Monitoring Bathing Waters - A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes
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ISBN 0-419-24390-1

Chapter 10*: CYANOBACTERIA AND ALGAE

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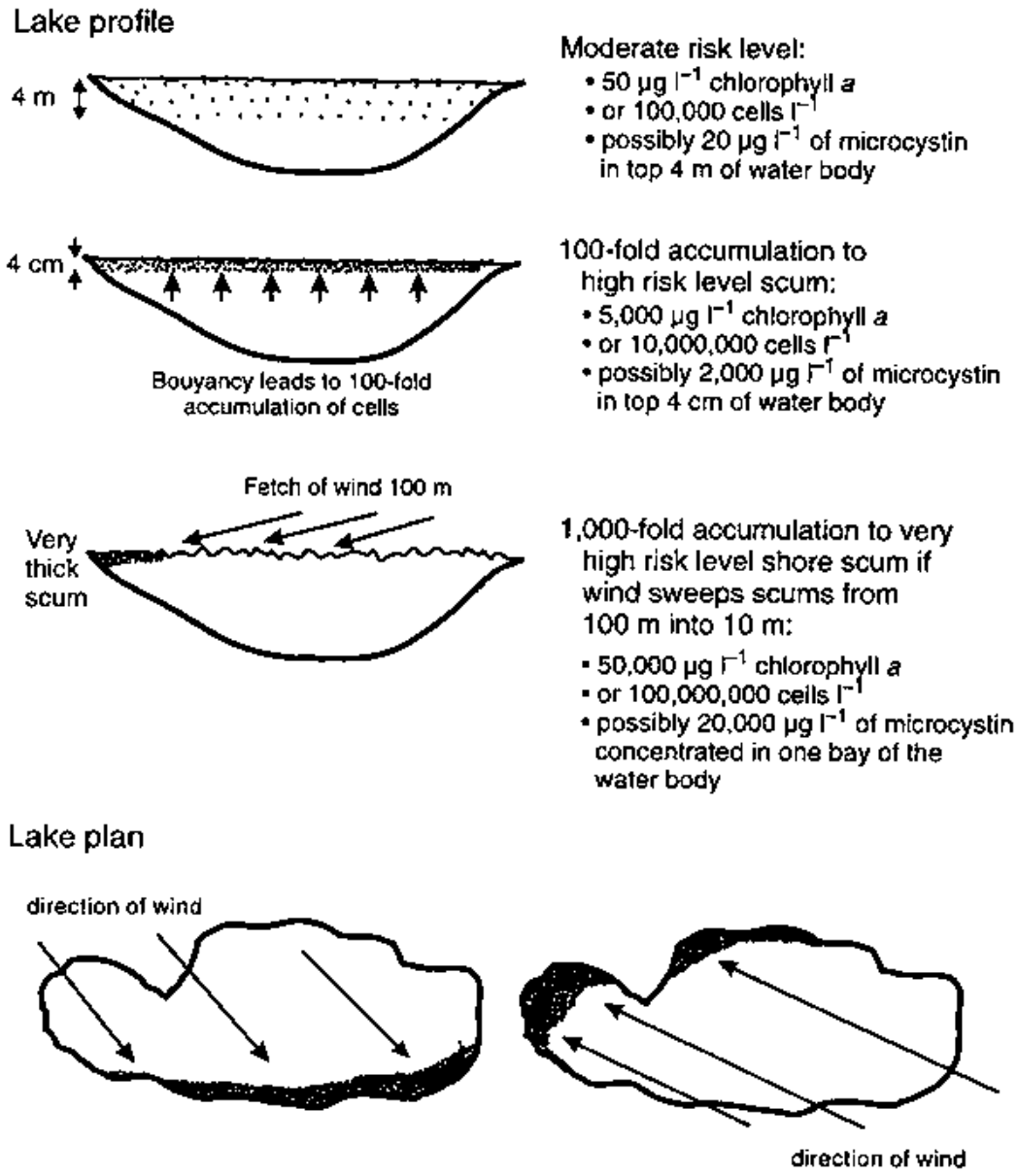
In freshwaters, scum formation by cyanobacterial phytoplankton is of concern to human health. Freshwater algae proliferate quite intensively in eutrophic waters and may contain irritative or toxic substances. Nevertheless, incidents of impairments of human or animal health caused by algae are rarely reported. One example was the closure of a number of bathing sites in Sweden because of mass occurrences of the flagellate *Gonyostomum semen* which causes skin irritations and allergies (Cronberg *et al.,* 1988). Incidents attributed to cyanobacteria are far more numerous and, in most cases, have been caused by species of cyanobacteria that may accumulate to surface scums of extremely high cell density. As a result, the toxins they may contain ("cyanotoxins") reach concentrations likely to cause health effects.

Surface aggregations of planktonic cyanobacteria occur because of their capability to regulate their buoyancy, enabling them to seek water depths with conditions optimal for their growth. Regulation of buoyancy is a slow process, and cells adapted to ambient turbulence may take several days to adapt their buoyancy when conditions change (e.g. turbulence is reduced). Thus, cells or colonies may show excessive buoyancy and accumulate at the water surface. Light winds drive such accumulations to leeward shores and bays, where the resulting scums become thick. In extreme cases, such agglomerations may become very dense, with cells frequently concentrated by a factor of 1,000 or more, eventually reaching in some cases, one million-fold concentrations with a gelatinous consistency. More frequently, surface accumulations are seen as streaks or slimy scums that may look like blue-green paint or jelly. Such situations can change rapidly within hours with changes in the wind direction. Monitoring strategies must take into account this highly dynamic variability of cyanotoxin occurrence in time and space.

Scum formation is influenced by the morphological conditions of the water body, such as the water depth from which cyanobacteria can rise to the surface (i.e. the thickness of the stratum in which they are dispersed) and the length of wind fetch over which surface aggregations can be swept together to form shoreline scums (Figure 10.1). Accumulated scum material may take a long time to disperse especially in shallow bays. Dying and lysing cells within the scum release their contents, including the toxins, into the water. However, toxin dissolved in the water is rapidly diluted and probably also degraded. Cell-bound cyanotoxin concentrations usually are the greater cause for concern in recreational waters.

Figure 10.1 Schematic illustration of scum-forming potential changing the cyanotoxin risk from moderate to very high (After Falconer *et al.,* 1999)



Most of the problems reported with nuisance and toxin-containing aquatic cyanobacteria in freshwaters have involved planktonic species, i.e. those distributed in the water body or forming surface scums. However, benthic (i.e. bottom-dwelling), species have occasionally surfaced, and been washed ashore where they have caused the death of dogs scavenging upon the material. Benthic cyanobacteria can grow as mats on sediments in shallow water. Some mats become detached and are driven onshore where they result in acutely toxic accumulations (Edwards *et al.,* 1992).

Severe illness due to direct dermal contact with such mats has been reported from tropical marine bathing sites (Kuiper-Goodman *et al.,* 1999). In coastal marine

environments many toxic species of dinoflagellates, diatoms, nanoflagellates and cyanobacteria occur, and have led to several forms of human health impacts mainly after consumption of shellfish and fish, i.e. syndromes such as Paralytic Shellfish Poisoning, Diarrhetic Shellfish Poisoning, Amnesic Shellfish Poisoning, Neurotoxic Shellfish Poisoning and Ciguatera. Nevertheless, there exists very little scientific evidence that marine toxic algal blooms cause health problems for recreational users of water. This evidence is reviewed in the WHO *Guidelines for Safe Recreational-Water Environments* (WHO, 1998).

As a result of the lack of evidence for effects of toxic algae on recreational users of marine waters, this chapter is concerned principally with monitoring and assessment of toxic cyanobacteria in freshwaters. Nevertheless, the methods given for cyanobacteria may be employed to assess the development of other planktonic algae. Although various toxic marine algae have been associated occasionally with human health effects, concern for human health centres on toxic cyanobacteria, and therefore these are the subjects of the remainder of this chapter. Further information concerning coastal phytoplankton blooms and associated monitoring strategies are available in Franks (1995), Smayda (1995) and in the UNESCO manual on this subject (UNESCO, 1996).

For lakes, reservoirs and rivers different levels of "alert" (for cyanobacterial cell concentrations and their toxin contents) have been proposed in *Toxic Cyanobacteria in Water* (Chorus and Bartram, 1999) and in the *Guidelines for Safe Recreational-water Environments* (WHO, 1998). These documents present a series of guideline values and situations associated with incremental severity and probability of greater effects in relation to cyanobacterial occurrence (Table 10.1).

10.1 Design of monitoring programmes

Many cyanobacterial species frequently forming mass developments may contain hepatotoxins or neurotoxins, and all of them contain lipopolysaccharides (LPS) in their cell wall. Lipopolysaccharides may be the cause of irritations of the skin, digestive tract, respiratory membranes, eyes and ears that are frequently associated with cyanobacteria. Research in pharmacology and ecotoxicology indicates that cyanobacteria contain a variety of substances not yet identified, but that may have a potential impact on people. The implications of the present state of knowledge for surveillance and management are that any mass development of cyanobacteria may be a potential health hazard. If the cyanobacterial cells contain hepatotoxic microcystins, cause for concern may be higher because of the chronic effects of this potent toxin. Therefore, monitoring should address primarily the occurrence of cyanobacterial mass developments, whereas microcystin analysis may be adequate in specific situations.

Table 10.1 Phase 3 monitoring - guidelines for safe practice in managing recreational waters according to three different levels of risk

Level of risk[1]	Health risks	Recommended actions
20,000 cells cyanobacteria per ml or 10 µg l^{-1} chlorophyll a with a dominance of cyanobacteria	Short-term adverse health outcomes (e.g. skin irritations and gastro-intestinal illness, probably at low frequency)	Post on-site risk advisory signs Inform relevant authorities
10^5 cells cyanobacteria per ml or 50 µg l^{-1} chlorophyll a with a dominance of cyanobacteria	Potential for long-term illness with some species Short-term adverse health outcomes (e.g. skin irritations and gastro-intestinal illness)	Watch for scums Restrict bathing and further investigate hazard Post on-site risk advisory signs Inform relevant authorities
Cyanobacterial scum formation in bathing areas	Potential for lethal acute poisoning Potential for long-term illness with some species Short-term adverse health outcomes (e.g. skin irritations and gastro-intestinal 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

[1] Expressed in relation to cyanobacterial density and given in order of increasing risk

Visual inspection of a bathing site is of crucial importance because it shows immediately whether cyanobacteria occur in potentially hazardous densities. However, as scum formation and dispersion may occur within hours and thus too frequently for monitoring, assessment of the risk of cyanobacterial exposure during recreational activities is greatly enhanced by an understanding of the population development of these organisms in a given water body (i.e. through background monitoring of variables that enable their proliferation). Good knowledge of the local growth conditions for cyanobacteria can greatly enhance predictability of bloom formation. As knowledge and understanding of a given site accumulate, regular patterns of cyanobacterial growth may be noticed, and surveillance may as a result be focused upon critical periods. Involving limnological expertise may be very useful, particularly during the development of monitoring programmes and for their periodic assessment for efficacy.

10.1.1 Monitoring strategy for freshwater cyanobacteria

A structured, quantitative investigation approach aims at focusing surveillance efforts upon those sites that are likely to present a risk. It further provides a scheme for immediate assessment and action by discerning three steps of action (Table 10.2).

- Determination of the carrying capacity of the ecosystem for cyanobacteria.

- Site inspection to detect mass developments.

- Quantitative assessment of biomass as a basis for risk assessment when mass developments occur.

Preliminary identification of water bodies potentially harbouring high densities of cyanobacteria is possible on the basis of simple transparency measurements with a Secchi disc. If transparency is high (greater than 2 m) and no significant discolouration of the water can be seen, cyanobacterial densities are unlikely to be high. It should be noted, however, that in large and deep lakes with a large volume from which cells can accumulate to scums, high densities in some parts of the lake cannot be excluded. In water bodies with Secchi disc readings of less than 2 m, the following three steps may be undertaken to assess the risk of toxic cyanobacteria.

Step 1. Determination of the carrying capacity of the ecosystem for cyanobacteria

Algal and cyanobacterial population growth requires phosphorus and nitrogen. The concentrations of these nutrients determine the maximum amount of algae and cyanobacteria that can develop in a given water body, or the "carrying capacity" of an ecosystem for these organisms. The carrying capacity is more often limited by the availability of phosphate but sometimes, particularly in marine ecosystems, it may also be limited by nitrogen. If total phosphorus is not limiting, it may be worthwhile to analyse nitrogen to check whether the carrying capacity may be lower than assumed from the total phosphorus concentrations. Total phosphorus should be measured rather than dissolved phosphate (soluble reactive phosphate (SRP), also known as orthophosphate) because algae and cyanobacteria can store sufficient amounts of phosphate to increase their population 10-fold, even if no dissolved phosphate can be detected. Thus, cell-bound phosphate (which is included when measuring total phosphorus but is missed when measuring only dissolved phosphate) is more meaningful for the assessment of carrying capacity.

Total phosphorus should be assessed several times during the cyanobacterial growth season in order to check temporal variability. If variability of the concentrations is low (less than 50 per cent), assessment twice a year may prove sufficient (in subtropical and temperate climates in spring at total overturn and in summer during the main bathing season). If total phosphate concentrations are below 0.01-0.02 mg l^{-1} P, mass developments of cyanobacteria are unlikely and high turbidities, (if present) may have other causes. If total phosphate concentrations are higher, monitoring should move to Step 2 in order to check for the presence of phytoplankton mass developments.

Table 10.2 Parameters to be measured or assessed for each of the three phases of the recommended monitoring strategy

Phase or activity	Rationale for monitoring	Variables
Monitoring phases		
Phase 1 (background)	Potential for cyanotoxin problems	Nutrient concentrations (total phosphorus, nitrate and ammonia) Transparency (Secchi disc) Hydrophysical conditions (e.g. flow regime and thermal stratification) Other biological complex interactions
Phase 2 (basic)	Site inspection for indicators of toxic cyanobacteria	Transparency (Secchi disc) Discolouration Scum formation Hydrophysical conditions Temperature Weather conditions (e.g. winds, light) Changes in turbulence (i.e. mixing) Other biological complex interactions
Phase 3 (cyanobacteria)	Qualitative/quantitative assessment of potentially toxic cyanobacterial assemblages	Transparency (Secchi disc) Qualitative microscopic analysis to identify dominant taxa (genus is sufficiently precise) Quantitative microscopic analysis (only as precise as needed for management)[1]
	Determination of cyanobacterial biomass	Chlorophyll a analysis (provides an estimate of cyanobacterial biomass in the case of rather monospecific blooms)
Additional activities		
Toxicity	Presence of toxicity	Bioassays
Toxin analysis	Presence of specific toxins (qualitatively and quantitatively)	Chemical analyses

[1] The ratio of the concentration of algal to cyanobacterial cells (or filaments or colonies) in the water sample (as determined by enumeration) may be converted to biomass values

Step 2. Monitoring to detect possible mass developments of phytoplankton (algae plus cyanobacteria) by visual inspection of bathing sites and immediate actions to prevent health hazards

Monitoring should generally be performed at fortnightly intervals. The areas most likely to be affected should be assessed first, such as the downwind shorelines. Information on changes in wind direction or strength in the preceding 24 hours may be valuable for understanding the movement of surface scums in the water body.

