



Condensed Laboratory Methods for Monitoring Phytoplankton, Including Cyanobacteria, in South African Freshwaters

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CONDENSED LABORATORY METHODS FOR MONITORING PHYTOPLANKTON, INCLUDING CYANOBACTERIA, IN SOUTH AFRICAN FRESHWATERS

**Annelie Swanepoel*, Hein du Preez*, Carl Schoeman*,
Sanet Janse van Vuuren** & Ashogan Sundram*****

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In association with
The North-West University** and
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The *Comprehensive Methods For Monitoring Phytoplankton, Including Cyanobacteria, In South African Freshwaters* (attached as a CD at the back of this manual) contains all aspects of the methods including the validations thereof. Both these manuals should be regarded and studied as a unit.

DISCLAIMER

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FOREWORD

Reservoirs provide the bulk of South Africa's raw potable, irrigation and livestock water. These reservoirs are variously impacted by eutrophication arising from their catchments. Certain dams, especially those located in the interior of the country, are eutrophic to hypertrophic. Associated with these conditions is the excessive development of phytoplankton, especially cyanobacteria. As is globally common, cyanobacteria are associated with many aesthetically-displeasing (algal blooms, scums, odours) and health-related problems (toxins) which can adversely affect humans and animals.

With respect to cyanobacteria, of greatest importance to the potable water industry is the production of cyanotoxins and taste and odour compounds such as geosmin and 2-methyl-isoborneol (2-MIB). The monitoring of phytoplankton, cyanobacteria and their related organic compounds, is essential to the production of water safe for human and animal consumption.

Various strategies have been launched by the South African Department of Water Affairs and Forestry (DWAF) to monitor the country's reservoirs. The monitoring of phytoplankton and cyanobacterial composition, abundance and the concentration of related organic compounds, form an essential component of such programmes.

A need for a comprehensive methods manual for phytoplankton was identified during encounters with South African laboratories tasked with water quality monitoring. Most of the smaller laboratories do not possess the capacity and/or expertise to develop methods essential for the effective monitoring of phytoplankton and cyanobacteria. In order to address this lack, a project was initiated in association with the Water Research Commission (WRC) that resulted in the publication of this methods manual.

It is envisaged that this publication will aid to the much needed capacity building in the South African drinking water industry.

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1. INTRODUCTION

1.1 BACKGROUND

Algal blooms (especially cyanobacterial) cause annual problems for the potable water production industry in South Africa. Taste and odours released during blooms result in a severe increase in production costs when these compounds have to be removed. Many blooms also result in clogging of filters, resulting in increased filter maintenance with associated cost implications. Probably the most serious and often unnoticed consequence of most blooms is the ability of the cyanobacteria to produce and release toxins that can be detrimental to the health of consumers and livestock.

Reservoirs provide the bulk of South Africa's raw potable, irrigation and livestock water. These reservoirs are variously impacted by eutrophication arising from their catchments. Certain dams, especially those located in the interior of the country, are eutrophic to hypertrophic. Associated with these conditions is the excessive development of phytoplankton, especially cyanobacteria. As is globally common, cyanobacteria are associated with many aesthetically-displeasing (algal blooms, scums, odours) and health-related problems (toxins) which can adversely affect humans and animals.

A national eutrophication monitoring programme was recently developed to provide a manual for implementing a eutrophication management strategy in South Africa (DWAF, 2002). This approach advocates standardizing the variables required to monitor eutrophication. An incident management framework for potable water suppliers was recently developed by Rand Water and partners, for the management of cyanobacterial incidents in source water reservoirs (Du Preez and Van Baalen, 2006 – refer to **Figures 1.1** and **1.2**). Monitoring of algal species composition, abundance and the concentration of organic compounds is an important requirement of both programmes. Very few organizations (laboratories), however, are currently equipped to analyze for these variables. The position is worse in terms of facilities able to monitor algal toxins, resulting in a critical lack of information on the incidence of these toxins in the South African water industry. This severely incapacitates the effective management of “safe” drinking water.

Enabling the effective implementation and management of a eutrophication management programme, coupled with the incident-based management programme for cyanobacteria in potable water supplies, required the development of this manual. No existing manuals were found to suit the requirements of the South African water industry and inter-laboratory calibrations and proficiency testing schemes have revealed a high variability of results.

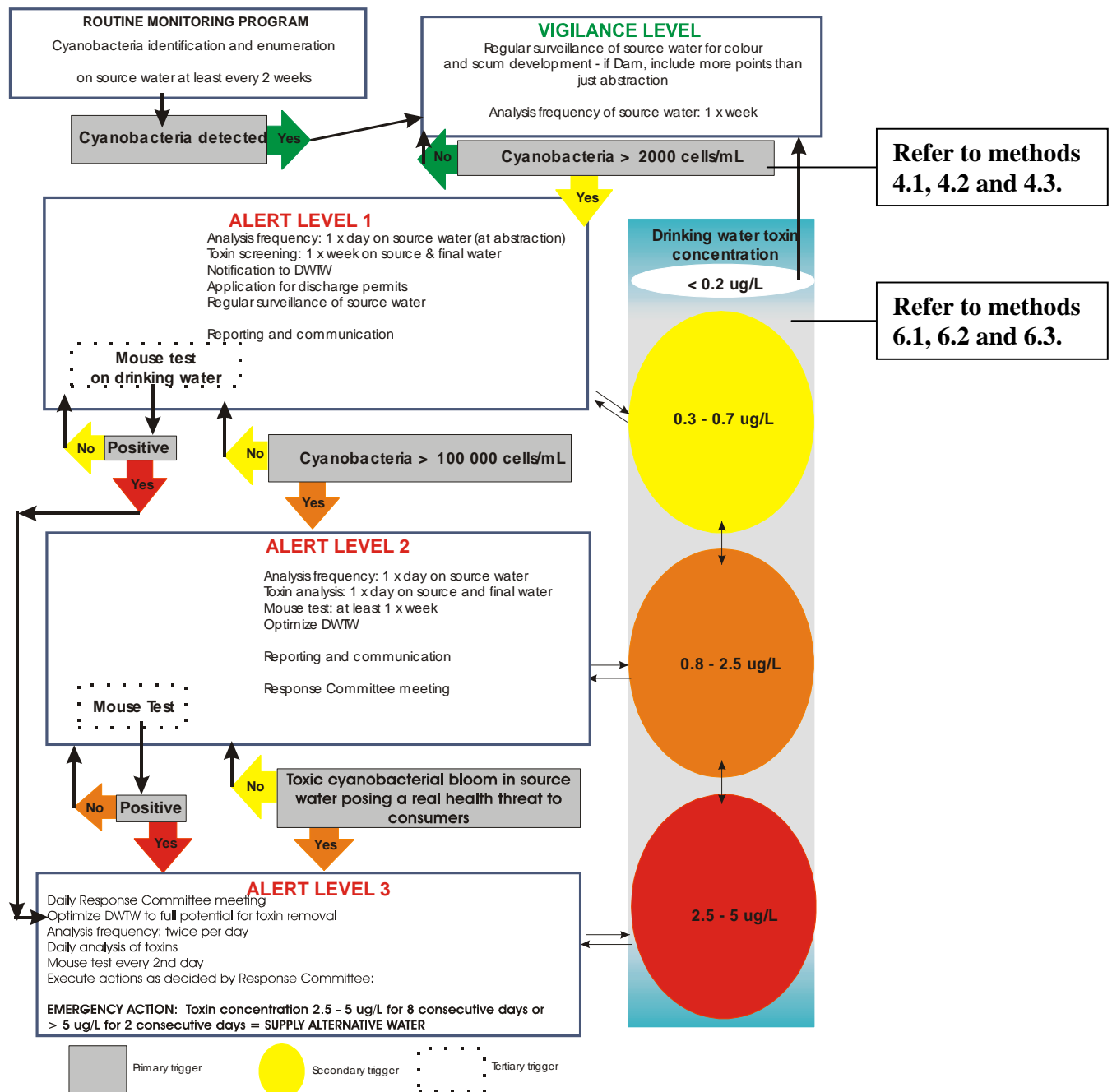


Figure 1.1 Cyanobacteria Incident Management Framework (CIMF) using cyanobacteria concentration as a primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).

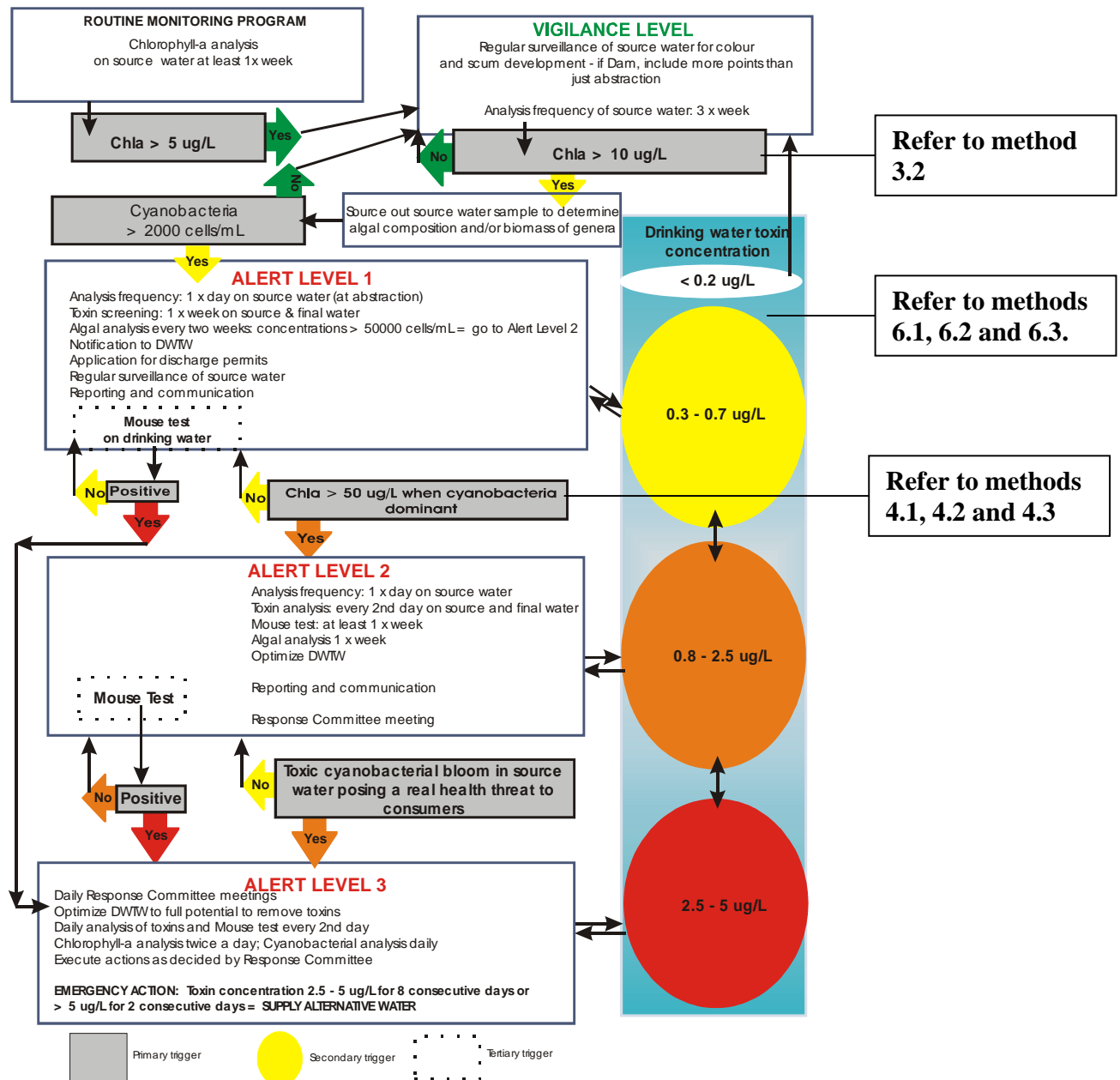


Figure 1.2 Cyanobacteria Incident Management Framework (CIMF) using chlorophyll-*a* as a primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).

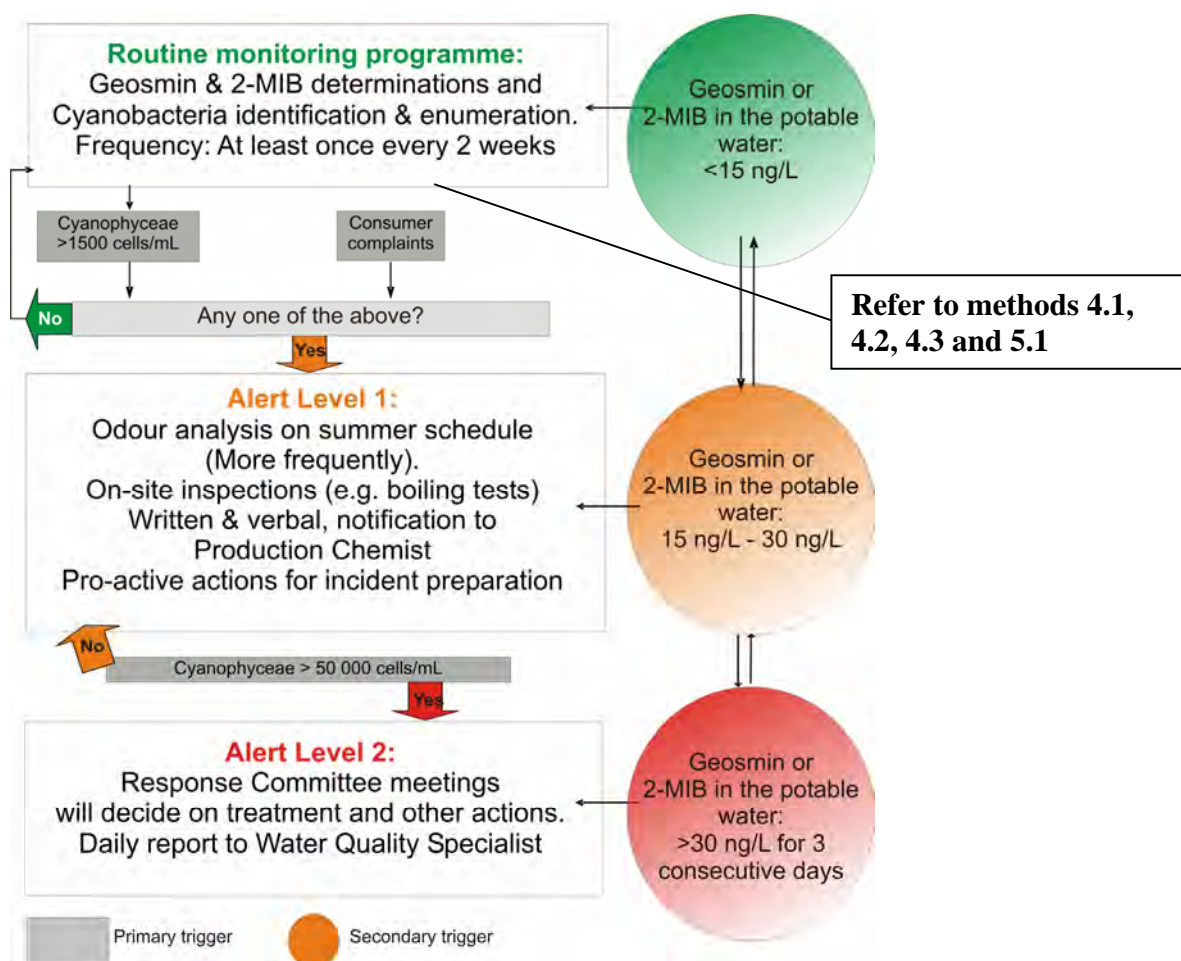


Figure 1.3 Cyanobacteria Incident Management Framework (CIMF) for the reaction to taste and odorous substances (as used by Rand Water).

The methods contained in this manual are written in the standard format prescribed by the International Organization for Standardization (ISO). Examples of method validation procedures are also included as part of the “Comprehensive Laboratory Methods for monitoring phytoplankton, including Cyanobacteria in South African Freshwaters”, which is included on the CD at the back of this document. It is, however, important to stress that each laboratory has to validate its own methods. Validations for a specific method (although copied exactly) cannot be accepted from another laboratory because differences between analysts, their competence, equipment and working environment all contribute to variability in the data generated by different laboratories.

For accreditation of a laboratory and the methods it employs, it is required that participation in proficiency testing schemes takes place regularly. This is the only way in which laboratories can evaluate their equipment, their analysts, their methods and the overall significance of the data they produce. It also supports the establishment of national standardization.

1.2 THE ROLE OF PHYTOPLANKTON

Phytoplankton may be broadly defined as photosynthetic, free-floating organisms which are mostly microscopic. This includes a large and diverse group of organisms, with a great range of shapes, sizes, pigmentation, structural complexities, and life cycles (AWWA, 1995). Phytoplankton is a common and normal component of surface waters and is present in every water source that is exposed to sunlight (Palmer, 1980). These organisms use light energy to convert carbon dioxide and water to sugars, and thereafter, to cell matter. Being part of the first level of the food web, phytoplankton are generally sensitive to the slightest change in the aquatic environment and can be used (to varying degrees) to indicate water quality, especially in terms of water pollution (Palmer, 1980).

Wherever conditions of temperature, light and nutrient availability are conducive, surface waters may support increased growth of phytoplankton. The presence of phytoplankton becomes most apparent in eutrophic, or nutrient-enriched, waters. In eutrophic waters excessive growths of certain phytoplankton species may occur to form a “water bloom”. During a bloom the water is generally coloured and aggregations (“scums”) may form on the water surface or accumulate at the water’s edge. The high concentrations of phytoplankton cells may also cause an unpleasant smell or taste (e.g. grassy, fishy or muddy). Tastes and odours are caused by the release of certain organic compounds (such as geosmin and 2-methyl-isoborneol) by both living, dead and decomposing phytoplankton. These problems are most commonly associated with cyanobacteria, but may also be caused by other taxa.

Elevated levels of phytoplankton can have negative consequences for the water purification industry. Potable purification costs are significantly increased when phytoplankton blooms occur, resulting in the need for the algal cells or their by-products, to be removed from the water. These costs arise from treatment plant downtime caused by shortened filtration cycles and a need for extended backwashing; use of additional chemicals and treatments; discarding of backwash water to reduce the risk of re-contamination; health risks due to the potential for formation of carcinogenic trihalomethanes during chlorination and the use of activated carbon to absorb toxins and taste and odour compounds.

The phytoplankton assemblage (composition) of a water body can provide an indication of the prevailing water quality. For example: Oligotrophic systems (very low nutrient concentrations) usually support minimal phytoplankton biomass with low species diversity and are generally dominated by nanoflagellates belonging to the Chrysophyceae and Cryptophytes, or by non-noxious cyanobacterial or chlorophyte picoplankton (Willèn et al., 1990). On the other hand, eutrophic and hyper-eutrophic systems sustain very high levels of phytoplankton biomass, often dominated by very few taxa, usually Cyanobacteria, Bacillariophyceae and, in some water bodies, chlorococcales or dinoflagellates (e.g. Padisak & Dokulil, 1994).

The correct identification and enumeration of phytoplankton in natural waters, together with the determination of the concentrations of their by-products, is therefore very important, not only because of the different problems related to individual species and genera, but also because of their properties to be good indicators of different water qualities and/or environmental and ecological conditions.

1.3 USING THE MANUAL

The target audience for whom this Methods Manual is anticipated to be of most use, is the smaller water laboratories with limited expertise and skills that need to develop similar methods (as described in this manual) in their own laboratories.

The “Condensed Laboratory Methods for the Analyses of Phytoplankton, including Cyanobacteria, in South African freshwaters” was written with the day-to-day laboratory use in mind, whereas the “Comprehensive Methods for Monitoring Phytoplankton, including Cyanobacteria, in South African Freshwaters” (on the CD at the back of this document) contain the same and additional information (such as validation reports etc.) not used in the laboratory every day.

Please note that wherever a blue text box like this one appears in the document, it refers the reader to more information available in the “**Comprehensive Methods for Monitoring Phytoplankton and Cyanobacteria in South African Freshwaters**”, that is available on the CD at the back of this document.

1.4 REFERENCES

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2. GUIDANCE FOR SAMPLING OF PHYTOPLANKTON AND CYANOBACTERIA

2.1 DESIGN OF PHYTOPLANKTON AND CYANOBACTERIA SAMPLING PROGRAMMES

2.1.1 INTRODUCTION

The overriding objective of collecting a water sample is to collect a relatively-small volume of water that is easily transported and handled in the laboratory in such a manner that the water quality variable (for example: chlorophyll concentration, phytoplankton and cyanobacteria species, cyanotoxin concentrations, etc.) still accurately represents the water quality variable being sampled i.e. that the sample is representative of the greater mass of water from which it was collected. This implies that the concentration or relative proportions of a specific water quality variable will be the same in the sample as in the material being sampled and that the sample will be handled in such a manner that no significant changes in composition occurs before the sample is analyzed (APHA, 2001). However, to achieve this certain aspects such as sample site selection, number of samples, sampling frequency, sampling techniques, sample preservation and sampling handling should be determined and should be documented in a well designed sampling programme (SANS 5667-1: 1980; SANS 5667-4: 1987; SANS 5667-2: 1991; SABS ISO 5667-6: 1990; SABS ISO 5667-3: 1994; APHA, 2001; Hötzel & Croome, 1998; ISO 5667-14: 1998; Olrik, et al.,1998).

For a list of references with useful information on the design of sampling programmes, refer to Section 3.1.1 in Chapter 3 of the “**Comprehensive Methods Manual**”.

2.1.2 SETTING OBJECTIVES FOR THE SAMPLING PROGRAMME

The design of specific sampling programme will depend on the specific objectives of the phytoplankton and cyanobacteria monitoring programme. The design and implementation of any sampling programme should therefore be a well thought through process with careful consideration of the specific objectives of the programme and the inherent and potential variability of the system being investigated (SANS 5667-1: 1980; APHA, 2001; Hötzel & Croome, 1998).

For examples of objectives for a monitoring programme, refer to Section 3.1.2 in Chapter 3 of the “**Comprehensive Methods Manual**”.

2.1.3 HISTORICAL AND PILOT SURVEY DATA

Data from previous investigations or pilot surveys constitute historical or baseline information that must be used to determine specific aspects such as sampling time and frequency (daily, weekly, every two weeks, monthly or variable), spatial distribution of sampling sites and type of samples (surface samples, integrated composite samples, depth interval samples) of the envisaged sampling programme (Hötzel & Croome, 1998). If no data are available for a specific water body or water system, it is advisable to conduct a pilot survey to obtain baseline data (for example, spatial and temporal variation in species composition and abundance, frequency of change in species composition and abundance and hydrodynamics of the system). This process is commonly known as ‘benchmarking’.

The historical data or data from the pilot survey are used to apply statistical techniques to aid in the determination of the required number of samples and the sampling frequency (SANS 5667-1: 1980; APHA, 2001; Hötzel & Croome, 1998). It would be an advantage if an experienced phycologist designs all phytoplankton and cyanobacteria sampling programmes with the appropriate advice and assistance from a statistician (as advised by Hötzel & Croome, 1998).

2.1.4 SAMPLE SITE SELECTION

The actual location of the sample sites is a vital aspect in the design of a specific monitoring programme. Some of the factors that will influence the selection of sites are:

- **The specific objectives of the monitoring programme.** For example, a national programme, a recreational monitoring or a programme monitoring the source water abstraction points and intakes to a treatment plant.
- **The availability of resources.** The availability of resources is one of the most important drivers of the number of samples sites and their location. It is recommended that a costing exercise is performed before the final selection of the location and number of sites.
- **The health and safety aspects of the monitoring and sampling staff.** All potential hazards (danger from people and animals, steep slopes) including sampling potential toxic cyanobacteria blooms.
- **Dealing with non-conservative water quality variables.** The consideration of non-conservative elements, both biotic and abiotic, requires especial attention to ensure that they reach the laboratory largely unchanged. This may require the use of preservatives in some cases.
- **Possible spatial (horizontal and vertical) distributions.** It is well known that phytoplankton and cyanobacteria can vary in both spatial dimensions - horizontally and vertically in a specific water body. The variation would be influenced by the

morphometric and hydro physical aspects of the water body (for example: the prevailing wind direction and strength).

