993) and to hydrophobic microcystins from freshwater cyanobacteria (Craig *et al.,* 1993). The method has also been successfully used for quantitation of microcystins in drinking water before and after water treatment (Lambert *et al.,* 1994). The detected amounts in raw and treated waters were estimated to be 0.12-0.87 and 0.09-0.18 µg l^{-1}, respectively. Although this method has been widely used in research, there is a reluctance in adopting it for the routine monitoring of microcystins because of the requirement to use radioactivity which necessitates specialised laboratory equipment and regulations.

An and Carmichael (1994) have used a colorimetric protein phosphatase inhibition assay which avoids the complications of using radioactive materials. Isobe *et al.* (1995) reported a firefly bioluminescence system for the detection of protein phosphatase 2A inhibitors, in which luciferin phosphate is hydrolysed to luciferin and inorganic phosphate by protein phosphatase 2A. The use of the protein phosphatase inhibition assay is extremely helpful to confirm biological activity, and hence toxicity, of microcystins in environmental samples. The non-radioactive bioassay may therefore be Used increasingly for the routine screening of water samples, as shown recently by Ward *et al.* (1997).

The biochemical activity of anatoxin-a(S) can be exploited in an enzyme-based assay to detect the inhibition of acetylcholinesterase (AChE), thereby providing an indication of the presence of this toxin (Mahmood and Carmichael, 1987). This is a sensitive method and is the only alternative to the mouse bioassay currently available for this toxin. The assay is not selective because it will also detect other toxicants, such as organophosphorus-based pesticides.

13.3.5 Immunological detection

The Enzyme-Linked Immuno Sorbent Assay (ELISA) technique is currently the most promising method for rapid sample screening for microcystins because of its sensitivity, specificity and ease of operation. Monoclonal antibodies raised against microcystin-LA were initially developed by Kfir *et al.* (1986) and offered a simple approach to a general immunoassay for microcystins. An ELISA technique was subsequently developed by Chu *et al.* (1989) as a practical method. This assay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR. The antisera showed good cross-reactivity with microcystins-LR, -RR, -YR and nodularin, but less with -LY and -LA. The sensitivity of the assay showed approximately 50 per cent binding at a toxin concentration of 1 ng ml^{-1} which is appropriate for normal water quality testing. In fact, this method has been successfully employed for quantitation of cyanobacterial hepatotoxins in domestic water supplies and biomass extracts with detection limits of 0.2 µg l^{-1} and 0.25 µg g^{-1} for water and biomass samples, respectively (Chu *et al.,* 1990).

Recently, Nagata *et al.* (1995) produced six monoclonal antibodies against microcystin-LR. Among them, M8H5 antibody showed cross-reactivity with microcystin-RR (106 per cent), microcystin-YR (44 per cent), microcystin-LA (26 per cent), [D-Asp^3]microcystin-LR (51 per cent), [Dha^7] microcystin-LR (48 per cent), glutathione conjugate of microcystin-LR (47 per cent), monomethyl ester of microcystin-LR (30 per cent), nodularin (46 per cent) and 6(Z)-ADDA microcystin-LR (< 4 per cent). Although the epitope of this antibody is not clear, the importance of the Adda moiety for antibody binding has been indicated. It should be noted that this antibody also reacts to the non-toxic monomethyl ester of microcystin-LR giving a false positive from the toxicological point of view. Using this monoclonal antibody, a more sensitive competitive ELISA method has been developed by Ueno *et al.* (1996) with detection limits of 0.05 µg l^{-1} for water samples. This method has been successfully applied to detect microcystins in drinking water in China (Box 13.3).

Box 13.3 Application of the ELISA method to the detection of microcystins in drinking water in China

Using an ELISA method, Ueno *et al.* (1996) analysed microcystin concentrations in drinking water collected in Haimen and Fusui in China, where the rates of primary liver cancer (PLC) do not correlate with PLC-causing agents such as aflatoxin and hepatitis-B virus. It had also been observed that people who drank pond and ditch water had a higher risk of PLC than people who drank well water. The authors investigated the levels of microcystins in four types of water (pond/ditch, river, shallow well and deep well) collected from 989 different sampling sites in Haimen. The results showed a positive detection of microcystin for 17,32,4 and 0 per cent of the total samples of pond/ditch, river, shallow well and deep well water, respectively. The average microcystin concentration in the pond/ditch and river water were 101 and 160 pg ml^{-1}, respectively, which were significantly higher than those of the shallow and deep well water. Among the samples examined, two samples from the river showed microcystin levels over 1,000 pg ml^{-1}. These data suggested that microcystin in drinking water from ponds/ditches and rivers, or both, is one of the risk factors for the high incidence of PLC in China. Furthermore, the results indicate that ELISA can be applied successfully to the monitoring of microcystins in environmental samples.