Visual inspection should consider the three following conditions. Each condition may be considered a prerequisite for the following condition.

1. Determine if Secchi transparency is less than 1 m or, in absence of a Secchi disc, if the bottom of the lake cannot be seen at 50 cm depth along the shore line; if so

2. Determine if cyanobacteria are visible as a greenish discolouration of the water or at the water's edge or as green or blue-green streaks on the water surface (note: evenly dispersed greenish discolouration may also be due to algal phytoplankton rather than to cyanobacteria, and microscopic assessment is necessary to determine the causative organism, but surface scums and streaks may be attributed to cyanobacteria); if so

3. Determine if a green or blue-green scum is visible on the water surface in any area.

If cyanobacteria are visible and if their population density exceeds one of the guideline values given in Table 10.1, then action appropriate for the given location should be taken, such as informing responsible authorities, initiating the posting and publication of warning notices, regular monitoring according to Step 3 below, and deciding whether or not to initiate immediate action such as marking or enclosing affected areas (if sufficiently small) and prohibiting access to such areas by water users; restricting access to the water edge that is affected, other than for launching boats; and regulating or restricting access by all recreational users where cyanobacterial blooms cover the general waters.

High nutrient inputs favour the development of cyanobacteria and algae. Therefore, if cyanobacteria are present, inspect the catchment area for signs of sewage outlets, excessive fertilisation close to the shoreline, erosion, or other potential sources of phosphate input. The identification of such sources provides the basis for measures addressing the cause of the problem.

Step 3. Quantitative assessment of cyanobacterial biomass and further actions to prevent health hazards

Upon detection of cyanobacteria at bathing sites, their quantification may be desirable for risk assessment. Two quantitative measures are equally valuable: microscopic cell counts or determination of chlorophyll *a* concentration as a simple measure for algal (including cyanobacterial) density. The choice of methods depends on equipment, expertise and personnel available. If chlorophyll *a* is used and its concentrations remain below 10 µg l^{-1}, hazardous densities of cyanobacteria are unlikely. At higher concentrations, chlorophyll *a* analysis must be supported by qualitative microscopic investigations for dominance of cyanobacteria.

If the results of the measurements exceed the guideline values, immediate management action are suggested (Table 10.1). Furthermore, the detection of cyanobacteria at potentially hazardous concentrations should initiate planning of measures for restoration of bathing water quality. Because of the complexity of factors leading to cyanobacterial proliferation, flexible approaches are important, involving further development of monitoring and of protection measures as information on a given water body is accumulated.

Regular measurements of transparency (Secchi readings) in Step 3 can greatly enhance understanding of the system. If transparency is high (greater than 2 m) and no significant discolouration of the water can be seen, cyanobacterial densities are unlikely to be high. However, samples should be taken to determine the phytoplankton community and the water chemistry, in order to estimate the capacity of the water body for cyanobacterial bloom formation. In deep and stratifying lakes, samples from different levels within the

vertical stratification are required because some cyanobacteria accumulate near the thermocline. If transparency is low (less than 1-2 m) and accompanied by a greenish to bluish discolouration, high cyanobacterial densities are likely. In addition, greenish streaks formed by buoyant cyanobacteria (e.g. *Microcystis, Anabaena*) during warm and calm weather may be visible on the water surface. Inspection of downwind areas of the water body is essential when these characteristics are observed, because wind action can readily lead to accumulations of these buoyant organisms. Samples for further analysis (taxonomical and toxicological) should be taken.

Assessment of toxicity or chemical analysis for specific cyanotoxins is not generally recommended for two reasons: (i) the results of toxin analysis provide only a partial basis for risk assessment because only some of the substances causing health outcomes are known and can be analysed, and (ii) the results of an epidemiological study indicate that some health outcomes are not due to known cyanotoxins (Pilotto *et al.,* 1997). Assessment of toxicity using a bioassay may circumvent this problem, but the results are not easily interpreted in terms of human exposure during swimming, particularly with respect to skin reactions. Many cyanotoxin survey studies in different parts of the world have shown that more than half of the field populations investigated did contain toxins, particularly microcystins. Therefore, cyanobacteria are likely to be toxic.

Toxin analysis or toxicity assays may be useful under specific circumstances, because if concentrations of the known cyanotoxins (particularly microcystins) prove to be low, some critical health outcomes (particularly liver intoxication due to microcystins) may be excluded. Warning notices regarding potential irritative effects of cyanobacteria (on the skin, gastrointestinal tract, ears, eyes, respiratory membranes) should nonetheless be posted while the cyanobacterial population density is above the guideline values (Table 10.1). Toxin analysis or toxicity assays may be required in advance of a sports event to improve understanding of the potential risk due to cyanobacteria. If cyanotoxin concentration is low, a local authority may decide to proceed with the event in spite of high cyanobacterial density. Recreational facilities may also choose to invest in monitoring of the known cyanotoxins in order to avoid temporary closure of the facility whenever cyanobacteria proliferate.

10.1.2 Sample site selection

In addition to the general guidance provided in Chapter 2, site selection for cyanobacteria sampling should take account of specific incidents, that are suspected to be associated with exposure to cyanobacteria or algae. Additional factors to be considered in site selection are: the history, if available, of cyanobacterial and algal population development and occurrence of toxins at the water body or coastal strip; local characteristics of the catchment and water body which (may) influence the development and fate of cyanobacterial populations, or even their current location in parts of the water body; and the wider knowledge of the characteristics of cyanobacterial and algal population development and fate and the production and fate of cyanobacterial and algal toxins.

The heterogeneous and dynamic nature of many planktonic population developments may require sampling several sites, such as locations that are prone to accumulation or scum formation, particularly if these are in areas used for recreation; locations at

beaches or in the open water body which are used for immersion sports or those involving accidental immersion (such as sail-boarding or water-skiing); 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 to assess the total population size and thus estimate accumulation or scum formation potential); and decaying accumulations for dissolved toxins. A useful approach for large water bodies is to sample individual zones that differ in some physical, chemical, geomorphological or biological features. Preliminary sampling may help to define these zones, based on their spatial variability and gradients in environmental and plankton properties.

Sampling to assess the total population size may require information on the horizontal distribution of cyanobacteria within the water body, as well as on their distribution with depth. Particularly in thermally stratified water bodies, some cyanobacteria form pronounced population maxima at the depth with optimal light intensity and/or nutrient concentrations. Stratification leads to a water body functioning as two separate masses of water (the epilimnion and the hypolimnion) with different physico-chemical characteristics, with a transitional layer (metalimnion) sandwiched between. Thermal stratification can be determined by measuring vertical profiles of temperature within the water body. In temperate climates, thermal stratification generally occurs seasonally in water bodies of appropriate depth, whereas in tropical climates it often follows diurnal patterns. Thermal stratification has important implications for the distribution of the concentrations of nutrients and the interpretation of phosphorus and nitrogen data. Usually, shallow (2-3 m), wind exposed lakes do not stratify, 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; the stratification is then disrupted by the next event of rain or wind. However, even if temperature is uniform throughout the water column, stratification of organisms can occur on calm days. Depth gradients of oxygen concentration and pH are good indicators of such stratification. Depth-integrated samples are more adequate than surface samples for the assessment of population size and nutrient concentrations in such situations. Approaches to optimising depth-integration are discussed by Utkilen *et al.* (1999) (see also section 10.3).

10.1.3 Monitoring frequency

Monitoring at time intervals should aim (i) to give warnings of developing cyanobacterial and algal populations and associated toxin levels; (ii) to provide information on the duration of cyanobacterial and algal populations and toxin levels that exceed guideline values; (iii) to provide information on the decline of cyanobacterial and algal populations and toxins due to natural processes; or (iv) to enable assessment of the persistence or reduction in cyanobacterial populations and toxin levels due to intervention, such as eutrophication control.

In recreational waters where bloom formation is suspected, the frequency of monitoring should be sufficient to provide data to enable an appropriate Alert Levels Framework system to operate (Chorus and Bartram, 1999). For example, monitoring may begin on a fortnightly basis and be increased to twice-weekly whilst alert levels are exceeded, before being reduced again after alert levels and guideline values for cyanobacterial cells and toxins are no longer exceeded. A scheme of suggested frequencies according to the steps of monitoring is presented in Table 10.3.

Table 10.3 Monitoring frequency and parameters for each of the three phases of the recommended monitoring strategy

Phase or activity	Parameters	Frequency
Monitoring phases		
Phase 1 (background)	Transparency (Secchi disc)	At least once a month
	Nutrient concentrations	At least twice a year (spring overturn and summer)
	Hydrophysical conditions (e.g. flow regime, thermal stratification)	At least once a month
Phase 2 (basic)	Transparency (Secchi disc)	Fortnightly
	Discolouration	Fortnightly
	Scum formation	Fortnightly
	Hydrophysical conditions	At least once a month
	Temperature	Possibly continuous
	Weather conditions (e.g. winds, light)	Continuous
	Changes in turbulence (i.e. mixing)	Possibly continuous
Phase 3 (cyanobacteria)	Transparency (Secchi disc)	Twice weekly
	Qualitative microscopic analysis	Twice weekly
	Quantitative microscopic analysis	Twice weekly
	Chlorophyll a analysis	Twice weekly
Additional activities		
Toxicity	Bioassays (to confirm the presence of toxicity in cells and/or released into the water)	Possibly at first appearance of situations (as in Phase 3) and in all cases when health problems are suspected or reported
Toxin concentration	Chemical analyses (to confirm the presence of specific toxins both qualitatively and quantitatively)	As above

If a water body prone to cyanobacterial mass development is to be used for water-contact sports on a seasonal basis, or for a single event, monitoring should begin not less than two weeks before the beginning of the season or the event. Monitoring should then continue, with the frequency adjusted to enable decisions to be made about access to the facility throughout the season, or whether to proceed with the event.

10.2 Laboratory and staff requirements

Monitoring for cyanobacterial and algal health hazards makes a range of demands upon analytical resources, some of which are different from those made by other aspects of water quality monitoring. Although a higher level of sophistication will provide more information, cyanobacterial and algal monitoring can be highly effective at a very low level of demand on facilities (Table 10.4).

Background monitoring of physical and chemical variables reflecting bloom-forming potential (such as transparency, nutrient concentrations and hydrophysical conditions) makes limited demands upon analytical resources and capacities and may be readily decentralised. While any capable chemical laboratory can carry out laboratory analysis, some limnological or oceanographic expertise is necessary for planning field work, quality control of data, and interpretation of results.

Basic monitoring for indicators of toxic cyanobacteria focuses mainly on critical site inspection and requires almost no facilities. If performed by local staff with observation skills and increasing experience, regular monitoring and recording of simple variables such as transparency, discolouration and scum formation, provides much information for management.

Monitoring of populations of cyanobacteria and algae requires a microscope and some skill in its use. Health authority staff with experience in microscopy can easily learn to recognise the most important toxin-producing cyanobacteria and algae in the waters under their responsibility, provided occasional training by experts is provided.

10.3 Sampling

Samples taken directly by immersion of a sample bottle or sampling device are termed "grab" samples; they are also known as "spot" or "snap" samples. For sampling cyanobacteria, grab samples are often taken from the surface. Composite or integrated samples consist of several sub-samples collected separately (e.g. from different parts of the water body) and then mixed together. They are taken for quantitative, representative samples when the variables to be assessed are unevenly distributed (but information on distribution is not required), for example, when assessing the total content of a substance in a water body (e.g. total phosphorus potentially available for phytoplankton growth) or the total population of an organism (e.g. taking into account the horizontal or vertical variations in distribution of cyanobacterial populations due to the presence of physico-chemical gradients). If knowledge of the precise distribution is required, each sub-sample can be evaluated individually. Composite samples may be:

• *Depth-integrated.* These are most commonly made up of two or more equal sub-samples collected with a sampler at predetermined depth intervals from the surface to just above the bottom. Selection of depths for subsampling must be adequate to account for stratification of temperature, substances and organisms in the water body (Utkilen *et al.,* 1999). Continuous depth integration can be obtained with a tube sampler or a pumping system (see Figures 10.2-10.4).

• *Area-integrated.* These are made by combining a series of samples taken at various sampling points spatially distributed in the water body (usually all at one depth or at predetermined depth intervals).

• *Time-integrated.* These are made by mixing equal volumes of water collected at a sampling station at regular time intervals.

• *Discharge-integrated.* These are integrated over time intervals adapted to the discharge at regular intervals over the period of interest. A common arrangement is to

sample every 3 hours over a 24-hour period. The composite sample is then made by mixing portions of the individual samples that are proportional to the rate of discharge at the time the sample was taken.

Table 10.4 Monitoring approaches, their requirements and options for their organisation

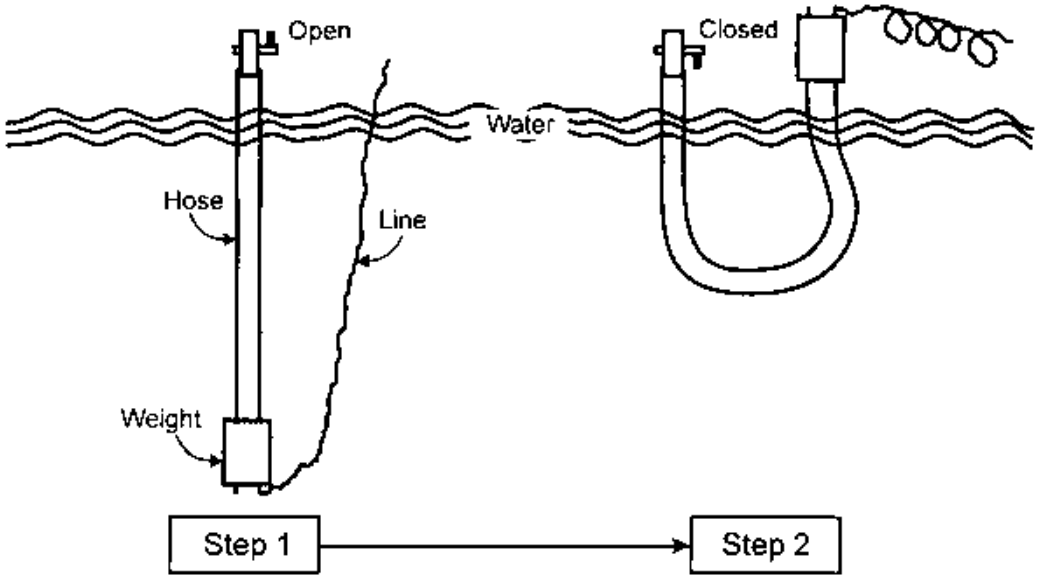
Monitoring type	Parameters of interest	Analytical demands	Who	Where	Notes
Background[1]	Nutrient concentrations (i.e. total phosphorus, nitrate and ammonia); flow regime; thermal stratification; transparency	Low, basic (i.e. photometer, boat, depth sampler and Secchi disc)	Environmental officers or consultants with limnological expertise	Local, regional	Readily incorporated into water resource monitoring
Basic[2]	Transparency; discolouration; scum formation	Minimal (i.e. Secchi disc and regular site inspection by trained staff)	Environmental or health officers	Local	Very high return in relation to input
Cyanobacteria	Dominant taxa - quantity (often determination of genus is sufficiently precise; quantification only as needed for management)	Low, basic (i.e. microscope; photometer is useful)	Environmental or health staff; consultants with limnological expertise	Local, regional	Specific training is required, but quite easily achieved; very high return in relation to input
Toxicity	Toxicity	Low, but skilled (i.e. biotests)	Toxicologists	Central	Demands on skills rather high
Toxin concentration	Toxin content	High[3]	Analytically skilled staff	Central	High return in relation to effort; enables de-warning if bloom proves to be non-toxic

