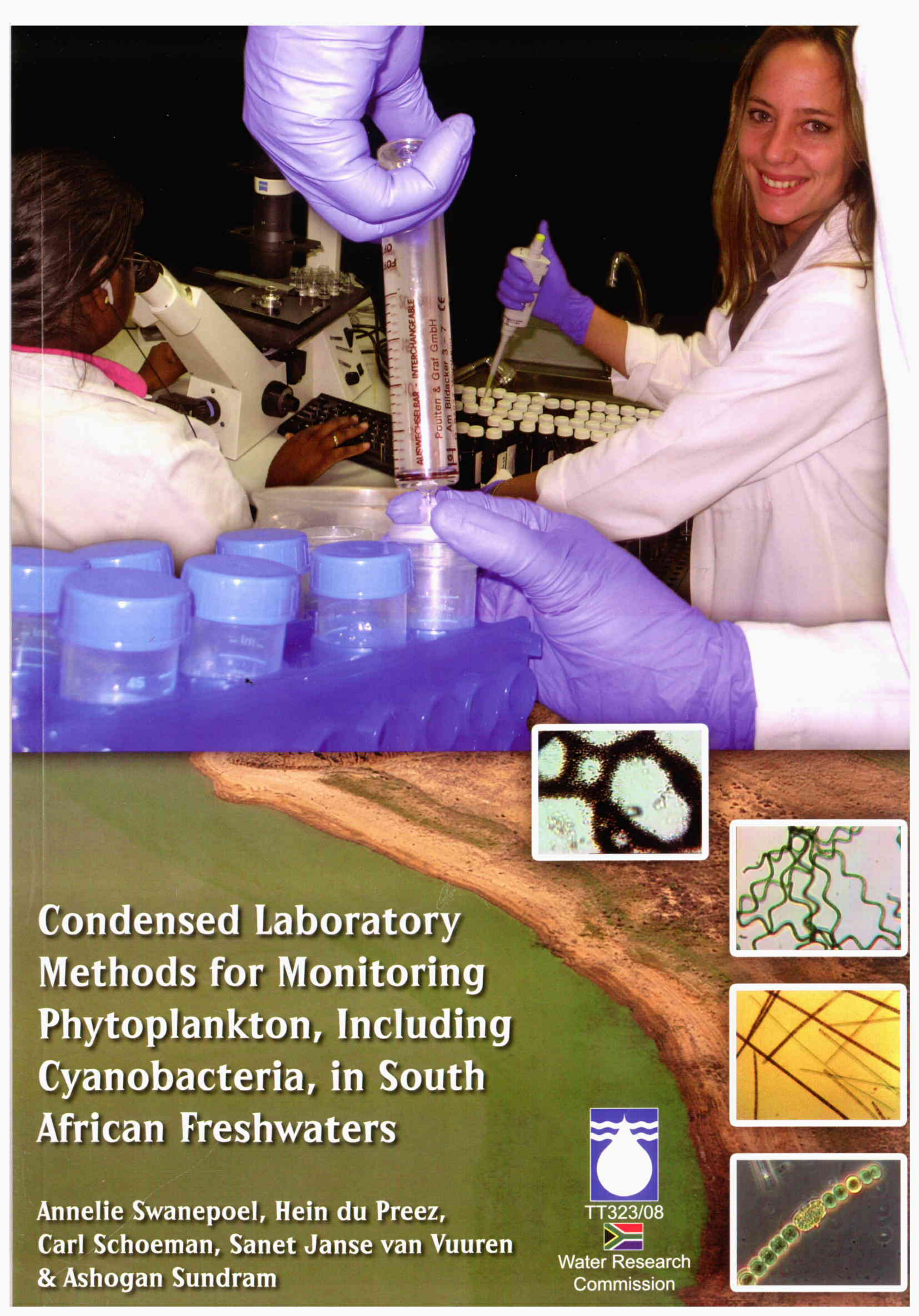
Condensed Laboratory Methods for Monitoring Phytoplankton, Including Cyanobacteria, in South African Freshwaters
Annelie Swanepoel, Hein du Preez,
Carl Schoeman, Sanet Janse van Vuuren
& Ashogan Sundram
TT323/08
Water Research
Commission

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

Annelie Swanepoel*, Hein du Preez*, Carl Schoeman*,
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Report to the Water Research Commission
by
Rand Water*
In association with
The North-West University** and
Umgeni Water***

WRC Report No TT 323/08
February 2008

Obtainable from:

Water Research Commission
Private Bag X03
Gezina
0031

The publication of this report emanates from a project entitled: *Laboratory methods for the monitoring of phytoplankton, including Cyanobacteria, in South African freshwaters*
(WRC Project No. K5/1533)

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

This report has been reviewed by the Water Research Commission (WRC) and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

ISBN 978-1-77005-684-8
Printed in the Republic of South Africa

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.

ACKNOWLEDGEMENTS

The authors wish to thank the following members of the Steering Committee for their valuable inputs to this project:

Ms. A Moolman (chairman)	Water Research Commission
Mss. Z Franken & A Schoeman (secretaries)	Rand Water
Dr. S du Plessis	North-West University
Ms. C van Ginkel	DWAF
Mr. P Grobler	LNIN
Dr. T Downing	Nelson Mandela Metropolitan University
Dr. WR Harding	DH Environmental Consulting
Mr. J Parsons	Rand Water
Ms. M Kruger	MidVaal Water Co
Dr. P Kempster	Department of Water Affairs and Forestry

The financial support received from the Water Research Commission and Rand Water to conduct the project is gratefully acknowledged.

Thank you to Mr. Hennie Slabbert for the valuable support with the use of the Rand Water SAP system.

Special thanks to Dr. WR Harding and his staff at DH Environmental Consulting, South Africa for their valued contribution towards editing the document.

Thank you to GP Kriel, Germarié van Zyl and Nicolene van der Walt for the validations of certain methods and their contributions towards the biovolume determination section in the manual.

A very special thanks to the current and former staff of Hydrobiology and Organic Chemistry, at Rand Water Vereeniging, especially Leoni van Baalen, Zelna Franken, Rita Guglielmi, Annelie Schoeman, Lindani Mkhize, Sibusiso Mdunge, Ishana Dusrath, Ashvita Ramcharan, Zinhle Ngwenya and Elmari de Kock for their contributions towards the project.

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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.