Commercially, a polyclonal ELISA kit is available for microcystins. The antibodies are fixed to the walls of the wells of a microtitre plate. The first step involves binding of the calibrators (a non-toxic microcystin-LR surrogate at 0.1, 0.4 and 1.6 µg l^{-1}) a negative control and the samples to the antibodies in the wells. This is followed by addition of a microcystin-enzyme conjugate which binds to the remaining antibodies. After thorough rinsing, the concentration of bound enzyme is measured colourimetrically in an ELISA plate reader. The microcystin concentration is inversely proportional to the colour intensity.

ELISA method for microcystins

Apparatus

- Filtration equipment for samples containing particles
- Automatic 100 µl pipette
- Timer
- Multichannel pipette for washing the microtitre plate

- Apparatus for shaking the microtitre plate
- ELISA reader with filter at 450 nm

Reagents

- ELISA test kit
- Distilled water

Procedure

1. Water samples to be analysed are treated twice by freeze-thawing followed by filtration through membrane or glass fibre filters.

2. Samples or standards are first mixed with antibody (M8H5) solution and then added to a 96-well microtitre plate that is pre-coated with a microcystin-LR bovine serum albumin conjugate.

3. After washing, bound monoclonal antibody is detected with horseradish peroxidase-labelled goat anti-mouse IgG and its substrate (0.1 mg ml^{-1} of 3,3',5,5'-tetramethlybenzidine, 0.005 per cent H_2O_2 in citrate buffer).

4. The optical density is measured at 450 nm and the microcystin concentration determined from a standard competitive curve of microcystin-LR.

Development of immunodiagnostic systems for the detection of saxitoxins have primarily been aimed at replacing the mouse bioassay for the routine monitoring of shellfish from the marine environment and are discussed in more detail elsewhere (Cembella *et al.,* 1995). Both polyclonal and monoclonal antibodies have been produced, although none have shown cross-reactivity with all the known variants. Antibodies tend to be raised to saxitoxin mainly because it has been the most extensively studied and because it is also the most readily available; hence methods reliably detect this variant but most notably fail to cross-react with neosaxitoxin which is of similar toxicity. Methods continue to be developed (e.g. Kralovec *et al.,* 1996) and may provide a suitable routine monitoring system in the future.

13.3.6 Mammalian cells

Bioassays using mammalian cells have received attention as suitable replacements for mouse toxicity tests. The well documented fact that microcystins have caused acute liver damage has prompted studies using hepatocytes (liver cells). Freshly isolated rat hepatocytes were first investigated by Aune and Berg (1986) who reported good correlation between toxicity measured by leakage of the enzyme lactate dehydrogenase (LDH) from hepatocytes and results from mouse bioassay. More recently this method has received renewed interest due to legislative restrictions in the use of the mouse LD_{50} (Heinze, 1996). Isolated rat hepatocytes have been incubated with pure toxin or bloom extracts for 4 h and 20 h and then the viability has been assessed using the MTT ((3,4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) test. Toxicity was found to be time-dependent with the LC_{50} for microcystin-LR being 0.20 µg ml^{-1} at 4 h reduced to 0.05 µg ml^{-1} after 20 h. Different LC_{50} values were found for microcystin variants, most notably microcystin-RR was found to be at least an order of magnitude less toxic in this assay. This is consistent with *in vivo* toxicity data.

An *in vitro* fibroblast cytotoxicity assay for microcystins has been investigated by Codd *et al.* (1989) as a replacement for the mouse bioassay. The assay, using V79 hamster

fibroblast cells, responded to microcystins with the results correlating reasonably well with those of the mouse bioassay (Lawton *et al.,* 1994b). However, the assay demonstrated a number of false positives and, of more concern, false negatives were also recorded.

Box 13.4 When is physicochemical analysis needed?

Physicochemical analysis of cyanotoxins is recommended if:

- Cyanobacterial species composition or bioassay results indicate which toxins to look for.
- Unambiguous identification of toxins is required.
- Quantification of toxins is required.
- Rapid screening of a large number of samples is required, especially for regular monitoring of sites where the toxin patterns are well established.
- Low toxin concentrations which may not be detected by a bioassay are expected (e.g. in drinking water).
- New toxic cyanobacterial metabolites are to be identified.