[1] Potential for cyanotoxin

[2] Site inspection for indicators of toxic cyanobacteria

[3] Methods with lower demands are currently under development, for example ELISA

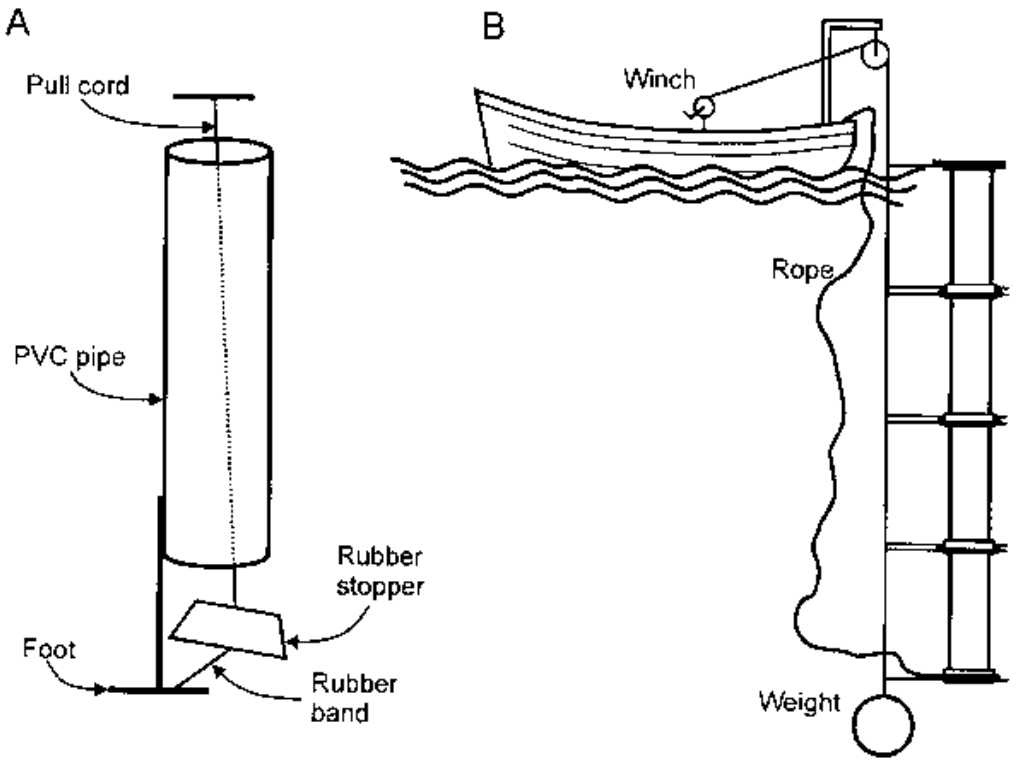
Figure 10.2 A hosepipe sampler for deep waters



A depth sampler, sometimes called a grab sampler or bottle (Van Dorn or Niskin type), is designed in such a way that it can retrieve a sample from any predetermined depth (Venrick, 1978). It consists of a tube that can be closed at its ends by spring-loaded flaps that are triggered by dropping a weight (called a messenger) down the lowering rope. A sample obtained in this way can be used for all chemical analyses except dissolved oxygen. This common sampler is relatively inexpensive, robust and can be deployed from almost any vessel. It gives samples for quantitative analysis.

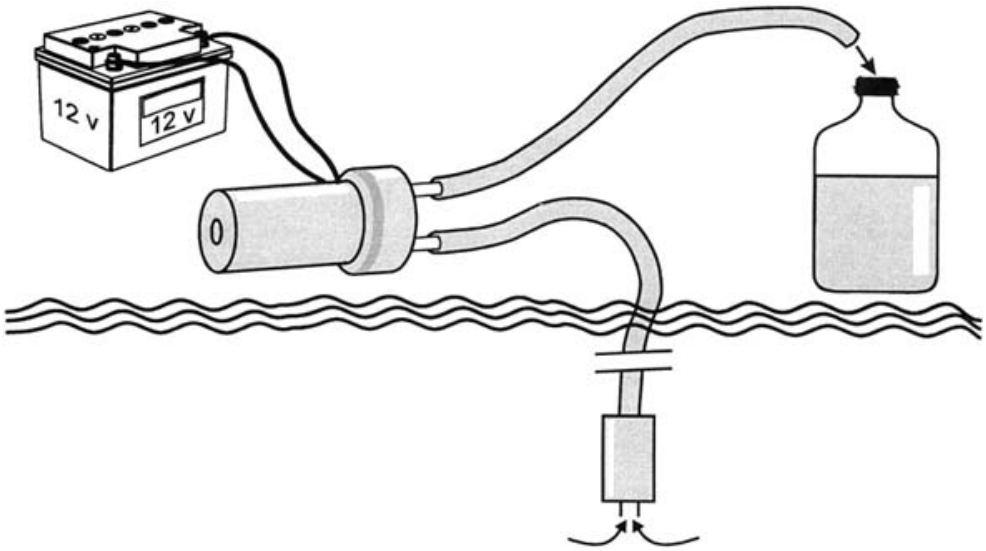
The hosepipe sampler is a piece of flexible plastic piping of several metres in length, weighted at the bottom, and provides a simple mechanism for collecting and integrating a water sample from the surface to the required depth in a lake. The hosepipe is lowered with its upper end open, trapping a water column as it is lowered (Figure 10.2, Step 1). The upper end is closed before hauling up the lower (open) end by means of an attached rope (Figure 10.2, Step 2). The total length of this tube can be up to 30-35 m and it is suitable for relatively calm waters. Another device suitable for taking depth-integrated samples for shallow water columns (less than 5 m deep) or surface waters of deeper water bodies is a simple pipe sampler (Figure 10.3A). The segmented tube sampler (Lindahl, 1986; Sutherland *et al.,* 1992) is a similar alternative for relatively shallow and calm waters. It consists of lengths (1-3 m each) of PVC (polyvinylchloride) pipe linked with valves, with a total length of up to 20 m (Figure 10.3B).

Figure 10.3 A. The pipe sampler: a simple device for depth integrated samples from shallow water bodies; B. The segmented tube sampler (After Sutherland *et al.,* 1992)



Integrated samples can also be obtained using a battery-operated water pump (electric diaphragm pumps are effective) and flexible plastic piping (Figure 10.4) which is operated at a steady pumping rate while the water inlet is drawn upwards between the desired depths at a uniform speed. This apparatus may also be operated to sample many litres of water from the same depth or to filter, for example through a plankton net, large quantities of water from a fixed depth for qualitative and for quantitative (volumes can be measured) analyses. General discussions are given in Beers (1978) and Powlik *et al.* (1991) for peristaltic pumps, and Voltolina (1993) and Taggart and Legget (1984) describe diaphragm pumps.

Figure 10.4 A pump sampling system



Sampling scums

Scums usually 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 their heterogeneous density must be taken into account. Two different approaches may be developed. The first aims to assess the maximum density of cyanobacteria and/or highest toxin level by taking a sample where the scum is thickest (move the bottle mouth along the surface to collect the dense mats of buoyant cyanobacteria). The second approach aims to simulate conditions where shallow waters are mixed by bathers and playing children (agitate the scum before submerging the bottle). Both types of approach may be used for comparison.

Plankton net sampling

Sampling with a plankton net (Tangen, 1978) is mainly performed when large quantities of cell material are required (e.g. for toxicity testing) or when only qualitative analysis of phytoplankton is necessary. Sampling with a net causes a bias according to the size and shape of the organisms, i.e. the finest mesh size of 10 µm will miss small cells, such as unicells of *Microcystis* spp. and picoplankton. Furthermore, filamentous cyanobacteria may be under-represented because some filaments may slip through the net.

The sampling depth is dependent on the taxa of algae and cyanobacteria present. Floating cells *(Microcystis, Anabaena)* are harvested within the upper few metres, whereas sampling of well mixed or stratified water bodies showing a depth distribution of cyanobacteria (e.g. *Planktothrix*) may include deeper water layers. For sampling the water column (or parts of it) lower the plankton net (25-50 µm mesh) to the desired depth, wait until the rope is taught, and then draw it back slowly to the surface. The net should

be drawn very slowly out of the water to allow the water to run through it because large nets are sometimes heavily loaded with water when the pores are clogged with plankton. Rinsing plankton off the netting can be assisted by shaking the net slightly while raising it out of the water slowly.

For sampling surface blooms horizontal net hauls are more appropriate in order to filter floating cells. The plankton net should be moved parallel to the water surface. It can also be towed behind a boat moving slowly.

A disadvantage of collecting material with a plankton net is that the water volume filtered through the net cannot be determined precisely. Calculations based on the area of the net opening and length and distance hauled are not recommended because they overestimate strongly the amount of water actually filtered (due to clogging of the pores, only a fraction of the water volume will actually have passed through the net).

10.3.1 Determination of phosphorus and nitrogen

Water samples are collected with a clean sampler, a water pump or directly with the sample bottle. In shallow, unstratified lakes the sampling depth is less important than in deep, stratified lakes, where at least one sample from the epilimnion, one from the metalimnion and one from the hypolimnion should be taken. If this is not possible, a single surface sample will provide useful information, but only an incomplete picture of the growth conditions for cyanobacteria and algae.

A 100 ml sample container (see section 10.3.4) should be filled, immediately closed and stored cool. If analysis aims at differentiating between the different forms of phosphorus and nitrogen, the storage time should be as short as possible, and no more than 24 hours if the samples can be stored cool. Risks during extended storage involve transformations between dissolved and particulate fractions as well as between nitrate and ammonia. Preferably, the samples should be filtered in the field using membrane filters (0.45 µm pore diameter) pre-washed with a few millilitres of sample, and the filtered fractions should be stored separately. Alternatively samples should be filtered within 4 hours after sampling.

During filtration of samples for analysis of the dissolved nutrient fractions it is particularly important to avoid contamination. Filtering devices may be contaminated with higher concentrations from previous samples, especially if nutrient-enriched deep water layers had been filtered previously. This is especially important for phosphate, because it tends to adsorb to materials. Rinsing the equipment with double-distilled water between localities or with the sample to be filtered is recommended. It is helpful to take samples with low concentrations first (e.g. usually surface water) and to move on to samples in which higher concentrations are expected (e.g. deep waters).

10.3.2 Quantitative and qualitative determination of cyanobacteria and algae

For microscopic determination of cyanobacteria and algae, and for their microscopic quantification grab samples of 50-200 ml are put into a brown glass bottle and fixed immediately with Lugol's iodine solution or formaldehyde solution. Lugol's solution renders cells heavier, thus facilitating enumeration. Addition of 1-2 ml of Lugol's iodine per 100 ml of sample results in a 1 per cent final concentration (note: very hypertrophic

waters may require more preservative). Formaldehyde should be avoided because it presents health risks to the user (it is a potent allergen), or it should be used only under conditions of excellent ventilation in the laboratory and at the microscope. It has the advantage of not causing discolouration of the sample, but the disadvantage of not enhancing settling in counting chambers as effectively as Lugol's solution. Additional fresh, unpreserved samples are useful for microscopic identification because the iodine in the Lugol's solution covers the characteristic colour of the cyanobacteria. Such samples need not be quantitative and may be collected with a plankton net (10 µm mesh) or as a grab sample at a site with high cyanobacteria and algal density. Unpreserved samples for identification may be stored for several hours without appreciable deterioration if kept cool during transportation to the laboratory.

If biomass is to be quantified by chemical analysis of chlorophyll *a* concentration, samples of 1 litre (or less if cell densities are high) of water are taken and filtered as soon as possible. Filtration in the field involves problems of filter transport, which is possible either on ice in an icebox, or submerged in ethanol used for extraction. Direct sunlight must be avoided during filtration and transport.

Materials and method

✔ Brown (or white) glass bottles: 50-200 ml, preferably pre-stocked with a few drops of Lugol's iodine solution.

✔ Brown glass bottles or dark plastic bottles of 1 litre (for chlorophyll a)

✔ Lugol's solution (Willén, 1962).

For chlorophyll a analysis, apparatus for field or laboratory filtration of the water samples includes:

✔ Electric vacuum pump (if filtration is to be performed in the field, a system using a 12V power supply or hand vacuum pump is necessary).

✔ Filtration device.

✔ Glass fibre filters (average pore size 0.7 µm, filter diameter 47 mm).

✔ Either ice and icebox or ethanol and glass vessels for filter transport.

Lugol's solution

Dissolve 20 g potassium iodide into 200 ml of distilled water, mix and add 10 g of sublimated iodine (the solution must not be supersaturated with iodine because this can result in crystal formation with consequent interference in counting). Supersaturation can be tested by diluting 1 ml of stock solution to 100 ml with distilled water to give concentrations similar to those in preserved 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 must be added. Store stock solution in a dark bottle and use within one year.

10.3.3 Cyanotoxin analysis

For toxicity testing, a large amount of cell material may be collected (without determining the water volume from which it originates) with a plankton net as described in section 10.3. If the concentration of cell-bound toxin is to be related to the water volume from which the cells were collected, the best approach is to filter a defined volume as described by Lawton *et al.* (1994) through a 0.45 µm mesh membrane filter. The volume chosen should be sufficient to provide a pronounced greenish layer of material on the filter without clogging the filter. Care must be taken to stir the sample to disperse the cells evenly every time immediately before a sample is poured onto the filter. The filtrate may be used for the analysis of toxins dissolved in the water.

10.3.4 Sample containers

The laboratory that conducts the analyses should ideally provide containers and bottles for the transport of samples. These should be pre-labelled and well-arranged in suitable containers (if cooling is not necessary, soft drink crates, with subdivisions for each bottle, are cheap and very practical). For routine sampling of the same sites, it is advisable always to use the same bottle for each site and each variable. This avoids cross-contamination, which is a particular concern for phosphorus analyses. For most samples glass containers are appropriate, but often plastic containers (which are more stable than glass) can be used instead. The following containers are recommended for the transport of cyanobacteria and related samples:

- *Phosphorus analysis.* 100 ml glass bottles pre-washed and stored with sulphuric acid (4.5 mol l^{-1}) or hydrochloric acid (2 mol l^{-1}) until use, rinse well before use.

- *Nitrate, ammonia and total nitrogen.* Glass or polyethylene bottles (100 ml).

- *Microscopic identification of cyanobacteria.* Wide-mouth polyethylene bottles (100 ml) are appropriate for studying living material in a fresh grab or net sample.

- *Microscopic identification and quantification of cyanobacteria.* Brown glass bottles (100 ml) already containing preservative. Clear (plastic or glass) bottles may be used if the samples can be stored in the dark.