- **Temporal variability.** The occurrence and growth of phytoplankton and cyanobacteria varies with season and can be linked to factors such as rainfall patterns (dry and wet seasons), environmental temperature, light intensity, and day/night length (Chorus & Bartram, 1999; DWAF, 2002).

For more information on sample site selection in a) streams and rivers, b) dams and lakes c) draw-off points at lakes and rivers and d) drinking water purification plants, refer to Section 3.1.4 in Chapter 3 of the “Comprehensive Methods Manual”.

2.1.5 FREQUENCY OF SAMPLING

The frequency of sampling will be guided by several factors including the following:

- **The specific objectives of the monitoring programme.** For example, if it is a national programme or a programme monitoring the source water intakes to a drinking water plant.
- **Dealing with non-conservative water quality variables.** The consideration of non-conservative elements, both biotic and abiotic, requires especial attention to ensure that they reach the laboratory largely unchanged. This may require the use of preservatives in some cases.
- **Temporal variability.** The occurrence and growth of phytoplankton and cyanobacteria varies with season and can be linked to factors such as rainfall patterns (dry and wet seasons), environmental temperature, light intensity, and day/night length (DWAF, 2002).
- **Specific situations.** The frequency of a specific monitoring programme, (for example, for cyanobacteria sampling frequency), can change as concentrations of the cyanobacteria changes in the water column (frequency of sampling: routine monitoring programme < vigilance level monitoring programme < alert level monitoring level programme).
- **Availability of historical data.** If historical data is not available on which to base statistical decisions related to the frequency of sampling, it is advisable to adopt a high frequency of sampling (for example, weekly) and for a limited period (for example, a hydrological year). This data is then used to optimize the frequency of sampling of the programme.
- **Serial correlation of data.** If frequency of sampling is too high (for example, daily or weekly) the data may show serial correlation and there may be some degree of redundancy of the consecutive data points.
- **Consideration of cost implications.** A balance between sampling frequency, the sampling objectives and the associated cost must be achieved. However, a decrease in

the obtained information content of the monitoring data, as a result of a decrease in sampling frequency, may increase the risk of not achieving the sampling programme objectives and thus the monitoring programme objectives. The relationship between the obtained information content and the sampling frequency is depicted in **Figure 2.1** (DWAF 2002; Du Preez & Van Baalen, 2006).

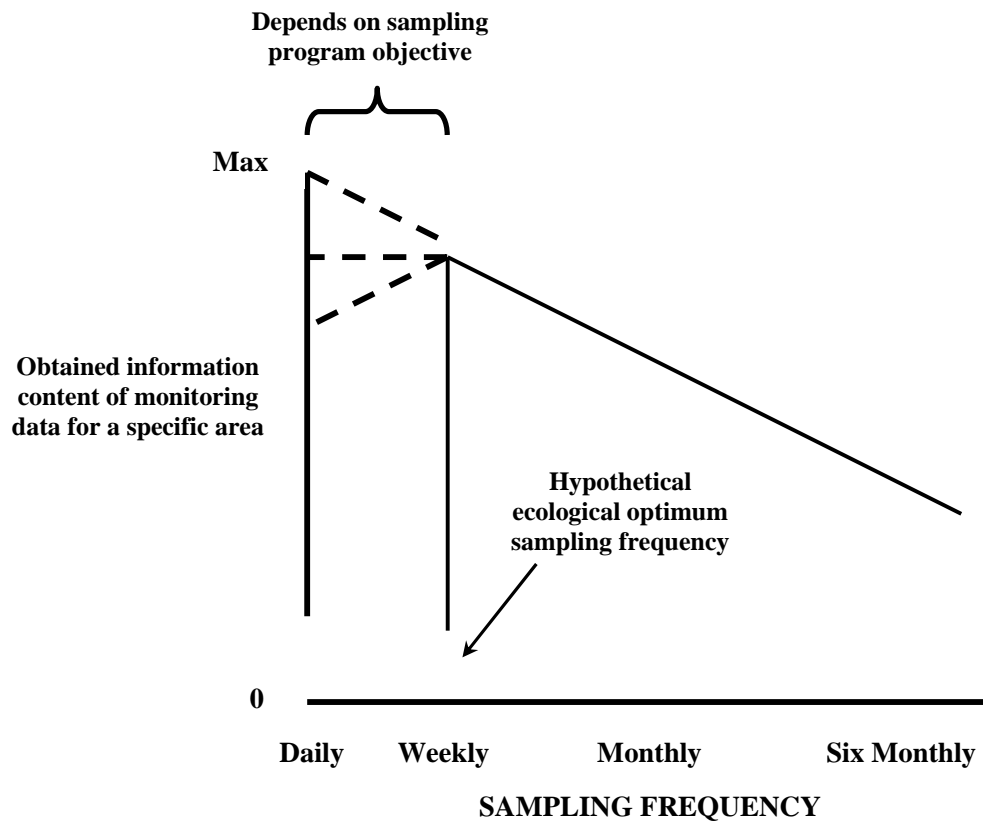


Figure 2.1 : The relationship between the obtained information content of the monitoring data (hypothetical) and the sampling frequency (adapted from DWAF, 2002.).

For more information on sampling frequency in a) streams and rivers, b) dams and lakes c) draw-off points at lakes and rivers and d) drinking water purification plants, refer to Section 3.1.5 in Chapter 3 of the “Comprehensive Methods Manual”.

2.2 TYPES OF SAMPLES

The following types of samples are generally collected:

- **Grab sample:** A discrete volume of water is taken at a specific site, depth and time, and is generally referred as a '*grab sample*'. This can be taken at the surface '*surface grab sample*', or at approximately 15 cm below the water surface '*subsurface sample*' or at different depths at a specific site '*discrete depth grab sample*'.
- **Depth-integrated grab sample:** A discrete volume of water collected by taking a sample that collects water from the surface to a specific depth below the surface or to just above the sediment.
- **Composite sample:** Appropriate known volumes of two or more samples or sub-samples mixed together and then a sample from this composite mixture is taken for analysis (SANS 5667-2: 1991; APHA, 2001; Hötzel & Croome, 1998; Chorus & Bartram, 1999; DWAF, 2002).

Discrete depth grab sampling or depth-integrated grab sampling is recommended when an estimation of the overall phytoplankton and cyanobacteria population is required. Discrete depth grab sampling is recommended when the source water body is used for drinking water purification and specifically at all source water abstraction points. Grab sampling or discrete depth grab sampling is usually performed when collecting samples for cyanotoxin analysis.

2.3 SAMPLING EQUIPMENT

2.3.1 APPARATUS

2.3.1.1 Water sampler

Hosepipe sampler: A standard clear PVC pipe (25 mm inner diameter) of 5 m length, with a weight and a 6 to 7 m rope tied at one end. The length of the rope may vary depending on the height from which the sample is taken. The length of rope will be at least high as the height from the water surface plus 5 m (Hötzel & Croome, 1998; DWAF, 2002). The length of the hosepipe (diameter 2 mm) can be increased to sample depths of 30 – 35 m (Chorus & Bartram, 1999). In practice, the feasibility of using a hosepipe sample with a length greater than 5 m is, however, questionable.

Van Dorn or Rittner sampler: These sampling devices consist of an open cylindrical tube with stoppers at each end, a closing device and a nylon rope or steel cable to lower it to the desired depth (APHA, 2001; Chorus & Bartram, 1999).

A dip-stick sampler: These sampling devices consist of a dip-stick (>3 m long) carrying the sample bottle at the end. This device is usually used to collect water from the shore (Hötzel & Croome, 1998).

A bucket sampler: These sampling devices consist of plastic bucket with a nylon rope or steel cable to lower it to the surface of the water or to collect samples from the shore. The bucket sampler is generally not the preferred sampling device (Hötzel & Croome, 1998).

2.3.1.2 Plankton net

Plankton net with a mesh diameter 20 µm: A plankton net (mesh diameter 20 µm) with a rope for the collection of large quantities of cyanobacteria required for toxicity testing or chemical analysis, or when additional samples are taken to supplement the phytoplankton and cyanobacteria larger species list (Ollrik, et al., 1998; APHA, 2001; Chorus & Bartram, 1999).

Plankton nets are unsuitable for taking quantitative or even presence/absence samples, as they do not collect picoplankton (<2 µm) and nanoplankton (2 – 20 µm) species (Hötzel & Croome, 1998).

2.3.1.3 Plastic container with lid

A plastic bucket (5 to 10 L) with lid for decanting a sample collected with a hosepipe, or Rittner or Van Dorn samples. A larger bucket (>20 L) is required for the mixing of composite samples.

2.3.1.4 Large plastic ladle or equivalent device

This is required for the mixing of the water in the bucket before a sub-sample is taken.

2.3.1.5 Filtration unit and handheld vacuum pump

The filtration unit for 250 mL (for example supplied by Millipore or Nalgene) and a hand vacuum pump (e.g. hand pump) are required for the filtering of samples on-site, for example, for chlorophyll analysis.

2.3.1.6 Cooler box and ice bricks

This is required for the storing and transporting of the samples.

2.3.1.7 Sample storage bottles

Microscopic identification and quantification of phytoplankton and cyanobacteria: Brown glass bottles (100 mL). Brown polyethylene bottles (100 mL) can be used, but if Lugol's iodine is used as a preservative, the bottles will be stained. If samples are collected from a purified drinking water point, then a 2 L bottle is required. If clear bottles are used, the samples must be stored in the dark.

Chlorophyll analysis: Brown glass bottles (1 L to 2.5 L) are preferred, but brown polyethylene bottles (1 L to 2.5 L) can be used. If samples are collected from a purified drinking water point a 2 L bottle is required. If clear bottles are used, the samples must be stored in the dark.

Cyanotoxin analysis: Glass bottles (1 L to 2 L) with lids are preferred, but polyethylene bottles (1 L) can be used.

Cyanotoxin analysis frozen: Samples to be frozen in liquid nitrogen can be stored in 50 mL polypropylene tubes. Samples to be freeze-dried can be frozen in specimen containers (100 mL) used for urine analysis.

Geosmin and 2-MIB analysis: Samples are collected in glass sample bottles (1 L).

2.3.1.8 Cooler box and ice bricks

Required to keep samples in the dark (when clear sample bottles are used) and to keep samples cool (<10°C) during transportation.

2.4 PRESERVATIVES

2.4.1 SAMPLES FOR THE IDENTIFICATION AND ENUMERATION OF PHYTOPLANKTON AND CYANOBACTERIA

Phytoplankton and cyanobacteria samples for later identification and enumeration should be preserved as soon as possible. If samples are analyzed immediately, it should also be fixed with the same preservatives as to render them non-motile for accurate enumeration. The most frequently used preservatives are Acid Lugol's solution, formalin and glutaraldehyde (SABS ISO 5667-3: 1994; APHA, 2001; Hötzel & Croome, 1998; Olrik, et al., 1998).

2.4.1.1 Acid Lugol's Solution

Please note that when Lugol's solution is used as a preservative/fixative, no pressure deflation of gas vacuoles is necessary during the preparation of samples for identification and enumeration of phytoplankton (see sections 4.1.2.1, 4.1.2.2 and 4.1.2.3)

Preparation

Dissolve 100 g potassium iodide (IK) in 1000 mL distilled water. Then dissolve 50 g pure iodine (I₂) in this solution. A few days before use add 100 g glacial acetic acid (96 – 100% CH₃COOH) to the solution.

To remain effective for at least a year, the Acid Lugol's solution must be stored in an amber/brown glass bottle kept in the dark.

Volume of preservation

Add 0.5 – 1 mL of the Acid Lugol's solution (that is 6 - 8 drops with a Pasteur pipette) per 100 mL sample or until the sample gains a colour like brandy (Olrik, et al., 1998).

Preservation for long-term storage of a sample is by adding 1) an additional 1 to 3 drops of Acid Lugol's' solution per 100 mL of an already preserved sample and 2) by adding 3 mL buffered formalin to the sample after an hour.

Storage

Samples must be stored in amber/dark bottles and, to prevent the iodine from escaping, an insert made of teflon should be placed in the sample bottle cap. Samples should be kept in darkness such as in a closed cupboard.

2.4.1.2 Acidified Formaldehyde Solution (20%)

Preparation

To prepare a 20% aqueous solution of acidified formaldehyde, mix 500 mL of formalin (40% HCHO) and 500 mL of acetic acid (mixture is thus 1:1).

Store in a glass or a high-density plastic bottle.

Volume of preservative

Add 2 mL of the acidified formaldehyde solution per 100 mL sample (final concentration of HCHO should be 0.4%).

Storage

Samples must be stored in amber/dark bottles and, to prevent the formaldehyde vapours from escaping, an insert made of teflon should be placed in the sample bottle cap.

2.4.1.3 Neutralized Glutaraldehyde

Preparation

Use glutaraldehyde (P.A.).

Volume of preservative

To preserve the phytoplankton sample (mainly picophytoplankton samples that are for electron microscope evaluations) add neutralized glutaraldehyde to a final concentration of 1 to 4%.

Storage

Samples can be stored in amber/dark bottles for several years.

2.4.2 SAMPLES FOR CHLOROPHYLL DETERMINATION

Samples are usually only cooled and stored in the dark where after they are analyzed with 8 h of sampling. If the samples cannot be analyzed within 8 hours of sampling, the sample is filtered and the residue (usually the filter paper with the residue) is stored in 90% ethanol and frozen. The sample container is usually wrapped in tinfoil to prevent light exposure.

2.4.2.1 Ethanol 95%

Preparation

Use analytical grade ethanol (95%).

Volume of preservative

To preserve the chlorophyll sample residue on the filter paper add 10 ml analytical grade ethanol (95%) and ensure the filter paper with the residue is submerged in the ethanol.

Storage

Samples extracted in ethanol can be stored for 1 month, in glass or amber/dark bottles, sealed (no evaporation) and wrapped in tinfoil, and kept below 8°C.

Filter papers can alternatively be wrapped in tinfoil and frozen (preferable in liquid nitrogen). These can be stored for one month.

For advantages and disadvantages of each type of preservative, refer to Section 3.4 in Chapter 3 of the
“Comprehensive Methods Manual”.

2.5 SAMPLING PROCEDURE

2.5.1 SURFACE GRAB SAMPLE USING THE SAMPLE BOTTLE

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample.
- Remove the lid and lower the sample bottle into the water (approximately 15 cm below the water surface). Perform a forward scooping motion to fill the bottle with water and discard the water (this process is required for rinsing the bottle).
- Fill the bottle again by lowering it into the water (approximately 15 cm below the water surface). Perform a forward scooping motion to fill the bottle.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis the sample bottle is usually filled to the lid leaving no free (air) space.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.5.2 SURFACE GRAB SAMPLE USING A BUCKET FIXED TO A NYLON ROPE OR STEEL CABLE

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample (DWAF 2004).
- Fix the nylon rope or steel cable to the bucket.
- Ensure the bucket is clean.
- Lower the bucket into the water. If the sample is taken from the shore, the bucket is thrown to the desired sampling area in the water. Perform a forward scooping motion to fill the bucket with water. Discard the collected sample (this process is required for rinsing the bucket).
- Lower the bucket into the water again. If the sample is taken from the shore, the bucket is thrown to the desired sampling area in the water. Perform a forward scooping motion to fill the bucket with water.
- Remove the lid from the sample bottle and immediately fill the sample bottle.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis, the sample bottle is usually filled to the lid leaving no free (air) space.

- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.5.3 SURFACE GRAB SAMPLE USING A DIP-STICK (RANGING POLE) SAMPLER

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample (DWAF 2004).
- Attach the sample bottle to the dipstick sampler (>3 m long).
- Remove the lid from the bottle and, with the aid of the dip-stick sampler, lower the sample bottle into the water. Perform a forward scooping motion to fill the bottle with water. Discard the collected sample (this process is required for rinsing the bucket).
- Repeat the procedure.
- Remove the sample bottle from the dip-stick sampler.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis, the sample bottle is usually filled to the lid leaving no free space.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.5.4 DEPTH-INTEGRATED GRAB SAMPLE USING A HOSEPIPE

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample (DWAF, 2004).
- Lower the weighted end of the pipe (a clear PVC pipe, 25 mm inner diameter of 5 m length, with a weight and a 6 to 7 m rope tied at one end) into the water until the whole pipe is suspended in the water.
- Close the top end and pull up the bottom end until the U-shape is formed and lift this end out of the water. Empty the pipe and discard the collected sample (this process is required for rinsing the pipe).
- Once again, lower the weighted end of the pipe into the water until the whole pipe is immersed in the water.
- Close the top end and pull up the bottom end until the U-shape is formed and lift this end out of the water.
- Empty the contents of the pipe into a clean bucket.

- Mix the sample in the bucket with a clean plastic paddle before the sub-sample is taken.
- Remove the lid from the sample bottle and immediately fill the sample bottle.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis, the sample bottle is usually filled to the lid leaving no free space.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.5.5 DISCRETE DEPTH GRAB SAMPLE

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample (DWAF, 2004).
- Ensure the depth water sampler (e.g. Rittner) is clean and in working order.
- Fix the nylon rope or steel cable to the grab sampler.
- Gently lower (never drop) the sampler into the water, allow to fill with water and remove. Empty the depth water sampler and discard the collected sample (this process is required for rinsing the depth water sampler).
- Lower the depth water sampler again into the water; ensure that water flows through the cylinder until the desired depth is reached.
- Avoid rough handling while lowering the grab sampler, as this will cause premature triggering of the closing device.
- At the desired depth, sharply pull the cord to trigger the closing device.
- Recover the depth water sampler and empty contents into the bucket.
- Mix the sample in the bucket with a clean plastic paddle before the sub-sample is taken.
- Remove the lid from the sample bottle and immediately fill the sample bottle.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis, the sample bottle is usually filled to the lid leaving no free (air) space.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.
- Repeat the process to collect the samples at the required depths.

2.5.6 GRAB SAMPLES FROM A FIXED SAMPLING POINT (FOR EXAMPLE, A TAP)

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample.
- Open tap and allow to run freely for ± 5 minutes.
- If sampling occurs where a tap is running continuously do not adjust the flow.
- Remove the lid from the sample bottle.
- Hold the sample bottle under tap to fill the sample bottle with water and discard the water (this process is required for rinsing the bottle).
- Fill the sample bottle again.
- Pour out a small volume (± 2 cm from the top of bottle) to allow proper mixing of the sample prior to analysis or to adding the required preservative. Note: when samples are collected for geosmin and/or cyanotoxin analysis, the sample bottle is usually filled to the lid leaving no free (air) space.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.5.7 SAMPLING USING A PLANKTON NET

Procedure

- Ensure the correctly marked bottle is used at the sampling site.
- Leave the lid on the bottle until you are ready to take the sample (DWAF, 2004).
- Ensure the plankton net is clean. It is very important to **clean the plankton net** thoroughly before using it to sample at another sampling site (reservoir, lake or river).
- The plankton net is pulled through the water (horizontally and/or vertically) until a suitable concentration of phytoplankton is collected.
- Remove the lid from the sample bottle and place a clean funnel into the bottle.
- Transfer the sample collected into the sampling bottle (via the funnel). Use a squeeze bottle filled with water from the sampling site to wash the collected plankton from the plankton net.
- Add the required preservative.
- Screw the top tightly onto the bottle and place in cooler box or dark container with ice bricks.

2.6 TRANSPORTING OF SAMPLES

Sample containers holding the samples must be protected and sealed to prevent leaking and deterioration of the sample during transport. During transportation the samples must be kept as cool as possible and protected from light (SABS ISO 5667-3: 1994).

The techniques generally applied for preservation and transporting of samples are summarized in **Table 2.1**.

Table 2.1 : Techniques generally suitable for the preservation, storage and transporting of samples

Parameter to be analyzed	Type of container	Preservation technique	Maximum recommended preservation time before analysis	Transport condition	Comments
Phytoplankton and cyanobacteria identification	Brown glass bottle (100 mL or 2 L for drinking water)	Add 0.5 to 1 mL Acid Lugol's per 100 mL sample	1 year stored in the dark	Cool to <8°C and keep in the dark	Samples should be preserved as soon as possible after collection.
	Brown polyethylene bottles (100 mL or 2 L for drinking water) if not preserved with Lugol's iodine	Add 0.5 ml Acid Lugol's and 3 mL buffered per 100 mL sample	More than a year stored in the dark		Keep in the dark if clear sample bottles are used.
		Add 2 mL acidified formaldehyde per 100 mL (final concentration 0.4% HCHO)	1 year stored in the dark		
		Neutralized Glutaraldehyde at a final concentration of 1 to 4%	1 year stored in the dark	Cool to <8°C and keep in the dark	Only for electron microscopic analysis Keep in the dark if clear sample bottles are used. Samples should be preserved as soon as possible after collection.
Chlorophyll	Brown glass bottle (1 L to 2 L)	Cool to <8°C and keep in the dark	8h after sampling and store in dark	Cool to <8°C and keep in the dark	Perform analysis as soon as possible.
	Brown polyethylene bottles (1 L or 2 L)	Filtered residue stored in 10 ml analytical grade (95%) ethanol and if possible frozen	1 month stored in the dark	Cool to <8°C, sample bottle wrapped in tinfoil and keep in the dark	Keep in the dark if clear sample bottles are used.

Table 2.1 (cont.): Techniques generally suitable for the preservation, storage and transporting of samples

Parameter to be analyzed	Type of container	Preservation technique	Maximum recommended preservation time before analysis	Transport condition	Comments
Geosmin and 2-MIB	Brown glass bottle (1 L)	Cool to <8°C and keep in the dark	24h after sampling and store in dark	Cool to <8°C and keep in the dark	
	Brown polyethylene bottles (1 L)				
Cyanotoxins	Brown glass bottle (1 L)	Cool to <8°C and keep in the dark	24h after sampling and store in dark	Cool to <8°C and keep in the dark	Different fractions can be analyzed (Total or cell bound or dissolved).
	Brown polyethylene bottles (1 L)	Samples frozen in liquid nitrogen stored in 50 mL polypropylene tubes	1 year	Frozen	Different fractions must be separated before freezing.

2.7 RECEPTION OF SAMPLES IN THE LABORATORY

The samples received should be individually inspected to ensure that the condition of the sample has not been compromised. For example, ensure that the containers were tightly sealed, sample bottles did not crack or break during transport, the samples were protected against sunlight and cooled to the desired temperature. Check that all the necessary documentation has been completed, including the sample collection data sheet (Comprehensive Methods Manual **Table 3.5**) and the chain of custody sheet (Comprehensive Methods Manual **Table 3.6**). Ensure that the samples that cannot be analyzed quickly are stabilized by cooling to below 8°C or preserved as indicated in **Table 2.1**. The quality assurance samples used for transportation, stabilization and storage should be processed in the same way as the samples to be analyzed (ISO 5667-14: 1998).

For examples of a field sampling form and a chain of custody form, refer to Section 3.7 in Chapter 3 of the **“Comprehensive Methods Manual”**.

2.8 QUALITY ASSURANCE OF WATER SAMPLING AND HANDLING

Quality control procedures must be implemented that will be used to identify and quantify errors associated with sampling. The implementation of the quality control procedures will have the following broad objectives:

- a. To provide a means of monitoring and detecting errors that will assist in improving the sampling process as well as providing a means of rejecting suspect data.
- b. To demonstrate that possible sampling errors have been controlled.
- c. To assess the variability of sampling and thus give an indication of the error (ISO 5667-14: 1998).

2.8.1 SELECTED QUALITY CONTROL TECHNIQUES

A detailed description of techniques is given in the ISO document 'Water quality sampling – Part 14: Guidance on quality assurance of environmental water sampling and handling (ISO 5667-14: 1998).