Another cell-based assay proposed in the past has used blood cells, with agglutination of the cells being reported as an indicator of microcystin level (Carmichael and Bent, 1981). Although this bioassay did appear promising, it has since been found to be a poor indicator for microcystins.

Two *in vitro* cell bioassays have been found to be successful in detecting saxitoxins and, like the immunoassay, they were developed primarily for monitoring toxins in shellfish. First, a neuroreceptor binding assay was developed that uses radiolabelled saxitoxin and works on the basis of competitive displacement (Davio and Fontelo, 1984). The initial protocol has subsequently been refined (Doucette *et al.,* 1994) and the data obtained correlate well with the mouse bioassay (Cembella *et al.,* 1995). A neuroblastoma cell line technique for sodium channel blocking activity has also been developed (Gallacher and Birkbeck, 1992; Jellett et al., 1992). This assay, which is currently undergoing evaluation for its suitability as a replacement for the mouse in shellfish monitoring, is now available as a commercial test kit.

13.4 Analytical methods for cyanotoxins

Analytical methods use the physicochemical properties of cyanotoxins such as molecular weight, chromophores and reactivities due to the functional groups in the molecules. Physicochemical methods used for cyanotoxin detection are summarised in Table 13.2, which also indicates that the initial capital expenditure to establish most of these methods is high. Appropriate use of such methods is discussed in Box 13.4.

Table 13.2 Physicochemical methods for the detection of cyanotoxins

Method[1]	Cost[2]			Comments	Reference(s)
	Cap.	Con.	Pers.		
Microcystins and nodularins					
HPLC-PDA	H	M	L	UV spectra can give tentative id	Lawton *et al.,* 1994b
LC/MS	VH	M	M/L	A number of different interfaces; mass confirmation; can have PDA	Kondo *et al*., 1992; Edwards *et al.,* 1992
TLC	L	L	M	Qualitative; requires standards and further confirmation of toxins	Harada, 1996
MMPB	H/VH	M	M	Detection by GC-MS or LC-MS detects total microcystin/nodularin	Sano *et al*., 1992; Harada *et al*., 1996
MALDI	VH	L	M/L	Initially poor but recent developments have improved accuracy	Erhardt *et al*, 1997
CE-MS	H	L	M	Requires further development but has future promise	
NMR	VH	M	M/H	Can characterise cyanotoxins; needs mg quantities and expert interpretation	Botes *et al*., 1984; Harada, 1996
Anatoxin-a and homoanatoxin-a					
HPLC-PDA	H	M	L	Characteristic UV spectra	Edwards *et al.,* 1992
GC-MS	H	M	L	Characteristic ion spectra	Smith *et al*., 1987
GC-ECD	H	M	L	Requires sample cleanup	Stevens *et al*., 1988
LC/MS	VH	M	M/L	Sensitive and specific	Harada *et al*., 1993
Anatoxin-a(S)					
HPLC	H	M	L	Very poor chromophore, not suitable for routine detection	Matsunaga *et al*., 1989
Cylindrospermopsin					
HPLC-PDA	H	M	L	Lack of available standards; give characteristic UV spectra	Harada *et al*., 1994; Hawkins *et al*., 1997
Saxitoxins					
HPLC-pre	H	M	H	Precolumn derivatisation; poor stability of derivative	Lawrence *et al*., 1995
HPLC-post	H	M	M	Three solvent systems required to analyse for all variants	Oshima *et al*., 1995
LC/MS	VH	M	M/L	Best method for all variants but equipment cost can be prohibitive	Quilliam *et al*., 1989; Hines *et al*., 1993

CE-MS	H	L	M	Poor detection limits; needs further development	Pleasance *et al*., 1992; Lock *et al*., 1994

[1] See text for an explanation of the different methods listed
[2] Cap. Capital; Con. Consumables; Pers. Personnel; L Low; M Medium; H High; VH Very high

Another approach for further confirmation and identification of microcystins has been proposed using a LC-linked protein phosphatase assay (Boland *et al*., 1993; Chen *et al*., 1993). Essentially, this method makes use of HPLC to separate and identify tentatively the cyanotoxins present, and then monitors the bioactivity of the detected peaks using the protein phosphatase inhibition assay. This provides excellent confirmation of protein phosphatase inhibiting cyanotoxins, especially in complex samples.