- *Chlorophyll a analysis.* 1 litre brown (plastic or glass) bottles are recommended to avoid degradation of chlorophyll by sunlight. Clear (plastic or glass) bottles may be used if the samples can be stored in the dark. If filtration for chlorophyll *a* analysis is performed in the field, moist filters are best transported either on ice, by folding them with the cell layer inside and wrapping them in aluminium foil, or by immersing them in ethanol immediately after filtration. For the latter, 50 ml wide-mouth brown glass bottles or 10 ml tightly sealing test tubes may be used.

For toxin analysis, cell material is either collected with a plankton net or by filtration. Material enriched with a plankton net is best transported in wide-mouth plastic

containers, in which it may be frozen for subsequent freeze-drying. Material on filters is best transported either dry, if filters can be dried rapidly without direct sunlight, or on ice by folding them with the cell layer inside and wrapping them in aluminium foil. Samples for analysis of dissolved cyanotoxins are acquired by filtration either in the laboratory or in the field. They are transported (filtered or unfiltered) in 1 litre plastic containers.

10.3.5 Sample transport and preservation

Samples must be labelled clearly with sampling site (station), date and time of sampling and depth of sampling. Preservation of samples, filtered volumes and any irregularity should be noted in the field record. In general, samples should be stored cool and dark in a storage box (if necessary, with solid coolant) during sampling and transportation. Storage of samples between 2°C and 5°C may preserve many types of samples, but checks should be made to confirm this with each sample type. Preferably, the samples should be analysed immediately after sampling. If a storage time longer than 12 hours is necessary, quick-freezing of samples to -20°C is recommended. Samples to be filtered must be filtered before freezing.

For the analysis of ammonia, storage is particularly critical. Samples can be cooled in a refrigerator but should be analysed within three hours of collection. Preservation for longer periods is not recommended. Filtration of samples should be avoided. It is nearly impossible to obtain filters free of ammonia and filtration may also evaporate the ammonia contained in the sample.

If samples for chlorophyll *a* are filtered in the field, filters must be either transported frozen, or submerged in ethanol. The ethanol should be boiling (at 75°C) when put onto the filters. If this is not possible in the field, transporting filters submerged in cold ethanol and heating the ethanol later in the laboratory may be preferable to the risk of degradation occurring on filters transported dry but poorly cooled. The suitability of this approach should be checked for any given situation.

Samples for microscopic enumeration preserved with Lugol's iodine at the time of collection (see section 10.3.2) are relatively stable and no special storage is required, although they should be protected from extreme temperatures and strong light. Nevertheless, it is recommended that samples are examined and counted within a few weeks because some species of phytoplankton are sensitive to prolonged storage and Lugol's iodine solution and may degrade if stored for many months or even years. Unpreserved samples for microscopic quantification require immediate attention, either by addition of preservative or by following alternative counting methods that do not use preserved cells. Where unpreserved samples cannot be analysed immediately they should be stored in the dark with the temperature kept stable, at about +4 °C.

10.4 On-site analysis

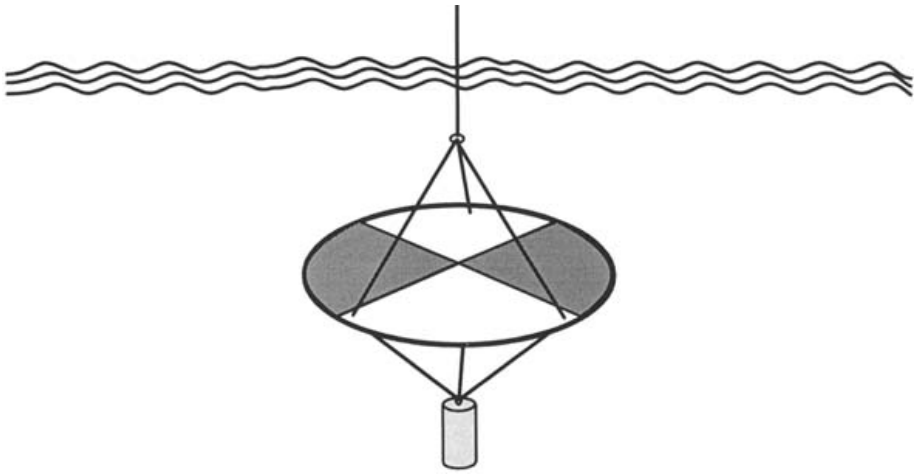
A significant advantage of on-site testing is that tests are carried out on fresh samples which have not been contaminated or the characteristics of which have not otherwise changed as a result of storage. Some analyses such as temperature and transparency can only be carried out in the field.

10.4.1 Transparency

The transparency of water is strongly influenced by turbidity due to particles such as phytoplankton (cyanobacteria and algae) or suspended silt. It can be measured easily in the field. If transparency is low (less than 1-2 m) and accompanied by greenish to bluish discolouration, streaks, or even scums, high cyanobacterial densities are likely. Such results may initiate immediate inspection of further downwind sites and the collection of samples for cyanobacterial analysis.

Transparency can be obtained approximately with a Secchi disc (see Figure 10.5). The disc is made of rigid plastic or metal, but the details of its design are variable. It may be 20 or 30 cm or even larger in diameter and is usually painted white. Alternatively, it may be painted with black and white quadrants. The disc is suspended on a light rope or chain so that it remains horizontal when it is lowered into the water. The suspension rope is graduated at intervals of 0.1 and 1 m from the level of the disc itself and usually the rope does not need to be more than 5 m in length. A weight fastened below the disc helps to keep the suspension rope vertical while the measurement is being made. Transparency estimated in this way (submersible photometers are also available for these measurements) is taken to be the mean of the depths at which the disc disappears when viewed from the shaded side of the boat and at which it reappears upon raising after it has been lowered beyond visibility. Observation of the disc through a tube (painted black inside) with a transparent pane at the lower end and held just below the surface improves precision, particularly if the water surface is perturbed (Wetzel and Likens, 1990).

Figure 10.5 The Secchi disc for measuring transparency



If cyanobacteria occur as floating streaks or mats on the water it is difficult to obtain representative transparency data. Depending on the measuring site, values can vary from 0 to greater than 2 m. It may be useful to determine transparency in areas without floating cells as well as within scums. The Secchi disc has to be lowered very carefully to prevent destroying the formation of accumulated cyanobacterial cells, and before taking

the Secchi disc measurement the surface scums should be given time to return to their original water coverage again.

An improvised transparency determination may be recommended by local authorities for users of recreational sites known to be affected frequently by cyanobacterial mass developments. If bathers cannot see their feet while standing knee-deep in the water because of greenish turbidity, bathing should be avoided.

10.4.2 Temperature

Temperature is best measured *in situ* with a probe because water samples gradually reach the same temperature as the surrounding air. If this is not possible, it may be measured with a thermometer or probe in a water sample of at least 1 litre, immediately after taking the sample. Graduations of 0.1 °C are appropriate.

Procedure

1. If measuring in a sample, immerse the thermometer in the water until the temperature reading is constant. Record the reading to the nearest 0.1 °C.

2. If using a probe, lower the probe to the required depth. Hold it at that depth until the reading on the meter is constant. If a complete profile of temperatures is to be taken, measurements should be made at 1 m intervals from the surface to the bottom. Near the surface, or in areas of large thermal discontinuities, measurements should be made at intervals of less than 1 m.

10.4.3 *In situ* fluorometric analysis of chlorophyll *a* and remote sensing

Submersible fluorometers exist which can provide fine scale profiles vertically and horizontally. This is valuable, particularly for monitoring large water bodies and coastal areas for highly variable patterns of chlorophyll concentration (algal and/or cyanobacterial biomass). This approach has been used successfully to monitor variability in phytoplankton biomass and species composition, as well as surface temperature, salinity and nutrient concentrations, in the Baltic Sea. Fully automated analyser systems are installed on three passenger ferries. The system allows high frequency sampling with a spatial resolution of about 100 m and a temporal resolution of 1-3 days. The project uses, especially during the cyanobacterial bloom period, satellite images to detect the extent of the algal surface accumulations. The data are used to provide information on the Baltic Sea phytoplankton on the Internet at http://www.fimr.fi. Another example of the use of a flow-through system deployed on ferries has been reported from Japan (Harashima *et al.,* 1997).

Real-time data for chlorophyll *a* distribution and concentrations, and potentially for cyanobacterial phycobiliprotein pigments in freshwaters, can be generated by the remote sensing of water optical properties 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 such as cloud cover. Nevertheless, the remote sensing of cyanobacterial populations as a contribution to water body management has excellent potential.

Visible satellite imagery provides a synoptic perspective but, with few exceptions, does not yet have the ability to discriminate between different phytoplankton taxa nor is it effective during inclement weather. New techniques like the use of Advanced Very High Resolution Radiometers (AVHRR) on board the polar orbiting National Oceanic Atmospheric Administration (NOAA) satellites have been applied to monitor large-scale algal blooms, for example in North America (Gower, 1997) and the Baltic Sea (Kahru, 1997).

Table 10.5 Differentiation of phosphorus fractions

Fraction	Definition
1. Soluble reactive phosphorus	Filtered sample
2. Dissolved organic phosphate	Digested filtered sample
3. Particulate phosphorus	Total phosphorus less the dissolved organic phosphate fraction (i.e. 4 minus 2)
4. Total phosphorus	Digested unfiltered sample

10.5 Determination of nutrients in the laboratory

Whereas for phosphorus, the method given in section 10.5.1 is widely accepted and easy to perform with common laboratory equipment, several methods may be considered appropriate for nitrate, depending on available equipment. The method given in section 10.5.2 demands the least equipment but, due to an evaporation step, reproducibility may be poorer than for the method given in section 10.5.3. Both methods use hazardous chemicals which require appropriate safety protection and hazardous waste collection. Ion chromatography, if available, evades this problem and may be the preferred option.

10.5.1 Phosphorus

Some widely accepted digestion methods for dissolving particles to release all of the phosphate achieve this aim only incompletely. The procedure of Koroleff (1983a) for determining total phosphate has proved to be simple and efficient and is the basis of the ISO/FDIS protocol 6878 (ISO, 1998). This method is applicable to many types of water including seawater, in a concentration range of 5-800 µg l^{-1} of P, or higher if the samples are diluted. Differentiation by the fractions shown in Table 10.5 is possible through filtration. Further information is available in Wetzel and Likens (1990) and APHA/AWWA/WPCF (1995).

Principle

Digestion or mineralisation of organophosphorus compounds to SRP (also known as 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 a household pressure cooker), or simply by gentle boiling. Polyphosphates and many organophosphorus compounds may also be hydrolysed with

sulphuric acid to molybdate-reactive orthophosphate. Many organophosphorus compounds are converted to SRP by mineralisation with persulphate. Orthophosphate ions are reacted with an acid solution containing molybdate and antimony ions to form an antimony phosphomolybdate complex. The complex is reduced with ascorbic acid to form a strongly coloured molybdenum blue complex. The absorbance of this complex is measured to determine the concentration of SRP present. An overview of the procedure, necessary equipment and chemicals is provided below (see also ISO, 1998).

Reagents

Only reagents of recognised analytical grade, and only distilled water having a phosphate content that is negligible compared with the smallest concentration to be determined in the samples, should be used.

✔ Sulphuric acid (1.84 g ml^{-1}) (Caution, eye protection and protective clothing are necessary).

✔ Sulphuric acid, solution, $c(H_2SO_4)$ = 9 mol l^{-1}. Add 500 ± 5 ml of water to a 2 litre beaker. Cautiously add, with continuous stirring, 500 ± 5 ml of sulphuric acid (1.84 g ml^{-1}) and mix well. (Caution, eye protection and protective clothing are necessary).

✔ Sulphuric acid, solution, $c(H_2SO_4)$ = 4.5 mol l^{-1}. Add 500 ± 5 ml of water to a 2 litre beaker. Cautiously add, with continuous stirring, 500 ± 5 ml of sulphuric acid solution (9 mol l^{-1}) and mix well. (Caution, eye protection and protective clothing are necessary)

✔ Sulphuric acid, solution, $c(H_2SO_4)$ = 2 mol^{-1}. Add 300 ± 3 ml of water to a 1 litre beaker. Cautiously add, with continuous stirring and cooling, 110 ± 2 ml of sulphuric acid solution (9 mol l^{-1}). Dilute to 500 ml ± 2 ml with water and mix well. (Caution, eye protection and protective clothing are necessary)

✔ Ascorbic acid, 100 g l^{-1} solution. Dissolve 10 g ± 0.5 g of ascorbic acid in 100 ml ± 5 ml of water. The solution is stable for two weeks if stored in an amber glass bottle in a refrigerator and can be used as long as it remains colourless.

✔ Sodium hydroxide, solution, c(NaOH) = 2 mol l^{-1}. Dissolve 80 g of sodium hydroxide pellets in water, cool and dilute to 1 litre with water. (Caution, eye protection and protective clothing are necessary).

✔ Acid molybdate, solution. Add the molybdate solution (I) to 300 ml ± 5 ml of sulphuric acid 9 mol l^{-1} with continuous stirring. Add the tartrate solution (II) and mix well. (Caution, eye protection and protective clothing are necessary).

✔ Molybdate solution (I). Dissolve 13 g ± 0.5 g of ammonium heptamolybdate tetrahydrate $[(NH_4)_6Mo_7O_{24}4H_2O]$ in 100 ml ± 5 ml of water.

✔ Tartrate solution (II). Dissolve 0.35 g antimony potassium tartrate hemihydrate [$K(SbO)C_4H_4O_6½H_2O$] in 100 ml ± 5 ml of water. The reagent is stable for at least two months in an amber glass bottle.

✔ Sodium thiosulphate pentahydrate, 12 g l^{-1} solution. Dissolve 1.2 g sodium thiosulphate pentahydrate ($Na_2S_2O_35H_2O$) in 100 ml water. Add about 50 mg of anhydrous sodium carbonate (Na_2CO_3) as preservative. This reagent is stable for at least four weeks if stored in an amber glass bottle.

✔ Potassium peroxodisulphate solution. Add 5 g potassium peroxodisulphate ($K_2S_2O_8$) to 100 ml water, stir to dissolve. The solution is stable for at least two weeks, if the supersaturated solution is stored in an amber borosilicate bottle, protected from direct sunlight.

✔ Soluble reactive phosphate (orthophosphate), stock standard solution corresponding to 50 mg of P per litre. Dry a few grams of potassium dihydrogenphosphate to constant mass at 105 °C. Dissolve 0.2197 g of KH_2PO_4 in about 800 ml of water in a 1,000 ml volumetric flask. Add 10 ml of 4.5 mol l^{-1} sulphuric acid and make up to the mark with water. The solution is stable for at least three months if stored in a well stoppered glass bottle. Refrigeration to about 4 °C is recommended.

✔ Soluble reactive phosphate (orthophosphate), standard solution corresponding to 2 mg of P per litre. Pipette 20 ml of SRP stock standard solution into a 500 ml volumetric flask.

Make up to the mark with water. Prepare this solution each day it is required. One millilitre of this standard solution contains 2 µg of P.

Apparatus

✔ Ordinary laboratory apparatus and filter assembly with membrane filters, 40-50 mm diameter with 0.45 µm pore size.