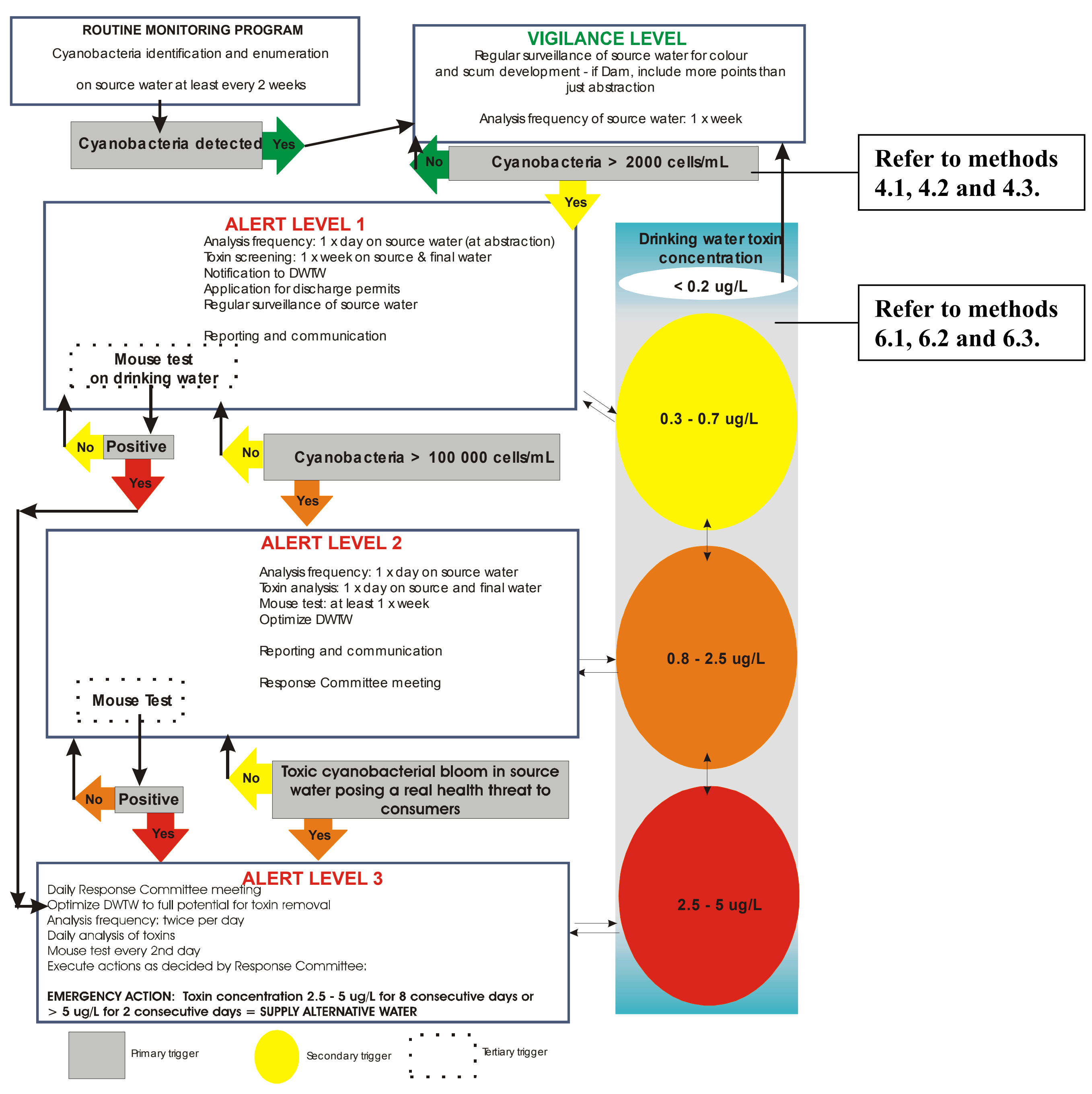

Figure 1.1 **Cyanobacteria Incident Management Framework (CIMF) using cyanobacteria concentration as a primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).**

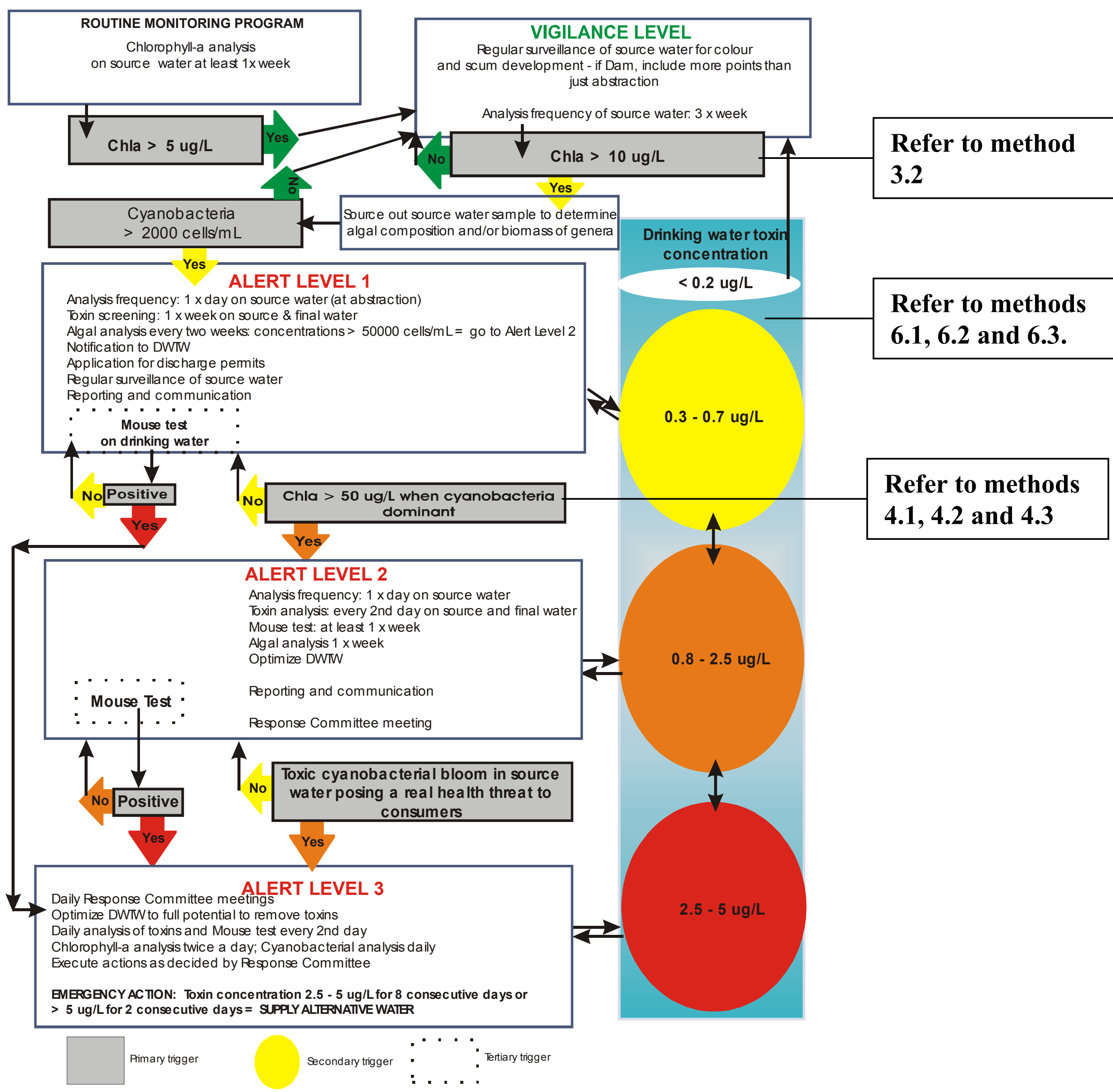

Figure 1.2 Cyanobacteria Incident Management Framework (CIMF) using chlorophyll-*a* as a primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).

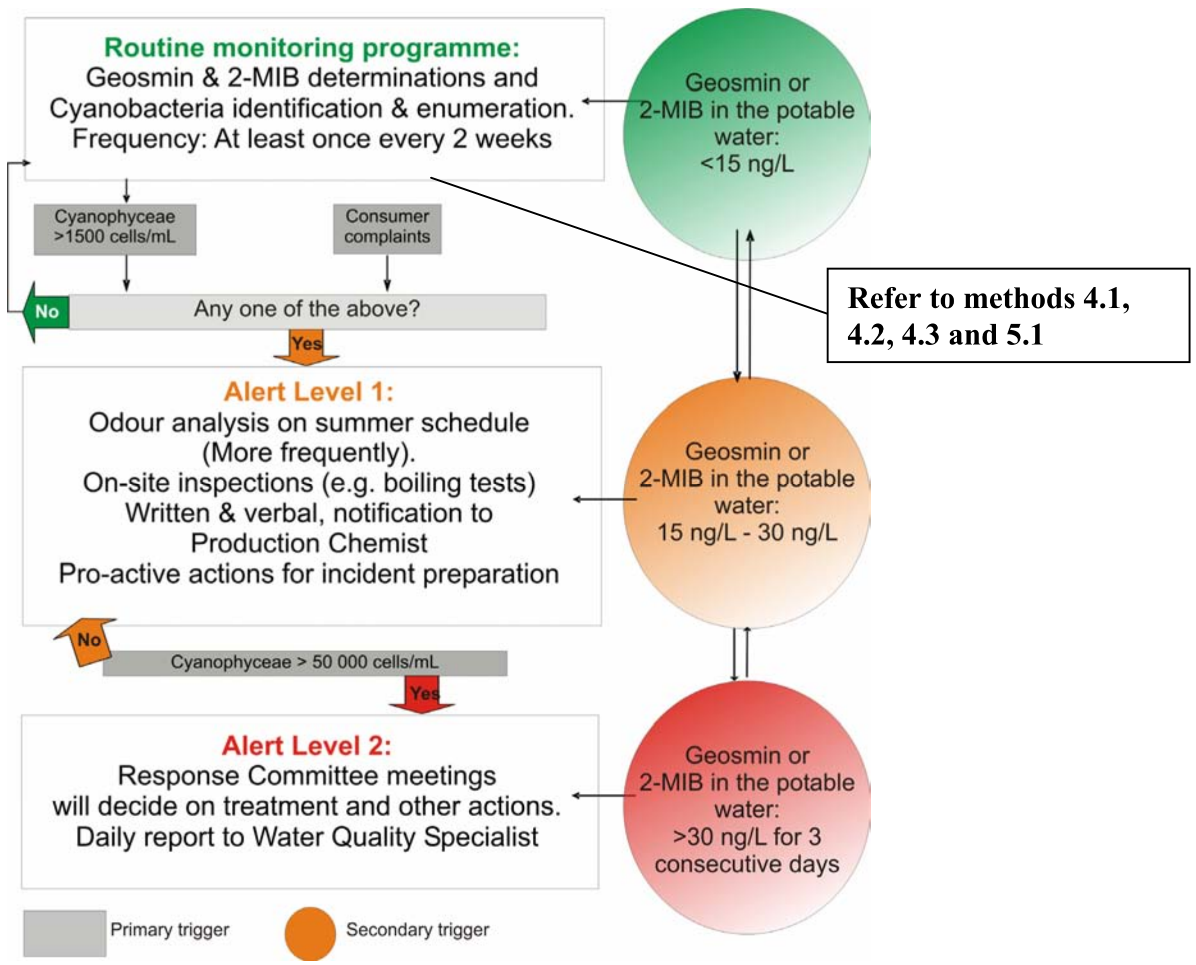

Figure 1.3 Cyanobacteria Incident Management Framework (CIMF) for the reaction to taste and odorous substances (as used by Rand Water).