13.4.1 Detection methods for microcystins and nodularins

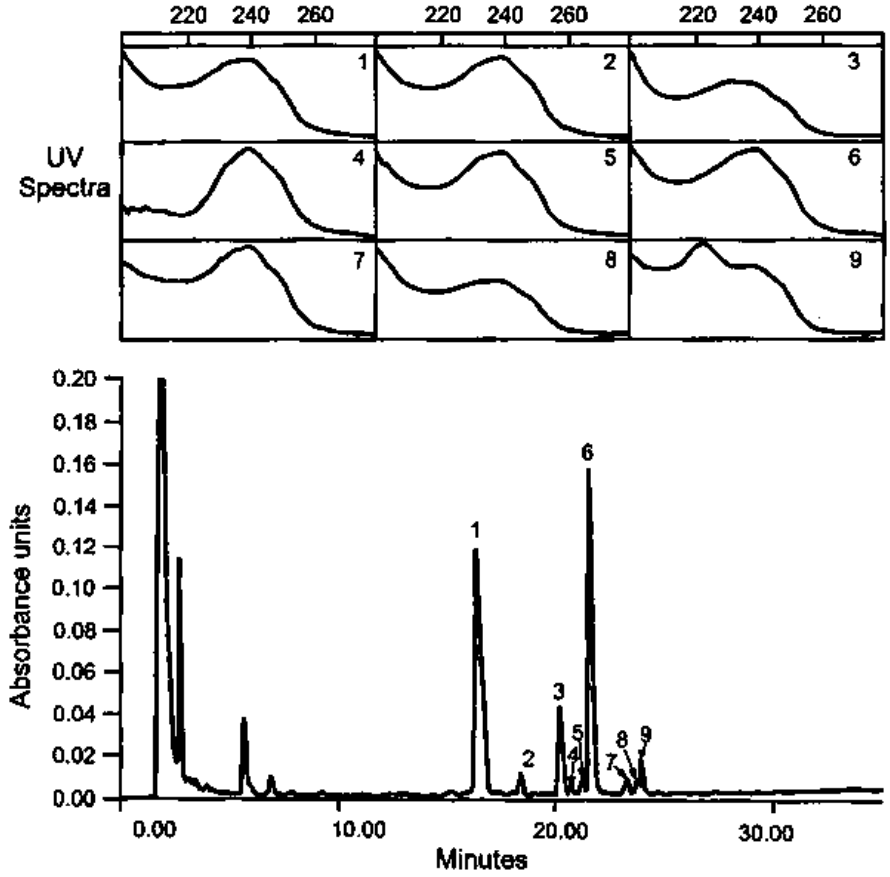
The majority of analytical methods have been developed primarily for microcystins although because both nodularins and microcystins show similar physicochemical properties, nodularins can easily be analysed by the same methods. The most commonly-used analytical system for this class of cyanotoxins is HPLC (see Box 13.5). Combined with UV detection, HPLC has been used extensively for the detection of microcystins, but because this method relies on retention time for identification, microcystin standards are required (Harada, 1996). Detection by UV can be made more specific by using a photodiode array (PDA) UV detector (Lawton *et al.,* 1994a) but it has very limited ability to identify individual microcystins because almost all microcystins show a similar UV spectrum. Recent advances in detector hardware can now provide high resolution spectra that detect very slight variations in chemical composition and can be used in conjunction with advanced spectral matching software. These developments may assist in the identification of microcystins by spectral match data in conjunction with retention times. However, a fundamental problem still exists in the availability of standards. With over 60 microcystins known, it is currently impossible to create a definitive spectral library, which is a limiting factor when using this method to identify unknown microcystins.

Typical HPLC analysis uses a reverse-phase C18 silica column with separation achieved over a gradient of water and acetonitrile, both containing 0.05 per cent triflouroacetic acid (TFA). The gradient has to cover a sufficient range of polarities (e.g. 30-70 per cent acetonitrile) to allow the analysis of all microcystins which are known to vary considerably in their polarities. Data is gathered at 238 nm and where PDA is used spectral information is collected between 200 and 300 nm. Use of HPLC-PDA can allow tentative identification of microcystins and this method was found to perform very well when over 20 samples were assessed by HPLC and compared with mouse bioassay data. No false negatives were observed and only one false positive was reported, the latter being attributed to a relatively low level of microcystin which failed to cause death in the mouse bioassay (Lawton *et al.,* 1994b). Other solvent systems have also been successfully used including methanol/water and ammonium acetate/acetonitrile.