✔ Pre-cleaned glass bottles for filtered samples.

✔ Spectrometer, suitable for measuring absorbance in the visible and near infrared regions. Capable of accepting optical cells with pathlengths from 1 cm to 5 cm. The most sensitive wavelength is 880 nm, but if a loss of sensitivity is acceptable, absorbance can be measured at the second maximum of 680-700 nm. The detection limit of the method is lower if a spectrometer capable of accepting 10 cm pathlength optical cells is available.

✔ Autoclave (or pressure cooker): used for digestion of samples at 115-120 °C.

✔ Borosilicate flasks, 100 ml, with glass stoppers tightly fastened by metal clips (heat resistant polypropylene bottles or conical flasks (screw capped) are also suitable). Before use, clean the bottles or flasks by adding about 50 ml of water and 2 ml of 1.84

gm l^{-1} sulphuric acid. Place in an autoclave for 30 minutes at 115-120°C, cool and rinse with distilled water. Repeat the procedure several times and store filled with distilled water and covered.

Before use, all glassware should be washed with 2 mol l^{-1} hydrochloric acid at 45-50 °C and rinsed thoroughly with water. Do not use detergents containing phosphate. Preferably, the glassware should be used only for the determination of phosphorus. After use it should be cleaned as above and kept covered until use. Glassware used for the colour development stage should be rinsed occasionally with sodium hydroxide solution to remove deposits of the coloured complex that has a tendency to stick (as a thin film) on the walls of glassware.

Procedure

If filtration is necessary for the determination of total soluble phosphorus and/or dissolved phosphate, filter the sample within 4 hours after sampling. If the sample was cooled, bring it to room temperature before filtration. Wash a 0.45 µm membrane filter to ensure it is free of phosphate by passing through it 200 ml of water, previously heated at 30-40 °C. Filter the sample discarding approximately the first 10 ml of filtrate and collecting 5-40 ml depending on the concentrations expected. The filtration time should not exceed 10 minutes. If necessary a larger diameter filter should be used. Add 1 ml of 4.5 mol l^{-1} sulphuric acid per 100 ml of test sample. The acidity should be about pH 1, if not, adjust with NaOH 2 mol l^{-1} or H_2SO_4 2 mol l^{-1}. Store in a cool dark place until analysis is possible.

The mineralisation method using potassium peroxodisulphate is described here. This method will not be efficient in the presence of large quantities of organic matter. In this case oxidation with nitric acid-sulphuric acid is necessary. This latter procedure must be carried out in an efficient fume cupboard.

1. Pipette up to a maximum of 40 ml of the test sample (appropriately prepared) into a 100 ml conical flask. If necessary, dilute with water to about 40 ml.

2. Add 4 ml of potassium peroxodisulphate solution and boil gently for 30 minutes. Periodically, add sufficient water so that the volume remains between 25 ml and 35 ml.

3. Cool, adjust pH to between 3 and 10, transfer to a 50 ml volumetric flask, and dilute with water to about 40 ml.

Thirty minutes is usually sufficient to mineralise phosphorus compounds; some polyphosphoric acids need up to 90 minutes for hydrolysis. Alternatively mineralise for 30 minutes in an autoclave at between 115 °C and 120 °C. Most ordinary kitchen pressure cookers are adequate if laboratory equipment is not available. Any arsenate present will cause interference. If arsenic is known or suspected to be present in the sample, eliminate the interference by treating the solution with sodium thiosulphate solution immediately after the mineralisation step. In the case of seawater mineralised in an autoclave, free chlorine must be removed by boiling before the arsenate is reduced by thiosulphate. Iron concentrations above 600 mg l^{-1} (e.g. in mining lakes) and sulphide (detectable by its smell) will also interfere.

Table 10.6 Selection of appropriate volumes for test portions in relation to the concentration of soluble reactive phosphorus

SRP concentration range (mg l^{-1})	Volume of test portion (ml)	Thickness of optical cell (cm)
0.0 to 0.2	40	4 or 5
0.0 to 0.8	40	1
0.0 to 1.6	20	1
0.0 to 3.2	10	1
0.0 to 6.4	5	1

4. Test portion after the mineralisation or filtration step. The maximum volume of test portion to be used is 40 ml. This is suitable for the determination of SRP concentrations of up to 0.8 mg l^{-1} when using an optical cell of 1 cm pathlength to measure the absorbance of the coloured complex formed by the reaction with acid molybdate reagent. Smaller test portions may be used as appropriate in order to accommodate higher phosphate concentrations (Table 10.6). Phosphate concentrations at the lower end of the calibration ranges are best determined by measuring absorbance in optical cells of 4, 5 or 10 cm pathlength.

Carry out a blank test in parallel with the determination, by the same procedure, using the same quantities of all the reagents as in the determination, but using the appropriate volume of water instead of the test portion.

5. Calibration solutions. (A1) To prepare the set of calibration solutions transfer, by means of a pipette, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of the SRP standard solution to 50 ml volumetric flasks. Dilute with water to about 40 ml. These solutions represent SRP concentrations from 0.04 mg l^{-1} to 0.4 mg l^{-1}. If total phosphate or total soluble phosphate is being determined, proceed according to the mineralisation method chosen. Then proceed to colour development. Proceed accordingly for other ranges of phosphate concentration (Table 10.6). Typically, the test portion volume will be in the range of 5-10 ml.

6. Colour development. (A2) Add to each 50 ml flask, while swirling, 1 ml of ascorbic acid 100 g l^{-1} and, after 30 seconds, 2 ml of acid molybdate solution I. Make up to the mark with water and mix well.

7. Spectrometric measurements. (A3) Measure the absorbance of each solution at 880 nm after 10-30 min, or if loss of sensitivity can be accepted at 700 nm. Use water in the reference cell.

8. Plotting the calibration graph. (A4) Plot a graph of absorbance against the phosphorus content (in mg l^{-1}) of the calibration solutions. The relationship between absorbance and concentration is linear. Determine the reciprocal of the slope of the graph. Check the graph from time to time, especially if new packages of chemicals are used. Run a calibration solution with each series of samples.

9. Determination. (B1) Colour development - proceed as in (A2) using the test portion appropriately processed. (B2) Spectrometric measurements - proceed as in (A3).

Expression of results

The concentration of total phosphorus expressed in mg l^{-1} is given by the equation:

$$P_{tot} = \frac{(A - A_0)V_{max}}{fV_s}$$

where:

A is the absorbance of the test portion
A_0 is the absorbance of the blank test
f is the slope of the calibration graph (e4), in litres per milligram
V_{max} is the reference volume of the test portion (50 ml)
V_s is the actual volume, in ml, of the test portion.

The test report should contain complete sample identification, reference to the method used, the results obtained and any further details likely to influence the results.

10.5.2 Spectrometric method for nitrate using sulphosalicylic acid

This method does not require sophisticated equipment and is suitable for surface and potable water samples (ISO, 1998). The method may be used up to nitrate-nitrogen concentrations of 0.2 mg l^{-1} using the maximum test portion volume of 25 ml, and can be expanded by using smaller test portions. The limit of detection lies within 0.003 and 0.013 mg l^{-1}, using cells of path-length 40 mm and a 25 ml test portion volume. A nitrate-nitrogen concentration of 0.2 mg l^{-1} gives an absorbance of about 0.68 units, using a 25 ml test portion and cells of 40 mm pathlength. The main interferences are chloride, SRP, magnesium and manganese (II). Interference problems can be avoided with other Spectrometric methods such as ISO 7890-1 and 7890-2 (ISO, 1986a,b).

Reagents

✔ Sulphuric acid ≈ 18 mol l^{-1} = 1.84 g ml^{-1} (Caution, eye protection and protective clothing are necessary).

✔ Glacial acetic acid ≈ 17 mol l^{-1} = 1.05 g ml^{-1} (Caution, eye protection and protective clothing are necessary).

✔ Alkali solution = 200 g l^{-1}. Dissolve with care 200 g ± 2 g of sodium hydroxide pellets in about 800 ml of water. Add 50 g ± 0.5 g of EDTA-Na_2 and dissolve. Cool to room temperature and make up to 1 litre with water in a measuring cylinder. Store in polyethylene bottle. This reagent is stable indefinitely. (Caution, eye protection and protective clothing are necessary).

✔ Sodium azide solution = 0.5 g l^{-1}. Dissolve with care 0.05 g ± 0.005 g of sodium azide in about 90 ml of water and dilute to 100 ml with water in a measuring cylinder. Store in a glass bottle. This reagent is stable indefinitely. (Caution: this reagent is very toxic if swallowed. Contact between the solid reagent and acid liberates very toxic gas).

✔ Sodium salicylate solution 10 g l^{-1}. Dissolve 1 g ± 0.1 g of sodium salicylate in 100 ml ± 1 ml of water. Store in a glass polyethylene bottle. Prepare this solution freshly on each day of operation.

✔ Nitrate, stock standard solution 1,000 mg l^{-1}. Dissolve 7.215 g ± 0.001 g of potassium nitrate (previously dried at 105 °C for at least 2 h) in about 750 ml of water. Transfer to a 1 litre volumetric flask and make up to one litre mark with water. Store the solution in a glass bottle for not more than two months.

✔ Nitrate, standard solution 100 mg l^{-1}. Pipette 50 ml of the stock standard solution into a 500 ml volumetric flask and make up to the 500 ml mark with water. Store the solution in a glass bottle for not more than one month.

✔ Nitrate, working standard solution 1 mg l^{-1}. Into a 500 ml volumetric flask, pipette 5 ml of standard nitrate solution. Make up to 500 ml mark with water. Prepare the solution freshly on each occasion of use.

Apparatus

Standard laboratory apparatus plus:

✔ Spectrometer, capable of operating at 415 nm with cells of 40 or 50 mm pathlength.

✔ Evaporating dishes, about 50-ml capacity. If the dishes are new or not in regular use, they should be rinsed first with water and taken through the procedure as in the colour development step (see below).

✔ Water bath, boiling, capable of accepting at least six of the evaporating dishes.

✔ Water bath, capable of thermostatic regulation to 25 °C ± 0.5 °C.

Procedure

Warning: This procedure involves the use of concentrated sulphuric acid, acetic acid, sodium hydroxide and sodium azide solutions. Eye protection and protective clothing are essential when using these reagents. They must never be pipetted by mouth.

The maximum test portion volume, which can be used for the determination of nitrate concentrations up to 0.2 mg l^{-1}, is 25 ml. Use smaller test portions as appropriate in order to accommodate higher nitrate concentrations. Because surface water samples contain suspended matter, allow them to settle, centrifuge them or filter them through a washed

glass fibre filter before taking the test portion. Neutralise samples with a pH value greater than 8 with acetic acid before taking the test portion.

Carry out a blank test in parallel with the determination, using 5 ml ± 0.05 ml of water instead of the test portion and designate the measured absorbance A_b.

1. Calibration. To prepare the set of calibration solutions add, to a series of clean evaporating dishes, using a burette, 1, 2, 3, 4 and 5 ml respectively of the working nitrate standard solution, corresponding to nitrate amounts of 1, 2, 3, 4 and 5 µg in the respective dishes.

2. Colour development. Add 0.5 ml ± 0.005 ml of sodium azide solution and 0.2 ml ± 0.002 ml of acetic acid. Wait for at least 5 minutes, and then evaporate the mixture to dryness in the boiling water bath. Add 1 ml ± 0.01 ml of sodium salicylate solution, mix well and evaporate the mixture to dryness again. Remove the dish from the water bath and allow the dish to cool to room temperature.

Add 1 ml ± 0.01 ml of sulphuric acid and dissolve the residue in the dish by gentle agitation. Allow the mixture to stand for about 10 minutes. Then add 10 ml ± 0.1 ml of water followed by 10 ml ± 0.1 ml of alkali solution.

Transfer the mixture to a 25-ml volumetric flask but do not make up to the 25 ml mark. Place the flask in the water bath at 25 °C ± 0.5 °C for 10 min ± 2 min. Then remove the flask and make up to the 25 ml mark with water.

3. Spectrometric measurements. Measure the absorbance of the solution at 415 nm in cells of pathlength 40 or 50 mm against distilled water as a reference. Designate the absorbance measured as A_s.

4. Plotting the calibration graph. Subtract the absorbance of the blank solution from the absorbances of each of the calibration solutions and plot a calibration graph.

5. Determination. Pipette the selected test portion of volume V, such that the aliquot contains a mass of nitrate-nitrogen between 1 µg and 5 µg, into a small evaporating dish. Then proceed as in the preceding "Colour development" and "Spectrometric measurements" steps.

6. Correction for test portion absorption. If absorption by the test portion at the analytical wavelength is known, or suspected, to interfere (as may arise with highly coloured samples), carry out the operations given in the preceding "Colour development" and "Spectrometric measurements" steps, on the duplicate test portion but omitting the addition of sodium salicylate solution. Designate the absorbance measured be A_c.

Table 10.7 The effect of other substances on the results obtained with the spectrometric method for nitrate using sulphosalicylic acid

Other substance	Amount of other substance in a 25 ml test portion (µg)	Effect of other substance in a 25 ml test portion	
		m(N) = 0.00 µg (µg N)	m(N) = 5.00 µg (µg N)
Sodium chloride	10,000	+ 0.03	- 0.73
Sodium chloride	2,000	+ 0.01	- 0.16
Sodium sulphate	10,000	+ 0.04	- 0.16
Sodium hydrogen carbonate	10,000	- 0.02	- 0.52
Sodium hydrogen carbonate	2,000	- 0.03	- 0.18
Calcium chloride	5,000	+ 0.23	+ 0.38
Calcium chloride	2,500	+ 0.02	- 0.14
Iron (III) sulphate	20	+ 0.08	- 0.02
Manganese (II) sulphate	20	+ 0.92	+ 0.99
Manganese (II) sulphate	5	+ 0.05	+ 0.13
Zinc sulphate	20	- 0.02	+ 0.07
Copper sulphate	20	+ 0.03	+ 0.19
Ammonium chloride	500	- 0.12	- 0.17

Expression of results

Calculate the absorbance due to nitrate in the test portion, A_t, from the equation:

$$A_t = A_s - A_b$$

or, when a correction for sample absorption has been made, from the equation:

$$A_t = A_s - A_b - A_c$$

In both equations A_s, A_b and A_c refer to the sample, blank and correction absorbances respectively (see relevant sections above). Read off from the calibration graph, the mass of nitrate, *m(N)* in micrograms, corresponding to the absorbance value A_t.

The nitrate content in the sample, in mg l^{-1}, is given by the formula: *m(N)/V* where *V* is the volume of the test portion (in ml). The effect of other substances on this method is provided in Table 10.7.

10.5.3 Spectrometric method for nitrate by reduction of nitrate to nitrite

Nitrates are reduced to nitrites almost quantitatively by amalgamated granulated cadmium. Separate methods for nitrate reduction and for nitrite determination are presented below.

Determination of nitrate: scope, field application and principle

This method is based on the reduction of nitrate ions to nitrite. Because it determines the sum of nitrite and nitrate ions, a separate determination of nitrite must be conducted, and its concentration subtracted from the sum of nitrate and nitrite. At concentrations higher than about 20 µM NO_3-N, calibration factors for a low and high range must be established. The reduction is carried out at a pH of about 8.5. Ammonium chloride buffer is used to control the pH and to complex the liberated cadmium ions (Carlberg, 1972).