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.

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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 is it 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).

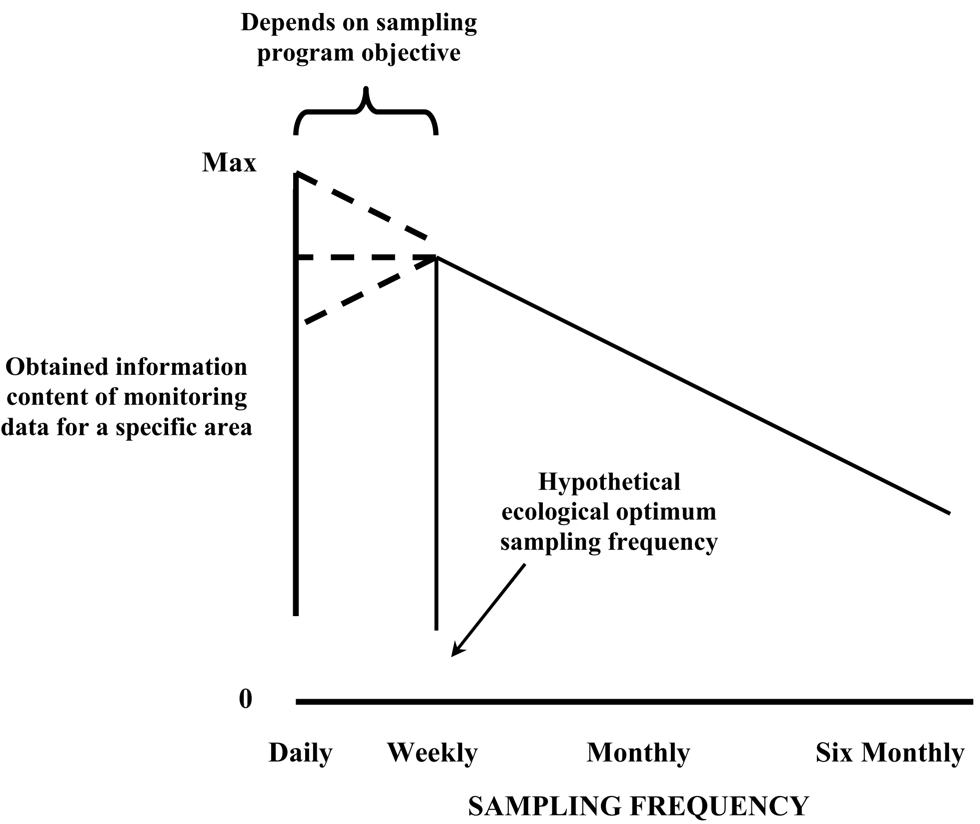

Figure 2.1 : The relationship between the obtained information content of the monitoring data (hypothetical) and the sampling frequency (adapted from DWAF, 2002.).

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 (Olrik, 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 to10 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 PHYTO-PLANKTON 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 (I_2) in this solution. A few days before use add 100 g glacial acetic acid (96 – 100% CH_3COOH) 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 place 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) Brown polyethylene bottles (100 mL or 2 L for drinking water) if not preserved with Lugol's iodine	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. Keep in the dark if clear sample bottles are used.
		Add 0.5 ml Acid Lugol's and 3 mL buffered per 100 mL sample	More than a year stored in the dark		
		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) Brown polyethylene bottles (1 L or 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. Keep in the dark if clear sample bottles are used.
		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	

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) Brown polyethylene bottles (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	
Cyanotoxins	Brown glass bottle (1 L) Brown polyethylene bottles (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).
		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-*a***
Analysis of total pigment	Analysis of chlorophyll-*a*
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 60ºC. 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 × 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 60ºC 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):

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

Where:

	E	=	Chlorophyll (phaeophytin)
	A	=	Absorption coefficient of 0.0133
	A_{665}	=	Absorbance at 660 nm
	A_{750}	=	Absorbance at 750 nm
	Ve	=	Volume of solvent (mL)
	Vm	=	Volume of sample (mL)
	R	=	Path length of cuvette (cm)
	x	=	Multiplication

- The chlorophyll-655 (total pigment) 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 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 analisi.
- 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 ±2ºC 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 100µL 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*:

$$\text{Chl}a\ (\mu g/L) = \frac{[(A_{665} - A_{750}) - (A_{665a} - A_{750a})] \times 28.66 \times Ve}{Vm}$$

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)
	Ve	=	Volume of ethanol used for extraction in mL (usually 10 mL)
	Vm	=	Volume of sample filtered in mL
	×	=	Multiplication

- The chlorophyll-*a* 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 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

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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. haemacytometer. 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 μm^3/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 haemacytometer	Inverted microscope (using a sedimentation chamber) / compound microscope when using a haemacytometer	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 haemacytometer, 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 90º.
- 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**).

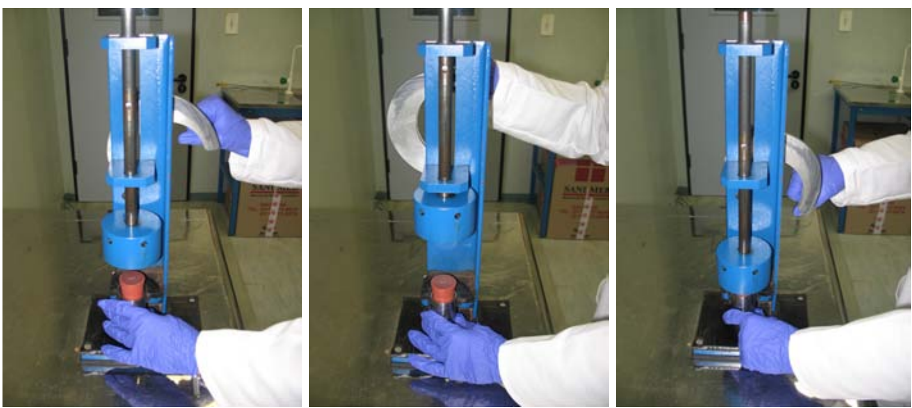


Figure 4.4: Deflation instrument (mechanical hammer), used to disrupt the gas vacuoles of the cyanobacteria, in order for buoyant cells to settle to the bottom of the counting chamber.

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 × 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 × dilution factor) × (10 × dilution factor) = 50 × 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 (±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 haemacytometer).
- Ensure that the 40× (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, CO_2, 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:**

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

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

<u>Example:</u>

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

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

Where π = 3.14
r = 8000 µm

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

- **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.

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.

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

$\therefore$ Area of a field $=$ Length $\times$ Length
$= 180\ \mu m \times 180\ \mu m$
$= \underline{32\ 400\ \mu m^2}$

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 40× 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.

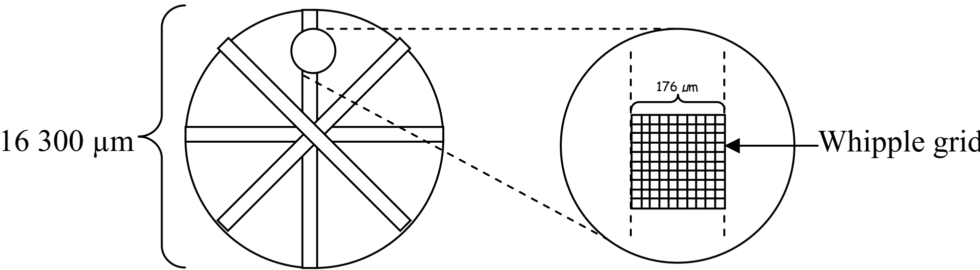


Figure 4.5: Line diagram showing the orientation of lanes and the Whipple grid.