The following quality control techniques can be considered:

Replicate quality control samples: When two discrete (separate) samples (A_1 and A_2) are taken at the sampling point at the same time, an estimate of the total sample variance (sampling, containers, storage and analysis) is obtained. When one bulk sample (B) is collected, from which two sub-samples (B_1 and B_2) are taken and subsequently used to take two additional sub-samples from each (B_{11} and B_{12} ; B_{21} and B_{22}), the difference between B_1 and B_2 (expressed as the mean (B_{11} & B_{12} and B_{21} & B_{22})) gives an indication of the analytical, plus sampling, variance (including storage, but excluding the sampling container). The difference between the replicate analysis of B_{11} & B_{12} and B_{21} & B_{22} gives an estimation of analytical precision. This technique can be applied to a sampling programme taking samples for chlorophyll, phytoplankton identification and enumeration, geosmin, 2-MIB and cyanotoxin analysis.

Field blank samples: This technique is used to identify errors resulting in contamination of samples and the sampling process. This technique can be applied to a sampling programme taking samples for chlorophyll, geosmin, 2-MIB and cyanotoxin analysis.

Rinsing of equipment: This technique is used to identify errors resulting in contamination of sampling devices and to errors in the sampling process due to incomplete cleaning of the sampling devices and sample containers. This technique can be applied to a sampling programme taking samples for chlorophyll, phytoplankton identification and enumeration, geosmin, 2-MIB and cyanotoxin analysis.

Filtration recovery equipment: This technique is used to identify errors resulting in contamination of sampling containers associated with the filtration of samples. This can include 1) the filtering of a spiked quality assurance sample and 2) the filtering of a de-ionized water blank. This technique can be applied to a sampling programme taking samples for chlorophyll, phytoplankton identification and enumeration, geosmin, 2-MIB and cyanotoxin analysis.

Spiking of samples: This technique is used for estimating errors in the sampling process which includes identifying the errors resulting in the contamination of sampling containers and as a result of errors of handling during the sampling process. This can include 1) of spiked environmental samples 2) and spiked de-ionized water samples. This technique can be applied to a sampling programme taking samples for geosmin, 2-MIB and cyanotoxin analysis.

2.8.2 ANALYSIS AND INTERPRETATION OF QUALITY CONTROL DATA

The quality control data are evaluated to ensure that the reliability of the sampling data adheres to the performance criteria required. The data are usually plotted in the form of a Shewhart chart (ISO 5667-14: 1998). The Shewhart is a chart on which the variable under investigation is plotted sequentially and the measured values are compared with the control value.

2.9 REFERENCES

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3. CHLOROPHYLL DETERMINATION IN WATER

3.1 INTRODUCTION

The expression of phytoplankton biomass in water is generally in the form of chlorophyll-*a* concentration. The analysis is relatively easy to perform and is therefore widely used in the analysis of water samples. The downside of chlorophyll-*a* analysis is that it is not suitable for water with low chlorophyll content, such as drinking water. In those cases, it is more appropriate to use the chlorophyll-665 method to determine the total pigment concentration.

All green plants contain chlorophyll-*a* and planktonic algae owe about 1 – 2% of its dry weight to chlorophyll-*a*. It is important to note that the chlorophyll-*a* content per cell varies between species and even more so between phyla. Low chlorophyll-*a* concentrations do not necessarily indicate low phytoplankton biomass, especially in cases of cyanobacterial dominance.

The Chlorophyll-665 method was introduced in water purification plants to determine the total pigment concentration in purified water, since the chlorophyll-*a* method is not sensitive enough at such low concentrations as those found in drinking water. Chlorophyll-665 is a quick method to determine total pigment concentrations in water within an hour or two of testing.

Determining chlorophyll concentrations is usually the first (and most basic) analysis done to determine phytoplankton-related problems in raw and potable waters.

3.1.1 DECISION WHETHER TO DO CHLOROPHYLL-665 OR CHLOROPHYLL-*a*

Table 3.1: Characteristics of the chlorophyll-665 and chlorophyll-*a* methods that will aid in determining the most appropriate method to use for a specific purpose

Chlorophyll-665	Chlorophyll-<i>a</i>
Analysis of total pigment	Analysis of chlorophyll- <i>a</i>
Turn-around time: 2 hours	Turn-around time: 24 hours
Sensitive at low concentrations (<2 µg/L)	Sensitive at higher concentrations (>2 µg/L)
Generally used for tap water	Generally used for source water

3.2 CHLOROPHYLL-665 (TOTAL PIGMENTS) (Adapted from Steynberg, 1986).

For the scope, definition of chlorophyll-665, field of application for this method, interferences with the determination of chlorophyll-665, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding chlorophyll-665, refer to Section 4.1.1 – 4.1.5 in Chapter 4 of the “Comprehensive Methods Manual”.

3.2.1 APPARATUS, MATERIALS AND REAGENTS

3.2.1.1 Instruments and equipment

- Centrifuge.
- Filtering apparatus (Refer to **Figure 3.1**).
- Printer (optional).
- Spectrophotometer.
- Uninterrupted power supply.
- Vacuum pump.
- Bottle top dispenser or 10 mL pipette.
- Balance.
- Vortex shaker.
- Refrigerator.
- Water bath that maintains a stable temperature at 60°C. Temperature is checked against a certified thermometer or one of equivalent accuracy.
- Thermostat.



Figure 3.1: Filtering apparatus.

3.2.1.2 Glassware

- Screw-capped test tubes.
- Test tubes - rimless, medium wall (100 mm x 14 mm).
- Volumetric flask – 1 L R A-grade.
- Thermometers - calibrated (with certificate).
- Measuring cylinders – 100 mL, 250 mL, 500 mL, 1000 mL.

3.2.1.3 Other materials

- Whatman glass fiber filters (GF/C) – 47 mm diameter.
- Trace-Klean.

3.2.1.4 Reagents

- Methanol – GR grade – pro analisi.
- Reagent water – Water that has been filtered by reverse osmosis, has a conductivity of less than 6.0 mS/m and turbidity of less than 2.0 nTU. This reagent water has no detectable salts or impurities.

3.2.2 PROCEDURE

- 3.2.2.1 Filter a known volume of sample (in duplicate) using a glass measuring cylinder (0.5 L to 2.5 L) depending on the density of the phytoplankton, through a glass fiber filter (Whatman GF/C). Before filtration the sample must be agitated to ensure uniformity. The glass measuring cylinder and the filtering cup must also be rinsed with reagent water.
- 3.2.2.2 Remove the filter and the entrapped phytoplankton without disturbing the phytoplankton or tearing the filter. Gently roll the filter without applying pressure.
- 3.2.2.3 Place the filter into a marked screw-capped test tube (20 mL) and add approximately 10 mL methanol using the methanol bottle top dispenser or appropriate pipette.
- 3.2.2.4 Place the test tubes in a water bath at 60°C for ± 1 hour.
- 3.2.2.5 After 1 hour shake the test tubes vigorously (using the vortex shaker at setting ± 7 for ± 15 seconds) before decanting the extract into marked centrifuge tubes.
- 3.2.2.6 Centrifuge the extract for ± 5 minutes at ± 4800 rpm (to clarify the extract). Ensure the test tubes in the baskets are balanced.
- 3.2.2.7 Read the absorbance, using the spectrophotometer at 660 nm and 750 nm wavelengths.
- 3.2.2.8 The absorbance reading taken at 750 nm is subtracted from the absorbance reading taken at 660 nm and the result is multiplied by a factor (see Section 3.2.4).

3.2.3 SAFETY PRECAUTIONS

3.2.3.1 Hazard warning



- Methanol (methyl alcohol) is **harmful and dangerous!!!** It may be fatal if swallowed or cause blindness. Methanol is harmful if inhaled or absorbed through the skin. It cannot be made non-poisonous, and is flammable in both liquid and vapor form. Methanol causes skin-irritations as well as irritations to the eyes and respiratory tract. It also affects the central nervous system and liver. **HANDLE WITH CARE!** (Mallinckrodt Chemicals, 2002).

- Ethanol – flammable liquid. Keep away from sources of ignition.

3.2.3.2 Clothing

- Always wear a laboratory coat when performing chlorophyll-665 analysis.
- Wear gloves when handling methanol.
- Wear gloves when handling potential hazardous water samples.

3.2.3.3 Safety instructions when working with methanol:



- Highly flammable, keep away from sources of ignition - no smoking.
- Avoid ingestion and contact with skin and eyes.



- Mark all containers very clearly toxic!
- Keep methanol container tightly closed.
- Never pipette methanol by mouth.

3.2.4 CALCULATIONS AND EXPRESSION OF RESULTS

- Use the following formula for the determination of chlorophyll-665 (total pigment):

$$E = \frac{10^6 \times A(A_{665} - A_{750}) \times V_e}{V_m \times L}$$

Where:	E	=	Chlorophyll (phaeophytin)
	A	=	Absorption coefficient of 0.0133
	A ₆₆₅	=	Absorbance at 660 nm
	A ₇₅₀	=	Absorbance at 750 nm
	V _e	=	Volume of solvent (mL)
	V _m	=	Volume of sample (mL)
	R	=	Path length of cuvette (cm)
	x	=	Multiplication

- The chlorophyll-655 (total pigment) values are "rounded off" as follows:

0 < Result < 1	Report to 2 decimal places
1 ≤ Result < 10	Report to 1 decimal place
10 ≤ Result	Report to nearest whole number

Note: It is important to note that rounding off should only occur in the final step (presentation phase) of calculation and not in the analytical phase.

For more information on records and data keeping, quality assurance and typical validations for the determination of chlorophyll-665 method, refer to Section 4.1.9 - 4.1.10 and Section 4.2.11 in Chapter 4 of the **“Comprehensive Methods Manual”**.

3.3 CHLOROPHYLL-*a* (adapted from Sartory, 1982).

For the scope, definition of chlorophyll-*a*, field of application for this method, interferences with the determination of chlorophyll-*a*, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding chlorophyll-*a* and disposal of hazardous material refer to Section 4.2.1 – 4.2.5 in Chapter 4 of the “**Comprehensive Methods Manual**”.

3.3.1 APPARATUS, MATERIALS AND REAGENTS

3.3.1.1 Instruments and equipment

- Centrifuge.
- Filtering apparatus (refer to **Figure 3.1**).
- Micropipette.
- Tecnomara pipetboy (or equivalent).
- Printer.
- Spectrophotometer.
- Uninterruptible power supply.
- Vacuum pump.
- Bottle top dispenser or equivalent pipette.
- Balance.
- Vortex shaker.
- Refrigerator.
- Water bath.

3.3.1.2 Glassware

- Screw-capped test tubes.
- Test tubes - rimless, medium wall (100 mm × 14 mm).
- Bulb pipettes - 4 mL A-grade.
- Graduated pipette - 10 mL A-grade.
- Volumetric flask – 1 L A-grade.
- Thermometer or thermostat - calibrated (with certificate).
- Measuring cylinders – 100 mL, 250 mL, 500 mL, 1000 mL.

3.3.1.3 Other materials

- Whatman glass fiber filters (GF/C) - 47 mm diameter.
- Trace-Klean.
- Safety glasses when working with acid.

3.3.1.4 Reagents

- Ethanol (95%) - AnalR grade - pro analysi.
- Hydrochloric acid (HCl).
0.3 mole/L hydrochloric acid made up as follows:
Make up 9.4 mL HCl (measured using a 10 mL A-grade graduated pipette) to 1 L with reagent water. Make up monthly.
- Reagent water - Water that has been filtered by reverse osmosis, has a conductivity of less than 6.0 mS/m and turbidity of less than 2.0 nTU. This reagent water has no detectable salts or impurities.

3.3.2 PROCEDURE

- 3.3.2.1 Filter a known volume of sample (in duplicate) using a glass measuring cylinder (0.5 L to 2.5 L), depending on the density of the phytoplankton, through a glass fibre filter (Whatman GF/C). Before filtration, the sample must be shaken thoroughly to ensure uniformity. The glass measuring cylinder and the filtering cup must also be rinsed thoroughly with reagent water.
- 3.3.2.2 Remove the filter and the entrapped phytoplankton without disturbing the phytoplankton or tearing the filter. Gently roll the filter without applying pressure.
- 3.3.2.3 Place the filter into a marked screw-capped test tube (20 mL) and add approximately 10 mL ethanol (95%), using the ethanol bottle top dispenser or equivalent pipette.
- 3.3.2.4 Place test tubes in the water bath at $78 \pm 2^\circ\text{C}$ for 5 minutes prior to placing in the dark at room temperature for 24 ± 7 hours.
- 3.3.2.5 After 24 ± 7 hours shake test tubes vigorously (using the vortex shaker at setting ± 7 for ± 15 seconds) before decanting the extract into marked centrifuge tubes.
- 3.3.2.6 Centrifuge the extract for ± 15 minutes at ± 4800 rpm (to clarify the extract) using the centrifuge. Ensure the test tubes in the baskets are balanced.
- 3.3.2.7 Carefully decant the supernatant into marked test tubes.
- 3.3.2.8 Accurately transfer 4 mL of the supernatant using a 4 mL A-grade bulb pipette into another set of marked test tubes used for the acidification process.
- 3.3.2.9 Read the absorbency of the remaining supernatant, using the spectrophotometer at 660 nm and 750 nm wavelengths.
- 3.3.2.10 Acidify the 4 mL extract by adding approximately 100 μL of a 0.3 mole/L HCl solution. Mix the content of the test tube by shaking (using the vortex shaker at setting ± 4 for ± 5 seconds) and allow standing for approximately 4 minutes. The acidification converts the chlorophyll-*a* to phaeophytin-*a*.
- 3.3.2.11 Read the acidified sample as for point 3.3.2.9.
- 3.3.2.12 The absorbency values obtained in 3.3.2.9 and 3.3.2.11 are used to calculate the chlorophyll-*a* concentration (see 3.3.2.10).

3.3.3 SAFETY PRECAUTIONS

3.3.3.1 Hazard warning

- Ethanol - flammable liquid.
- Hydrochloric acid - corrosive, causes burns and irritation to respiratory system.

3.3.3.2 Clothing

- Always wear a laboratory coat when performing chlorophyll-*a* analysis.
- Always wear protective eye-wear when making up acids.
- Wear gloves when handling water samples, if necessary.

3.3.3.3 Safety instructions when working with ethanol

- Highly flammable, keep away from sources of ignition - no smoking.
- Mark all containers very clearly toxic!
- Keep ethanol container tightly closed.

3.3.3.4 Safety instructions when working with acid

- Always wear an acid-resistance laboratory coat or -apron.
- Always wear protective eye-wear when making up acids.
- Always add acid to water, never water to acid! The density of water is less than that of acid. If water is added to acid the water will collect on the surface, increasing the contact surface and thus increasing the severity of the reaction.
- Wear acid-proof gloves when handling acids.
- Wear protective shoes.

3.3.4 CALCULATION AND EXPRESSION OF RESULTS

- Use the following formula for the determination of chlorophyll-*a*:

$$\text{Chla } (\mu\text{g/L}) = \frac{[(A_{665} - A_{750}) - (A_{665a} - A_{750a})] \times 28.66 \times V_e}{V_m}$$

Where: A_{665}	=	Absorbance at 660 nm before acidification
A_{750}	=	Absorbance at 750 nm before acidification
A_{665a}	=	Absorbance at 660 nm after acidification
A_{750a}	=	Absorbance at 750 nm after acidification
28.66	=	Constant (taking into account: ethanol with its specific absorption coefficient and path length of the cuvette)
V_e	=	Volume of ethanol used for extraction in mL (usually 10 mL)
V_m	=	Volume of sample filtered in mL
\times	=	Multiplication

- The chlorophyll-*a* values are “rounded off” as follows:

$0 < \text{Result} < 1$	Report to 2 decimal places
$1 \leq \text{Result} < 10$	Report to 1 decimal place
$10 \leq \text{Result}$	Report to the nearest integer

Note: It is important to note that rounding off should only occur in the final step (presentation phase) of calculation and not in the analytical phase.

For more information on records and data keeping, quality assurance and typical validations for the determination of chlorophyll-*a* method, refer to Section 4.2.9 - 4.2.11 in Chapter 4 of the “**Comprehensive Methods Manual**”.

3.4 REFERENCES

- Mallinckrodt Chemicals, J.T. Baker., 2002. Material Safety Data Sheet. Available on Internet: <http://www.jtbaker.com/msds/M2015htm> [Date of access: 6 Apr. 2006]
- Sartory, D.P., 1982. *Spectrophotometric analysis of chlorophyll-a in freshwater phytoplankton*. Department of Environmental Affairs Technical Report TR 115.
- Steynberg, M.C., 1986. Aspekte van die invloed van eutrofikasie op die Vaalrivierbarrage. Dissertation submitted as fulfillment for the degree, Magister Scientiae in the Faculty of Natural Science, Department of Botany, University of the Free State, pp. 52-53.

4. PHYTOPLANKTON AND CYANOBACTERIA IDENTIFICATION AND ENUMERATION

4.1 INTRODUCTION

Two techniques are commonly used in South Africa (as determined by the phytoplankton identification and enumeration proficiency testing scheme which has been operating since 1998) to perform phytoplankton identification and enumeration analysis. These are the sedimentation (using either gravity or centrifugation to sediment phytoplankton) and the membrane filtration techniques (using vacuum pump to sediment phytoplankton onto a membrane filter). Both the sedimentation and membrane filtration techniques are the only ones that will be discussed in this manual, as they are appropriate to use in South Africa and are routinely used by several laboratories within the country.

There are many variations on the above-mentioned sedimentation technique. One prominent method, used by some international laboratories, is to sediment the phytoplankton by gravity in a container, e.g. measuring cylinder. After the appropriate sedimentation time has elapsed, most of the water column is siphoned off. The sedimented phytoplankton is re-suspended in the smaller volume and a certain volume thereof, placed onto a counting chamber, e.g. haemocytometer. The sample is then analyzed by using a compound microscope and usually by making use of phase contrast.

The identification of phytoplankton needs to be performed by a competent and experienced analyst. Courses in phytoplankton identification are presented from time to time at institutions like the North-West University (Potchefstroom campus) and internal staff training is also done at Rand Water and Umgeni Water. The training at Rand Water and Umgeni Water may also be available on request from outside institutions, when new staff members need to be trained in algal identification and enumeration. Depending on the method used, it may be advisable to contact Rand Water when using the sedimentation technique and Umgeni Water when using the membrane filtration technique.

When phytoplankton identification is mastered the enumeration procedure becomes relatively easy. The most important step in the enumeration process is to determine the area of the counting chamber or membrane filter and determine the correct factor by which the physical counts should be multiplied to express results as cells/mL. However although not widely applied in South Africa, the best way to express phytoplankton biomass is by determining the biovolume of the different cells and expressing the results as $\mu\text{m}^3/\text{mL}$.

If a phytoplankton identification and enumeration method is introduced into a laboratory for the first time, careful consideration should be given to the purchase of equipment, since the two mainstream methods require totally different equipment, with advantages and disadvantages to both (refer to **Table 4.1**).

4.1.1 DECISION WHETHER TO USE THE SEDIMENTATION OR MEMBRANE FILTRATION TECHNIQUES

Table 4.1: Characteristics of the sedimentation and membrane filtration techniques

Sedimentation technique - gravity -	Sedimentation technique - centrifugation -	Membrane filtration technique
Phytoplankton has to settle at a rate ± 24 hours per 1 cm height of sedimentation chamber	Phytoplankton is settled by centrifugation in less than 20 minutes	Phytoplankton is settled onto membrane filter by rapid filtration
Inverted microscope (using a sedimentation chamber) / compound microscope when using a haemocytometer	Inverted microscope (using a sedimentation chamber) / compound microscope when using a haemocytometer	Compound microscope / inverted microscope
A low volume can be sedimented – algae more visible in turbid samples	A low volume can be sedimented – algae more visible in turbid samples	A higher volume of sample is needed for this method – algae can be obscured in turbid samples
Turn-around time is dependent on sedimentation time (>24 hours)	Same day turn-around time	Same day turn-around time
Algal cells not deformed	Algal cells not deformed	If filtering not done properly, algae can be difficult to recognize
Because of low volume more fields should be counted to increase sensitivity	Because of low volume more fields should be counted to increase sensitivity	Because of higher volume less fields should be counted for adequate sensitivity
Cells distribution on chamber floor naturally patchy	Cell distribution on chamber floor can be more concentrated in certain areas	Cells distribution on membrane naturally patchy

4.2 PHYTOPLANKTON IDENTIFICATION AND ENUMERATION, THE SEDIMENTATION TECHNIQUE USING CENTRIFUGATION (METHOD USED AND VALIDATED BY RAND WATER)

The basic principle is the same as the sedimentation technique using gravity for settling (see Section 4.2), but the turn-around time of the analysis is reduced by up to 24h. Reduced turn-around time is necessary if same-day results are required for effective management of water purification processes.

This method makes use of a sedimentation chamber containing anything from 1 – 5 mL of centrifuged sample, to allow algal cells to settle to the bottom and which are then identified and enumerated using an inverted light microscope.

For the scope, definition of the term phytoplankton, field of application for this method, interferences with the identification and enumeration of phytoplankton, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding phytoplankton and disposal of hazardous material refer to Section 5.1.1 – 5.1.5 in Chapter 5 of the “**Comprehensive Methods Manual**”.

4.2.1 APPARATUS, MATERIALS AND REAGENTS

4.2.1.1 Instrument and equipment

- Homogenizer, used to break up loosely aggregated flocs like *Microcystis* to improve counting accuracy. The drawback of using this instrument is that once cyanobacterial colonies are broken up, it may be difficult to accurately identify species and even genera. This is optional for taxonomy labs, but if a homogenizer is not used, it is important to count more fields or strips.
- Inverted light microscope (when using a sedimentation chamber; refer to **Figure 4.2** Lund et al., 1958); compound light microscope (when using counting chambers such as a haemocytometer, refer to **Figure 4.3**).



Figure 4.1: Homogenizer with variable speed



Figure 4.2: Inverted light microscope.

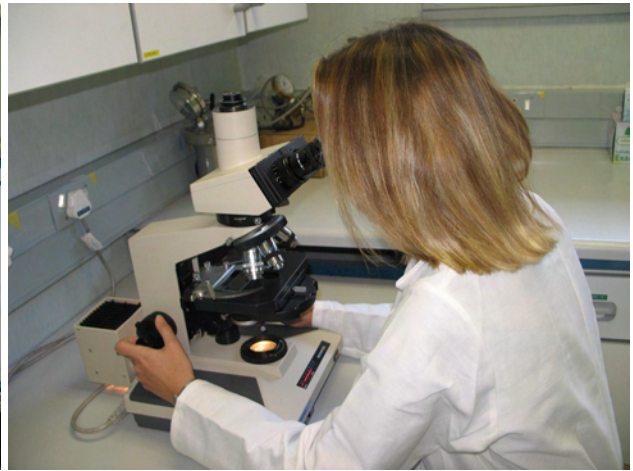


Figure 4.3: Compound light microscope.

- Centrifuge where the buckets can swing out 90°.
- Humidifier.
- Dispenser pipette (500 - 5 000 μL).
- Stage micrometer.
- PC with standard spreadsheet or SCS (scientific counting software). This is optional, because other counting devices can also be used.
- Deflation instrument / mechanical hammer (refer to **Figure 4.4**).