Box 13.5 Possibilities and limitations of HPLC with UV spectra for microcystin analysis

High pressure liquid chromatography can be used routinely to identify and quantify microcystins, but not to differentiate between structural variants of most microcystins. For example, in a sample from Radeburg Reservoir (1 July 1996, monospecific population of *Microcystis* spp.), UV-spectra indicated nine microcystins, three of which could be identified with commercially available standards by their retention times to be microcystin-RR (peak 1), microcystin-YR (peak 3), and microcystin LR (peak 6). However, six other minor microcystins could not be further specified by this method alone. For a tentative assessment of the toxicity of this sample, a "worst case" approach was chosen by calculating the sum of all of the nine microcystins and assuming them to be as toxic as -LR and -YR, the most toxic variants currently known.

For preliminary microcystin screening and for routine monitoring, HPLC with photodiode array detection is an excellent approach because it efficiently provides an overview of toxin content, and a worst-case toxicity estimate can be derived. Therefore, if a local or regional authority must deal regularly with microcystin monitoring, establishment of HPLC techniques with photodiode array detection of UV spectra is recommended. Further identification of microcystins can then be performed with selected samples. In many cases, it will be advisable to subcontract this to laboratories with specialised expertise.



Results of HPLC analysis of a sample from Radeburg Reservoir (1 July 1996, monospecific population of *Microcystis* spp.) showing nine microcystins identified by their characteristic UV-spectra (Fastner, unpublished data)

Analysis of microcystins and nodularins by HPLC-PDA

Apparatus

- Gradient HPLC system with photodiode array detection
- Data acquisition system
- Auto-sampler, recommended for high sample throughput
- C18 column e.g. 4.6 × 250 mm Symmetry (Waters)
- Column oven, 40 °C
- Pipette, 0.1-0.5 ml

Laboratory conditions

Effective ventilation, taking into account that acetonitrile is heavier than air and accumulates at ground level.

Reagents

- Eluent A, water plus TFA, 0.5 ml TFA added to 1,000 ml water
- Eluent B, acetonitrile plus TFA, 0.5 ml TFA added to 1,000 ml water
- Helium gas
- Methanol

Note: all reagents must be high purity, HPLC grade.

Procedure

1. Prepare solvents and degas in a stream of helium gas

2. Program a linear gradient (1 ml min^{-1}) as follows:

Time (mins)	0	10	40	42	44	46	55
Eluent A%	70	65	30	0	0	70	70
Eluent B%	30	35	70	100	100	30	30

3. Equilibrate the column at the desired temperature and gradient starting conditions.

4. Set photodiode array detector to monitor between 200 and 300 nm.

5. Running a blank sample first, i.e. injecting only methanol, helps the system settle and ensures reproducible retention times.

6. Samples and standards are usually prepared in methanol. However, some microcystins are less soluble in methanol (e.g. microcystin-YR). Check product information where available. Samples should be centrifuged or filtered to remove particulates before carrying out HPLC analysis.

7. It is advisable always to run a standard at the beginning and end of a set of analyses, because this helps to confirm correct operation of the system and indicates the degree of retention time drift.

8. A calibration curve should be performed when establishing the method and at regular intervals, especially after changing a column or lamp.

9. Chromatograms are best viewed, and integration carried out, at 238 nm because this is the absorption maximum of most microcystins and nodularins.

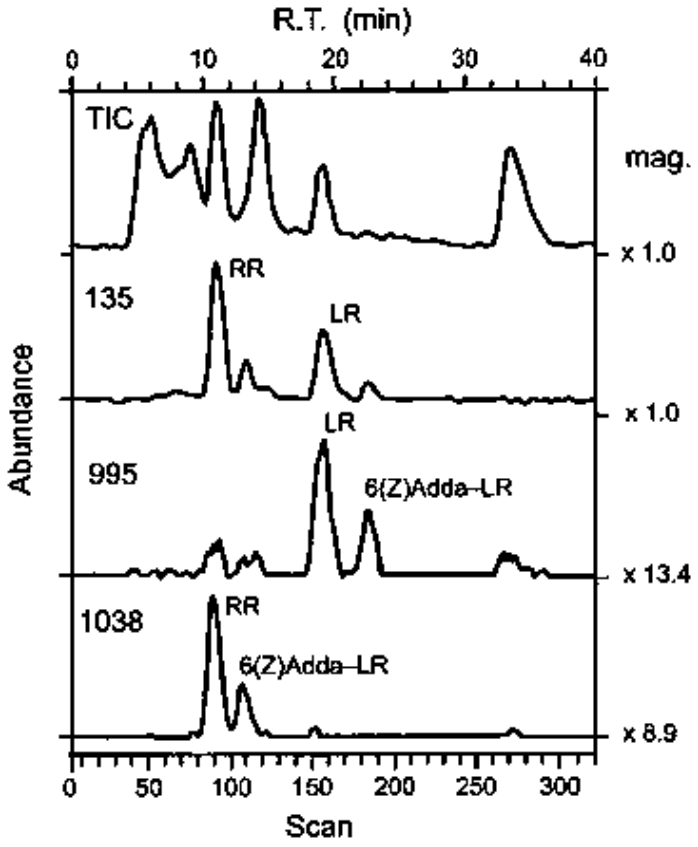
10. Microcystin congeners can be identified where they have the same retention time and spectrum as a standard, but for many microcystins no standards exist. These can be identified as microcystins, but the respective congener may only be tentatively inferred from published retention times in relation to identified congeners. However, advances in spectral matching software provide increased confidence in microcystin identification by providing a numeric indication of how similar an unknown is to a range of microcystins in a spectral library.