- 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 that 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, CO_2, 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:

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

- Calculate the area of one rectangular lane:

$$\text{Lane area} = \text{diameter of sedimentation chamber} \times \text{width of Whipple grid}$$
$$= 16\ 300\ \mu m \times 176\ \mu m$$
$$= 2\ 868\ 800\ \mu m^2$$

- 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.

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

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.

$$\text{Final conversion factor} = \frac{\text{Conversion factor}}{\text{Volume of sample used}}$$

$$= \frac{72.739}{3 \text{ mL}}$$

$$= 24.246$$

- 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.

$$\text{Biomass} = \text{Count} \times \text{Final conversion factor}$$

$$= 78 \times 24.246$$

$$= 1891.188$$

$$\approx 1891 \text{ cells/mL (rounded to the nearest integer)}$$

4.3.4.2 Calculating the percentage composition of a taxon

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

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. <u>Never</u> 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.

$$\text{Conversion factor (CF)} = \frac{\text{Area of filter}}{\text{Area of view under microscope}}$$

$$\text{Algae number} = \frac{\text{CF} \times \text{no. of individuals counted}}{\text{No. of fields} \times \text{volume filtered (mL)}}$$

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 (μm^3/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× - 1000×) these errors can be minimised to less than 1µm. 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 *Tetraedron* 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	d, h, d (cross view), h, d (apical view) **Cylinder** (Hillebrand et al., 1999)	$V = \pi/4 \cdot d^2 \cdot h$ **OR** $V = \pi \times radius^2 \times h$	*Arthrospira* *Aulacoseira* *Cyclostephanos* *Cyclotella* *Cylindrospermopsis* *Melosira* *Oscillatoria* *Spirogyra* *Spirulina* *Stephanodiscus*

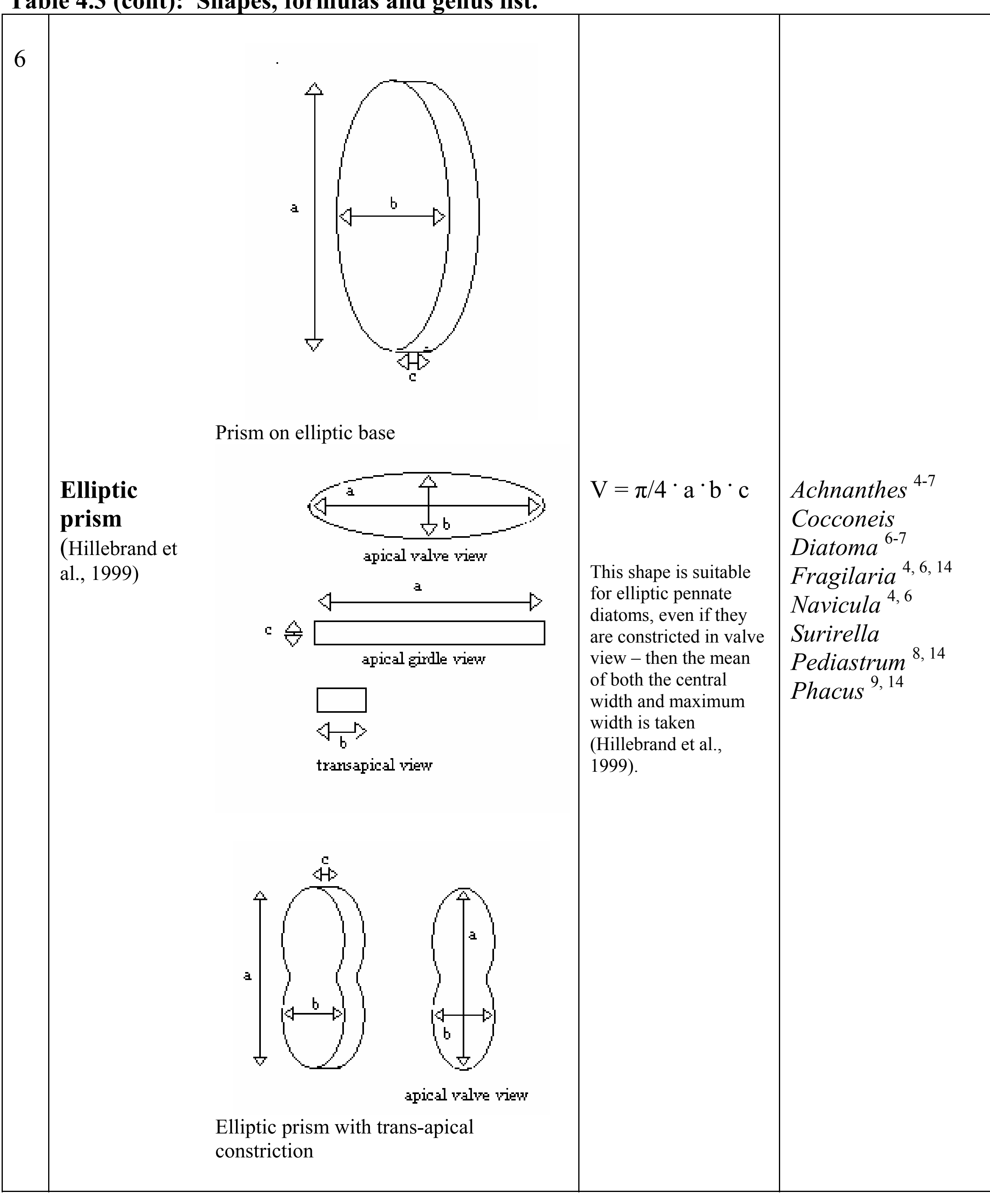
Table 4.3 (cont): Shapes, formulas and genus list.

2	**Sphere** (Hillebrand et al., 1999) d d cross view	$V = \pi/6 \cdot d^3$	*Anabaena* [1, 2, 14] *Aphanocapsa* *Carteria* [1, 3, 14] *Chlamydomonas* [3, 14] *Chlorella* *Chlorococcum* [3, 14] *Coelastrum* [3, 14] *Dictyosphaerium* [3, 14] *Eudorina* *Golenkinia* *Micractinium* [4] *Microcystis* *Sphaerocystis* *Tetrastrum* [14] *Volvox*
3	**Prolate spheroid** (Hillebrand et al., 1999) h d d cross view d h apical view	$V = \pi/6 \cdot d^2 \cdot h$ Sub-spherical body with circular cross-section and elliptical apical section. (Hillebrand et al., 1999).	*Cryptomonas* [1, 14] *Dinobryon* [1, 13, 14] *Mallomonas* *Oocystis* *Pandorina* [14] *Scenedesmus* [14]

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

4	**Ellipsoid** (Hillebrand et al., 1999) h a b a b cross view a h apical view	$V = \pi/6 \cdot a \cdot b \cdot h$ This body is sub-spherical with three different dimensions, i.e. prolate spheroid with elliptical cross-sections (Hillebrand et al., 1999).	*Characium* [14] *Lagerheimia* *Peridinium* *Strombomonas* [13] *Trachelomonas* [13]
5	**Prism on parallelogram** (Hillebrand et al., 1999) a b c a b apical valve view a apical girdle view a b transapical view	$V = ½ . a . b . c$ Rhombic diatom species belong for example to the genera *Pleurosigma* and *Gyrosigma*, the basic parallelogram is even-sided (Hillebrand et al., 1999).	*Gyrosigma* *Nitzschia* [10, 14] *Pleurosigma*

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



6	**Elliptic prism** (Hillebrand et al., 1999) Prism on elliptic base Elliptic prism with trans-apical constriction	$V = \pi/4 \cdot a \cdot b \cdot c$ 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).	*Achnanthes* [4-7] *Cocconeis* *Diatoma* [6-7] *Fragilaria* [4, 6, 14] *Navicula* [4, 6] *Surirella* *Pediastrum* [8, 14] *Phacus* [9, 14]