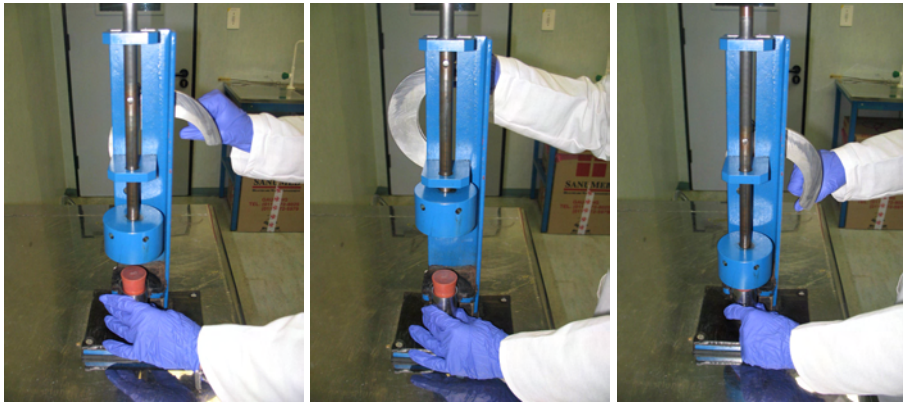


Figure 4.4: Deflation instrument (mechanical hammer), used to disrupt the gas vacuoles of the cyanobacteria, in order for buoyant cells to settle to the bottom of the counting chamber.

4.2.1.2 Glassware

- Glass tube (approximately 16.5 mm diameter) to make sedimentation chambers.
- Cover slip, No. 1 thickness.

4.2.1.3 Other materials

- Whatman lens cleaning tissue.
- 0.45 μm membrane filter (for concentrating potable water samples).

- Sample bottles (100 mL – 2 L).
- Latex gloves.
- Laboratory coat.
- Extraction cabinet.
- Safety glasses.

4.2.1.4 Reagents

- Formaldehyde solution / Lugol's acidic iodine solution (refer to Chapter 2 for the preparation of solutions).
- Ethanol (95%).
- Reagent water - water that has been filtered by reverse osmosis.

4.2.2 PROCEDURE

4.2.2.1 Sample preparation

- Samples should be fixed when sampled or once they are received in the laboratory. The ratio of formaldehyde to add to a sample is 2:100. This should be done on a down flow bench or well ventilated area. The ratio Lugol's solution to add to a sample is 1:100 (the sample should be a weak tea colour).
- Samples may be diluted if problems are experienced due to sample turbidity, algal composition or high algal biomass, by using the following dilution factors:

Table 4.2: Dilution of samples.

Dilution factor	Sample volume (mL) in a 200 mL volumetric flask
2	100
4	50
5	40
10	20

If the sample is very turbid or concentrated, first dilute twice ($2 \times$ dilution factor) and then use the made up dilution to dilute further, using **Table 4.2** above. Example: dilute sample two times and then select from Table e.g. 10 times dilution i.e. ($2 \times$ dilution factor) \times ($10 \times$ dilution factor) = $50 \times$ dilution factor. The final result (after multiplication with the conversion factor) will then have to be multiplied by 50. Ensure that the sample is properly mixed before commencing sub-sampling.

- Mark the sedimentation chamber (sample name, date and volume of sample added), which will be used for quantification.

- Gas vacuoles of cyanobacteria must be pressure deflated to ensure sedimentation. Agitate the sample to ensure uniform distribution of algal cells. Pour the sample into a marked (sample name and date) thick walled container ($\pm 10 - 50$ mL) and close container with a rubber stopper. Use a deflation instrument (like a mechanical hammer, a high pressure deflation apparatus or a rubber hammer) to apply pressure to the sample (if making use of a rubber hammer, deflate at least ten times. When Lugol's solution is used as a fixative, no deflation is needed).
- After pressure deflation, homogenise the sample with a homogenizer to ensure an even distribution of cells aggregated in loose colonies (± 20 seconds). Rinse the shaft thoroughly with reagent water to prevent contamination of other samples.
- Agitate the sample before pipetting a known volume of sample (0.5 mL - 5 mL) into a sedimentation chamber (depending on the concentration of algal cells in the water). Use separate pipette tips for every sample to prevent contamination.
- Centrifuge the sample (inside the sedimentation chamber) for 10 minutes at 3500 rpm. Ensure that the centrifuge is balanced before starting.
- Remove the sedimentation chamber carefully from the centrifuge, making sure not to disturb the sedimented phytoplankton. If immediate analysis is not possible, place the sedimented samples in a humidifier (filled with water at the bottom) to prevent evaporation.

4.2.2.2 Identification and enumeration

- Place the sedimentation chamber on the stage of an inverted light microscope (compound light microscope when using a counting device such as a haemocytometer).
- Ensure that the 40 \times (or other suitable magnification) objective is in place for analysis.
- Identify and enumerate the algal genera present on the surface of the sedimentation chamber in random fields (one field is the area within the Whipple grid). A minimum of 60 fields must be analyzed per sample. Alternatively, analysis can be stopped when at least 100 cells (of the dominant species) have been counted before 60 fields have been analyzed.
- When only part of a cell is located within the Whipple grid, then it must be counted only when more than half of the cell is within the grid and ignored if less than half of the cell is within grid.
- Every algal cell must be counted as one, whether it is part of a colony, filament or a single cell.
- Identify phytoplankton to genus and/or species level with suitable taxonomic keys. (Refer to the list of recommended keys under REFERENCES – Section 4.6).

4.2.3 SAFETY PRECAUTIONS

4.2.3.1 Hazard warning



- Formaldehyde – Flammable, irritant liquid. Toxic ☠ by inhalation, in contact with skin and if swallowed.
- Lugol's solution – for external use only. Do not swallow.
- Ethanol – flammable liquid. Keep away from sources of ignition.

4.2.3.2 Clothing

- A laboratory coat should be worn while preparing and analyzing the sample.
- Latex gloves should be worn whenever formaldehyde is handled.

4.2.3.3 Safety instructions when working with formaldehyde



- Formaldehyde should always be kept separate from other chemicals in an allocated locked cupboard.
- Personal protective equipment must be worn when working with undiluted formaldehyde i.e. gloves and protective clothing.
- Work with concentrated formaldehyde in a fume extraction cabinet (preferably a down-flow extraction cabinet, since formaldehyde is heavier than air). Ensure that the extraction fan is switched on before opening the formaldehyde container.
- Do not pipette by mouth. Use either a dispenser pipette or a pipette-boy.
- Replace bottle cap as soon as possible, and place the formaldehyde in the allocated cupboard after use.
- After completing the analysis, rinse the plastic- and glassware thoroughly under running water.
- In case of accidental contact, dilute formaldehyde immediately with plenty of water.
- In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- In case of fire, use fog-water spray (in the absence of fog equipment, a fine spray of water may be used) to control the fire.
- In case of spillage, dilute or wash away with plenty of water.

4.2.3.4 Safety instructions when working with ethanol

- Ethanol is highly flammable! Keep away from sources of ignition.
- Store in an allocated locked cupboard, away from other chemicals especially nitric acid.
- Keep the container tightly closed when not in use.
- In case of fire, water, CO₂, foam or powder may be used to extinguish the fire.

- In case of spillage, ethanol may be washed to drain with plenty of water.

4.2.4 CALCULATIONS AND EXPRESSION OF RESULTS

4.2.4.1 Calculating the algal biomass as cells/mL

The algal biomass concentration is expressed as algal cells/mL. It is derived from multiplying the actual count with a conversion factor, which includes certain variables.

- **Calculating the conversion factor:**

$\text{Conversion Factor} = \frac{(\text{Area of the sedimentation chamber floor})}{(\text{Area of a field}) \times (\text{Number of fields counted}) \times (\text{Volume sedimented})}$

<i>Note: Round the final conversion factor to 3 decimal places, but round the final algal concentration (cells/mL) to the nearest integer.</i>
--

Example:

- **Calculating the area of the sedimentation chamber floor:**

$\text{Area} = \pi r^2$

Where π = 3.14
 r = 8000 μm

$$\begin{aligned} \therefore \text{Area of the sedimentation chamber floor} &= \pi r^2 \\ &= 3.14 \times (8000 \mu\text{m})^2 \\ &= \underline{200\,960\,000 \mu\text{m}^2} \end{aligned}$$

- **Calculating the area of a field (area of the Whipple grid):**

This can only be determined by means of a stage micrometer, where the micrometer is placed on the stage of the microscope and the dimensions of the Whipple grid are measured.

<i>Note: It is of utmost importance to make sure that the same magnification used for counting, is used for the determination of the Whipple grid dimensions.</i>

For example: The area of the Whipple grid is a square with Length = 180 μm

$\begin{aligned} \therefore \text{Area of a field} &= \text{Length} \times \text{Length} \\ &= 180 \mu\text{m} \times 180 \mu\text{m} \\ &= \underline{32\,400 \mu\text{m}^2} \end{aligned}$
--

4.2.4.2 Calculating the percentage composition of a species

$$\frac{(\text{Algal biomass concentration of species in cells/mL}) \times 100}{(\text{Total biomass concentration in cells/mL})} = x\%$$

4.2.4.3 Reporting phytoplankton results

- Phytoplankton concentration is expressed as cells/mL and is rounded to the nearest integer. It is recommended that results be reported to genera level, except when the analyst is a qualified taxonomist and has the skill to identify phytoplankton to species level.
- Percentage composition may be useful to determine if dominant species are to be identified.
- Phytoplankton biomass can also be expressed as biovolumes that take the size, shape and volume of each organism into account. Refer to Section 4.5 for the details on how to calculate the biovolumes of different species.

For more information on records and data keeping, quality assurance and typical validations for the Phytoplankton identification and enumeration, the sedimentation technique using centrifugation, refer to Section 5.1.9 - 5.1.11 in Chapter 5 of the “**Comprehensive Methods Manual**”.

4.3 PHYTOPLANKTON IDENTIFICATION AND ENUMERATION, THE SEDIMENTATION TECHNIQUE USING GRAVITY (METHOD USED AND VALIDATED BY NORTH-WEST UNIVERSITY – POTCHEFSTROOM CAMPUS)

For the scope, definition of the term phytoplankton, field of application for this method, interferences with the identification and enumeration of phytoplankton, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding phytoplankton and disposal of hazardous material refer to Section 5.2.1 – 5.2.5 in Chapter 5 of the “**Comprehensive Methods Manual**”.

4.3.1 APPARATUS, MATERIALS AND REAGENTS

4.3.1.1 Instruments and equipment

- Inverted light microscope with a 40x objective and a Whipple grid in the eyepiece.
- Dispenser pipette.
- Deflation instrument.
- Humidifier.
- Computer with spreadsheet- and phytoplankton counting software. Other counting devices may also be used.
- Calibrated mass balance.

4.3.1.2 Glassware

- Perspex or glass sedimentation chambers.
- Cover slips, No. 0 thickness.
- Glass beaker.

4.3.1.3 Other materials

- Lens cleaning tissue.
- Lens cleaning liquid.

4.3.1.4 Reagents

- Formaldehyde solution.
- Lugol's iodine solution.
- Distilled water.

4.3.2 PROCEDURE

4.3.2.1 Sample preparation

- Note, that before any work is undertaken, it is imperative that the analyst is familiar with the safety precautions found in section 4.3.3 of this report.

- The sample should be preserved immediately at the site or in the laboratory when the samples are received. Lugol's iodine solution is added at a ratio of 1:100 to give the sample a weak tea colour. Formaldehyde is added to a ratio of 2:100 (Hötzel & Croome, 1999).
- After preservation, the gas vacuoles of the cyanobacteria need to be pressure deflated to allow these organisms to settle out. Deflating is done by placing a sub-sample in a thick-walled metal container to a volume where there is no air left in the container when it is closed with a rubber stopper. Apply pressure on the rubber stopper with a hammer or similar instrument. However, when Lugol's solution is used as fixative, no deflation is needed.
- The sample is then shaken to ensure the uniform distribution of cells.
- With a calibrated dispenser pipette transfer 1 mL of the sample (or sub-sample) into a sedimentation chamber labelled with the sample name and date. Leave it to settle for approximately 30 minutes on a bench free from any vibrations and disturbances. It is important to use a new pipette tip for each sample, as this will reduce the chances of cross contamination.
- Place the sedimentation chamber on the inverted light microscope and briefly examine for turbidity, as well as density and distribution of phytoplankton in the sample.
- In the event of the sample being too turbid or too dense in algal concentration it will need to be diluted. Start by diluting the known volume of the preserved (and deflated) sample to half the volume. This is done by adding one part sample to one part distilled water, giving a dilution factor of 2. Re-examine the chamber briefly for turbidity, if still too turbid or dense in algal concentration, add one part of the diluted sample to one part distilled water, giving a dilution factor of 4. Re-examine the chamber briefly for turbidity. This process is repeated until phytoplankton cells are visible enough to identify and enumerate accurately.
- In the event of the sample being too low in algal concentration, a greater volume can be settled out. This is done by estimating the volume of sample necessary to identify algal taxa without any phytoplankton cells or particles obscuring each other. This would then be the final volume of sample added to the sedimentation chamber. It should be noted that accurate estimation of this volume is gained with experience. For example: After 1 mL is added and the sample examined briefly, the analyst feels that more of the sample could be added without hampering the identification process, and an estimate of 4 mL is made. An additional 3 mL of sample is then added to the 1 mL already in the sedimentation chamber. The factor with which the counts are multiplied will then be divided by the amount of sample (mL) present in the sedimentation chamber.
- Make sure that the final volume of sample in the sedimentation tube is recorded on the sedimentation chamber.

- The sedimentation chamber is then filled to the top with distilled water and covered with a cleaned cover slip so that no air is left in the sedimentation chamber.
- Place the sedimentation chamber in a humidifier with water in the bottom section to prevent evaporation of sample water.
- The height of the sedimentation chamber will determine the time necessary for the phytoplankton to settle. For every 1 cm of the chamber, the phytoplankton should be allowed to settle for a period of 24 hours.

4.3.2.2 Identification and enumeration

- Remove the sedimentation chamber from the humidifier, taking care not to disturb the settled material at the bottom of the sedimentation chamber.
- Place it in the round slot on the microscope table and switch on the inverted light microscope.
- For identification of phytoplankton, 400× magnification is recommended.
- Identify and enumerate the settled phytoplankton to at least genus level, and where possible, to species level. Start counting on the left hand side of the sedimentation chamber on a line running through the centre of the sedimentation chamber. Identify all the phytoplankton taxa in the Whipple grid. Move one grid at a time from left to right, identifying all the phytoplankton species within the grid (refer to **Figure 4.5**). Continue counting in this manner until at least one lane is completed. Note that a minimum of 200 cells need to be identified.

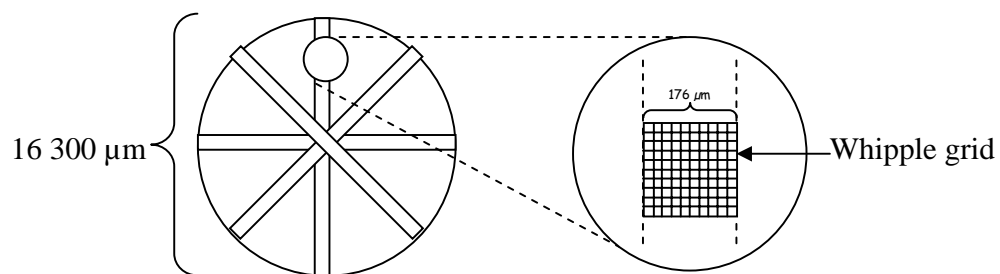


Figure 4.5: Line diagram showing the orientation of lanes and the Whipple grid.

- If the count is less than 200 cells at the end of the first lane, rotate the sedimentation chamber to a cross section that has not yet been analyzed and continue as above, this time from right to left. Continue these steps until a total greater than 200 cells is achieved. Do not stop in the middle of a lane if this value is reached, but always finish the lane, so that the exact area analyzed is known.
- Every phytoplankton cell is counted as one, whether it is part of a colony/filament or not. The amount of colonies/filament per taxon is also counted.

- If a cell is located on the edge of the Whipple grid, it is only counted if more than half of the cell is located within the Whipple grid. If not, the cell is not counted. When counting cells in a colony/filament, only those cells falling within the Whipple grid are counted.
- Record the counts on a well marked sheet with space for the sample name, date sampled, date of analysis, the amount of lanes enumerated, objective used, the conversion factor, name of the analyst and the count of each species/genus.
- Any of the literature under REFERENCES (Section 4.6) is recommended for accurate identification of phytoplankton. Some other references not listed, may also be useful.

4.3.3 SAFETY PRECAUTIONS

4.3.3.1 Hazard warning



- Formaldehyde – Flammable, irritant liquid. Toxic ☠ by inhalation, contact or ingestion.
- Lugol's solution – for external use only. Do not swallow.
- Ethanol – flammable liquid. Keep away from sources of ignition.

4.3.3.2 Clothing

- Laboratory coat.
- Latex gloves.
- Safety glasses.

4.3.3.3 Safety instructions when working with formaldehyde (Merck, 2004)



- Formaldehyde is toxic by inhalation, in contact with skin and if swallowed it could lead to serious irreversible effects. It could also cause burns, lead to sensitivity during skin contact and there is evidence suggesting carcinogenicity.
- Formaldehyde should always be stored at 15°C - 25°C in a tightly closed container in a well ventilated place.
- When handling this substance, personal protective equipment, such as latex gloves, a laboratory coat and safety glasses, should be used.
- Formaldehyde is heavier than air and should always be used in a suitable extraction cabinet, that is, one with a down flow extraction system.
- Never inhale the substance and avoid any generation of vapours of this substance. The inhalation of fresh air is best after inhalation of formaldehyde.
- After contact with the skin or the eyes, the affected area should be washed thoroughly with plenty of water. Contaminated clothing should be removed. Immediately call a physician/ophthalmologist.



- Should swallowing occur, drink plenty of water and call a physician.
- Formaldehyde vapours are combustible, as it forms explosive mixtures with air at ambient temperatures. In the case of fire, extinguish with water, CO₂, foam or powder, whilst remaining at a safe distance.
- Formaldehyde, and solutions containing formaldehyde, should always be disposed of using a proper waste disposal system.
- Also see Section 4.2.3.3.

4.3.3.4 Safety instructions when working with ethanol (Merck, 2006)

- It should be noted that this colourless liquid forms highly combustible vapours, as it mixes with air at ambient temperatures and backfiring could occur. Measures should also be taken to prevent electrostatic charging.
- Also see Section 4.2.3.4.

4.3.4 CALCULATIONS AND EXPRESSION OF RESULTS

4.3.4.1 Calculation of the phytoplankton biomass as cells/mL

Phytoplankton biomass is expressed as the amount of algal cells per millilitre (cells/mL). This value is calculated below (values used in the calculation are for example purposes only).

- Calculate the area of the circular sedimentation chamber floor:

Sedimentation chamber floor area	=	πr^2
	=	$\pi \times (8150 \mu\text{m})^2$
	=	208 672 438 μm^2

- Calculate the area of one rectangular lane:

Lane area	=	diameter of sedimentation chamber × width of Whipple grid
	=	16 300 μm × 176 μm
	=	2 868 800 μm^2

- Calculate the conversion factor

The conversion factor is calculated by dividing the total sedimentation chamber floor area by the total lane area. Note that the total lane area is the area of one lane multiplied by the amount of lanes analysed. For this example 1 lane was analyzed.

Conversion factor	=	$\frac{\text{Sedimentation chamber floor area}}{\text{Total lane area}}$
	=	$\frac{208\,672\,438 \mu\text{m}^2}{(2\,868\,800 \mu\text{m}^2 \times 1)}$
	=	72.739

At this stage it is important to remember the volume of the original sample that was sedimented as mentioned in 4.2.2.1. The conversion factor is divided by the volume (mL) of sample that was used.

Final conversion factor =	$\frac{\text{Conversion factor}}{\text{Volume of sample used}}$
=	$\frac{72.739}{3 \text{ mL}}$
=	24.246

- Calculate the biomass as cells/mL

The biomass, expressed in cells/mL, is calculated by multiplying the count of each taxon with the final conversion factor.

Biomass = Count × Final conversion factor
= 78 × 24.246
= 1891.188
≈ 1891 cells/mL (rounded to the nearest integer)

4.3.4.2 Calculating the percentage composition of a taxon

% composition = $\frac{(\text{biomass concentration of the taxon in cells/mL}) \times 100}{\text{Total biomass concentration in cells/mL}}$

4.3.4.3 Reporting phytoplankton results

- Phytoplankton concentration is expressed as cells/mL and is rounded to the nearest integer. It is recommended that results be reported to genus level, except when the analyst is a qualified taxonomist and has the skill to identify phytoplankton to species level.
- Percentage composition may be useful to determine the dominant species.
- Phytoplankton biomass can also be better expressed in terms of biovolume that takes the size, shape and volume of each organism into account. It will be shown how to calculate the biovolumes of different organisms in Section 4.5.

For more information on records and data keeping, quality assurance and typical validations for Phytoplankton identification and enumeration, the sedimentation technique using gravity, refer to Section 5.2.9 - 5.2.11 of Chapter 5 of the “**Comprehensive Methods Manual**”.

4.4 PHYTOPLANKTON IDENTIFICATION AND ENUMERATION USING THE MEMBRANE FILTRATION TECHNIQUE (METHOD USED AND VALIDATED BY UMGENI WATER)

For the scope, definition of the term phytoplankton, field of application for this method, interferences with the identification and enumeration of phytoplankton, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding phytoplankton and disposal of hazardous material refer to Section 5.3.1 – 5.3.5 in Chapter 5 of the “**Comprehensive Methods Manual**”.

4.4.1 APPARATUS, MATERIALS AND REAGENTS

4.4.1.1 Instruments and equipment

- Microscope with a mechanical stage, 10×, 40× and 100× objective lenses and preferably also with a Plan-Neofluar 63× oil immersion lens or other similar lenses (refer to **Figure 4.3**).
- Vacuum manifold fitted with membrane filter holders capable of holding 47 mm diameter or other similar membrane filters (refer to **Figure 4.6**). Vacuum pump with a vacuum gauge and adjustable vacuum connected (via a collection vessel) to the vacuum manifold.
- Homogenizer, variable speed (**Figure 4.1**).



Figure 4.6: Vacuum manifold fitted with 47 mm membrane filter holders.

4.4.1.2 Glassware

25 mL, 50 mL and 2000 mL measuring cylinders.

4.4.1.3 Other materials

0.45 µm filters of appropriate quality.

4.4.1.4 Reagents

- Lugol's solution - 20 g potassium iodide (AR) with 10 g iodine crystals (AR) in 200 mL water with 20 mL glacial acetic acid (minimum assay 98% m/m). Store in a dark glass bottle. The solution is stable for 3 years.
- Buffered formalin - 20 g sodium borate (AR) in 1 L formaldehyde (minimum assay 37% m/m AR). This solution is prepared fresh as required.

4.4.2 PROCEDURE

4.4.2.1 Sample preparation

- Samples should be filtered on the day of collection. Where necessary, bottled samples may be stored between 1 - 8°C for a maximum of three days. In special circumstances, whole samples may be preserved by adding 40 mL/L buffered formalin or 3 mL/L Lugol's solution. Dried filters may be kept in the dark at room temperature for a maximum of 20 days but only if unavoidable.
- Ensure all taps on the vacuum apparatus are turned off.
- Ensure the filter holder is clean. Squirt sufficient water onto the filter holder to wet the surface to prevent the formation of air bubbles. Place the numbered filter onto the filter holder and position the graduated filter funnel.
- Mix the sample well by inverting and shaking the sample bottle several times (See *Note 1*). Using a measuring cylinder, measure a predetermined volume of sample into the graduated filter funnel for filtering (See *Note 2*). The use of the measuring cylinder is more accurate than the use of the graduated filter funnel. The volume will depend on algal densities and also turbidity but commonly falls between 20 mL for dam water and 1200 mL for potable water. (Previous volumes used may give an indication of the volume needed). See *Note 3* for highly turbid and algal dense samples.
- The tap on the filtering apparatus is turned on and the sample allowed to filter under suction. The suction must not exceed 80kPa.
- Once the sample has nearly finished filtering through, turn off the suction at the tap and let the remainder filter through passively. Never suck the filter dry using suction as this distorts cells and breaks colonial forms.
- Remove the membrane filter and place on a clean surface or tray and leave to dry in the dark at room temperature.
- The sample number and the volume of sample filtered are entered into the relevant laboratory record book.
- The graduated filter funnels must be rinsed thoroughly between samples to avoid contamination. The funnels must be washed with detergent, cold water and a brush once a week or whenever a deposit is noticed or when extremely dense samples are filtered.