When further confirmation and identification of microcystins is required, more advanced methodology must be used. Liquid chromatography/mass spectrometry (LC/MS) is a very promising method because it enables the simultaneous separation and identification of microcystins in a mixture (Kondo *et al.,* 1992; Edwards *et al.,* 1993; Poon *et al.,* 1993). Figure 13.6 shows the Frit-FAB (fast atom bombardment) LC/MS analysis data of a toxin from a bloom sample collected in Japan. The toxic fraction contains mainly two microcystins as shown by the mass chromatogram monitored at the characteristic ion m/z 135 derived from Adda, which has proved to be useful for the discrimination of microcystins from other types of compounds (Kondo *et al.,* 1992). The two peaks were readily identified as microcystins-RR and -LR according to the mass spectra and mass chromatograms at their $[M+H]^+$. An advanced Frit-FAB LC/MS method using a microbore column (0.3 mm internal diameter) enabled the identification of nanogram levels of microcystins in water and biological samples (Kondo *et al.,* 1995, 1996). This increase in sensitivity was achieved by splitting the mobile phase between the pump and the injector so that the total effluent could be introduced into the mass spectrometer.

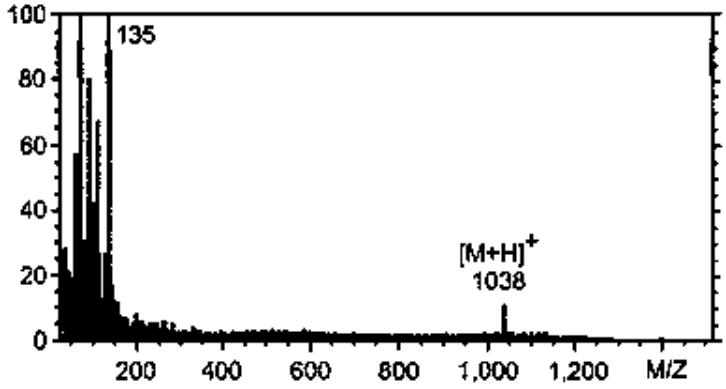
A physicochemical screening method that is based on the detection of 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) as an oxidation product of microcystins has been reported. The MMPB was initially prepared by Lemieux oxidation, followed by analysis by gas chromatography (GC) with a flame ionisation detector or HPLC with fluorescence detection (Sano *et al.,* 1992). However, it required tedious procedures such as extraction, cleanup, oxidation and post-treatment in order to eliminate the reagents used, and derivatisation for GC and HPLC analysis. An improved method using ozonolysis made it possible to reduce significantly the formation times of MMPB because the previously required extraction, cleanup and other procedures could be entirely eliminated (Harada *et al.,* 1996). The resulting intact MMPB was directly analysed by thermospray (TSP) interface LC/MS and EI-GC/MS using selected ion monitoring. This new procedure, from the ozonolysis of the microcystins to analysis of MMPB at picomol levels, took only 30 minutes to perform. The quantification of bloom samples achieved by this method were consistent with those obtained by HPLC analysis, showing that the method provided a means of screening for microcystins, as well as for their accurate quantification. Additionally, the most remarkable feature of this method is the applicability to complex sample matrixes, including solid material such as animal tissue, without the requirement for any complicated processing.

The structural determination of microcystins and nodularins has been carried out by nuclear magnetic resonance (NMR) spectroscopy and recent advances in 2D NMR techniques have proved to be essential for the structural determination of known and unknown microcystins. Fast atom bombardment MS and liquid secondary ion (LSI) MS give a protonated molecule $[M+H]^+$, providing information about molecular weight with further structural information obtained by Tandem FAB MS (FAB-MS/MS) as used in recent studies (Namikoshi *et al.,* 1995). However, NMR and MS (except LC/MS) usually require relatively large amounts of sample (milligram quantities) and completely purified microcystins, therefore they are not used in routine monitoring (Botes *et al.,* 1984; Namikoshi *et al.,* 1995; Harada, 1996).