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

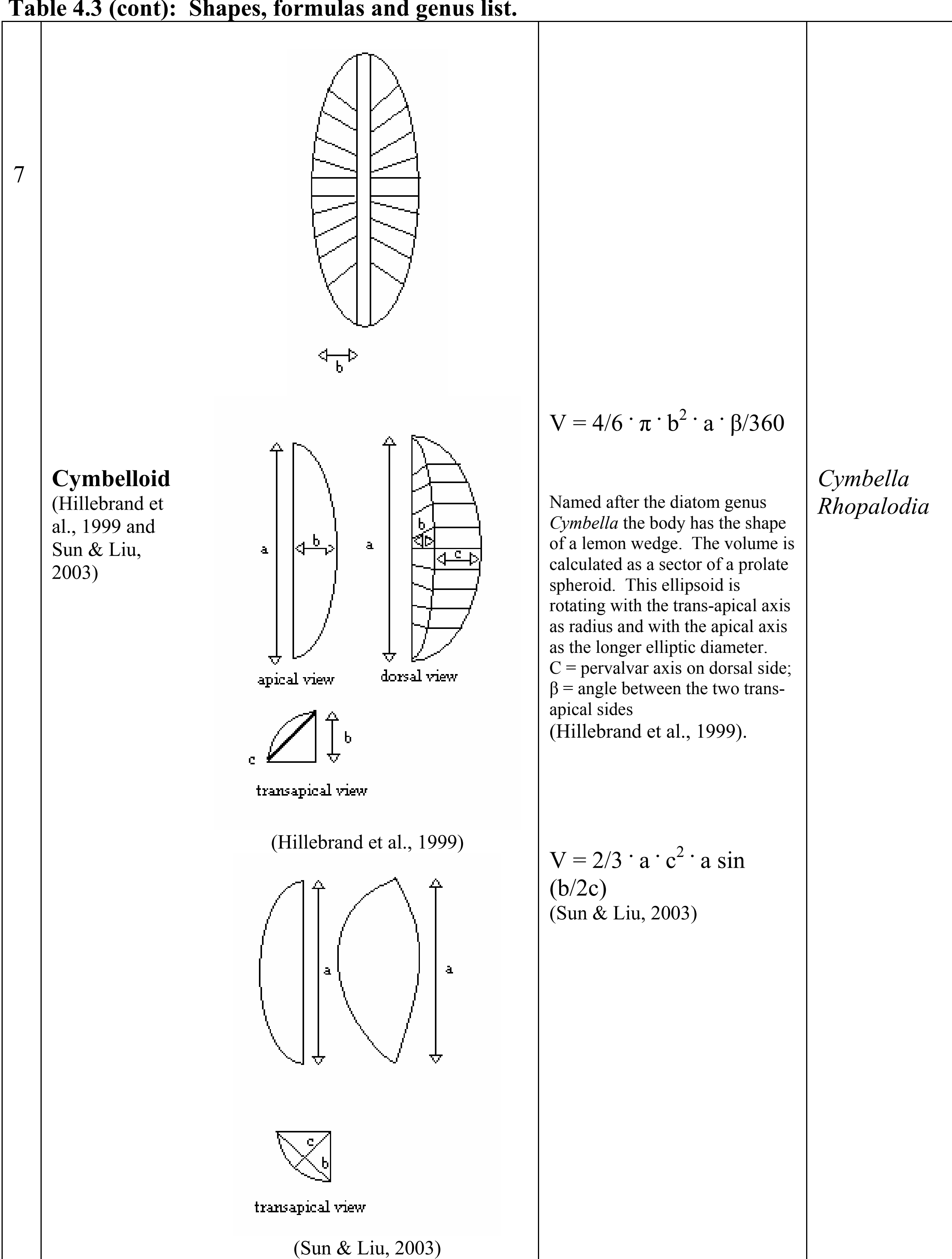
7	**Cymbelloid** (Hillebrand et al., 1999 and Sun & Liu, 2003)  (Hillebrand et al., 1999) (Sun & Liu, 2003)	$V = 4/6 \cdot \pi \cdot b^2 \cdot a \cdot \beta/360$ Named after the diatom genus *Cymbella* the body has the shape of a lemon wedge. The volume is calculated as a sector of a prolate spheroid.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

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

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

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

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

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

No.	Shape	Formula	Genus
12	**Sickle-shaped monoraphidioid** (Hillebrand et al., 1999)	$V = d^2/8 \cdot (2b - d + a) \cdot (\pi^2/6 + 1)$ A special case is lunate bodies which are circular in cross-section. The chlorophyte genera *Monoraphidium* and *Kirchneriella* are examples. The maximum diameter of the body is given as d (= $b - b_2$), all other abbreviations as given (Hillebrand et al., 1999).	*Kirchneriella* *Monoraphidium/ Selenastrum*
13	**Box** (Modified from Hillebrand et al., 1999)	$V = a \cdot b \cdot c$	*Tetraedron* [14]
14	**Cube** (Modified from Hillebrand et al., 1999)	$V = a^3$ A cube is a special case of the shape in 3.13 where a=b=c, then $V=a^3$ (Hillebrand et al., 1999).	*Crucigenia* [14] *Merismopedia* [14]

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

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

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

17	**Half ellipsoid** + **cone on elliptic base** (Constructed from Hillebrand et al., 1999) For half ellipsoid see 9 Cone on elliptic base **OR** **OR** **Cone** + **half sphere** + **cylinder** (Sun & Liu, 2003) cross view apical view	See: 9 + $V = \pi/12 \cdot x \cdot z \cdot c$ (Constructed from Hillebrand et al., 1999) **OR** See: 11 + 1	*Euglena* [1, 9, 14]

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

18	**2 truncated cones** Truncated cone:	Formula for one truncated cone: $V = \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2)$	*Staurastrum* [14] *Staurodesmus*
19	**Cylinder** + **ellipsoid** + **3 cones** (Modified from Hillebrand et al., 1999 and Sun & Liu, 2003) cross view of ellipsoid	$V = \pi/4 \cdot h \cdot d^2$ + $\pi/6 \cdot a \cdot b \cdot c$ + $\pi/12 \cdot (e + f + g) \cdot b_2^2$	*Ceratium* [1, 12]

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 × 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.

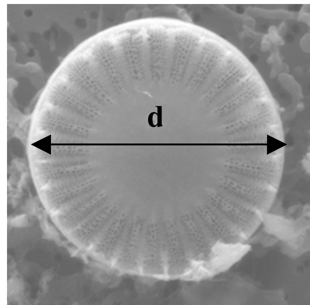
10. The genus *Nitzschia* is quite variable in its shape. The sigmoid and rhombic cells can be calculated as prism on a parallelogram as described. Elliptic species are to be calculated as elliptic prisms, linear species as boxes.
11. These genera include some species which are straight and others which are bent. The latter cells should be calculated as Monoraphidiods (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-thecal 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*



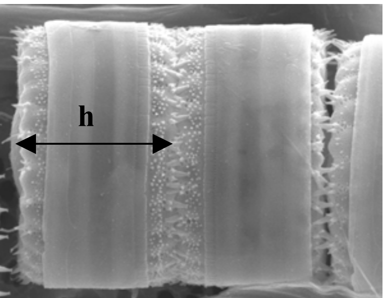


Figure 4.7: Scanning electron microscope photo demonstrating linear measurements of *Cyclotella meneghiniana*.

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:

$$V\ (\mu m^3) = \pi/4 \cdot d^2 \cdot h$$
$$= \pi/4 \cdot 9.84^2 \cdot 6.27$$
$$= 476.81$$

4.5.4.2 Calculating the biovolume of *Staurastrum tetracerum*:

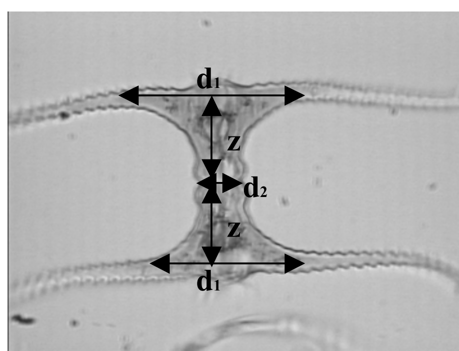


Figure 4.8: Light microscope photo demonstrating linear measurements of *Staurastrum tetracerum*.

Table 4.5: **Dimensions of *Staurastrum tetracerum*:**

Repetition	First truncated cone			Second truncated cone	
	z	d_1	d_2**	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:

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

Biovolume of first truncated cone:

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

Biovolume of second truncated cone:

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

Total biovolume:

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

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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.

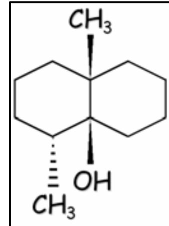

Figure 5.1: Biochemical structure of geosmin

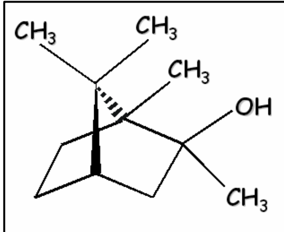

Figure 5.2: Biochemical structure of 2-MIB

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.