- Clean or replace the plastic filter holder grid if it becomes blocked. This will be evident by an uneven distribution of sample on the membrane filter.
- A check must be kept on the water level in the reservoir to prevent water from being drawn into the vacuum manifold. When the water level is high the vacuum must be closed and the reservoir drained.

Note 1: Sample bottles should not be completely filled as this prevents thorough mixing when the bottle is shaken.

Note 2: When Microcystis is present in samples, it is necessary to break up colonies into individual cells but without destroying the cells. To do this, homogenize approximately 100 mL sample for approximately 10 seconds using the homogenizer on speed 13 500 rpm. Thereafter continue with filtering the sample (adapted from Zohary and Pais-Madeira, 1987).

Note 3: If a very turbid sample, or a sample with an exceptionally high algal density is to be filtered, it may be necessary to dilute the sample. The sample is mixed vigorously (especially when buoyant algae are present) and the necessary volume of sample made up to at least 50 mL with distilled water using a calibrated measuring cylinder; this ensures an even distribution of sample on the filter. (Refer to Section 4.4.2.1).

4.4.2.2 Identification and enumeration

- The membrane filter must be completely dry before being viewed. This is essential if clarity is to be obtained. To test for dryness a small spot of immersion oil can be applied to the edge of the filter. If the filter becomes transparent, then it is dry. If the filter is damp, the oil area will remain opaque.
- Once dry, the filter is placed on a drop of immersion oil on a microscope slide and a second drop of oil placed gently on top of the filter. This will clear the filter enabling light to shine through.
- The slide and filter are then placed on the microscope stage.
- To ensure an even distribution of the sample, the filter is examined briefly under low magnification. The higher magnification oil immersion lens is then carefully swung into position for enumeration.
- Identify and count the algae in a number of fields which must be totally randomly selected. The easiest way of achieving this is to avoid looking down the microscope when the field is moved, or use an accepted random cell selection technique.
- SCS (standard counting software) is available commercially for the enumeration of organisms like invertebrates and phytoplankton (see Addendum A for supplier's details). The SCS has its data storage facility from which results are

exported to LIMS (Laboratory Information Management System) once all samples for the day are complete. Throughout the counting, data can be copied to an Excel worksheet on the analyst's C-drive as a temporary file. The SCS will indicate when sufficient fields have been counted to reach a pre-determined level of statistical confidence. This level may only be set by the Section Head and is recorded together with the data. In the event of a failure in the counting software, a manual count can be done using a minimum of 15 fields that would yield a count with acceptable precision.

- In order to identify the algae observed, reference could be made to any applicable phytoplankton identification book (refer to Section 4.6 for a detailed reference list).
- Turbid samples should be read just like the non-turbid samples. If no algae are visible, a comment to that effect should be captured on LIMS.

4.4.3 SAFETY PRECAUTIONS

4.4.3.1 Hazard warning



- Glacial acetic acid (and thus Lugol's solution) is dangerous and should be handled with care in a fume cupboard. Do not pipette by mouth.
- Ensure that you are familiar with the dangers and treatment associated with each of the substances mentioned above.

4.4.3.2 Clothing

- Always wear a laboratory coat.
- Wear gloves when handling water samples, if necessary.

4.4.4 CALCULATION AND EXPRESSION OF RESULTS

The actual number of algae observed is converted to numbers per milliliter.

Conversion factor (CF)	=	$\frac{\text{Area of filter}}{\text{Area of view under microscope}}$
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Algae number	=	$\frac{\text{CF} \times \text{no. of individuals counted}}{\text{No. of fields} \times \text{volume filtered (mL)}}$
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Under normal circumstances the SCS (algal counting software) performs the final calculation. The conversion factor should be checked and changed if necessary if a new microscope or different optics is used.

The results are expressed as counts per mL.

Sources of error may arise from the following:

- Poor mixing of sample before filtering.
- Incorrect identification to genus level.
- Inadequate selection of random fields.
- Incorrect optics.
- Uneven distribution of algae on membrane filters due to clogged holder.
- Damage to cells during dispersion of colonies.
- Loss of cell detail due to damage/desiccation on filter.
- Incorrect counts due to cells being clumped.
- Very high turbidity/silt obscures algae.

For more information on records and data keeping, quality assurance and typical validations for Phytoplankton identification and enumeration, the sedimentation technique using gravity, refer to Section 5.3.9 - 5.3.11 of Chapter 5 of the “**Comprehensive Methods Manual**”.

4.5 BIOVOLUME DETERMINATIONS

4.5.1 INTRODUCTION

Cell volume (biovolume) determination is one of several common methods used to estimate biomass of algae in aquatic systems. Cell numbers of algae are used frequently in aquatic surveys as indicators of algal production. However, cell numbers alone cannot represent true biomass because of considerable cell-size variation among algal species. If, for instance, a sample is taken and *Microcystis* sp. and *Euglena* sp. are present, it may be found that a cell count of *Microcystis* sp. results in a higher cell number than that of *Euglena* sp. This, however, does not mean that the smaller cells of *Microcystis* sp. contribute more to the biomass than the larger cells of *Euglena* sp.

Cell volume (μm^3) is determined by obtaining critical cell measurements or cell dimensions (for example, length, width, height, or radius) for 20 to 50 cells of each important taxon to obtain an average biovolume per cell. Cells are categorised according to the correspondence of their cellular shape to the nearest geometric solid or combinations of simple solids (for example, spheres, cones or cylinders). From cell volume, total algal biomass expressed as biovolume ($\mu\text{m}^3/\text{mL}$) is thus determined by multiplying the number of cells of a given species by its average cell volume and then summing these volumes for all taxa present in the sample.

4.5.2 METHODS FOR CALCULATING BIOVOLUME

Although time consuming, the use of a light microscope (LM) is the most preferred method when calculating biovolume. A standard calibrated scale bar, with which linear measurements of cells can be made, is mounted in the eyepiece of a microscope. Light-halos around cells may be responsible for incorrect measurements of cell dimensions. By using high magnification (400 \times - 1000 \times) these errors can be minimised to less than 1 μm . Smayda, in Hillebrand et al. (1999), suggested that 25 randomly selected, individual cells per genus/species should be measured to give a standard error (SE) less than 5%. The more random the measurements are, the smaller the SE will be. However, in some cases the SE might be <10% because of the high variability in size of individual cells of the same species.

Automated and semi-automated methods for estimating biovolume have been developed, but these technologies have many drawbacks. To use these technologies for measuring, expensive equipment needs to be obtained, and some are just as time-consuming as measuring with a microscope. Therefore time and cost should be taken into consideration when choosing a suitable technique for the determination of biovolume.

4.5.3 SHAPES AND FORMULAE

Hillebrand et al. (1999) suggested the use of 20 geometric shapes and mathematical equations for over 850 pelagic and benthic marine and freshwater microalgal genera. The equations were proposed for individual cells of colonial or chain-forming species.

The use of a combination of basic geometrical shapes may be used to construct the shape that best suits the shape of the observed cell. Variation in shape may however occur between different species of a particular genus, for example *Euglena* sp. may be in a contracted or expanded state, or different species of *Tetradron* may be in the form of different shapes (e.g. *T. minimum* is box-like, *T. mediocris* is triangular). The discretion of the analyst should be used when selecting the appropriate shape. **Table 4.3** gives a guideline of the shapes and formulas for the most common species encountered in South African freshwaters. Most of the shapes and formulas used in Table 1 were taken, and in some cases modified, from Hillebrand et al. (1999) and Sun & Liu (2003).

Table 4.3: Shapes, formulas and genus list.

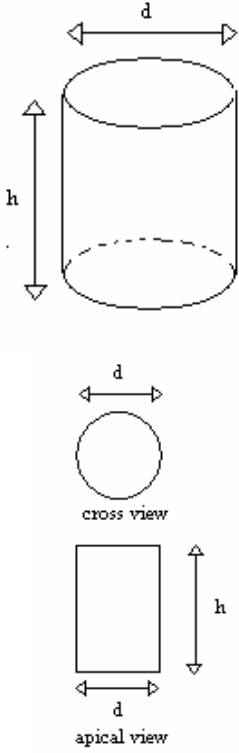
	Shape	Formula	Genus
1	 <p>Cylinder (Hillebrand et al., 1999)</p>	$V = \pi/4 \cdot d^2 \cdot h$ <p style="text-align: center;"><u>OR</u></p> $V = \pi \times \text{radius}^2 \times h$	<i>Arthrospira</i> <i>Aulacoseira</i> <i>Cyclostephanos</i> <i>Cyclotella</i> <i>Cylindrospermopsis</i> <i>Melosira</i> <i>Oscillatoria</i> <i>Spirogyra</i> <i>Spirulina</i> <i>Stephanodiscus</i>

Table 4.3 (cont): Shapes, formulas and genus list.

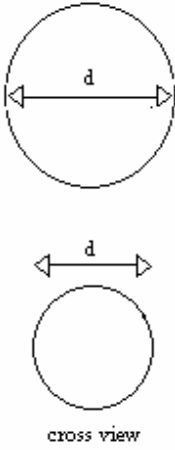
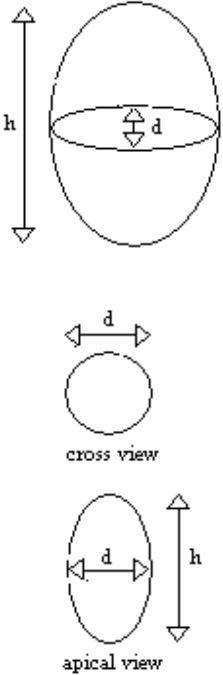
2	<p>Sphere (Hillebrand et al., 1999)</p>  <p>The diagram shows a sphere with a horizontal double-headed arrow across its center labeled 'd'. Below it is a smaller circle labeled 'cross view' with a horizontal double-headed arrow across its center also labeled 'd'.</p>	$V = \pi/6 \cdot d^3$	<p><i>Anabaena</i>^{1, 2, 14} <i>Aphanocapsa</i> <i>Carteria</i>^{1, 3, 14} <i>Chlamydomonas</i>^{3, 14} <i>Chlorella</i> <i>Chlorococcum</i>^{3, 14} <i>Coelastrum</i>^{3, 14} <i>Dictyosphaerium</i>^{3, 14} <i>Eudorina</i> <i>Golenkinia</i> <i>Micractinium</i>⁴ <i>Microcystis</i> <i>Sphaerocystis</i> <i>Tetrastrum</i>¹⁴ <i>Volvox</i></p>
3	<p>Prolate spheroid (Hillebrand et al., 1999)</p>  <p>The diagram shows a prolate spheroid with a vertical double-headed arrow on the left labeled 'h' and a horizontal double-headed arrow across its center labeled 'd'. Below it is a circle labeled 'cross view' with a horizontal double-headed arrow across its center labeled 'd'. At the bottom is an ellipse labeled 'apical view' with a horizontal double-headed arrow across its center labeled 'd' and a vertical double-headed arrow on the right labeled 'h'.</p>	$V = \pi/6 \cdot d^2 \cdot h$ <p>Sub-spherical body with circular cross-section and elliptical apical section. (Hillebrand et al., 1999).</p>	<p><i>Cryptomonas</i>^{1, 14} <i>Dinobryon</i>^{1, 13, 14} <i>Mallomonas</i> <i>Oocystis</i> <i>Pandorina</i>¹⁴ <i>Scenedesmus</i>¹⁴</p>

Table 4.3 (cont): Shapes, formulas and genus list.

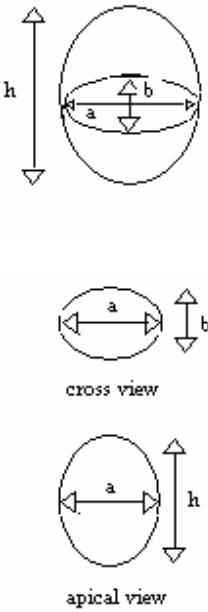
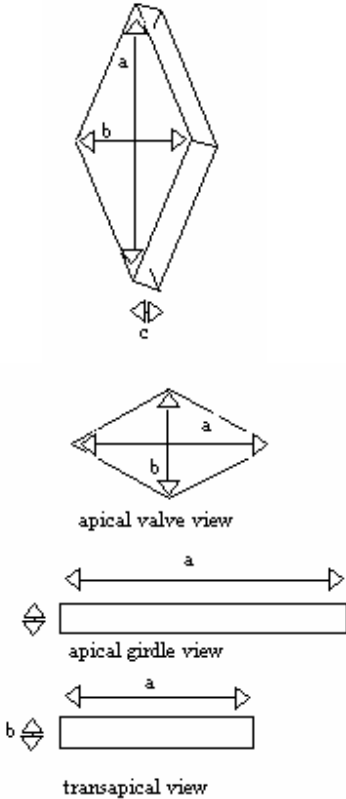
4	<p>Ellipsoid (Hillebrand et al., 1999)</p>  <p>cross view</p> <p>apical view</p>	$V = \pi/6 \cdot a \cdot b \cdot h$ <p>This body is sub-spherical with three different dimensions, i.e. prolate spheroid with elliptical cross-sections (Hillebrand et al., 1999).</p>	<p><i>Characium</i>¹⁴ <i>Lagerheimia</i> <i>Peridinium</i> <i>Strombomonas</i>¹³ <i>Trachelomonas</i>¹³</p>
5	<p>Prism on parallelogram (Hillebrand et al., 1999)</p>  <p>apical valve view</p> <p>apical girdle view</p> <p>transapical view</p>	$V = 1/2 \cdot a \cdot b \cdot c$ <p>Rhombic diatom species belong for example to the genera <i>Pleurosigma</i> and <i>Gyrosigma</i>, the basic parallelogram is even-sided (Hillebrand et al., 1999).</p>	<p><i>Gyrosigma</i> <i>Nitzschia</i>^{10, 14} <i>Pleurosigma</i></p>

Table 4.3 (cont): Shapes, formulas and genus list.

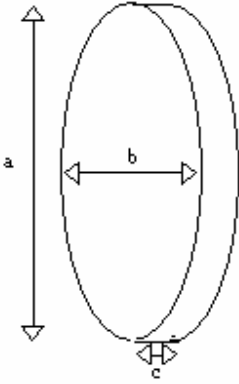
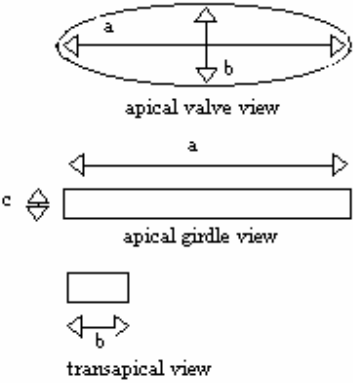
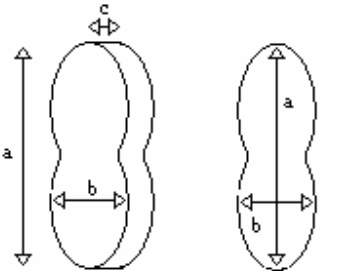
6	<p style="text-align: center;">  </p> <p style="text-align: center;">Prism on elliptic base</p> <p>Elliptic prism (Hillebrand et al., 1999)</p> <p style="text-align: center;">  </p> <p style="text-align: center;">  </p> <p style="text-align: center;">Elliptic prism with trans-apical constriction</p>	$V = \pi/4 \cdot a \cdot b \cdot c$ <p>This shape is suitable for elliptic pennate diatoms, even if they are constricted in valve view – then the mean of both the central width and maximum width is taken (Hillebrand et al., 1999).</p>	<p> <i>Achnanthes</i>⁴⁻⁷ <i>Cocconeis</i> <i>Diatoma</i>⁶⁻⁷ <i>Fragilaria</i>^{4, 6, 14} <i>Navicula</i>^{4, 6} <i>Surirella</i> <i>Pediastrum</i>^{8, 14} <i>Phacus</i>^{9, 14} </p>
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Table 4.3 (cont): Shapes, formulas and genus list.

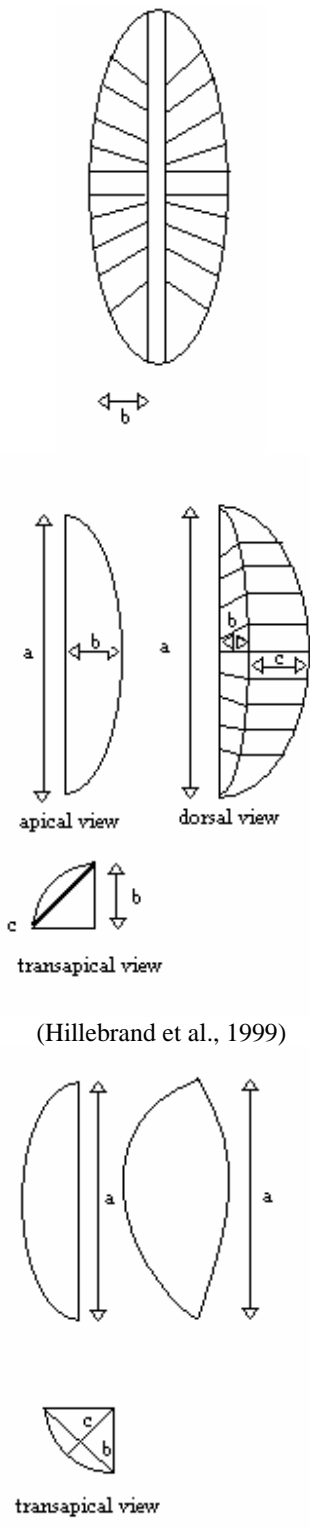
7	<p>Cymbelloid (Hillebrand et al., 1999 and Sun & Liu, 2003)</p>  <p>(Hillebrand et al., 1999)</p> <p>(Sun & Liu, 2003)</p>	$V = 4/6 \cdot \pi \cdot b^2 \cdot a \cdot \beta/360$ <p>Named after the diatom genus <i>Cymbella</i> the body has the shape of a lemon wedge. The volume is calculated as a sector of a prolate spheroid. This ellipsoid is rotating with the trans-apical axis as radius and with the apical axis as the longer elliptic diameter. C = peralvar axis on dorsal side; β = angle between the two trans-apical sides (Hillebrand et al., 1999).</p> $V = 2/3 \cdot a \cdot c^2 \cdot a \sin(b/2c)$ <p>(Sun & Liu, 2003)</p>	<p><i>Cymbella</i> <i>Rhopalodia</i></p>
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Table 4.3 (cont): Shapes, formulas and genus list.

8	<p>Gomphonemoid (Sun & Liu, 2003)</p>	$V = (a \cdot b) / 4 \cdot [a + (\pi/4 - 1) \cdot b] \cdot a \sin(c/2a)$	<p><i>Gomphonema</i></p>
9	<p>2 Half ellipsoids (Modified from Hillebrand et al., 1999)</p>	<p>Formula for one half-ellipsoid:</p> $V = \pi/12 \cdot a \cdot b \cdot h$	<p><i>Cosmarium</i></p>

Table 4.3 (cont): Shapes, formulas and genus list.

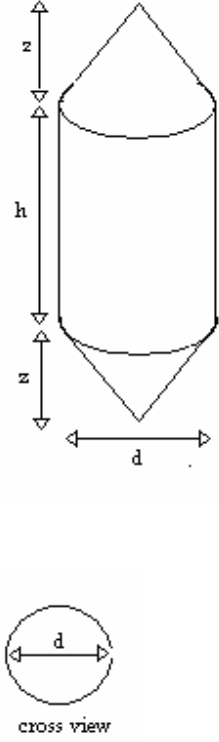
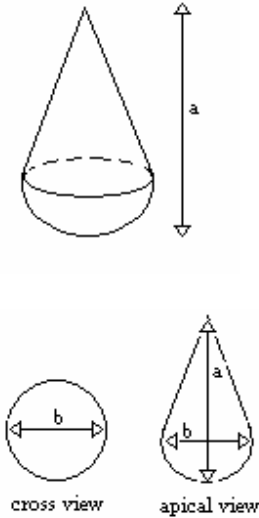
10	<p>Cylinder</p> <p>+</p> <p>3 cones (Hillebrand et al., 1999)</p>  <p>cross view</p>	$V = \pi/4 \cdot d^2 \cdot (h + z/2)$ <p>This body refers to cylindrical species, but here the cells have acute apices (Hillebrand et al., 1999).</p>	<p><i>Actinastrum</i> <i>Ankistrodesmus</i>^{11,14} <i>Chlorolobion</i></p>
11	<p>Cone</p> <p>+</p> <p>half sphere (Sun & Liu, 2003)</p>  <p>cross view apical view</p>	$V = \pi/12 \cdot d^2 \cdot (z + d)$	<p><i>Rhodomonas</i>^{1, 14}</p>

Table 4.3 (cont): Shapes, formulas and genus list.

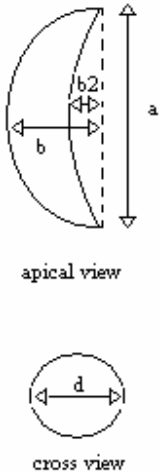
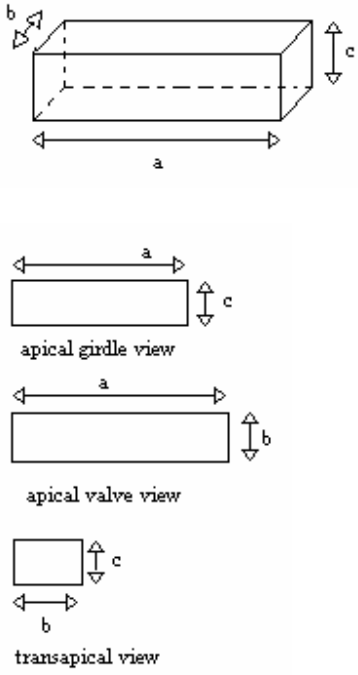
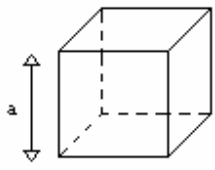
12	<p>Sickle-shaped monoraphidioid (Hillebrand et al., 1999)</p>  <p>apical view</p> <p>cross view</p>	$V = d^2/8 \cdot (2b - d + a) \cdot (\pi^2/6 + 1)$ <p>A special case is lunate bodies which are circular in cross-section. The chlorophyte genera <i>Monoraphidium</i> and <i>Kirchneriella</i> are examples. The maximum diameter of the body is given as $d (= b - b_2)$, all other abbreviations as given (Hillebrand et al., 1999).</p>	<p><i>Kirchneriella</i> <i>Monoraphidium</i>/ <i>Selenastrum</i></p>
13	<p>Box (Modified from Hillebrand et al., 1999)</p>  <p>apical girdle view</p> <p>apical valve view</p> <p>transapical view</p>	$V = a \cdot b \cdot c$	<p><i>Tetraedron</i>¹⁴</p>
14	<p>Cube (Modified from Hillebrand et al., 1999)</p> 	$V = a^3$ <p>A cube is a special case of the shape in 3.13 where $a=b=c$, then $V=a^3$ (Hillebrand et al., 1999).</p>	<p><i>Crucigenia</i>¹⁴ <i>Merismopedia</i>¹⁴</p>

Table 4.3 (cont): Shapes, formulas and genus list.


15	<p>See:</p> <p>Box 13</p> <p>+</p> <p>2 cylinders 1 (Hillebrand et al., 1999)</p>	<p>See:</p> <p>13</p> <p>+</p> <p>1</p>	<i>Asterionella</i>
16	<p>2 cones (Modified from Hillebrand et al., 1999)</p>  <p>apical view</p> <p>cross view</p>	$V = \pi/6 \cdot d^2 \cdot z$	<i>Closterium</i> ¹¹

Table 4.3 (cont): Shapes, formulas and genus list.