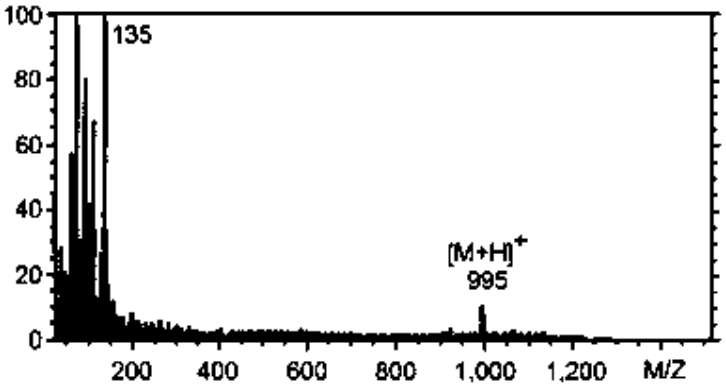
Figure 13.6 Frit-FAB LC/MS analysis of a toxic fraction from a bloom sample collected in Japan (Data supplied by Ken-ichi Harada and Fumio Kondo)



Microcystin-RR (MW 1037)



Microcystin-LR (MW 994)



For identification of microcystins with very small sample volumes (less than 1 mm^3 freeze-dried material). Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) has recently been developed (Erhardt *et al.,* 1997). This method provides the molecular mass of all of the peptides in a sample and thus gives strong indications of the microcystin variants present. Post Source Decay (PDS) spectra may be obtained, which are characteristic for different microcystins. A library is currently being established. For rapid qualitative assessment of microcystins and other cyanobacterial peptides, this method is highly promising. Quantitative assessment is not yet possible.

Different methods provide different and often complementary information, therefore combined use of suitable methods is recommended according to the purpose and type of data required. This is necessary because none of the methods currently available provides all the information which may be required. Furthermore, individual laboratories must identify the techniques that are both suitable for their analytical requirements and use their own expertise and available technology (see Meriluoto (1997) for a recent review of chromatographic methods for microcystins). Initial screening of samples can check rapidly for the presence of microcystins in a small amount of sample using sensitive and simple methods. Figure 13.1 summarised the relationship between sensitivity and selectivity of analytical methods for microcystins, showing that different methods provide different and complementary information.

For studies requiring enhanced precision, accuracy and sensitivity in detection of individual toxins, a multistage procedure is required. This may comprise initial screening of samples to check for the presence of microcystins in a small amount of sample using sensitive and simple methods, such as bioassays (e.g. ELISA, protein phosphatase inhibition assay) and MMPB method. The use of screening helps to reduce the number of samples which require full analytical investigation and thus reduces the laboratory commitment. Furthermore, rapid results from an initial screen may aid prompt regulatory responses regarding the suitability of water for human use. If a sample is positive in a screening test, it will be necessary to follow through with identification and quantitative analysis. Prior to this, cleanup and sample concentration is often very important for many samples because relatively low levels of microcystins, around 1-2 µg l^{-1}, are usually present in water samples. A tandem cleanup system using ODS silica gel and silica gel cartridges, facilitates the accurate analysis of trace amounts of microcystins in water. Finally, quantitation of microcystins is usually essential, and although the screening methods can provide an indication of total microcystin concentrations in samples, a separation and spectroscopic method such as HPLC with UV detection should be applied at this final step. Availability of authentic standards would strengthen the capability of HPLC with UV detection (preferably with photodiode array detection) but otherwise LC/MS should be the method of choice despite the requirement of a more specialised technique.