Parameter	Setting
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, (effecting 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 µL – 125 µL) 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 mm) 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 (µg/L) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 0.6ppb (µg/L) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.

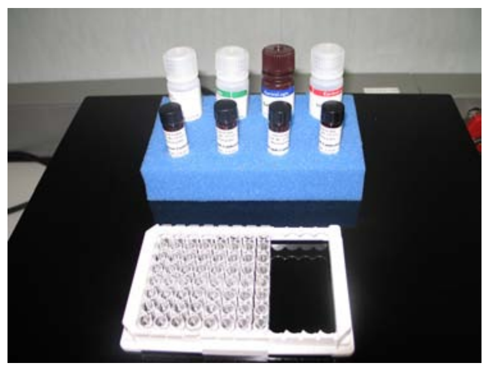


Figure 6.1 : Example of an ELISA screening test kit (e.g. Envirologix).

- 2.5ppb (µg/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.

6.2.2 PROCEDURE

6.2.2.1 Sample preparation

- Determine the presence of total chlorine.
- Should total chlorine be present (>0.1 mg/L) add sodium thiosulphate to sample in the ratio 800 µL 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:

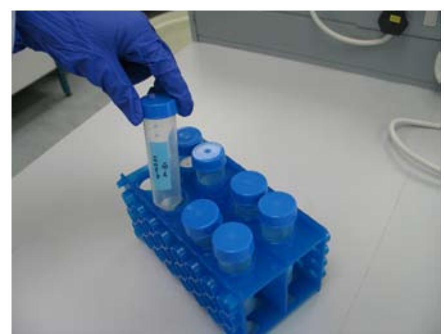


Figure 6.2 : Polypropylene tubes used for sample preparation.

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**).

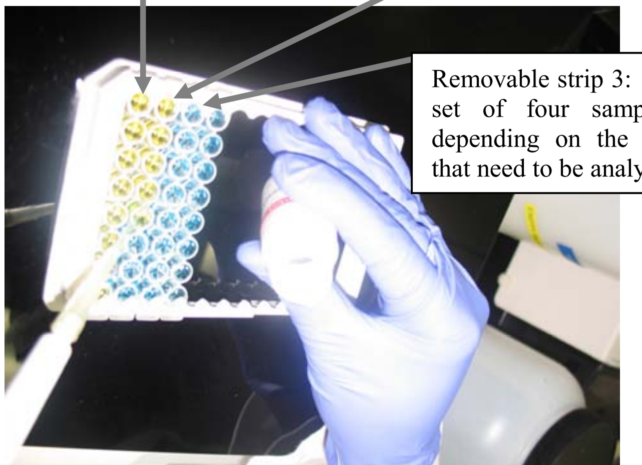


Figure 6.3 : Photograph of the Envirologix Microcystin ELISA test plate with four removable strips mounted onto the frame. The first strip (vertical on the left hand side of the frame) may be used for the four <u>calibrators</u> (standards) to be placed in duplicate below one another. The second strip may be used for the first four <u>samples</u> 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 µL 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 µL 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 µg/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 µg/L.
- If the microcystin concentration of a sample is lower than 0.18 µg/L the results should be reported as <0.18 µg/L or when it is higher than that of the highest calibrator it should be reported as >2.5 µg/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, $C_2HF_3O_2$, 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 ($Na_2S_2O_3$) 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 Phenomanex 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_{Sample}(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, $V_{supernatant}$) 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 [80-10 (*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* µg for the 0.5 mL extract (from 1 mL of the supernatant).

Concentration in the supernatant	=	y µg × $V_{supernatant}$ (mL)
To take into account sample volume	=	y µg × $V_{supernatant}$ / V_{Sample} (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 µg/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: CH_3OH, HPLC grade.
- Acetonitrile: CH_3CN, HPLC grade.
- Water: H_2O, HPLC grade.
- Trifluoroacetic acid: $C_2HF_3O_2$, analytical grade.
- Re-dissolving solvent: Methanol/water [50/50 (*V/V*)].
- Sodium thiosulphate solution: Dissolve 1 g of sodium thiosulphate, $Na_2S_2O_3$, in 100 mL of water.
- SPE elution solution: Methanol containing 0.1% (*V/V*) TFA.
- Acetic acid: CH_3COOH, HPLC grade. 1% in HPLC grade H_2O (*V/V*).
- Ammonium hydroxide solution: Dissolve 2 g of ammonium hydroxide, NH_4OH, in 100 mL water.
- **Solid phase extraction cartridges (SPE) for anatoxin-a and cylindrospermopsin enrichment:** Reversed phase C_{18} SPE cartridges are used to

extract and concentrate Anatoxin-a and Cylindrospermopsin. Any suitable C_{18} 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 Phenomanex 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)

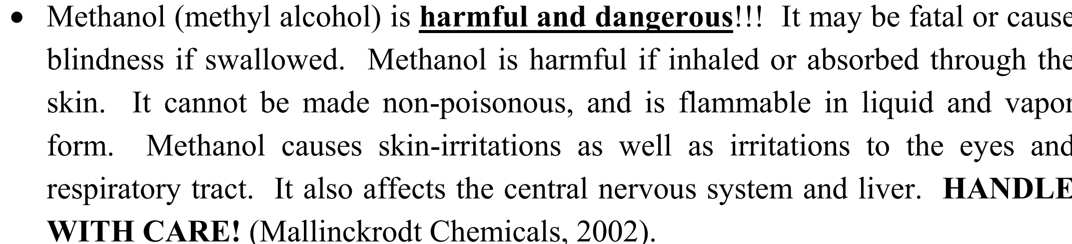
Separate the Anatoxin-a and Cylindrospermopsin by HPLC at 40°C with a reversed phase column. Adjust the flow rate and the injection volume according to the column dimensions (inner diameter, particle size) to obtain the optimal peak shape and resolution. Use a wavelength of 227 and 262 nm to detect the 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 µ/L to 2.34 µg/L for the Anatoxin-a and 1.0 µg/L to 4.0 µg/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**".

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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 (± 5 – 50 mL)	Merck	011 345-9000 www.merck.co.za
Glass tube (± 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 µm)	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)				✓				

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

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

Report to the Water Research Commission
by
Rand Water*
In association with
The North-West University** and
Umgeni Water**

February 2008

(This manual is available on CD only, which can be obtained with WRC Report TT 323/08 "CONDENSED LABORATORY METHODS FOR THE ANALYSES OF PHYTOPLANKTON, INCLUDING CYANOBACTERIA, IN SOUTH AFRICAN FRESHWATERS."

Both manual and Report should be regarded and studied as a unit.

DISCLAIMER

This report has been reviewed by the Water Research Commission (WRC) and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC, nor does mention of trade names or commercial products constitute endorsement or recommendation for use

EXECUTIVE SUMMARY

BACKGROUND AND MOTIVATION

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, therefore, is essential to the production of water which is 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), with the following objectives:

AIMS OF THE PROJECT

- The documentation of current methods for chlorophyll determinations, phytoplankton identification and enumeration, geosmin and 2-MIB analyses and cyanobacterial toxin analyses, internationally and nationally.
- The compilation of a condensed methods manual for the analysis of phytoplankton, geosmin and 2-MIB and cyanobacterial toxins in South African freshwaters.

- The compilation of a comprehensive document that includes all aspects of the above mentioned methods, including validations for reference purposes.

METHOD DEVELOPMENT

After a thorough literature research was undertaken, the identified methods were evaluated and validated by scientists from Rand Water, in collaboration with Umgeni Water and the Botany Department of the North-West University (Potchefstroom Campus). These methods have been incorporated into a methods manual, written in the format prescribed by SANAS (South African National Accreditation System) and inclusive of the validation data.

The following methods were evaluated and validated:

- spectrophotometric methods for the determination of **chlorophyll-*a*** (usually performed on raw water samples) and **chlorophyll-665** (total pigment – usually performed on drinking water)
- filtration (one method) and two sedimentation methods used for **phytoplankton and cyanobacterial identification and enumeration**
- a reference for the **determination of biovolumes** for all phytoplankton and cyanobacteria found in South Africa
- a “purge and trap” procedure, coupled to a GC/MS method, for the determination of **geosmin** and **2-methylisoborneol (2-MIB)**
- ELISA method for the determination of **microcystin** concentrations
- HPLC method for the determination of **microcystin** and **nodularin** concentrations
- HPLC method for the determination of **anatoxin-a** and **cylindrospermopsin** concentrations.