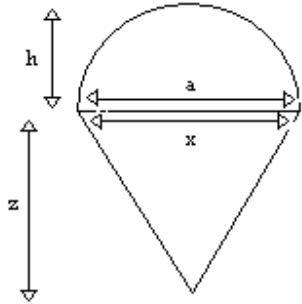
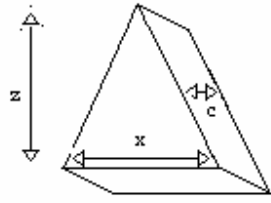
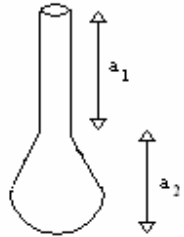
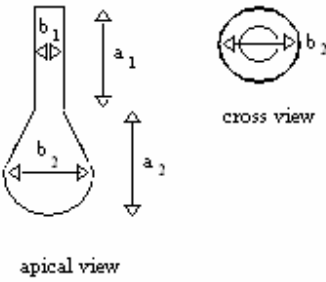
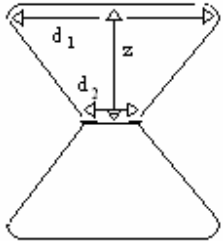
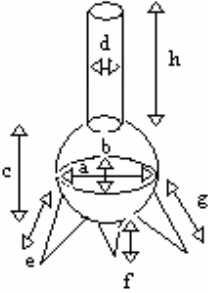
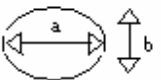
17	<p>Half ellipsoid</p> <p>+</p> <p>cone on elliptic base (Constructed from Hillebrand et al., 1999)</p>  <p>For half ellipsoid see 9</p>  <p>Cone on elliptic base</p> <p>OR</p> <p>OR</p> <p>Cone</p> <p>+</p> <p>half sphere</p> <p>+</p> <p>cylinder (Sun & Liu, 2003)</p>   <p>apical view</p> <p>CROSS VIEW</p>	<p>See:</p> <p>9</p> <p>+</p> <p>$V = \pi/12 \cdot x \cdot z \cdot c$ (Constructed from Hillebrand et al., 1999)</p> <p>OR</p> <p>See:</p> <p>11</p> <p>+</p> <p>1</p>	<p><i>Euglena</i>^{1, 9, 14}</p>
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Table 4.3 (cont): Shapes, formulas and genus list.

18	<p>Truncated cone:</p>  <p>2 truncated cones</p>	<p>Formula for one truncated cone:</p> $V = \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2)$	<p><i>Staurostrum</i>¹⁴ <i>Staurodesmus</i></p>
19	<p>Cylinder</p> <p>+</p> <p>ellipsoid</p> <p>+</p> <p>3 cones (Modified from Hillebrand et al., 1999 and Sun & Liu, 2003)</p>   <p>cross view of ellipsoid</p>	$V = \pi/4 \cdot h \cdot d^2$ <p>+</p> $\pi/6 \cdot a \cdot b \cdot c$ <p>+</p> $\pi/12 \cdot (e + f + g) \cdot b^2$	<p><i>Ceratium</i>^{1, 12}</p>

1. Cross section may be elliptic rather than round. In this case the squared diameter of the equation should be replaced by the product of smaller \times greater diameter.
2. Some cells or species are elongated and should be calculated as cylinders or prolate spheroids.
3. Some species are apically elongated. They should be calculated as prolate spheroids.
4. Species with a rhombic valve view should be calculated as prisms on a parallelogram.
5. In species which are genuflexed in girdle view, the apical axis can be calculated more precisely if the length of the two straight parts is summed.
6. Species with a linear valve view should be calculated as boxes.
7. Some species have great capitate poles, these can be added as cylinders. In this case, the apical axis means the apical length without the capitae.
8. Elliptic prism refers to the colony of *Pediastrum*, not to single cells.
9. The euglenoid algae are variable in shape and cross-section (Rott, 1981). Most *Euglena* species are not round, but flattened in cross-section. Therefore the obtuse pole is calculated as a half ellipsoid, the acute pole as cone with an elliptical base. Sicko-Goad *et al.* (1977) propose a similar shape with a cylinder instead of a cone. The smaller and wider diameters have to be measured as well as the height of the cone and the length of the obtuse pole. Some *Euglena* species are so flat that they resemble a flat elliptic prism. The genus *Phacus* is leaf-flat (Leedale, 1967), sometimes the cells are circular and can be calculated as cylinders. Note, that these elliptic prisms are based on the apical section.

10. The genus *Nitzschia* is quite variable in its shape. The sigmoid and rhombic cells can be calculated as prism on a parallelogram as described. Elliptic species are to be calculated as elliptic prisms, linear species as boxes.
11. These genera include some species which are straight and others which are bent. The latter cells should be calculated as Monoraphidioids (see number 12 in **Table 4.3**).
12. The genus *Ceratium* is quite variable in shape. The general proposal is: to calculate the central cell body as ellipsoid, to add the hypo-theical horns as cones and the apical horn as cylinder.
13. The shape is suggested for the cell inside of the lorica.
14. The shape may vary between different species. Choose a geometric shape, or a combination of shapes, that most closely resembles that of the particular cell observed.

Note: Most footnotes are modified from Hillebrand et al. (1999).

4.5.4 EXAMPLES OF BIOVOLUME CALCULATIONS

For biovolume calculations, 20 measurements of *Cyclotella meneghiniana* and *Staurastrum tetracerum* were made. Dimensions of *Cyclotella meneghiniana* were measured using a FEI Quanta 200 ESEM (scanning electron microscope). Cells of *Staurastrum tetracerum* were measured using a Zeiss light microscope and photo micrographs were taken with a Motic Moticam 2000 Camera with Motic Images Plus 2.0 ML software. The average (mean) of the linear measurement should be used to calculate biovolume and not as a mean of a set of calculated average biovolumes.

4.5.4.1 Calculating the biovolume of *Cyclotella meneghiniana*

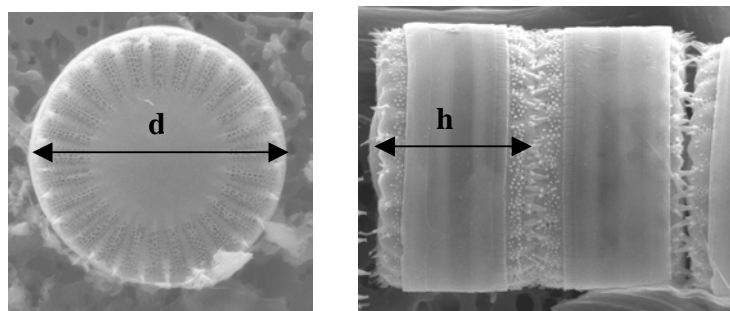


Figure 4.7: Scanning electron microscope photo demonstrating linear measurements of *Cyclotella meneghiniana*.

Table 4.4: Dimensions of *Cyclotella meneghiniana*.

Repetition	d (μm)	h (μm)
1	10.30	6.76
2	9.73	9.76
3	8.80	4.84
4	8.57	4.77
5	7.18	5.00
6	10.46	5.38
7	10.52	9.45
8	10.30	7.09
9	8.10	5.30
10	9.70	5.48
11	9.43	4.93
12	8.12	4.74
13	10.85	4.75
14	9.33	5.71
15	11.09	5.59
16	11.20	7.08
17	9.94	7.20
18	13.13	4.75
19	7.99	8.35
20	11.96	8.39
Average	9.84	6.27

The geometrical shape used for calculating the biovolume of *Cyclotella meneghiniana*: Cylinder (see **Table 4.3**).

Formula for calculating biovolume of a cylinder:

$$\begin{aligned}
 V (\mu\text{m}^3) &= \pi/4 \cdot d^2 \cdot h \\
 &= \pi/4 \cdot 9.84^2 \cdot 6.27 \\
 &= 476.81
 \end{aligned}$$

4.5.4.2 Calculating the biovolume of *Staurastrum tetracerum*:

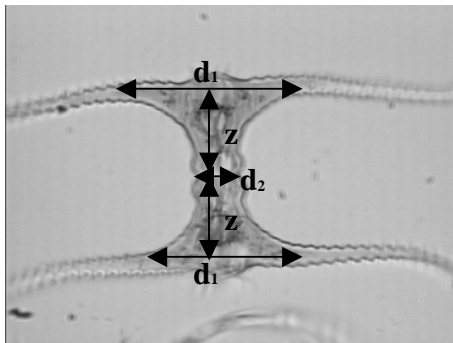


Figure 4.8: Light microscope photo demonstrating linear measurements of *Staurastrum tetracerum*.

Table 4.5: Dimensions of *Staurastrum tetracerum*:

Repetition	First truncated cone		d_2^{**}	Second truncated cone	
	z	d_1		z	d_1
1	11.3	17.5	6.9	13	19.1
2	9.4	13.7	7.1	9.9	13.9
3	11.6	15.3	7.2	9.7	12.4
4	10.6	14.2	7.7	11.3	14.9
5	9.4	13	6.6	11.4	14.6
6	10.7	13.7	8	10.4	15.5
7	10.6	13.2	7.6	11.1	8.1
8	9.4	13.8	7.3	11.3	14.7
9	9.9	14.3	9.1	10.7	15.1
10	10.3	13.7	9.3	10.3	15.3
11	11.2	14.8	6.8	10.9	15.4
12	9.1	14.3	7.2	9.8	15.1
13	10.2	13.5	7.7	10.6	15.1
14	10.7	13.9	6.8	10.9	15.4
15	9.6	13.1	7.3	10.2	13.6
16	11.1	16.6	7.9	10.6	14.1
17	10.6	14.3	8.4	10.5	13.7
18	10.5	13.9	7.8	10.5	14.2
19	9.9	13.9	7.6	10.2	14.5
20	10.6	14.2	6.7	10.3	13.4
Average	10.335	14.245	7.55	10.68	14.405

**** d_2** is the same for both truncated cones, thus it is not necessary to be measured twice.

The geometrical shape used for calculating the biovolume of *Staurastrum tetracerum*: 2 truncated cones (see **Table 4.3**).

Formula for calculating the biovolume of one truncated cone:

$$V_1 (\mu\text{m}^3) = \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2)$$

Biovolume of first truncated cone:

$$\begin{aligned}
 V_1 (\mu\text{m}^3) &= \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2) \\
 &= \pi/12 \cdot 10.335 \cdot (14.245^2 + (14.245 \cdot 7.55) + 7.55^2) \\
 &= 994.269
 \end{aligned}$$

Biovolume of second truncated cone:

$$\begin{aligned}
 V_2 (\mu\text{m}^3) &= \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2) \\
 &= \pi/12 \cdot 10.68 \cdot (14.405^2 + (14.405 \cdot 7.55) + 7.55^2) \\
 &= 1043.653
 \end{aligned}$$

Total biovolume:

$$\begin{aligned}
 V_{\text{total}} (\mu\text{m}^3) &= V_1 + V_2 \\
 &= 994.269 + 1043.653 \\
 &= 2037.922
 \end{aligned}$$

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5. GEOSMIN AND 2-METHYLISOBORNEOL (2-MIB)

5.1 INTRODUCTION

Geosmin and 2-methylisoborneol (2-MIB) are the two most important compounds responsible for the earthy/musty odour problem in drinking water in South Africa. Both these compounds may be produced by cyanobacteria and/or actinomycetes. Of the cyanobacteria, *Microcystis* sp., *Anabaena* sp., *Aphanizomenon* sp. and *Oscillatoria* sp. are known as the major contributors to the geosmin concentrations in raw and treated water, while *Oscillatoria* sp., *Pseudanabaena* sp. and *Synechococcus* sp. are known to produce 2-MIB, (Knappe et al., 2004). In most cases *Anabaena* is most commonly found to be responsible for geosmin production in South Africa.

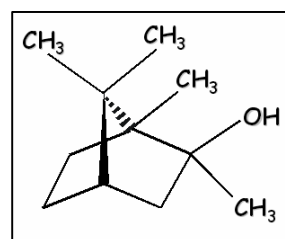
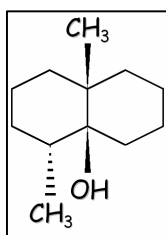


Figure 5.1: Biochemical structure of geosmin **Figure 5.2: Biochemical structure of 2-MIB**

Taste and odour substances penetrating into the final drinking water, is regarded as one of the biggest problems (if not the biggest) that the water treatment industry face currently. Where cyanobacterial toxins (especially microcystins and nodularins) are can be treated with the addition of extra free chlorine for the oxidation thereof (Acero et al., 2005; Knappe et al., 2004; Chorus & Bartram 1999), geosmin and 2-MIB are much more resistant to oxidation and, once it is released into the water, cannot be removed without the use of advanced treatment like activated carbon. The occurrence of geosmin and 2-MIB in drinking water (although not at all harmful) is aesthetically unacceptable to consumers and the one complaint that water treatment facilities come across very often. In Rand Water's case, the occurrence of taste and odour substances is one of the largest contributors to the reduction of consumer confidence in tap water.

The most common method employed to determine geosmin and 2-MIB concentrations in water samples is the gas chromatography mass spectrometry (GC/MS) method (APHA, 2001), as described in this chapter. This method, however, is technically specialized and the instruments expensive and very sensitive. The handling of the GC/MS should therefore be restricted to competent analysts with sufficient experience in gas chromatography.

Instruments manufactured by different companies have different specifications and the criteria specified in the method described in this chapter was particularly validated for the instrument used, and therefore may not necessarily be the same in other similar instruments.

5.2 THE DETERMINATION OF GEOSMIN AND 2-METHYLISOBORNEOL (2-MIB) BY PURGE AND TRAP COUPLED TO GAS CHROMATOGRAPHY-MASS SPECTROMETRY

For the scope, definition of geosmin and 2-methylisoborneol (2-MIB), field of application for this method, interferences with the method, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding geosmin and 2-MIB and disposal of hazardous material refer to Section 6.1.1 – 6.1.5 in Chapter 6 of the “**Comprehensive Methods Manual**”.

5.2.1 APPARATUS, MATERIALS AND REAGENTS

All instruments are operated in accordance with the manufacturers instructions.

5.2.1.1 Instruments and equipment

- Hewlett-Packard GC/MSD.
- Column type: Cross-linked methyl siliconed gum.
- Column used: HP-5MS (Crossed 5% ME Siloxane: 30 m × 0.25 mm × 0.25 µm film thickness), or of similar phase.
- Large volume extraction tubes.
- Recommended operating conditions for the GC and oven:

Table 5.1: Parameters for GC Operating conditions

Parameter	Value
Oven temperature Program	70°C to 160°C at 50°C/min, 180°C at 4°C/min, 200°C at 10°C hold for 6 minutes
Inlet B : Initial temperature	250°C
Detector B: Temperature	280°C
Injection B: Pressure	Electronic pressure control constant flow
Run time	11.8 minutes
Flow rate	1.20 mL/min

5.2.1.2 Glassware

- Measuring cylinder.
- 10, 50 and 100 mL calibrated volumetric flasks.
- Microsyringe.

5.2.1.3 Other materials

- Maintenance book for the GC-MSD.
- Software for MS chemstation - User guide.
- Software for MS chemstation - Handbook.
- GC: User manual.

5.2.1.4 Reagents

- Geosmin and 2-MIB standards.
- Carrier Gas: Helium gas.
- Methanol.

5.2.2 PROCEDURE

The procedure outlines the extraction and analysis of samples for the presence of geosmin (target ion 112amu and qualifier ion 125) and 2-MIB (target ion 109amu and qualifier ion 95amu).

5.2.2.1 Sample preparation

- Decant samples (and calibration standards, verification standards and method blanks) into the vials and seal the vials using a new septum each time to ensure the seal and to minimise cross-contamination.
- Load the vials into the autosampler.

5.2.2.2 Extraction and analysis

- Switch on the external heating element to heat the purge vessel to 70°C.
- Set up the analysis sequence on the Teclink PC.
- AUTOTUNE the MS as required.
- Set the split flow on the GC to 20 mL/min.
- Set up and start the sample sequence on the GC-MS PC.
- Start the analysis sequence on the Teclink PC.

5.2.2.3 Calibration procedure

- A calibration curve is generated by analyzing spiked Milli-Q water.
- Calibration curves will only be accepted if the correlation coefficients are greater than or equals to 0.950, a quadratic regression analysis may also be used.
- Prepare the calibration standards as indicated in **Table 5.2**, by spiking 1000 mL milli-Q water with 0.2 ng/μL.

Table 5.2: Preparation of calibration standards.

Standard concentration (ng/L):	Volume of working stock to be spiked in μL:
Method Blank	0
Std. 10	50
Std. 20	100
Std. 30	150
Std. 40	200

- The calibration is performed prior to each analysis.

5.2.2.4 Setting the instrument parameters

Make sure the instrument parameters are set as indicated in **Tables 5.3** and **5.4**.

Table 5.3: Instrument parameters.

Rinse water temperature	90°C
Sample cup temperature	40°C
Sample needle temperature	40°C
Transfer line temperature	100°C
Soil valve temperature	100°C
Sample sweep time	0.50 min
Needle rinse volume	7 mL
Needle sweep time	0.50 min
Bake rinse volume	7 mL
Bake sweep time	0.50 min
Bake drain time	0.50 min
Number of bake rinses	1
Valve oven temperature	200°C
Transfer line temperature	200°C
Sample mount temperature	80°C
Purge ready temperature	45°C
Dry flow standby temperature	175°C
Standby flow	2 mL/min
Pre-purge time	0.00 min
Pre-purge flow	40 mL/min
Sample heater	Off
Sample pre-heat time	0.00 min
Pre-heat temperature	40°C
Purge time	8.00 min
Purge temperature	0°C
Purge flow	40 mL/min
Dry purge time	1.00 min
Dry purge temperature	40°C
Dry purge flow	300 mL/min
GC start	Start of Desorb
Desorb pre-heat temperature	40°C
Desorb drain	On
Desorb time	3.00 min
Desorb temperature	250°C
Desorb flow	300 mL/min
Bake time	1.00 min
Bake temperature	260°C
Dry flow bake temperature	300°C
Bake flow	400 mL/min

Table 5.4: HP 5973 Parameters

Parameter	Value
GC Column	RTX 624, 30 m × 0.25 mm × 0.25 mm id × 1.4 µm
Oven temperature Program	70°C to 160°C at 50°C/min, 180°C at 4°C/min, 200°C at 10°C hold for 3 minutes
Inlet B: Temperature	250°C
Detector B: Temperature	280°C
Injection B:	Electronic pressure control, constant flow
Split flow	15 mL/min
Run time	11.8 minutes
Flow rate	1.20 mL/min

5.2.2.5 Preparation of geosmin and 2-MIB standards

The preparation of the standards solution should be done in the fumehood.

- **Stock standards**

- From the commercially available (100 µg/mL) mixed Geosmin and 2-MIB standard, prepare stock standard solution by making up 1 mL into 10 mL of methanol in a calibrated volumetric flask.
- Put a stopper on the flask and mix. Transfer the contents into an appropriate glass container and label with the date prepared, concentration (10 µg/mL), analyst signature, batch or lot number of original stock standards and expiry date.
- Store the standard solution in a refrigerator at 5°C (plus or minus 2°C).

- **Working standard**

- From the stock standard prepare a working stock by transferring 1 mL of stock into a 50 mL calibrated volumetric flask and make up to the mark with methanol.
- Put a stopper on the flask and mix. Transfer the content into an appropriate glass container and label with the date prepared, concentration (0.2 ng/µL), analyst signature, batch or lot number of original stock standards and expiry date.
- Store the solution in a refrigerator.

5.2.3 SAFETY PRECAUTIONS

5.2.3.1 Hazard warning



- Methanol (methyl alcohol) is **harmful and dangerous!!!** It may be fatal or cause blindness if swallowed. Methanol is harmful if inhaled or absorbed through the

skin. It cannot be made non-poisonous, and is flammable in liquid and vapor form. Methanol causes skin-irritations as well as irritations to the eyes and respiratory tract. It also affects the central nervous system and liver (Malinckrodt Chemicals, 2002). **HANDLE WITH CARE!**

5.2.3.2 Clothing

- Always wear a laboratory coat when performing the geosmin 2-MIB analysis.
- Wear gloves when handling methanol.
- Wear gloves when handling potential hazardous water samples.

5.2.3.3 Safety instructions when working with methanol



- Highly flammable, keep away from sources of ignition - no smoking.
- Avoid ingestion and contact with skin and eyes.
- Mark all containers very clearly toxic!
- Keep methanol container tightly closed.
- **NEVER** pipette methanol by mouth.

5.2.4 CALCULATIONS AND EXPRESSION OF RESULTS

5.2.4.1 Identification of geosmin and 2-MIB

- Identify a compound by matching both the retention times and the presence and ratio of the qualifying ion.
- Use internal standards calculation to calculate concentrations. (The software normally used for this is CHEMSTATION. It is used to run the GC-MS, integrate peaks, do data analysis and generate results from chromatographic parameters.)
- Quantification of compounds is done by means of the calibration curve generated as described in Section 5.2.2.3 of this method.

For more information on records and data keeping, quality assurance and typical validations for geosmin and 2-MIB analyses, refer to Section 6.1.9 - 6.1.11 in Chapter 6 of the “**Comprehensive Methods Manual**”.

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6. CYANOBACTERIAL TOXIN ANALYSES

6.1 INTRODUCTION

Water blooms of harmful cyanobacteria are a natural phenomenon especially in eutrophic waters. Such blooms are generally composed of only a few (often one or two) dominant genera e.g. *Microcystis*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Nostoc*, *Anabaenopsis*, *Arthrospira* and *Cylindrospermopsis* (Knappe et al., 2004; Meriluoto & Codd, 2005). Blooms of potentially toxic cyanobacteria are a common occurrence in surface supplies of drinking water in both lentic and lotic water bodies. The occurrence of cyanobacteria in raw water is important to water treatment facilities because taste and odour substances, as well as toxins, may penetrate into the final drinking water. Cyanotoxins have been shown to cause acute toxicity and lethality to animals and humans and may also cause chronic poisoning, including tumor promotion (Carmichael, 2001). Based on differences in their chemical structure and mechanism of toxicity, cyanotoxins can be classified as hepatotoxins (affecting the liver), neurotoxins (affecting the nervous system), cytotoxins, (affecting the kidney and liver) and dermatotoxins (affecting the skin), (Knappe et al., 2004).

Because all bloom-forming cyanobacteria genera are potentially toxic, any cyanobacterial bloom in the raw water should be viewed with caution. Appropriate diagnostic procedures are therefore needed; these include:

- Microscopic identification of the predominant phytoplankton taxa present (see Chapter 4).
- Laboratory analysis for the presence of toxins.
- Verification of toxic responses (clinical signs, survival times) in laboratory test animals (intraperitoneal [i.p.] and oral dosed) to verify that the clinical responses are compatible with the properties of the algal toxins detected. (The laboratory at Onderstepoort is able to do these analyses - contact Mr. Thulani Masango or Ms. Leonie Labuschagne on 012-529 9256 or 012-529 9220).

This is of special importance for the implementation of a Cyanobacterial Incident Management Framework (CIMF) as part of most potable water suppliers' water safety plans (Du Preez & Van Baalen, 2006).

Two mainstream methods are used to test for cyanotoxins, namely the ELISA (enzyme-linked immuno sorbent assay) method, and the HPLC (high performance liquid chromatography) method. Previously ELISA kits for only the screening of microcystins were available, but since the beginning of 2007, ELISA kits for the determination of Cylindrospermopsin, Saxitoxin and Anatoxin-a has also been developed. The ELISA method can be implemented easily, with relatively low initial cost equipment in any laboratory and the execution thereof is also relatively

easy and does not involve high skilled expertise like in the case of the HPLC method (both described in this chapter). However, the HPLC method is regarded as the preferred reference method and, if expertise and equipment are available, water samples can be analyzed at a fairly low cost in comparison to the ELISA method (refer to **Table 6.1**).