Reagents

✔ Sulphanilamide (SAN) (Caution: possibly hazardous, use fume cupboard, solution must not be discharged to a public sewer). Dissolve 10 g SAN, $NH_2C_6H_4SO_2NH_2$, in a mixture of 100 ml concentrated hydrochloric acid, HCl (36 per cent) and 600 ml bi-distilled water (BdW). After cooling dilute the solution to 1 litre. At room temperature, when stored in glass bottles, the reagent is stable for several months.

✔ Naphtylamine solution (NED) (Caution: possibly hazardous, use fume cupboard, solution must not be discharged to a public sewer). Dissolve 1 g N-(1-naphthyl)-ethylene-diamine dihydrochloride, $C_{10}H_7NHCH_2NH_22HCl$, in BdW and dilute to 1 litre. The solution should be stored in a tightly closed dark bottle, with 3-4 drops of saturated $HgCl_2$ solution in a refrigerator (Kirkwood, 1992). The solution contains 10 µmoles ml^{-1}.

✔ Buffer solutions:

25 per cent stock buffer. Dissolve 250 g ammonium chloride, NH_4Cl in BdW and

25 ml concentrated ammonium hydroxide 25 per cent. Dilute to 1 litre.

2.5 per cent work buffer (WB). Dilute 100 ml of stock buffer with BdW to 1 litre.

Wash buffer solution (WbS). Dilute 20 ml of 2.5 per cent WB with BdW to 1 litre.

✔ Hydrochloric acid 2M. Dilute 165 ml of concentrated commercial HCl (37 per cent) with BdW to 1 litre.

✔ Mercuric chloride solution 1 per cent (Caution, highly toxic). Dissolve 5 g mercuric chloride, $HgCl_2$, in 500 ml BdW.

✔ Synthetic seawater (SSW). Dissolve 36 g sodium chloride, NaCl, 12 g magnesium sulphate heptahydrate, $MgSO_47H_2O$, and 0.25 g sodium carbonate, $NaHCO_3$, with BdW

and dilute to 1 litre. For analytical purposes this is equivalent to a salinity of 40 psu. For calibration work the SSW may be diluted to the desired salinity.

✔ Nitrate standard stock solution (NO_3-N SSS). Dissolve 0.25278 g potassium nitrate, KNO_3 (molecular weight 101.11) dried at 110 °C to constant weight, in BdW and dilute to 250 ml. Store in a tightly closed dark bottle with 2-3 drops of a saturated $HgCl_2$ solution in a refrigerator (Kirkwood, 1992). The solution contains 10 µmoles ml^{-1}.

✔ Cadmium coarse pulver. Sieve commercially available granulated cadmium and retain and use the fraction between 35 and 40 mesh, i.e. around 0.5 to 0.42 mm. (Caution: Cadmium is a poisonous metal. It should be handled with great care. All operations on the dry metal, particularly the granules, must be carried out in a well-ventilated area, e.g. a fume cupboard. Never inhale the dust. Cadmium must be treated as hazardous waste).

✔ Amalgamated cadmium. The required amount of cadmium metal is about 35 g per reduction column (RC) (Figure 10.6). The sieved granules are rinsed from the oxides by washing with 2M HCl. Then they are washed with plenty of water to eliminate all HCl. All the washed metal is transferred to a round-bottom flask that is filled with 1 per cent $HgCl_2$ solution. The flask is closed with a glass stopper. After this step all contact between air and the metal should be avoided. The flask is rotated for 90 minutes in a horizontal position or shaken with suitable equipment. Finally the flask is opened and the turbid sublimate solution rinsed out with BdW. (Caution: the used $HgCl_2$ solution must not be discharged to a public sewer). When a suitable volume of $HgCl_2$ is collected, 25 ml concentrated HCl is added per litre and then precipitated with hydrogen sulphide, H_2S, or sodium sulphide, Na_2S. The liquid is filtered and the precipitate stored, discarding the clear filtrate.

Apparatus and equipment

✔ Test tubes with glass or plastic stoppers, graduated or marked at 25 ml volume.

✔ Automatic syringe pipettes of 1 ml.

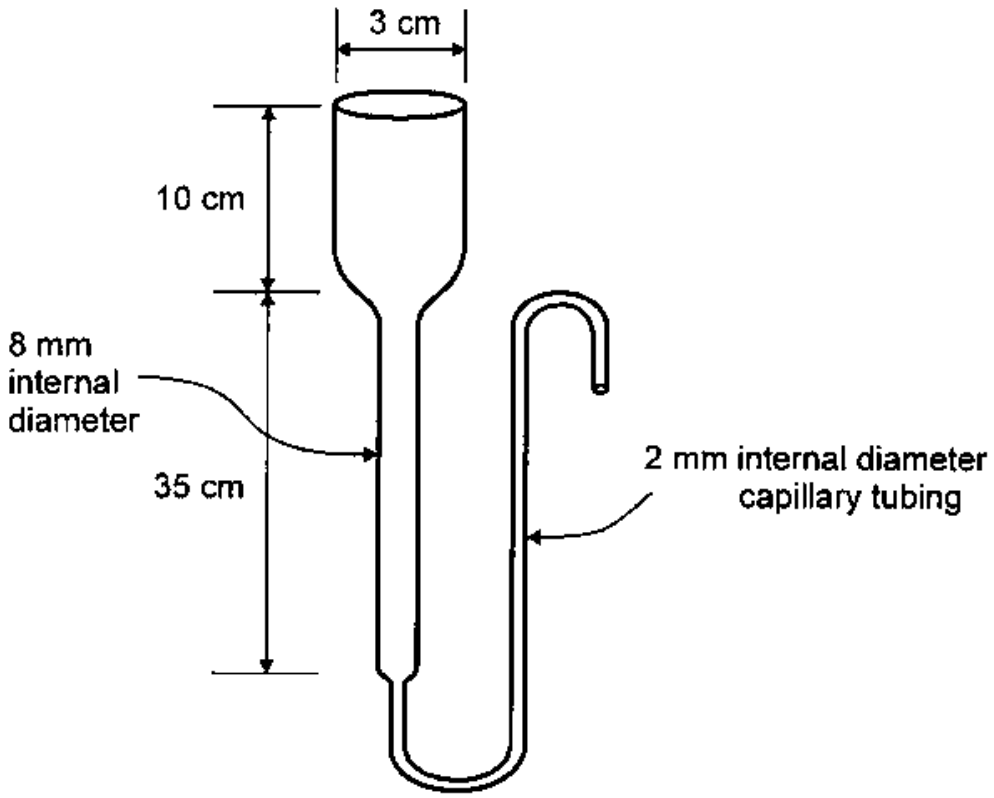
✔ 25 ml automatic pipette.

✔ 500 ml round-bottom flask.

✔ Reduction columns (Figure 10.6).

✔ Spectrometer, with 1, 5 and 10 cm pathlength cells.

Figure 10.6 A reduction column



Preparation of the reduction columns

A small ball of thin copper wire is placed at the bottom of the RC and above the wire a small ball of glass wool. The RC is filled entirely with water. The metal granules are poured into the RC, with a small plastic spoon (making sure that no cavities are formed in the column) and filled to about 1 cm below the reservoir. The amalgamated metal is activated by passing through about 150 ml of Wash buffer Solution (WbS) containing about 100 µM NO_3-N then rinsed thoroughly. The RC is packed with WbS only, before being used for analysis. A newly prepared RC reduces nitrate with an efficiency of 95-100 per cent.

Table 10.8 Preparation of working standard solutions of nitrate by dilution with bi-distilled water or synthetic seawater

Volume of solution D[1] (ml)	Total volume (ml)	Resultant concentration (µM NO_3^--N)
25.0	500	10.00
25.0	1,000	5.00
5.0	1,000	1.00

[1] Solution "D" is prepared by diluting 5 ml of an SSS in 250 ml, giving a solution which contains 0.20 µmoles NO_3^--N per ml

Calibration

There is a significant salinity effect in the calibration for nitrate measurements by manual methods using Hg-Cd reduction columns. Freshly amalgamated RC show a salinity effect of less than 10 per cent while the same RC, after several weeks use, shows a higher discrepancy (up to 30 per cent) when calibration against Working Standard Solution (WSS) made up from BdW and compared with standards in SSW of 35 psu. Working Standard Solution should therefore be made from SSW or the magnitude of the salinity effect should be recorded frequently, where after proper correction should be applied to the data. A series of WSS is prepared from the NO_3-N SSS by dilutions with BdW (or SSW) using volumetric flasks (Table 10.8).

Triplicates of WSS and the blank samples with BdW are analysed as described below. Each RC should be calibrated using blanks and calibration solutions. The linear regression of the absorbances measured in the spectrometer against the concentrations of the WSS (including absorbances of the blank samples, concentration equals 0) gives the calibration factor (cf). Using 5 cm cells, a cf of approximately 4.3 should be obtained.

Analysis

Pour 25 ml of sample into the reservoir. Immediately add 1 ml of WB using an automatic syringe pipette, followed by another 25 ml of the sample. Let this pass through the amalgamated metal. Collect drops in a test tube: the first 25 ml are discarded (use them as washer); the second 25 ml are the nitrite sample. The turbidity reference samples are unnecessary. The RC is now ready to receive the next sample. After every analytical batch, the RC must be flushed with WbS. It should never be left to dry. The concentration of the nitrate in the samples is calculated by multiplying their absorbances (A_s) by the cf:

$$\text{concentration } NO_3\text{-N} = cf \times A_s \text{ [µM]}$$

Control of the reduction efficiency

The reduction efficiency of each RC must be controlled from time to time, preferably for every analytical batch. Duplicates of WSS for nitrite must be analysed, followed by WSS for nitrate of the same concentration:

$$\text{Reduction efficiency}(\%) = \frac{\text{Absorbance of the nitrate WSS} \times 100}{\text{Absorbance of the nitrite WSS}}$$

If the reduction efficiency decreases below 85 per cent, empty the RC, wash the filings quickly with 2M HCl and rinse well with water. Dry the filings, sieve and reamalgamate as described above.

Determination of nitrite: scope, field application and principle

This method is specific for nitrite ions and is applicable to all types of marine waters. It is not appreciably affected by salinity, small changes in reagent concentrations, or by

temperature (Grasshoff, 1983). Using 5 cm cells, the detection limit is about 0.02 µM and shows linearity up to about 10 µM. The determination is based on the reaction of nitrite ions with sulphanilamide with the formation of a diazonium compound which, coupled to a second aromatic amine, forms a coloured azo dye.

Reagents

✔ Sulphanilamide (SAN) - as in nitrate determination.

✔ Naphtylamine solution (NED) - as in nitrate determination.

✔ Nitrite standard stock solution (NO_2-N SSS). Dissolve 0.17250 g sodium nitrite, $NaNO_2$ (molecular weight: 69.00), dried at 110 °C to constant weight, in BdW and dilute to 250 ml. Store in a tightly closed dark bottle with 2-3 drops of a saturated $HgCl_2$ solution in a refrigerator (Kirkwood, 1992). The solution contains 10 µM ml^{-1}. (Note: aged solid reagent, even if it is of analytical grade, may contain less than 100 per cent $NaNO_2$ because it is unstable in air, and therefore should not be used for the preparation of SSS).

Apparatus and equipment

✔ Test tubes with glass or plastic stoppers, graduated or marked at 25-ml volume.

✔ Automatic syringe pipettes of 1 ml.

✔ 25 ml automatic pipette.

✔ 500 ml round-bottom flask.

✔ Reduction columns (Figure 10.6).

✔ Spectrometer, with 1, 5 and 10-cm pathlength cells.

Sampling

Nitrite is an intermediate compound in the simplified redox sequence from ammonia to nitrate, and therefore it cannot be preserved properly. Avoid filtration of samples. If samples are slightly turbid or have a visible natural colouration and contain no other disturbing substances, (such as samples taken from nearshore areas), analyse them together with turbidity blanks, but do not filter.

Calibration

Perform calibration in solutions made with BdW. A series of WSS from the NO_2-N SSS is prepared by dilution (Table 10.9). From each of the WSS above, transfer 25-ml triplicates into the tubes. In addition, prepare with BdW one set of triplicates of "blank samples", adding reagents to all tubes as described later. The linear regression of the

absorbances against the concentrations of the WSS (including absorbances of the blank samples, concentration equals 0) gives the calibration factor, cf.

Analysis

Transfer 25 ml of sample into the test tubes. If the turbidity has to be determined, transfer 25 ml of sample to a second tube. Pipette 0.5 ml of SAN into the tubes, mix well, and, after 2 minutes but not exceeding 8 minutes, add 0.5 ml of NED into one of the tubes. Stopper and shake. After 8 minutes read at 543 nm in a 5-cm pathlength cell, using the tube without NED as a reference. Colour is stable for two hours.

$$\text{Concentration } NO_2\text{-N} = cf \times A_s \text{ [µM]}$$

Table 10.9 Preparation of working standard solutions for nitrite by dilution with bi-distilled water

Volume of solution D[1] (ml)	Total volume (ml)	Resultant concentration (µM NO_2^--N)
5.0	250	1.00
5.0	500	0.50
1.0	200	0.25
1.0	500	0.10
1.0	1,000	0.05

[1] Solution "D" is prepared by diluting 5.0 ml of a SSS in 1 litre, giving a solution which contains 0.05 µmoles NO_2^--N per ml

10.5.4 Determination of ammonium

Scope, field of application and principle

This method is specific for ammonium and applicable to all kinds of natural waters (Koroleff, 1983b). Ammonium refers to the sum of ammonia and ammonium ions, because the original proportion of each in a water sample is pH dependent. The detection limit is about 0.10 µM (in 5 cm pathlength cells). The Lambert-Beer's law is followed up to about 40 µM. Interferences from amino acids and urea can be neglected. To compensate for the influence of salinity on the developed colour, a correction factor has to be applied (see below).

Reagents

Most of the reagents are caustic and very toxic, therefore mouth pipetting should not be used.

✓Ammonium-free water (AFW). If the ammonium blank concentrations are higher than 0.3 µM, the water should be treated. Therefore, water that has been passed through an acidic deionisation cation exchange resin should be used. Alternatively, 2 ml of concentrated sulphuric acid (96 per cent) and 1 g of potassium peroxodisulphate, $K_2S_2O_8$, can be added per litre. The solution should be boiled for 10 minutes (without the

condenser) to remove ammonium, and then distilled to give a residue of 150 ml. Ammonium-free water should be stored in a tightly sealed plastic container with thick walls.

✔ Citrate buffer solution. Dissolve 67 g of trisodium citrate dihydrate, $Na_3C_6H_5O_72H_2O$, 34 g boric acid, H_3BO_3, 19 g citric acid dihydrate, $C_6H_8O_72H_2O$, and 30 g sodium hydroxide, NaOH, in AFW and dilute to 1 litre. The solution is stable and should be stored in a well-stoppered glass bottle at room temperature.