The condensed methods manual (“**Condensed Laboratory Methods for the Analyses of Phytoplankton, including Cyanobacteria, in South African Freshwaters**”) mainly include the step-by-step execution of all the methods, and is aimed to be used in the lab. This condensed methods manual is supplemented by a comprehensive methods manual (“**Comprehensive Laboratory Methods for Monitoring Phytoplankton, including Cyanobacteria, in South African Freshwaters**”) that include all aspects of the methods as well as their validations, to serve as reference when methods are being developed or additional information regarding the methods are required.

It is envisaged that these two manuals will support and facilitate capacity building in South African water laboratories.

CAPACITY BUILDING AND INFORMATION TRANSFER

Capacity building and information transfer concerning method development and the execution of the methods included in the manual were performed on various occasions. The capacity building focused on skills transfer to staff members working for Rand Water as well as externally to the organisation. The internal capacity building focused on chlorophyll determinations, cyanobacteria and phytoplankton identification, and cyanotoxins analysis. The project team managed to secure funds from the Finnish Embassy to enhance the capacity of cyanotoxins analysis. This enabled two representatives (Messrs C. Schoeman, and L. Sihlobo,) to attend a training programme at the University of Helsinki, Finland during 2005. The funds were also used to present a training workshop (presenters: Dr S. De Kock, SAAMS and Mr. C Schoeman, Rand Water) on the analysis of cyanotoxins (microcystins, nodularin, anatoxin-*a* and cylindrospermopsin) at Analytical Services, Rand Water. Although representatives of many drinking water companies were invited only representatives from Rand Water and Johannesburg Water attended the course.

A second workshop, held at Rand Water from 19 to 22 February 2007 (presenters: Prof. Hein du Preez, Drs Sanet Janse van Vuuren and Jonathan Taylor as well as Mss Carin van Ginkel and Annelie Swanepoel), focused on phytoplankton and cyanobacteria identification and enumeration as well as microcystin determination with the ELISA method. Sixteen delegates attended, mostly from the water purification industry (Mhlathuze Water, MidVaal Water Company, NamWater, Rand Water, Sedibeng Water and Umgeni Water) but also from other analytical laboratories (Buckman Laboratories, Sasol and DWAF).

The details of persons involved in the capacity building are summarised in the table below.

Person involved	Organization	Capacity building focus	Type of intervention
A. Ramcharan	Rand Water	Chlorophyll-*a* Chlorophyll-665 ELISA screening for microcystins	- In-house training at Rand Water as required by ISO 17025
Z. Ngwenya	Rand Water	Chlorophyll-*a* Chlorophyll-665 ELISA screening for microcystins	- In-house training at Rand Water as required by ISO 17025
P. Maseti	DWAF Former employee of Rand Water	Chlorophyll-*a* Chlorophyll-665	- In-house training at Rand Water as required by ISO 17025
P. Mofokeng	Rand Water	Chlorophyll-665 Chlorophyll-*a*	- In-house training at Rand Water as required by ISO 17025

Person involved	**Organization**	**Capacity building focus**	**Type of intervention**
I. Dusrath	Rand Water B. Tech student at VUT	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins Chlorophyll-*a* Chlorophyll-665	- In-house training at Rand Water as required by ISO 17025 - External training course at NWU (algal id) - Training during workshop 2 held at Rand Water
E. de Kock	Rand Water	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins Chlorophyll-*a* Chlorophyll-665	- In-house training at Rand Water as required by ISO 17025 - Training during workshop 2 held at Rand Water
L. Bungu	Rand Water	ELISA screening for microcystins	- In-house training at Rand Water as required by ISO 17025
L. van Baalen	Former employee of Rand Water	Cyanotoxin analysis	- Training in Finland
C. Schoeman	Former employee of Rand Water	Cyanotoxin analysis	- Training in Finland
L. Sihlobo	Afrox Germiston Former employee of Rand Water	Cyanotoxin analysis Geosmin and MIB analysis	- In-house training at Rand Water as required by ISO 17025 - Training in Finland - Training workshop 1 held at Rand Water
L. Mkhize	Rand Water	Cyanotoxin analysis – HPLC Geosmin and MIB analysis – GC/MS	- In-house training at Rand Water as required by ISO 17025
S. Mdunge	Rand Water	Cyanotoxin analysis – HPLC Geosmin and MIB analysis – GC/MS	- In-house training at Rand Water as required by ISO 17025
A. Swanepoel	Rand Water	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins Chlorophyll-*a* Chlorophyll-665	- In-house training at Rand Water as required by ISO 17025 - External training course at NWU (algal id) - Presenter at workshop 2
M. Nkosi	Rand Water	Geosmin and MIB analysis	- In-house training at Rand Water as required by ISO 17025

Person involved	Organization	Capacity building focus	Type of intervention
L. Sebola	Municipality in Limpopo Former employee of Rand water	Phytoplankton and cyanobacteria identification and enumeration	- In-house training at Rand Water as required by ISO 17025
R. Avis	Johannesburg Water	Cyanotoxin analysis	- Training workshop 1 held at Rand Water
L. Hanyane	Mhlathuze Water	Phytoplankton and cyanobacteria identification and enumeration	- 1 week training at Rand Water - Training at workshop 2 held at Rand Water
E. Fosso	M.Sc. student at UJ	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training at Rand Water
G.P. Kriel	M.Sc. students at NWU	Phytoplankton and cyanobacteria identification and enumeration	- Training and participating in validation of methods included in document - Participation in workshop 2
N. v.d. Walt	Hons student at NWU	Phytoplankton and cyanobacteria identification and enumeration	- Training and participating in validation of methods included in document
G. van Zyl	Hons student at NWU	Phytoplankton and cyanobacteria identification and enumeration	- Training and participating in validation of methods included in document - Biovolume determinations
Z. Franken	Rand Water B. Tech student at VUT	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- In-house training at Rand Water as required by ISO 17025 - Training during workshop 2 held at Rand Water
C. Mchabeleng	DWAF	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water held at Rand Water
E. Viljoen	SASOL	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
E. Rava	Buckman Gauteng	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water

Person involved	Organization	Capacity building focus	Type of intervention
E. Almirall	NamWater	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
E. Barnes	Sedibeng Water	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
F. Dlamini	Umgeni Water	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
I. Kangootui	NamWater	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
K. Milford	Umgeni Water	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
M. Nel	SASOL	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
P. Mahlatsi	MidVaal Water Company	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
S. Letshwene	DWAF	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water
S. Morrison	MidVaal Water Company	Phytoplankton and cyanobacteria identification and enumeration ELISA screening for microcystins	- Training during workshop 2 held at Rand Water

RECOMMENDATIONS

The recommendations arising from this project are as follows:

- Technical courses and or workshops in the field of identification and enumeration of phytoplankton, including cyanobacteria, should be presented frequently.
- The monitoring of phytoplankton and its related substances should be encouraged and the importance thereof stressed, especially in the potable water industry in South Africa. Legislation by DWAF (Department of Water Affairs and Forestry in South Africa) may be necessary for the effective implementation of a monitoring programme by potable water suppliers, to be able to supply safe drinking water to all South African consumers.
- The current methods manual should be updated when new research is done and additional methods are developed or existing methods are modified/optimised.

ACKNOWLEDGEMENTS

The authors wish to thank the following members of the Steering Committee for their valuable inputs to this project:

Ms. A Moolman (Chairman)	Water Research Commission
Mss. Z Franken & A Schoeman (Secretaries)	Rand Water
Dr. S du Plessis	North-West University
Ms. C van Ginkel	DWAF
Mr. P Grobler	LNIN
Dr. T Downing	Nelson Mandela Metropolitan University
Dr. WR Harding	DH Environmental Consulting
Mr. J Parsons	Rand Water
Ms. M Kruger	MidVaal Water Co
Dr. P Kempster	RQS DWAF

The financial support received from the Water Research Commission and Rand Water to conduct the project is gratefully acknowledged.

Thank you to Mr. Hennie Slabbert for the valuable support with the use of the Rand Water SAP system.

Special thanks to Dr. WR Harding and his staff at DH Environmental Consulting, South Africa for their valued contribution towards editing the document.

Thank you to GP Kriel, Germarié van Zyl and Nicolene van der Walt for the validations of certain methods and their contributions towards the biovolume determination section in the manual.