The decision of which of the two methods to use is solely based on the availability of apparatus. The HPLC method is preferred one, as it is the oldest and more often than not used as the reference for other methods. The ELISA method on the other hand, is quick and easy to use.

6.1.1 DECISION WHETHER TO USE THE ELISA OR HPLC METHODS FOR CYANOTOXIN ANALYSIS

Table 6.1: Characteristics of the ELISA and HPLC techniques

	ELISA Technique	HPLC Technique
Apparatus needed:	Automatic plate reader (spectrophotometric)	HPLC chromatograph and applicable column
Cost of apparatus needed:	Relatively low	High
Technical expertise required:	Moderate level	High level
Availability of kits and or standards (as at publication date in 2007):	Kits for Microcystin, Cylindrospermopsin & Saxitoxin are readily available	Microcystin is readily available, but other standards for toxins are very hard to get hold of especially outside of Europe and the USA
Analysis cost per sample:	High	Relatively low

6.2 ENZYME-LINKED IMMUNO SORBENT ASSAY (ELISA) METHOD FOR DETERMINING MICROCYSTIN CONCENTRATIONS IN RAW AND POTABLE WATER

For the scope, definition of microcystin, field of application for this method, interferences with the method, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding microcystin and disposal of hazardous material refer to Section 7.1.1 – 7.1.5 in Chapter 7 of the “**Comprehensive Methods Manual**”.

6.2.1 APPARATUS, MATERIALS AND REAGENTS

6.2.1.1 Instruments and equipment

- Air displacement pipette (or otherwise called a dispenser pipette) with disposable tips (able to measure 20 μ L – 125 μ L) as supplied by Merck or equivalent supplier.
- Microtiter plate reader as supplied by Envirologix inc. or equivalent supplier.
- Microtiter plate washer as supplied by Envirologix inc. or equivalent supplier.
- Universal Calibration Test Plate as supplied by Envirologix inc. or equivalent supplier.
- Orbital plate shaker (incubator) as supplied by Envirologix inc. or equivalent supplier.
- Timer as supplied by Merck or equivalent supplier.
- Liquid nitrogen storage container supplied by Fedgas or equivalent supplier.
- Vortex shaker as supplied by Labretoria or equivalent supplier.

6.2.1.2 Glassware

Glass syringes (± 5 mL) as supplied by Merck or equivalent supplier.

6.2.1.3 Other materials

- Universal plate kit as supplied by Envirologix inc. or equivalent supplier.
- Laboratory marking pen.
- Parafilm/masking tape as supplied by Merck or equivalent supplier.
- Pipette tips as supplied by Merck or equivalent supplier.
- Syringe filters (0.45 μ m) as supplied by Merck or equivalent supplier.
- Polypropylene tubes (± 2 mL) as supplied by Merck or equivalent supplier.
- Polypropylene bottles (500 mL – 5 L) as supplied by Merck or equivalent supplier.

6.2.1.4 Reagents (all but the reagent water and liquid nitrogen is supplied with the kit)

- Negative Control – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 0.16ppb ($\mu\text{g/L}$) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 0.6ppb ($\mu\text{g/L}$) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 2.5ppb ($\mu\text{g/L}$) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Assay diluent – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Microcystin-enzyme conjugate – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Substrate – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Stop solution – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Buffer solution – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- Reagent water – water that has been filtered by reverse osmosis.
- Liquid nitrogen – as supplied by Fedgas or equivalent supplier.

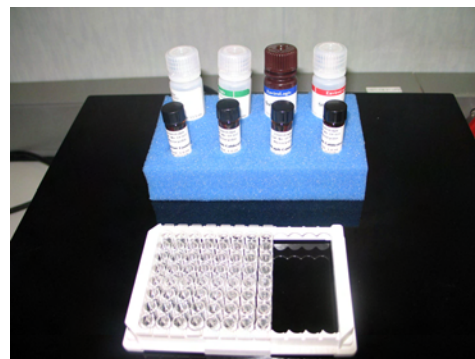


Figure 6.1 : Example of an ELISA screening test kit (e.g. Envirologix).

6.2.2 PROCEDURE

6.2.2.1 Sample preparation

- Determine the presence of total chlorine.
- Should total chlorine be present ($>0.1 \text{ mg/L}$) add sodium thiosulphate to sample in the ratio $800 \mu\text{L}$ to 1 L of sample before analysis and shake the sample to ensure uniform distribution.
- Prepare polypropylene tubes (refer to **Figure 6.2**) for every sample to be analyzed by marking them with sample name (or number), date and the type of treatment it requires. Use the table below to determine the type of treatment required:

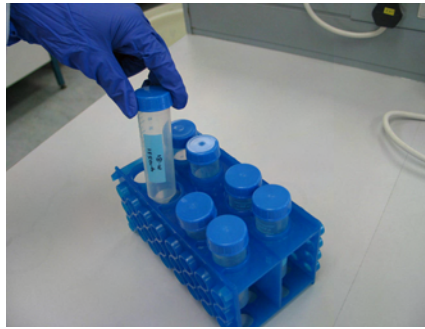


Figure 6.2 : Polypropylene tubes used for sample preparation.

Table 6.2: Treatment required for different samples.

TREATMENT		Freeze thaw (FT)	Filter (F)
RESULT REQUIRED (µg/L)	SAMPLE TYPE		
Extra-cellular Microcystin	Potable	N/A	N/A
	Source	✗	✓
Total Microcystin	Potable	✗	✗
	Source	✓	✓

N/A = Not applicable ✓ = Treatment required ✗ = Treatment not required

Note 1: When intracellular toxin concentration is requested then extra-cellular and total toxin concentrations must be determined for that sample. The difference between total and extra-cellular concentrations will be the intracellular toxin concentration.

Note 2: Total toxin concentration should be determined on all routine samples except when the customer requests otherwise. Potable water and samples that have been frozen overnight (and not filtered) are only suitable for total toxin determination.

- Agitate sample to ensure homogeneity and immediately fill the marked polypropylene tube destined for freeze thawing (approximately 1.5 mL) with the sample.
- Samples where extra-cellular microcystin concentration should be determined or where all the algal cells have been removed should/need not be agitated.
- Break up algal cells to release the microcystin by freeze thawing the sample with liquid nitrogen as follows:
 - Wear protective equipment (cryogenic gloves and face shield).
 - Gently lower the sample in the polypropylene tube into liquid nitrogen until it is frozen and then remove from nitrogen.

- Defrost sample in a water bath or other container with hot water until it has warmed to ambient temperature.

Note: Freeze thawing is not necessary if sample was stored in liquid nitrogen.

- Filter the sample as follows:
 - Use one glass syringe per sample and extract a minimum of 50 µL of sample.
 - Attach filter to syringe and dispense the sample into the marked polypropylene tube destined for the filtrate. More than one filter may be necessary per sample.
 - Close the lid of the polypropylene tube.

6.2.2.2 Microcystin toxin determination

- Allow all reagents to reach ambient temperature (18°C to 24°C) before commencing with the test (at least 30 minutes with un-boxed strips and reagents at ambient temperature – do not remove the strips from the bag with desiccant until it has reached ambient temperature).
- Calibrate the microtiter plate reader before commencing with the analysis.
- Set-up the automated washer and incubator respectively.
- Arrange all samples, reagents and pipettes so that pipetting can be performed in 10 minutes or less (as per instruction received with each kit).
- Determine how many removable strips will be used and place them on a separate frame. Reseal the unused strips and the desiccant in the plastic bag provided.
- Mark the strips with the sample names.
- One strip can accommodate four samples in duplicate. Thus, when analyzing four samples in duplicate, two strips will be needed as the negative control and three calibrators will occupy the first removable strip and the actual samples the second removable strip (refer to **Figure 6.3**).
- Complete the analysis details on a form as the test proceeds.
- Mix all the reagents on a vortex shaker for approximately ten seconds before using them for the analysis.
- Ensure the pipette is set at 125 µL and rapidly pipette 125 µL of microcystin assay diluent to each well that will be used (direction: top to bottom, from left to right).
- Replace all unused test kit components into cooler box immediately after use.
- Reset the pipette volume to 20 µL, start the timer and add 20 µL of negative control, 20 µL of each calibrator and 20 µL of each sample into their respective wells (each with their own pipette tip). This is done in duplicate (two wells below one another assigned to one sample refer to **Figure 6.3**).

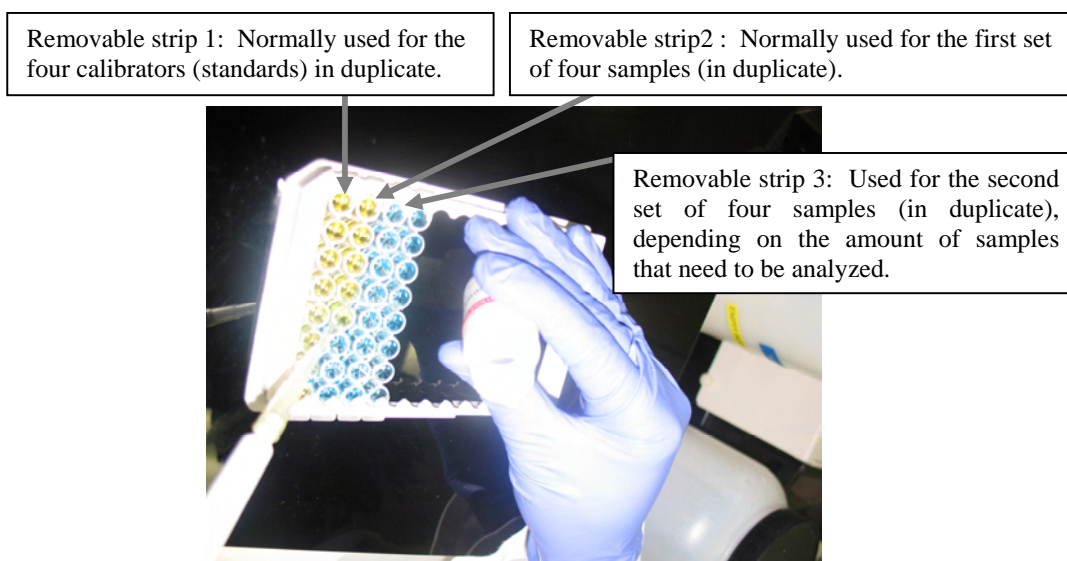


Figure 6.3 : Photograph of the Envirologix Microcystin ELISA test plate with four removable strips mounted onto the frame. The first strip (vertical on the left hand side of the frame) may be used for the four calibrators (standards) to be placed in duplicate below one another. The second strip may be used for the first four samples to be placed in duplicate below one another etc. During the analysis captured in the picture, 12 samples were analyzed - 1 strip used for the calibrators (standards) and 3 strips used for the 12 samples in duplicate. The difference in colour between the wells is due to the addition of the stop solution that causes a colour reaction from blue to yellow.

*Note: The ABRAXIS and EnviroGuard kits may not necessarily have the same number of calibrators (standards) as the Envirologix kit that is displayed in **Fig. 6.5**. However, all the different kits have a standard set of eight wells per strip and therefore the placement of calibrators and samples in the strip may vary from kit to kit.*

- Cover the wells with parafilm or tape to prevent evaporation and incubate at ambient temperature while thoroughly mixing the contents of the wells at 200 rpm for approximately 30 minutes.
- Reset the timer after incubation of approximately 30 minutes.
- Reset the pipette to 100 μL , start timer and then add 100 μL of microcystin-enzyme conjugate to each well. Cover the wells with parafilm or tape to prevent evaporation and incubate at ambient temperature (preferably between 20°C-25°C) while thoroughly mixing the contents of the wells at 200 rpm for approximately 30 minutes.
- After incubation, reset timer, remove the plate covering and then wash plate with the automated microtiter plate washer with wash solution.

- Start the timer and add 100 μ L of substrate to each well. Cover the wells with parafilm or tape to prevent evaporation and incubate at ambient temperature while thoroughly mixing the contents of the wells at 200 rpm for approximately 30 minutes.
- Add 100 μ L of stop solution to each well and mix thoroughly for approximately 30 seconds on the bench-top. This will turn the well contents yellow.
- The plate must be read with the microplate reader within 30 minutes of the addition of stop solution (as per instruction received with each kit).

6.2.3 SAFETY PRECAUTIONS

6.2.3.1 Hazard warning



- If the samples are suspected or proven to contain microcystin, the samples itself may be toxic ☠ and should not be disposed untreated via the drainage system, but be autoclaved before disposal. Microcystin is toxic by skin contact and if swallowed.
- Liquid nitrogen should be handled with the utmost care: It can spatter (possibly in the eyes) while being poured and also causes tissue damage (due to freeze burns) and is very dangerous. Contact with liquid nitrogen should be avoided at all costs.

6.2.3.2 Clothing

- Laboratory coat – as supplied by Ray Jennings or equivalent supplier.
- Latex gloves – as supplied by Merck or equivalent supplier.
- Cryogenic gloves – as supplied by Merck or equivalent supplier.
- Face shield – as supplied by Merck or equivalent supplier.

6.2.3.3 Safety instructions when working with the microcystin calibrators



- Always wear a laboratory coat and latex gloves when working with the microcystin calibrators.
- Avoid contact with the skin and do not swallow!

6.2.3.4 Safety instruction when working with liquid nitrogen

- Always wear a laboratory coat and especially cryogenic gloves and a face shield when working with liquid nitrogen.

6.2.4 CALCULATIONS AND EXPRESSION OF RESULTS

- The microplate reader is set up to read the optical density; calculate the toxin concentration, standard deviation and percentage coefficient of variance. Manual

calculation can also be done by drawing up a standard curve from the 4 calibrators and reading the absorbance of the samples from the standard curve.

- Microcystin concentration is expressed as $\mu\text{g/L}$.
- The percentage coefficient of variance of each pair of calibrators or pair of samples should not exceed 20%. To avoid a high percentage coefficient of variance, make sure all samples and calibrators are very well mixed before pipetted into each well.
- If the microcystin toxin concentration exceeds the concentration of the highest calibrator the sample may preferably be diluted with reagent water to fall in the range of the calibrators and re-analyzed or the concentration reported as $>2.5 \mu\text{g/L}$.
- If the microcystin concentration of a sample is lower than $0.18 \mu\text{g/L}$ the results should be reported as $<0.18 \mu\text{g/L}$ or when it is higher than that of the highest calibrator it should be reported as $>2.5 \mu\text{g/L}$.

For more information on records and data keeping, quality assurance and typical validations for the microcystin screening by means of the ELISA method, refer to Section 7.1.9 - 7.1.11 in Chapter 7 of the **“Comprehensive Methods Manual”**.

6.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR DETERMINING MICROCYSTIN AND NODULARIN CONCENTRATIONS IN RAW AND POTABLE WATER

For the scope, definition of microcystin and nodularin, field of application for this method, interferences with the method, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding microcystin and disposal of hazardous material refer to Section 7.2.1 – 7.2.5 in Chapter 7 of the “Comprehensive Methods Manual”.

6.3.1 APPARATUS, MATERIALS AND REAGENTS

6.3.1.1 Instruments and equipment

- Adjustable horizontal shaker: Needed only if samples contain phytoplankton.
- SPE Manifold.
- Laboratory Centrifuge: The use of an explosion-safe centrifuge is strongly advised due to the use of flammable extraction solvents.
- Ultrasonic probe.
- Ultrasonic bath.
- Heating block with temperature control and nitrogen–gas delivery unit: Block-temperature: 30°C to 50°C, gas temperature: ~20°C, and gas-purity: >99.996%.
- Filter unit: pore size <1.0 µm. Prior to use, verify that no microcystin losses occur during filtration (recovery testing).

Note: There is a possibility that various filter materials may retain microcystins.

- HPLC System:
 - Binary HPLC pump: Suitable for flow rates between 0.3 mL/min and 1.0 mL/min.
 - HPLC column oven.
 - Injection system: Injection volumes between 5 µL and 100 µL.
 - HPLC column: C18 or ODS-2, particle size 3 µm to 5 µm inner diameter 2 mm to 4.6 mm, length 250 mm. Alternative columns that ensure baseline resolution of MCYST-LR and –RR standards may be used. A suitable guard-column will assist in prolonging the column life.
 - UV / Photo Diode Array (PDA) detector: Microcystins are detected at a wavelength of 238 nm.

6.3.1.2 Glassware

- Laboratory glassware and equipment may be used. Avoid the use of plastic where possible, microcystins may adsorb to it, resulting in an under-estimation of toxin concentrations.
- Sampling bottles and glassware should all be pre-cleaned and sterile.

6.3.1.3 Other materials

- Glass micro fiber filter paper. Retention size 1 μm to 2 μm , needed only for the analysis of samples containing phytoplankton.

6.3.1.4 Reagents

Use only reagents of recognized analytical grade ensuring that no interferences or contaminants are introduced.

- Methanol, CH_3OH , HPLC grade.
- Acetonitrile, CH_3CN , HPLC grade.
- Water, H_2O , HPLC grade.
- Trifluoroacetic acid, $\text{C}_2\text{HF}_3\text{O}_2$, analytical grade.
- Standard dilution solution, SPE rinsing solvent, and re-dissolving solvent. Methanol/water [20/80 (V/V)].
- Extraction solution. Methanol/water [75/25 (V/V)].
- SPE elution solution. Methanol/water [80/20 (V/V)] containing 0.1% (V/V) TFA.
- Sodium thiosulphate solution. Dissolve 1 g of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) in 100 mL of water.
- Ammonium hydroxide solution. Dissolve 2 g of ammonium hydroxide, NH_4OH , in 100 mL water.
- Solid phase extraction cartridges (SPE) for microcystin enrichment: Reversed phase C_{18} SPE cartridges are used to extract and concentrate microcystins and nodularin. Any suitable C_{18} cartridges may be used provided that they are evaluated prior to use.

Note: The recovery strongly depends on the SPE cartridge material/brand, material specifications such as carbon load, particle size etc. The SPE cartridges used were Phenomenex STRATA-X 33 μm polymeric reversed phase, 200 mg/6 mL.

- HPLC mobile phase solution (A). Add approximately 800 mL of acetonitrile in a 1 L volumetric flask, add 500 μL of TFA and 10 mL HPLC grade water. Fill up the flask with acetonitrile to the mark. Degas and filter the solution before use. This solution is stable at room temperature for about three weeks.
- HPLC mobile phase solution (B). Add approximately 800 mL of HPLC grade water to a 1 L volumetric flask; add 500 μL of TFA and 10 mL acetonitrile. Fill

up the flask with water to the mark. Degas and filter the solution before use. This solution is stable at room temperature for about two weeks.

- **Microcystins and Nodularin:** Standards are prepared at a 10 µg/mL concentration. Pre-prepared standards may also be used. To prepare calibration curves, increasing volumes of this standard are added to 1000 mL aliquots of method blanks and extracted as samples. It is important to use water that is similar to the samples to be analyzed as extraction methodologies may vary for different water types.

Table 6.3: Preparation of calibration standards

Standard solution	Volume standard added (mL)			Concentration in 1000 mL spiked water (µg/L)		
	MCYST-RR 10.326 (µg/L)	MCYST-LR 10.107 (µg/L)	Nodularin 0.200 (µg/L)	MCYST-RR	MCYST-LR	Nodularin
1	0.050	0.050	2.0	0.517	0.506	0.400
2	0.100	0.100	4.0	1.033	1.011	0.800
3	0.150	0.150	6.0	1.549	1.516	1.200
4	0.200	0.200	8.0	2.065	2.022	1.600

6.3.2 PROCEDURE

6.3.2.1 Sampling and preservation

Collect water samples in 1 L glass bottles. For potable water samples it is necessary to add 1 mL sodium thiosulphate solution (refer to section 6.3.1.4) and store at 4°C.

6.3.2.2 Sample preparation

- **Treated water / tap water:** Extract and concentrate microcystins and nodularin in water samples using solid phase extraction (section 6.3.1.4).
- **Raw water containing phytoplankton:** Filter the sample [recommended volume: 50 mL to 100 mL, $V_{\text{Sample}}(\text{mL})$] to separate the biomass from the liquid fraction. If floating layers of algae are present, one filter may be insufficient, replace the filter as soon as it has become clogged. Extract the microcystins and nodularin in the filtrate (add 500 µL sodium thiosulphate solution per 500 mL filtrate) by solid phase extraction (section 6.3.1.4). Extract the biomass on the filter separately (section 6.3.2.3) followed by clean-up (section 6.3.2.5) of the extract prior to HPLC analysis (section 6.3.2.6).

Note: If a gravimetric filter is used, dry weight of biomass is determined and content of microcystins expressed also as µg/g as dry weight.

6.3.2.3 Extraction of microcystins from the cells on the filter

Extract the cells on the filter (if more than one filter is used combined the filters) three times with 3 mL methanol/water [75/25 (V/V)]. Sonicate the solution on ice for 2 min with an ultrasonic probe or in an ultrasonic bath. After centrifugation, pool the supernatants (record this volume, $V_{\text{supernatant}}$) and blow 1 mL of this solution to dryness under a nitrogen stream (40°C). Prior to clean-up, re-dissolve the extract in 500 μL of methanol/water [20/80 (V/V)].

6.3.2.4 SPE for microcystin extraction and enrichment

- To avoid losses, ensure that the pH of the water sample is in the range between 5.0 and 8.0 - adjust with TFA or ammonium hydroxide solution, respectively. Add 5 mL of methanol, shake well, and allow standing for 5 min. For SPE cartridge conditioning, refer to the suppliers' recommendations. If not indicated, elute 4 mL of methanol and 4 mL of water through the cartridge. Let the solvent pass at a speed of <10 mL/min through the column and make sure that a small portion of the solvent remains on top of the column until the sample is applied. Pass the sample through the conditioned cartridge not exceeding a flow rate of 10 mL/min (visible drops).
- Elute the microcystins and nodularin with 3 successive 1000 μL aliquots of methanol/water [80/20 (V/V) containing 0.1% (V/V) TFA] into an HPLC autosampler vial (allow the solvent to soak the cartridge bed for 1 minute). Evaporate the eluate to dryness with a nitrogen stream (40°C), re-dissolve in 500 μL of methanol/water [20/80 (V/V)]. Sonicate the extract for 5 min and analyze using HPLC.
- It is necessary that a method blank (unspiked water sample) be analyzed to ensure that interferences from reagents do not compromise the integrity of the results.

6.3.2.5 SPE for microcystin clean-up

- Apply the extract from Step 6.3.2.3 to the conditioned cartridge (section 6.3.2.4) reservoir. Rinse the vial with an additional 500 μL of methanol water [75/25 (V/V)] and add to the cartridge reservoir. Pass the extract through the cartridge and discard the eluate. Elute the microcystins and nodularin with 3 successive 1000 μL aliquots of methanol/water [80/20 (V/V) containing 0.1% (V/V) TFA] into a test tube (allow the solvent to soak the cartridge bed for 1 minute). Evaporate the eluate to the dryness with a nitrogen stream (40°C), re-dissolve in 500 μL of methanol/water [20/80 (V/V)]. Sonicate the purified extract for 5 min and analyze.
- If dilution of the sample is necessary, dilute 100 μL of the purified extract with 900 μL of methanol/water [20/80 (V/V)]. If clean-up with cartridges does not

reduce the co-elution, size exclusion chromatography or clean-up with immuno-affinity columns may be used as an alternative (Kondo et al., 2002).

6.3.2.6 High performance liquid chromatography (HPLC)

Resolve the microcystins by HPLC at 40°C with a reversed phase column. Adjust the flow rate and the injection volume according to the column dimensions (inner diameter, particle size) to obtain the optimal peak shape and resolution. The microcystins elute in the order of MCYST-RR first and then MCYST-LR (should be baseline resolved). Nodularin elutes between the MCYST-RR and MCYST-LR. Use a wavelength of 238 nm to detect the microcystins and Nodularin. Acquire absorption spectra between 200 nm and 300 nm to confirm the identification.