13.4.2 Anatoxin-a

Three methods for analysis of anatoxin-a have been reported to date: GC/MS, GC with electron capture detection (ECD) and HPLC. One GC/MS method has been published as a confirmation of anatoxin-a in material taken from a toxic bloom that resulted in the deaths of 16 cows (Smith and Lewis, 1987). In the procedure, following liquid-liquid extraction with *N*-acetylation, the resulting acetylated toxin was analysed by a capillary GC/MS. Although confirmation was based on interpretation of the mass spectrum, the detection limit was unclear. Another GC/MS method has also been established for *N*-acetylated anatoxin-a (Himberg, 1989) and it was successfully employed for preliminary characterisation of neurotoxic cyanobacteria from Finland (Sivonen *et al.,* 1989). The GC/ECD method provided a higher sensitivity than other analytical methods, it used an internal standard for accurate quantification, and it could be applied to analysis of field samples (Stevens and Krieger, 1988). Although GC/ECD requires a considerably more complicated cleanup operation and derivatisation prior to analysis, it has been used successfully for stability studies on anatoxin-a (Stevens and Krieger, 1991). Derivatisation with N-pentafluorobenzylbromide was shown to achieve a sensitivity of 2.5 pg (Bumke-Vogt *et al.,* 1996). It is possible to use HPLC with UV detection to analyse intact anatoxin-a which has a strong absorption at 227 nm. After extraction of a cyanobacterial suspension with chloroform, followed by re-extraction with 0.01N hydrochloric acid, the resulting extract is separated under the following conditions: column, ODS silica gel; mobile phase, methanol-0.01M perchloric acid (7:3); detection, UV (227 nm). Harada *et al.* (1989) reported an alternative approach using a reversed phase HPLC method with a methanol-10.01M ammonium chloride (1:9) solvent system.

Mass spectrometry is a very effective method for identification of anatoxin-a and its derivatives. Conventional electron ionisation (EI) and chemical ionisation (CI) can yield molecular ions and protonated molecules, respectively. Ross *et al.* (1989) evaluated secondary ion mass spectrometry (SIMS), GC/MS, desorption CI and TSP-MS for

detection of the toxin and its derivatives. The use of HPLC coupled with mass spectrometry using thermospray interface (TSP-LC/MS) has also been investigated (Harada *et al.,* 1993). The latter method made possible a sensitive, specific and reproducible analysis of anatoxin-a and its non-toxic oxidation product when used in combination with a cleanup method including a solid phase extraction with a reversed phase carboxylic acid cartridge. Using this method, trace amounts of anatoxin-a were detected in three strains and two bloom samples collected in Japan.

13.4.3 Anatoxin-a(S)

Matsunaga *et al.* (1989) purified anatoxin-a(s) and determined its structure, although no analytical method has been developed. Traditional HPLC plus UV detection is not suitable because this cyanotoxin lacks a strong chromophore. Liquid chromatography/mass spectrometry may prove useful, but this analytical technique has not been evaluated yet for anatoxin-a(S).

13.4.4 Cylindrospermopsin

The first analytical method reported for this toxin consisted of a combination of a cleanup step using HP-20 and ODS silica gel cartridges followed by HPLC with photodiode array detection. This method was applied to a bloom sample collected in Japan which was thought to contain Cylindrospermopsin (Harada *et al.,* 1994). However, the authors suggested that the mobile phase used in this study was not satisfactory because Cylindrospermopsin showed poor retention power on the ODS column and slight tailing, and their method requires further development to allow precise analysis.

A recently published method (Hawkins *et al.,* 1997) describes the extraction of cells in 5 per cent aqueous acetic acid and analysis using Spherisorb ODS-2 with a 10 minute linear gradient from 0 to 5 per cent methanol followed by a further 10 minutes at 5 per cent methanol. This gave good retention, separation and peak shape. Cylindrospermopsin was found to have a characteristic UV absorbance spectrum between 200 and 300 nm, with a maximum absorbance at 262 nm.

13.4.5 Saxitoxins

The methods mentioned here have been developed primarily for the analysis of saxitoxins in the marine environment, particularly in shellfish. However, they have been found to be equally suitable for cyanobacterial samples. The most commonly used analytical method for the saxitoxins is HPLC with on-line post-column oxidation and fluorescence detection (Oshima *et al.,* 1995). Although this method has been found to be the most satisfactory to-date, it requires three different mobile phase systems to allow analysis of all the saxitoxins. Furthermore, there is limited availability of analytical standards for all saxitoxin variants, which are necessary for peak confirmation. Mass spectrometry with FAB (Mirocha *et al.,* 1992), TSP (Wils and Hulst, 1993), electrospray (Hines *et al.,* 1993) and ion-spray ionisation (Quilliam *et al.,* 1989) has been reported for the paralytic shellfish poison (PSP) toxins. The application of an LC/MS method for saxitoxins has been reported, although it appears that it will be difficult to achieve analysis of all PSP toxins within one LC/MS run. The combination of capillary electrophoresis (CE) with ion-spray ionisation has proved to be successful for the saxitoxins (Pleasance *et al.,* 1992; Locke and Thibault, 1994). Analysis using CE was

found to give poor detection limits due to the very small injection volumes (< 10 nl), but it may be possible to overcome this problem with an on-column sample preconcentration system. Furthermore, developments in CE technology are anticipated in the near future which will increase flow cell volume, hence enhancing detection limits.

13.5 References

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