✔ Reagent A, phenol-nitroprusside solution. Dissolve 35 g phenol, C_6H_5OH and 0.4 g of sodium nitroprusside dihydrate, $Na_2Fe(CN)5NO2H_2O$, in AFW and dilute to 1 litre. Store in a tightly closed bottle in a refrigerator. The solution is stable for several months. (Caution, highly toxic and must be treated as hazardous waste).

✔ Reagent B, hypochlorite solution. Dissolve 4 g of the sodium salt of the dichloro-isocyanuric acid and 15 g NaOH in AFW and dilute up to 1 litre. Store in a tightly closed bottle in a refrigerator. The solution is stable for several months.

✔ Ammonium Standard Stock solution (NH_3-N SSS). Dissolve 0.13373 g ammonium chloride, NH_4Cl, (molecular weight: 13.49), dried at 110 °C to constant weight, in AFW and dilute to 250 ml. Store in a tightly closed bottle with some drops of chloroform, in a refrigerator. The solution contains 10 µM ml^{-1}.

Table 10.10 Preparation of working standard solutions for ammonia by dilution with ammonium-free water

Volume of solution D[1] (ml)	Total volume (ml)	Resultant concentration (µM NH_3-N)
20.0	250	4.0
5.0	250	1.0
5.0	500	0.5
2.0	500	0.2

[1] Solution "D" is prepared by diluting 5.0 ml of a SSS to 1 litre, giving a solution which contains 0.05 µmoles NH_3-N per ml

Apparatus and equipment

✔ 25 ml test tubes with ground glass stoppers.

✔ Automatic syringe pipettes of 1 ml and 2 ml.

✔ 25 ml automatic pipettes.

✔ Spectrometer with cells of 1, 5 or 10 cm pathlength.

Analytical procedures

Test tubes should be carefully cleaned according to the following procedure: every tube is filled with about 25 ml water and reagents are added as described later. All ammonium contained in the tubes (dissolved in the water or adhered to the glass walls) will react. Tubes are then rinsed with AFW and stored filled with AFW. The tubes should be kept stoppered when not used. They should not be washed between the different

sets of calibrations or analyses, merely rinsed with AFW (Caution: serious contamination from the air can result from smoking).

Calibration

In order to avoid disturbances from variations in pH and salinity in the samples, the calibration can be carried out in two ways. In areas where the salinity variations are small, WSS are diluted with ammonium-free seawater (AFSW) (i.e. surface seawater preferably collected shortly after a plankton bloom) (Table 10.10). For work in estuaries, where the brackish water displays large salinity variations, a calibration in AFW, followed by corrections for the salinity (Table 10.11) of each sample, is preferred. A series of WSS from the NH_3-N SSS is prepared by dilution with AFW or AFSW, using volumetric flasks.

From each of the WSS above, 25 ml triplicates are transferred to the test tubes. In addition, two sets of "blank samples" are prepared, also in triplicate, but with AFW only. To all the tubes, the reagents are added as described below in "Analysis of the samples", but to one of the blank sets a double volume of reagents is added. The blank samples here correct for the absorbance caused by the residual ammonium impurities in the AFW. The second set of blanks, those with double volume of reagents, corrects for the ammonium impurities in the reagents only. The linear regression of absorbances measured against the concentrations of the WSS (including absorbances of the first set of blanks, concentration equals 0) gives the cf. The product of cf and the absorbance of the second sets of blanks will be a constant (K), which is deduced from the results obtained with the samples and may vary from analysis to analysis because of influences such as age of solutions and contaminants from chemicals or air. Using a 5 cm cell, the current cf is approximately 11.

Table 10.11 Salinity correction factors (cf) for ammonia analysis

	Salinity (psu)									
	<8	**11**	**14**	**17**	**20**	**23**	**27**	**30**	**33**	**36**
pH	0.8	10.6	10.5	10.4	10.3	10.2	10.0	9.95	9.90	9.80
cf	1.00	1.01	1.02	1.03	1.04	1.05	1.06	1.07	1.08	1.09

Source: Koroloff, 1983b

Analysis of the samples

1. With the automatic pipette, dispense 25 ml of the sample into the test tubes.

2. With the automatic syringe pipette, add 1.5 ml of citrate buffer and mix (a vortex mixer works very well).

3. Add 0.7 ml of reagent A and using the automatic syringe pipette mix well.

4. Add 0.7 ml of reagent B and with the automatic syringe pipette mix well.

5. Stopper the test tubes, shake and keep in the dark for at least 8 hours until colour has developed. The absorbance will become constant during a maximum of 48 hours.

6. Read at 630 nm in a cell with suitable pathlength. As a reference, AFW is used.

$$\text{Concentration } NH_4^+\text{-N} = cf \times A_s - K \text{ [µM]}$$

If the WSS was diluted with AFW and the samples are seawater, the results must be corrected using the correction factors given Table 10.11, depending on the salinity of the sample.

Alternative methods

A manual spectrometric method is given in ISO (1984a) where a blue compound, formed by reaction of ammonium with salycilate and hypo-chlorite ions in the presence of sodium nitrosopentacyanoferrate (III) is analysed at a limit of detection of 0.003-0.008 mg l^{-1}. An automated procedure is given by ISO (1986c) and distillation and titration method in ISO (1984b). Further details are also given in Wetzel and Likens (1990) and APHA/AWWA/WPCF (1995).

10.6 Algal and cyanobacterial identification and quantification

Approaches to the determination of cyanobacterial and algal taxa, numbers and biomass present in a sample are not yet internationally harmonised. The methods used are very variable and can be undertaken at very different levels of sophistication. Rapid and simple methods addressing the composition of a sample at the level of genera (rather than species) are 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 of whether cyanobacteria are present above a threshold density. More detailed taxonomic resolution and biomass analyses will be required if population development or toxin content is to be predicted. Distinction between these approaches is important because managers must decide how available staff hours can be most effectively invested. In many cases, the priority will be evaluation of a larger number of samples at a lower level of precision. Furthermore, investing time in regular intralaboratory calibrations encompassing these steps is likely to be more effective than investing time in counting protocols that reduce error, e.g. from 20 per cent to 10 per cent, but at quadrupled effort. The choice of methods also requires informed consideration of sources of variability and error at each stage of the monitoring process, from sampling to counting.

10.6.1 Identification

Microscopic examination of a bloom sample is very useful, even when quantification 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. Most cyanobacteria can be distinguished readily from other phytoplankton and particles under the microscope at 200-400 times magnification by their morphological features.

Cyanobacterial and algal 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, for example, with respect to microcystin production, and these cannot be differentiated microscopically. The distinction of genera is very important for assessing potential toxicity, but 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* and *Anabaena*) is frequently sufficient. It may be preferable to give only the genus name especially if differentiation between species by microscopy is uncertain on the basis of current taxonomic knowledge, 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 led to numerous published misidentifications of species. Practitioners in health authorities with some experience in using a microscope can easily learn to recognise the major cyanobacterial genera and some prominent species that occur in the region they are monitoring. Such efforts should not be deterred by the pitfalls of current scientific work in cyanobacterial taxonomy which targets differentiation to the species level.

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 of the toxin with different toxicity.

For establishing cyanobacterial identification in a laboratory, initial consultation and later occasional co-operation with experts on cyanobacterial identification is helpful. Training courses for beginners should focus on the genera and species relevant in the region to be monitored. Experts can assist in deriving an initial list of these taxa and the criteria for their identification. In the course of further monitoring experience, experts should be consulted periodically for quality control and for updating such a list. Helpful publications for determination of genera and species are presented in Box 10.1.

10.6.2 Quantification by direct counting methods

Microscopic enumeration of cyanobacterial cells, filaments or colonies has the advantage of assessing directly the potentially toxic organisms. Little equipment is required other than a microscope. The method may be rather time-consuming, ranging from 10 minutes to 4 hours per sample depending upon the accuracy required and the number of species to be differentiated. Precise and widely accepted counting procedures are time consuming and require a moderate level of expertise, but serve as a basis to assess performance of simplified methods developed to suit the expertise and requirements of sampling programmes tailored to the assessment of toxic cyanobacteria. A summary of methods is provided in Table 10.12. Detailed information on sampling and on counting marine toxic phytoplankton is given in UNESCO (1995, 1996), and for marine cyanobacteria in Falconer (1993).

Sample concentration by sedimentation or centrifugation

Direct counting of preserved cells is typically carried out by Utermöhl's counting techniques (Utermöhl, 1958) using a counting chamber and inverted microscope. Cells

from a sample preserved in Lugol's iodine are allowed to sediment onto the glass bottom of the chamber where they can be counted.

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 contain 2-10 ml of sample. These can fit easily on the inverted microscope stage. If larger volumes of water have to be sedimented (for example, when cell density is low) then the height of the tube has to be increased. These 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 can be overcome using a tube in two sections, which allows the supernatant to be removed after settling without disturbing the sedimented cells on the bottom glass. The amount of sedimented water required depends on the density of cells, on the counting technique (fields or transects) and on the magnification being used.

Table 10.12 A summary of methods for the quantification of algae

Method	Volume (ml)	Sensitivity (cells per litre)	Preparation time (minutes)
Compound microscope			
Sedgewick-Rafter Cell (counting cell)	1	1,000	15
Palmer-Malloney Cell (counting cell)	0.1	10,000	15
Drops on slide		5,000-10,000	1
Inverted microscope			
Utermöhl (sedimentation chamber)	2-50	20-500[1]	2-24[2]
Epifluorescence microscopy			
Counting on filters (fluorochrome: Calco Flour)	1-100	10-1,000	15

[1] Cells per ml
[2] Hours

Source: UNESCO, 1996

Samples for sedimentation must be equilibrated to room temperature before they are placed in the settling chamber, to prevent air-bubbles from developing. The water sample must be gently inverted several times to ensure even mixing of the particles before being poured into the sedimentation chamber. The chamber must be placed on a horizontal surface to settle making sure that the content is not disturbed or exposed to temperature changes or direct sunshine. Sedimentation times vary depending on the height of the sedimentation tube and the preservative used. Various sedimentation times have been recommended in the literature (Lund *et al.,* 1958). Samples preserved in Lugol's iodine should be allowed at least 3-4 hours per centimetre height of liquid to settle. For samples preserved in neutralised formaldehyde, twice this time is required. Buoyant cyanobacterial cells (e.g. of *Microcystis* spp.) occasionally do not settle unless their gas vesicles are destroyed by applying hydrostatic pressure. Once the samples have settled, phytoplankton density can be determined by counting the organisms on the bottom of the chamber.

If an inverted microscope is not available, and samples with low cyanobacterial density need to be counted, it is possible to concentrate samples sufficiently to enable a drop (of defined volume by using a micropipette) to be counted under a standard microscope. A 100 ml measuring cylinder can be used to sediment the sample, allowing 4 hours per centimetre of sedimentation height. The supernatant can then be carefully abstracted down to the bottom 5 ml. The sample is thus concentrated by a factor of 20. Gentle centrifugation may be applied for further concentration.

Box 10.1 Sources of information for identification of cyanobacteria and algae

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Bourelly P. 1970 Les algues d'eau douce, Tome III. *Les algues d'eau douce. Initiation a la systématique. Tome III. Les algues bleues et rouges. Les Eugléniens, Peridiniens et Cryptomonadines.* Boubée, Paris, 512 pp.

Bourelly, P. 1972 Les algues d'eau douce. T.I. *Les algues d'eau douce. Initiation a la systématique. Tome III. Les algues vertes.* Boubée, Paris, 572 pp.

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Carr, N.G. and Whitton, B.A. 1982 The biology of Cyanobacteria. *Botanical Monographs* **17**, Blackwell, Oxford, 688 pp.

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Fogg, G.E., Stewart, W.D.P., Fay, P. and Walsby, A.E. 1973 *The Blue-Green Algae.* Academic Press, London, 459 pp.

Hasle, G.R. and Fryxell, G.A. 1995 Taxonomy of Diatoms. In: G.M. Hallegraeff, D.M. Anderson and A.D. Cembella [Eds] *Manual on Harmful Marine Micro-algae.* IOC Manuals and Guides No. 33, UNESCO, United Nations Educational, Scientific and Cultural Organization, Paris, 339-364.

Komárek, J. and Anagnostidis, K. 1985 Modern approach to the classification system of cyanophytes. I. Introduction. *Archives Hydrobiology* Supplement **71** (Algol. Studies 38/39), 291-302.

Komárek, J. and Anagnostidis, K. 1986 Modern approach to the classification system of cyanophytes. *Archives Hydrobiology* Supplement **73** (Algol. Studies 43), 157-226.

Skulberg, O.M., Carmichael, W.W., Codd, G.A. and Skulberg, R. 1994 Taxonomy of toxic cyanophyceae (Cyanobacteria). In: I.R. Falconer [Ed.] *Algal Toxins in Seafood and Drinking*

Water. Academic Press, London, 145-164.

Starmach, K. 1966 *Cyanophyta. Flora slodkowodna Polski 2.* Polska Akademia, Warszawa, 807 pp.

Taylor, F.J.R., Fukuyo, Y. and Larsen, J. 1995 In: G.M. Hallegraeff, D.M. Anderson and A.D. Cembella [Eds] *Manual on Harmful Marine Microalgae.* IOC Manuals and Guides No. 33, UNESCO, United Nations Educational, Scientific and Cultural Organization, Paris, 283-317.

Tomas, C.R. 1993 *Marine Phytoplankton: A Guide to Naked Flagellates and Coccolithophorids.* Academic Press, Inc., New York.

Tomas, C.R. 1996 *Identifying Marine Diatoms and Dinoflagellates.* Academic Press, Inc., New York.

Where sedimentation is not possible, centrifugation (360× g for 15 minutes using 10-20 ml sample) can offer a rapid and convenient method of concentrating a sample (Ballantine, 1953). Centrifugation may be aided by addition of a precipitating agent, such as potassium aluminium sulphate (1 per cent solution) added at 0.05 ml per 10 ml sample. Fixation with Lugol's solution enhances susceptibility to separation by centrifugation. However, buoyant cells may still be difficult to pellet and may require disruption of gas vesicles prior to centrifugation by applying sudden hydrostatic pressure (Walsby, 1994), for example in a well sealed syringe or by banging a cork into the bottle very tightly.

Counting cyanobacteria and algae

When counting cyanobacteria the units to be counted must be defined. 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 separately. 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 heavy bloom where only several dozen 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, for example stating that 1 ml of sample contains an average of 2.43 colonies of *Microcystis aeruginosa,* gives little information on the quantity of cyanobacteria.

Typically unicellular species are counted as cells per millilitre and filamentous species can be counted as the number of filaments, with an average number of cells per filament quoted (often the cells per filament in the first 30 filaments encountered are counted and averaged), or they can be measured as total filament length by estimating the extension of each filament within a counting grid placed in the ocular of the microscope. The latter is more precise if 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. Colonies sometimes disintegrate after several days

when fixed with Lugol's iodine solution. For more stable colonies, disintegration can be achieved by ultrasonication. This often separates cells very effectively and, in cases where colonies do not totally break down into single cells, their size may be reduced sufficiently to allow single cells to be counted. Sometimes, this is not successful and it is necessary to estimate the geometric volume of individual colonies. If colonies are relatively uniform in size, the average number of cells per colony may be determined and then the colonies may be counted. Generally, the use of values for numbers of cells per colony published in the literature is not recommended because the size of colonies varies greatly.