Table 6.4: HPLC mobile phase gradient

Time (Min)	HPLC mobile phase solution (A) Acetonitrile with 0.05% TFA (%)	HPLC mobile phase solution (B) Water with 0.05% TFA (%)	Total flow, depending on the column (mL/min)
2	30	70	0.3 – 1.0
8	70	30	0.3 – 1.0
8.1	95	5	0.3 – 1.0
12	95	5	0.3 – 1.0
12.01	30	70	0.3 – 1.0
15	30	70	0.3 – 1.0

6.3.3 SAFETY PRECAUTIONS

6.3.3.1 Hazard warning



- Methanol (methyl alcohol) is **harmful and dangerous!!!** It may be fatal or cause blindness if swallowed. Methanol is harmful if inhaled or absorbed through the skin. It cannot be made non-poisonous, and is flammable in liquid and vapor form. Methanol causes skin-irritations as well as irritations to the eyes and respiratory tract. It also affects the central nervous system and liver. **HANDLE WITH CARE!** (Mallinckrodt Chemicals, 2002).



- If the samples are suspected or proven to contain microcystin, the samples itself may be toxic and should not be disposed of untreated via the drainage system, but be autoclaved before disposal. Microcystin is toxic by skin contact and if swallowed.



- Acetonitrile (**methyl cyanide**) is toxic by inhalation, ingestion or skin absorption. It may cause serious damage to the eyes (Mallinckrodt Chemicals, 2002).



- Ammonium hydroxide. The concentrated solution is extremely damaging to the eyes. Even contact with dilute ammonia solution can cause serious eye damage. Toxic if swallowed, harmful if inhaled and in contact with the skin. Very

destructive to mucous membranes. Corrosive, can cause burns (Mallinckrodt Chemicals, 2002).

6.3.3.2 Clothing

- Laboratory coat – as supplied by Ray Jennings or equivalent supplier.
- Latex gloves – as supplied by Merck or equivalent supplier.

6.3.3.3 Safety instructions when working with cyanotoxin standards



- Always wear a laboratory coat and latex gloves when working with standards.
- Avoid contact with the skin and do not swallow!

6.3.7.3 Safety instructions when working with methanol



- Highly flammable, keep away from sources of ignition - no smoking.
- Avoid ingestion and contact with skin and eyes.
- Mark all containers very clearly toxic!
- Keep methanol container tightly closed.
- Never pipette methanol by mouth.

6.3.3.5 Safety instructions when working with acetonitrile



- Avoid ingestion and contact with skin and eyes.
- Always use latex glove when working with this chemical!
- Mark all containers very clearly toxic!
- Keep acetonitrile container tightly closed.
- Never pipette acetonitrile by mouth.

6.3.3.6 Safety instructions when working with ammonium hydroxide



- Avoid ingestion and contact with skin and eyes.
- Always use latex glove when working with this chemical!
- Never pipette ammonium hydroxide by mouth.

6.3.4 CALCULATIONS AND EXPRESSION OF RESULTS

6.3.4.1 Calibration curve and calculations

The spiked microcystin calibration standards (**Table 6.4**) should be used to prepare the calibration curve. The standards span the range of 0.5 µg/L to 2.0 µg/L for microcystins, and 0.4 µg/L to 1.6 µg/L for Nodularin. No recoveries need to be determined as losses occur from either the incomplete adsorption onto the cartridges during extraction or from partial desorption during the elution into the HPLC autosampler vials. These losses will be the same for samples and calibration

standards. All chromatographic calculations are carried out using automated proprietary software associated with the HPLC.

6.3.4.2 Water calculations

A further advantage of spiked calibration standards is that no calculations are required for water samples as the calibration standards and samples are of the same volume. It is however necessary to spike untreated waters to ensure that recoveries are acceptable - as their (the untreated waters) chemical matrix is quite different from that of drinking waters.

6.3.4.3 Microcystin concentration calculations

Assume that the concentration determined from the calibration curve is $y \mu\text{g}$ for the 0.5 mL extract (from 1 mL of the supernatant).

Concentration in the supernatant	=	$y \mu\text{g} \times V_{\text{supernatant}} \text{ (mL)}$
To take into account sample volume	=	$y \mu\text{g} \times V_{\text{supernatant}} / V_{\text{Sample}} \text{ (mL)}$

6.3.4.4 Expression of results

- Report results for filtered water and biomass separately. They may be summed up for samples containing for phytoplankton. Under natural conditions the majority of microcystins are included in the particulate material, and usually less than 20% is dissolved in the water.
- Microcystins **other than** MCYST-RR and MCYST-LR may be identified/recognized by their UV spectra. Their mass concentrations can be estimated using the MCYST-LR calibration curve. Report these results as MCYST-LR equivalents. Report the mass concentrations of each microcystin in terms of $\mu\text{g/L}$ to one significant figure.

Note: When purified water samples were extracted, microcystin-RR and –LR and nodularin were all desorbed from the SPE cartridge using 90/10 (V/V) methanol/water as described in ISO/CD 20179. Difficulties were experienced when raw waters were extracted, 90/10 (V/V) methanol/water only desorbed microcystins-LR and nodularin from the SPE cartridge. Similar problems have been reported by Nicholson and Burch (2001). To ensure recovery of both microcystin-RR and –LR together with nodularin raw waters desorption had to be carried out with 80/20 (V/V) methanol/water. This is consistent with the results obtained by Rapala et al. (2002) where it was shown that the best overall recoveries for the microcystins and nodularin were obtained with between 70 and 90% (V/V) methanol/water solutions. Refer to the validations regarding the recovery of cyanotoxins Section 7.2.11 in the “Comprehensive Methods Manual”).

For more information on records and data keeping, quality assurance and typical validations for the HPLC microcystin and nodularin method, refer to Section 7.2.9 - 7.2.11 in Chapter 7 of the “**Comprehensive Methods Manual**”.

6.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR DETERMINING ANATOXIN-A AND CYLINDROSPERMOPSIN CONCENTRATIONS IN RAW AND POTABLE WATER

Note: The method was validated for potable water. Raw dam water was also analyzed using this method and yielded similar recoveries to potable water. Prior to any analyses being carried out, blank water of a similar nature should be spiked to ensure that recoveries are suitable.

Liquid Chromatography is generally used to separate algal toxins and the preferred method of detection for both Anatoxin-a and Cylindrospermopsin determinations are Mass Spectrometry (Rapala & Lahti, 2002, Maizles & Budde, 2004, Mazure et al., 2003; Hawkins, 2007). Derivatisation of Anatoxin-a for subsequent analysis by either Liquid Chromatography-Fluorescence Detection (Maizles & Budde, 2004), Gas Chromatography-Mass Spectrometry (Rapala & Lahti, 2002) and Capillary Electrophoresis (Rapala & Lahti, 2002) has also been reported.

These techniques are both more selective and more sensitive than the UV detection employed in this study. Liquid Chromatography-UV detection is however not as expensive to purchase and to run and is capable of reaching the required limits of detection. Staff to operate this equipment is also far more readily available.

For the scope, definition of Anatoxin-a and Cylindrospermopsin, field of application for this method, interferences with the method, method range, principle on which the method is based, significance of the analysis, potable water quality guidelines regarding cyanotoxins and disposal of hazardous material refer to Section 7.3.1 – 7.3.5 in Chapter 7 of the “**Comprehensive Methods Manual**”.

6.4.1 APPARATUS, MATERIALS AND REAGENTS

6.4.1.1 Instruments and equipment

- SPE Manifold.
- Ultrasonic probe.
- Ultrasonic bath.
- Heating block with temperature control and nitrogen–gas delivery unit: Block-temperature: 30°C to 50°C, gas temperature: ~20°C, and gas-purity >99.996%.

- **HPLC System:**
 - Binary HPLC pump: Suitable for flow rates between 0.3 mL/min and 1.0 mL/min.
 - HPLC column oven.
 - Injection system: Injection volumes between 5 µL and 100 µL.
 - HPLC column: C18 or ODS-2, particle size 3 µm to 5 µm inner diameter 2 mm to 4.6 mm, length 250 mm. Alternative columns that ensure baseline resolution of Anatoxin-a and Cylindrospermopsin standards may be used. A suitable guard-column will assist in prolonging the column life.
 - UV / Photo Diode Array (PDA) detector: Anatoxin-a are detected at a wavelength of 227 nm and Cylindrospermopsin at 262 nm.

6.4.1.2 Glassware

- Laboratory glassware and equipment may be used. Avoid the use of plastic where possible, cyanotoxins may adsorb to it, resulting in an under-estimation of toxin concentrations.
- Amber coloured sampling bottles are needed, because anatoxin-a breaks-down rapidly in the presence of direct sunlight.
- Glassware should all be pre-cleaned and sterile.

6.4.1.3 Other materials

- Glass micro fiber filter paper. Retention size 1 µm to 2 µm, needed only for the analysis of samples containing phytoplankton.

6.4.1.4 Reagents

Use only reagents of recognized analytical grade ensuring that no interferences or contaminations are introduced.

- Methanol: CH₃OH, HPLC grade.
- Acetonitrile: CH₃CN, HPLC grade.
- Water: H₂O, HPLC grade.
- Trifluoroacetic acid: C₂HF₃O₂, analytical grade.
- Re-dissolving solvent: Methanol/water [50/50 (V/V)].
- Sodium thiosulphate solution: Dissolve 1 g of sodium thiosulphate, Na₂S₂O₃, in 100 mL of water.
- SPE elution solution: Methanol containing 0.1% (V/V) TFA.
- Acetic acid: CH₃COOH, HPLC grade. 1% in HPLC grade H₂O (V/V).
- Ammonium hydroxide solution: Dissolve 2 g of ammonium hydroxide, NH₄OH, in 100 mL water.
- **Solid phase extraction cartridges (SPE) for anatoxin-a and cylindrospermopsin enrichment:** Reversed phase C₁₈ SPE cartridges are used to

extract and concentrate Anatoxin-a and Cylindrospermopsin. Any suitable C₁₈ cartridge may be used provided that they are evaluated prior to their use.

Note: The recovery strongly depends on the SPE cartridge material/brand, material specifications such as carbon load, particle size etc. The SPE cartridges used were Phenomenex STRATA-X 33 µm polymeric reversed phase, 200 mg/6 mL.

- **HPLC mobile phase solution (A)**

Put about 800 mL of acetonitrile in a 1 L volumetric flask, add 500 µL of TFA and 10 mL HPLC grade water. Fill up the flask with acetonitrile to the mark. Degas and filter the solution before use. This solution is stable at room temperature for about three weeks.

- **HPLC mobile phase solution (B)**

Put about 800 mL of HPLC grade water in a 1 L volumetric flask, add 500 µL of TFA and 10 mL acetonitrile. Fill up the flask with water to the mark. Degas and filter the solution before use. This solution is stable at room temperature for about two weeks.

- **Anatoxin-a and Cylindrospermopsin standards**

Standards are prepared at approximately 20 µg/mL concentration. Pre-prepared standards may also be used. To prepare calibration curves increasing volumes of standards are added to 1000 mL aliquots of method blanks and extracted as samples. It is important to use water that is similar to the samples to be analyzed as extraction methodologies vary for different water types.

Note: The Anatoxin-a standard was purchased as 1 mg of Anatoxin-a fumarate salt, the actual mass of Anatoxin-a must be calculated when preparing the standard. Water and methanol respectively were used as diluents for the Anatoxin-a and Cylindrospermopsin.

Table 6.5: Preparation of calibration standards

Standard solution	Volume standard added (µL)		Concentration in 1000 mL spiked water (µg/L)	
	Anatoxin-a 23.4 (µg/mL)	Cylindrospermopsin 20 (µg/mL)	Anatoxin-a	Cylindrospermopsin
1	25	50	0.590	1.00
2	50	100	1.17	2.00
3	75	150	1.76	3.00
4	100	200	2.34	4.00

6.4.2 PROCEDURE

6.4.2.1 Sampling and preservation

Collect water samples in 1 L amber coloured glass bottles, add 1000 µL sodium thiosulphate solution (section 6.4.1.4) and store at 4°C.

6.4.2.2 SPE for Anatoxin-a and Cylindrospermopsin extraction and enrichment

- To avoid losses, ensure that the pH of the water sample is in the range from 6,0 to 8,0 – adjust with 1% acetic acid (V/V) or ammonium hydroxide solution, respectively. Add 10 mL of methanol shake well and allow standing for 5 min. For SPE cartridge conditioning, refer to the suppliers' recommendation. If not indicated, elute 4 mL of methanol and 4 mL of water through the cartridge. Let the conditioning solvents pass at a speed of <10 mL/min through the column and make sure that a small portion of the solvent remains on top of the column until the sample is applied. Pass the sample through the conditioned cartridge not exceeding a flow rate of 10 mL/min (visible drops).
- Elute the Anatoxin-a and Cylindrospermopsin with 3 successive (allow the solvent to soak the cartridge bed for 1 minute) 1000 µL aliquots of methanol into test tubes. Evaporate the eluate to the dryness with a nitrogen stream (40°C), re-dissolve in 500 µL of methanol/water [50/50 (V/V)]. Sonicate the extract for 5 min and analyze on the HPLC (8).
- It is necessary that a method blank (an unspiked water) be analyzed to ensure that interferences from reagents don't compromise the integrity of the results.

6.4.2.3 High performance liquid chromatography (HPLC)

Separate the Anatoxin-a and Cylindrospermopsin by HPLC at 40°C with a reversed phase column. Adjust the flow rate and the injection volume according to the column dimensions (inner diameter, particle size) to obtain the optimal peak shape and resolution. Use a wavelength of 227 and 262 nm to detect the Anatoxin-a and Cylindrospermopsin. Acquire absorption spectra between 200 and 300 nm to confirm the identification.

Table 6.6: HPLC mobile phase gradient

Time (Min)	HPLC mobile phase solution (A) Acetonitrile with 0.05% TFA (%)	HPLC mobile phase solution (B) Water with 0.05% TFA (%)	Total flow, depending on the column (mL/min)
0	95	5	0.3 – 1.0
10	75	25	0.3 – 1.0
14	0	100	0.3 – 1.0
18	0	100	0.3 – 1.0
20	95	5	0.3 – 1.0

6.4.3 SAFETY PRECAUTIONS

6.4.3.1 Hazard warning



- Methanol (methyl alcohol) is **harmful and dangerous**!!! It may be fatal or cause blindness if swallowed. Methanol is harmful if inhaled or absorbed through the skin. It cannot be made non-poisonous, and is flammable in liquid and vapor form. Methanol causes skin-irritations as well as irritations to the eyes and respiratory tract. It also affects the central nervous system and liver. **HANDLE WITH CARE!** (Mallinckrodt Chemicals, 2002).



- If the samples are suspected or proven to contain anatoxin-a or cylindrospermopsin, the samples itself may be toxic and should not be disposed untreated via the drainage system, but be autoclaved before disposal.



- Acetonitrile (**also called methyl cyanide**) is toxic by inhalation, ingestion or skin absorption. It is an irritant that may cause serious damage to the eyes (Mallinckrodt Chemicals, 2002).



- Ammonium hydroxide. The concentrated solution is extremely damaging to the eyes. Even contact with dilute ammonia solution can cause serious eye damage. Toxic if swallowed, harmful if inhaled and in contact with the skin. Very destructive to mucous membranes. Corrosive, can cause burns (Mallinckrodt Chemicals, 2002).

6.4.3.2 Clothing

- Laboratory coat – as supplied by Ray Jennings or equivalent supplier.
- Latex gloves – as supplied by Merck or equivalent supplier.

6.4.3.3 Safety instructions when working with cyanotoxin standards



- Always wear a laboratory coat and latex gloves when working with the cyanotoxin standards.
- Avoid contact with the skin and do not swallow!

6.4.3.4 Safety instructions when working with methanol



- Highly flammable, keep away from sources of ignition – no smoking.
- Avoid ingestion and contact with skin and eyes.
- Mark all containers very clearly toxic!
- Keep methanol container tightly closed.
- Never pipette methanol by mouth.

6.4.3.5 Safety instruction when working with acetonitrile

- Avoid ingestion and contact with skin and eyes.



- Always use latex glove when working with this chemical!
- Mark all containers very clearly toxic!
- Keep acetonitrile container tightly closed.
- Never pipette acetonitrile by mouth.

6.4.3.6 Safety instructions when working with ammonium hydroxide



- Avoid ingestion and contact with skin and eyes.
- Always use latex glove when working with this chemical!
- Never pipette ammonium hydroxide by mouth.

6.4.4 CALCULATIONS AND EXPRESSION OF RESULTS

6.4.4.1 Calibration curve and calculations

The spiked Anatoxin-a and Cylindrospermopsin calibration standards (**Table 6.6**) are used to prepare the calibration curve. These standards cover the range of 0.59 $\mu\text{g/L}$ to 2.34 $\mu\text{g/L}$ for the Anatoxin-a and 1.0 $\mu\text{g/L}$ to 4.0 $\mu\text{g/L}$ for Cylindrospermopsin. No recoveries need to be determined as losses from either the incomplete adsorption onto the cartridges during extraction or only partial desorption will be the same for samples and calibration standards.

6.4.4.2 Water calculations

A further advantage of spiked calibration standards is that no calculations are required for water samples as the calibration standards and samples are of the same volume. It is however necessary to spike untreated waters to ensure that recoveries are acceptable as their matrix is quite different from that of drinking waters.

For more information on records and data keeping, quality assurance and typical validations for the HPLC Anatoxin-a and Cylindrospermopsin method, refer to Section 7.3.9 - 7.3.11 in Chapter 7 of the “Comprehensive Methods Manual”.

6.5 REFERENCES

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ADDENDUM A

LIST OF SUPPLIERS FOR CHEMICALS AND INSTRUMENTS USED IN THE METHODS DESCRIBED IN THE MANUAL

Apparatus and materials	Supplier (Vendor or equivalent supplier)	Contact details (Telephone number / website)
Beckman DU-650 spectrophotometer	Beckman Coulter	011 805-2014 beckman@intekom.co.za
Bottle top dispenser	Merck	011 345-9000 www.merck.co.za
Bulb pipettes – 4 mL A-grade	Glass World	011 474-6580
Centrifuge – Eppendorf	Merck	011 345-9000 www.merck.co.za
Centrifuge – Heraeus Multifuge 3 s-r	Stargate	011 674-2440
Cover slip (round glass Ø 22 mm thickness: 1)	Merck	011 345-9000 www.merck.co.za
Cryogenic gloves	Merck	011 345-9000 www.merck.co.za
Deflation instrument	University of the North- West Instrument makers	018 299-2200/1 www.puk.co.za
Humidifier	Merck	011 345-9000 www.merck.co.za
Ethanol (95%)	Merck	011 345-9000 www.merck.co.za
Face shield	Merck	011 345-9000 www.merck.co.za
Formaldehyde solution	Merck	011 345-9000 www.merck.co.za
GC and HPLC consumables	Stargate Scientific	011 674-2440 www.stargatescientific.co.za
Glass syringes (\pm 5 – 50 mL)	Merck	011 345-9000 www.merck.co.za
Glass tube (\pm 16.5 mm)	Merck	011 345-9000 www.merck.co.za
Graduated pipette – 10 mL A-grade	Glass World	011 474-6580
Homogenizer	Eureka	016 421-3335
Hydrochloric acid	Merck	011 345-9000 www.merck.co.za
HPLC and GC consumables	Stargate Scientific	011 674-2440 www.stargatescientific.co.za
Inverted light microscope	Zeiss	011 886-9510
Life jacket	Vaalgas	016 422-3581
Liquid nitrogen	Air Liquide	011 389-7000

Apparatus and materials	Supplier (Vendor or equivalent supplier)	Contact details (Telephone number / website)
Liquid nitrogen storage container	Fedgas	011 389-7181
Marking pen	Pen on Paper	016 422-2505
Measuring cylinders – 100 mL, 250 mL, 500 mL, 1000 mL	Labchem	011 452-1116
Memmert oven	Labotec	011 315-5434
Methanol	Merck	011 345-9000 www.merck.co.za
Mettler AE 240 balance	Micron Lab. Services	013 690-1532
Micro plate reader	Stargate	011 674-2440
Micro plate washer	Stargate	011 674-2440
Micrometer	Zeiss	011 886-9510
Millipore filtering apparatus and vacuum gauge	Millipore	011 444-2280
Orbital plate shaker (incubator)	Stargate	011 674-2440
Parafilm/masking tape	Merck	011 345-9000 www.merck.co.za
Computers	TAGG computers	011 907-1147
Pipette dispenser – Socorex micropipette	Labotec	011 315-5434
Pipette dispenser – Tecnomara pipetboy	Labotec	011 315-5434
Pipette dispenser (20 – 125 mg/L) with disposable tips	Merck	011 345-9000 www.merck.co.za
Pipette dispenser (500 – 5000 mL) with tips	Merck	011 345-9000 www.merck.co.za
Pipette tips	Merck	011 345-9000 www.merck.co.za
Polypropylene bottles (500 mL – 5 L)	Merck	011 345-9000 www.merck.co.za
Polypropylene tubes (± 2 – 50 mL)	Merck	011 345-9000 www.merck.co.za
Printer	Datacentrix	011 461-2034
Refrigerator	Eureka	016 421-3335
Safety glasses	Merck	011 345-9000 www.merck.co.za
Sample bottles (100 mL– 2 L)	Labchem	011 452-1116
Screw-capped test tubes	Labotec	011 315-5434
Scientific Counting Software (SCS)	Rezolve Information Management Solutions	(011) 678-2518 www.rezolve.co.za
Standard ELISA screening test kit for microcystins	Stargate Scientific	011 674-2440 www.stargatescientific.co.za
Syringe filters (0.45 μ m)	Merck	011 345-9000 www.merck.co.za

Apparatus and materials	Supplier (Vendor or equivalent supplier)	Contact details (Telephone number / website)
Test tubes – rimless, medium wall (100 mm x 14 mm)	Glassblowing Industries	011 493-6656
Thermometer or thermostat	Glass World	011 474-6580
Timer	Merck	011 345-9000 www.merck.co.za
Trace-Klean	Beckman Coulter	011 805-2014 beckman@intekom.co.za
Universal plate kit	Stargate Scientific	011 674-2440 www.stargatescientific.co.za
Vacuum pump	AFROX	011 490-0400
Volumetric flask – 1 L A-grade	Glass World	011 474-6580
Vortex shaker	Labretoria	012 460-6943
Water bath	Labotec	011 315-5434
Whatman glass fibre filters (GF/C) – 47 mm diameter	Merck	011 345-9000 www.merck.co.za
Whatman lens cleaning tissue	Wirsam	011 482-1060
Whatman membrane filter (0.45 µm)	Merck	011 345-9000 www.merck.co.za

ADDENDUM B

LIST OF SOUTH AFRICAN LABORATORIES ABLE AND/OR CERTIFIED TO PERFORM THE ANALYSES MENTIONED IN THE MANUAL

	Chl-665	Chl-a	Phyto id & enum	Geosmin/ 2-MIB	Microcystin	Nodularin	Anatoxin-a	Cylindrospermopsin
Amatola Water (East London)	✓							
Biocrop (Krugersdorp)					✓	✓	✓	✓
Buckman Laboratory (Johannesburg)	✓	✓						
Cape Metropolitan Council (Cape Town)		✓						
DWAF RQS (Roodeplaat Dam)		✓	✓		✓			
Jhb Water (Johannesburg)	✓	✓		✓				
Magalies Water (Pretoria)		✓						
Mhlathuze Water (Richards Bay)	✓	✓						
MidVaal Water Company (Klerksdorp)	✓	✓						
Municipality of East London		✓						
NamWater (Windhoek)	✓	✓	✓	✓				
Nelson Mandela Metropolitan University (Port Elizabeth)					✓	✓	✓	✓
North-West University (Potchefstroom)		✓	✓					
Rand Water (Vereeniging)	✓	✓	✓	✓	✓			✓
Sedibeng Water (Bothaville)		✓						
Umgeni Water (Pietermaritzburg)		✓	✓	✓	✓			
University of Pretoria (Pretoria)					✓			
University of Jhb (Johannesburg)				✓				