There are several methods for counting organisms. Most approaches aim at counting only a defined part of the sample and calculating back to the volume of the entire sample. The most common methods are: total surface counting, counting in transects and counting in fields. Counting the total chamber bottom may be very time consuming. It is usually only appropriate for very large counting units (cells, colonies, and filaments) at low magnification. Counting cells in transects from one edge of the chamber to the other, passing through the central point of the chamber, is more efficient. Some inverted microscopes are equipped with special oculars that enable the transect width to be adjusted as required. However, in many cases, horizontal or vertical sides of a simple counting grid can be used to indicate the margin of the transect. Back-calculating to 1 ml of sample can be done by measuring the area of the transects and of the chamber bottom, together with the volume of the counting chamber.

Cyanobacteria and algae occurring in randomly selected fields may be counted. When changing the position of the chamber to find the next field, it is preferable to avoid looking through the microscope to ensure random choice of fields. Microscopic field area covered by a counting grid is usually considered as one field. However, if no counting grid is available, the total spherical field can be considered. Back-calculating to 1 ml of sample can be done by measuring the area of the field and of the chamber bottom together with 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 sizes of the species, and therefore it is necessary to select the counting method that is adequate for the sample. Total chamber surface counting with low magnification (100×) may be useful for large species whereas transect or field counting with higher (200× to 400×) magnification is used for smaller or unicellular cyanobacteria and algae. Accurate enumeration using transects or fields assumes even distribution of cyanobacteria and algae on the bottom of the chamber surface after sedimentation. Due to the inevitable convection currents in the sedimentation chamber, that are very difficult to avoid, cells very rarely settle evenly on the surface of the bottom glass - they are almost always more dense either in the middle or around the circumference of the chamber. In some cases, density also varies between opposite edges of the bottom glass. The misestimation that arises from uneven distribution can be minimised by transect counting or by taking a fairly even distribution of randomly selected field. Counting four perpendicular diameters can minimise this error. The relation between counting time and accuracy is best if about 100 counting units (cells, colonies, and filaments) are settled in one transect. This may be achieved by diluting or concentrating samples so that the number of units of the important species lies in this range.

Specimens occurring exactly on the margin of the counting area (transect or field) present the common problem of whether to count them or not. For transect counting, those specimens that lie across the left margin are ignored, while those that cross the right margin are included. In field counting, two predetermined sides of the grid are included, the other two are ignored.

There are different recommendations in the published literature concerning how many specimens per species should be counted for reliable results. Mass developments of phytoplankton populations are generally characterised by dominance of 1-3 species. It is unusual for more than six to eight species to contribute to the majority of biomass. Therefore, it is suggested that 400-800 specimens in each sample are counted, leading to a maximum error of the total count of 7-10 per cent. In this situation 10-20 per cent of the error is accounted for by the few dominant species, 20-60 per cent is accounted for by 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.

Other counting chambers (e.g. Sedgewick-Rafter or haemacytometers) are available for use with a standard microscope. Samples might require prior concentration or dilution. It can also be useful to monitor samples under high magnification with oil-immersion (1,000×) to check the sample for the presence of very small forms that may be overlooked during normal counting.

The use of mechanical or electronic counters for recording cell counts can shorten counting time considerably, especially if only a few species are counted.

Simplifications

One alternative 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. Water samples (10 ml) are filtered through a membrane filter disc (13 mm) contained in a filtration device. The filter with the captured phytoplankton is dried at room temperature, and then placed on a drop of immersion oil on a microscope slide. A further drop of immersion oil is placed onto the surface of the filter, which makes the filter transparent. The sample is observed under a standard microscope (200× or 400×) without using a micro-cover slip. All cells on the surface of the filter are counted and the number of cells per litre can be calculated.

For optimising the relationship between the time spent and the information gained, various simplifications are possible. No method of enumeration is definitive, and personal creativity as well as understanding of potential pitfalls may compensate for lack of the ideal equipment or time. For each method applied (for improvisations as well as for "benchmark methods") it is crucial to check for reproducibility and comparability of the method established in the laboratory (parallel counts should not deviate by more than 20 per cent). Furthermore, clear statements of the units in which the results are given are of critical, but often unrecognised, importance. Unfortunately, many reported results are unclear about the size of units quoted, i.e. "one colony", or the size of "one filament". Such terminology varies between laboratories and makes it impossible to compare results.

For estimation of error, UNESCO (1996) gives the following equation (see Table 10.12): at a 95 per cent level of confidence, the relative limits of expected concentrations = ± (2 × 100%)/($n^{0.5}$/n). For example, if in a sample volume of 10 ml only 50 cells of species "x" are counted, the result is 5,000 cells per litre. Assuming a deviation of 28 at counting 50, this results in ± 2,800 cells per litre. If the sample was concentrated 10 fold, so that 500 cells were counted, this would result in a higher accuracy of 5,000 ± 900 cells per litre. As a result of the extremely dynamic changes of cyanobacterial density in many water bodies (often amounting to more than 10 fold within a few hours) the precision obtained in counting 50 cells may, in many cases, be quite sufficient for estimating the potential risk involved.

10.6.3 Determination of cyanobacterial biomass

Cell size can vary considerably within species and by a factor of 10 to 100 or more 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: estimation from cell counts and average cell volumes, and estimation from chemical analysis of pigment content (chlorophyll *a*).

Cyanobacterial and algal 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 by the cell number present in the sample to give the total volume of each species. The specific weight of plankton cells is almost 1 mg mm^{-3} and therefore biovolume corresponds quite closely to biomass. Average volumes can be determined by assuming idealised geometric bodies for each species (e.g. spheres for *Microcystis* cells, cylinders for filaments), measuring the relevant geometric dimensions of 10-30 cells (depending upon variability) of each species, and calculating the corresponding mean volume of the respective geometric body.

Simplification for biomass estimates

If the deviation of numbers of dominant species counted in two perpendicular transects is less than 20 per cent between both transects, it is not necessary to count further transects. If the standard deviation of cell dimensions measured on 10 cells is less than 20 per cent, it is not necessary to 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.

Chlorophyll a *analysis*

The concentration of chlorophyll *a* may be used as a sensitive approximation of algal biomass and as an alternative to counting and measuring biovolumes. Chlorophyll *a* concentrations of mixed phytoplankton populations give an overestimation of the

biomass of the cyanobacteria and algae of interest. This degree of overestimation can be assessed by a brief microscopic estimate of the share of cyanobacteria and algae in relation to other phytoplankton biomass. Nevertheless during cyanobacterial mass developments chiefly consisting of one species, chlorophyll *a* may be a good measure of biomass. The method requires relatively simple laboratory equipment and is considerably less time-consuming than microscopic enumeration. A useful analytical protocol is given in ISO (1992). This method involves an extraction procedure with hot ethanol to inactivate chlorophyllase and accelerate the lysis of pigments.

Apparatus

✔ Spectrometer, for use in the visible range up to 800-900 nm, with a resolution of 1 nm, a bandwidth of 2 nm or less, sensitivity less or equal to 0.001 absorbance units and with optical cells of pathlength between 1 cm and 5 cm.

✔ Vacuum filtration device, filter holder with clamp.

✔ Vacuum water pump or electric vacuum pump (in the laboratory).

✔ Glass fibre filters free of organic binder (average pore size 0.7 µm, 47 mm diameter).

✔ Filters for filtration of the extracts (average pore size 0.7 µm, 25 mm diameter), or as an alternative a centrifuge (possibly refrigerated), with an acceleration of 6,000 g and a swinging rotor suitable for extraction tubes.

✔ Extraction vessels, e.g. wide-necked amber glass vials with polytetrafluorethylene (PTFE) lined screw caps, typically of 30 ml to 50 ml capacity and suitable for centrifugation at 6,000 g.

✔ Water bath, adjustable to 75 °C ± 1 °C with a rack for extraction vessels.

Filtration

1. Samples must be shaken before filtration in order to mix thoroughly. Filter a measured volume of the sample (normally between 0.1 and 2 litres, depending on the concentration of algae and cyanobacteria). Pour into the filter cup, drop by drop, recording the volume, to avoid filter clogging.

2. Filter continuously and do not allow the filter to dry during filtration of a single sample. Vacuum pressure during filtration should not exceed 0.5 atmospheres.

3. The vacuum pressure should be reduced just before the filters become dry, in order to leave a thin layer of water and avoid rupture of the algal or cyanobacterial cells.

4. Some analysts recommend adding 0.2 ml of magnesium carbonate suspension (1 per cent (w/v) $MgCO_3$, shaken before use) to the final few millilitres of water in the filter cup. Avoid touching the filter with fingers. It preferable to use forceps. Direct sunshine must also be avoided because chlorophyll degrades rapidly.

5. Filters must either be frozen immediately (see below) or covered with hot ethanol to avoid pigment degradation.

6. Filters must be folded so that the cell layer is protected from rubbing off onto packaging materials. Wrapping folded filters in aluminium foil is a practical solution because it protects the filter and enables labelling on the foil.

Procedure

If extraction is not performed immediately, filters should be placed in individual labelled bags or Petri dishes and stored at -20 °C in darkness. Samples are readily transported in this form.

Extract the filters with a total volume of 10-40 ml (the volume to be used depends on the size of the photometer cuvette) of boiling 90 per cent ethanol (v/v) at 75 °C and leave overnight (24 hours) at +4 °C or in darkness at approximately 20 °C for 24-48 hours. Ethanol containing a denaturant is used successfully in many laboratories. However, denaturants vary and it is prudent to use ethanol without a denaturant or to run comparative analyses to assess the effect of the denaturant. A comparative determination with 90 per cent pure ethanol is recommended.

Homogenisation either by ultrasonication or with a tissue grinder, may be performed to disrupt cells and enhance extraction, after having poured part of the boiling ethanol onto the filter and having used the rest to rinse the apparatus. However, homogenisation is not likely to be essential for extraction of cyanobacteria.

Clarification of the slurry

1. Centrifuge the ethanol and the filter for 15 minutes at 6,000 g. This should result in a clear supernatant.

2. Carefully decant the clear supernatant with a Pasteur pipette into a calibrated flask with stopper. Fill to the mark with ethanol, stopper and mix. This is the extract volume V_e in millilitres.

As an alternative, filter the slurry through a filter (see Apparatus section above) into a calibrated flask with a stopper. Wash the extraction vessel with ethanol and transfer quantitatively into the calibrated flask. Fill to the mark with ethanol, stopper and mix. This is the extract volume V_e.

3. Store the flasks in darkness and proceed promptly to the measurement step.

Measurement

Blank the spectrometer with the same ethanol at each wavelength before reading sample.

1. Transfer the clear extract into the cuvette using a pipette, either a) leaving sufficient volume in the cuvette for the addition of HCl (if it is preferred to proceed by adding HCl

directly into the cuvette) or b) leaving a sufficient volume of extract in the flask for a second measurement after acidification of the extract left in the flask.

2. Record the absorbance at 750 nm (750a) and 665 nm (665a) against a reference cell filled with ethanol. The absorbance at 665 nm should fall between 0.01 and 0.8 units. This may be achieved by choosing a suitable volume of water to be filtered, extractant volume, dilution, or pathlength, etc. To start with, take 0.5 litre of sample, 20 ml of ethanol and a 5 cm cuvette.

3. Proceed with the acidification step, either a) adding HCl directly into the cuvette, or b) acidifying the extract left in the flask. In either case, add 0.01 ml of HCl 3 mol l^{-1} per 10 ml of extract volume and agitate gently for 1 minute.

4. Record absorbance at 750 nm (750b) and 665 nm (665b) after between 5 minutes and 30 minutes.

Calculation and expression of results

1. Calculate absorbance of the extract before acidification: 665_a-750_a = A_a

Calculate absorbance of the extract after acidification: 665_b-750_b = A_b

2. Calculate chlorophyll a concentration (Chla) in mg m^{-3}

$$\text{Chla} = 29.6 \times (A_a - A_b) \times V_e / V_s \times d$$

where:

V_e is the volume, in millilitres, of the extract
V_s is the volume, in litres, of the filtered water sample
d is the pathlength, in centimetres, of the cuvette
3. Phaeopigment concentration (Phaeo) in mg m^{-3} may be calculated to indicate the portion of inactive cyanobacterial and algal biomass:

$$\text{Phaeo} = 20.8 \times A_b \times V_e / V_s \times d - \text{Chla}$$

Note: The ratio of chlorophyll a to phaeophytin a gives an indication of the effectiveness of sample preservation, as well as of the condition of the cyanobacterial algal population. 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 extracted rapidly with hot ethanol, or frozen, chlorophyll a concentrations start to reduce. Occasionally, other factors disturb this method, resulting in very low or even negative values for chlorophyll a. If this occurs, the following calculation should be made:

$$\text{Chlorophyl } a + \text{Phaeophytin } a = \frac{12.2(663a) \times V_e}{V_s \times 1} \text{ mg m}^{-1}$$

This should result in a similar value as for the sum of the concentrations of both pigments determined separately, as above.

10.7 Detection of toxins and toxicity

Laboratory methods used to evaluate toxins can vary greatly in their degree of sophistication and the information they provide. Relatively simple, low cost methods can be employed which rapidly evaluate the potential hazard and allow management decisions to be taken. In contrast, highly sophisticated analytical techniques determine precisely the identity and quantity of cyanotoxins. 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 that will be adequate. Currently, there is no single method that can be adopted that will provide adequate monitoring for all cyanotoxins in the different sample types that might have to be evaluated. The increasing variety and number of individual cyanotoxins being discovered make the goal of very specific and sensitive analytical methods that would detect all relevant toxins increasingly complex and ultimately unachievable (Yoo *et al.,* 1995).

In conclusion, it is strongly suggested that a monitoring programme of toxin concentrations should not be adopted as a matter of course but only when specifically indicated as discussed in section 10.1. For most recreational sites, monitoring the development of blooms rather than toxins is a more rational approach. A comprehensive review of methods and approaches is given in Lawton *et al.* (1999) and for marine algal and cyanobacterial toxins in UNESCO (1995).

10.8 Elements of good practice

• Monitoring of recreational water-use areas should be sufficient to identify the risk of blooms, taking into account actual or potential accumulation of toxic cyanobacteria and algae.

• Sampling points should be located to represent different water masses (stratified waters, waters coming from river mouths, etc.) in the investigation area and the sources of nutrients (discharges, upwellings, etc.). Possible transport mechanisms of toxic phytoplankton should be considered, possible physical forcings should be identified and sampling schemes arranged accordingly.

• In areas of high risk, sampling for algae should be carried out at least weekly. During development of blooms, sampling should be intensified to daily.

• Monitoring of toxicity (using bioassays, chemical or immunological procedures) is only justified where reason exists to suspect that hazards to human health may be significant. In such cases, long-term information on phytoplankton populations (toxic, harmful and others) should be collected where appropriate.

• Analysis of toxins should only be undertaken where standard, replicable and reliable analyses can be performed.

• Where conditions are such that monitoring is considered essential, temperature, salinity (in marine coastal areas), dissolved oxygen, transparency, presence of surface water stratification, phytoplankton biomass (chlorophyll), surface current circulation (transport of algae) and meteorological patterns (such as seasonal rainfall, storms and special wind regimes) should be considered.

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