A very special thanks to the current and former staff of Hydrobiology and Organic Chemistry, at Rand Water Vereeniging, especially Leoni van Baalen, Zelna Franken, Rita Guglielmi, Annelie Schoeman, Lindani Mkhize, Sibusiso Mdunge, Ishana Dusrath, Ashvita Ramcharan, Zinhle Ngwenya and Elmari de Kock for their contributions towards the project.

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CHAPTER 1

INTRODUCTION

1.1 PROJECT 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). 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 an 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.

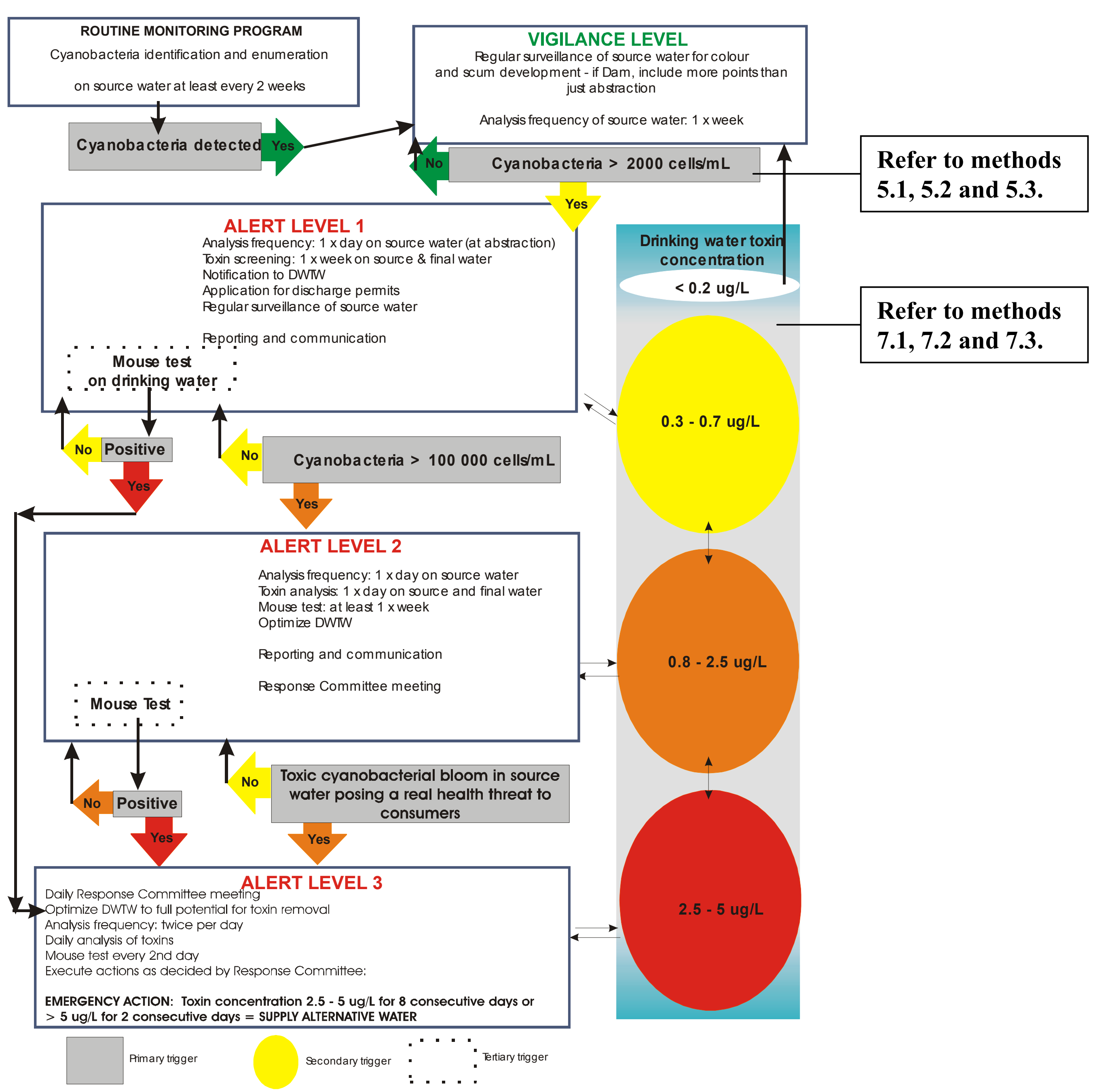

Figure 1.1 Cyanobacteria Incident Management Framework (CIMF) using cyanobacteria concentration as the primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).

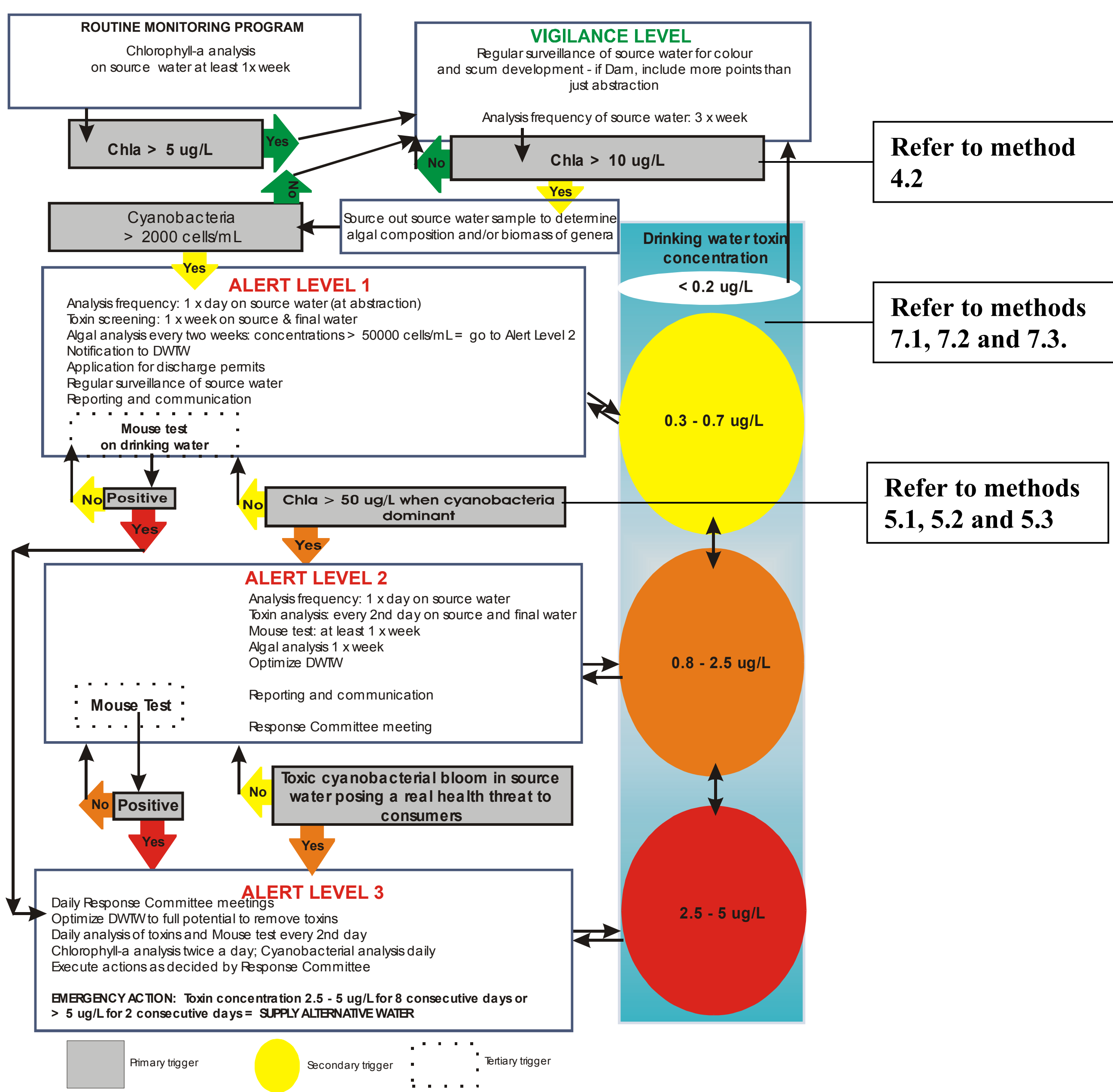

Figure 1.2 Cyanobacteria Incident Management Framework (CIMF) using chlorophyll-*a* as the primary trigger (adapted from Du Preez & Van Baalen, 2006 – WRC Report Number TT 263/06).

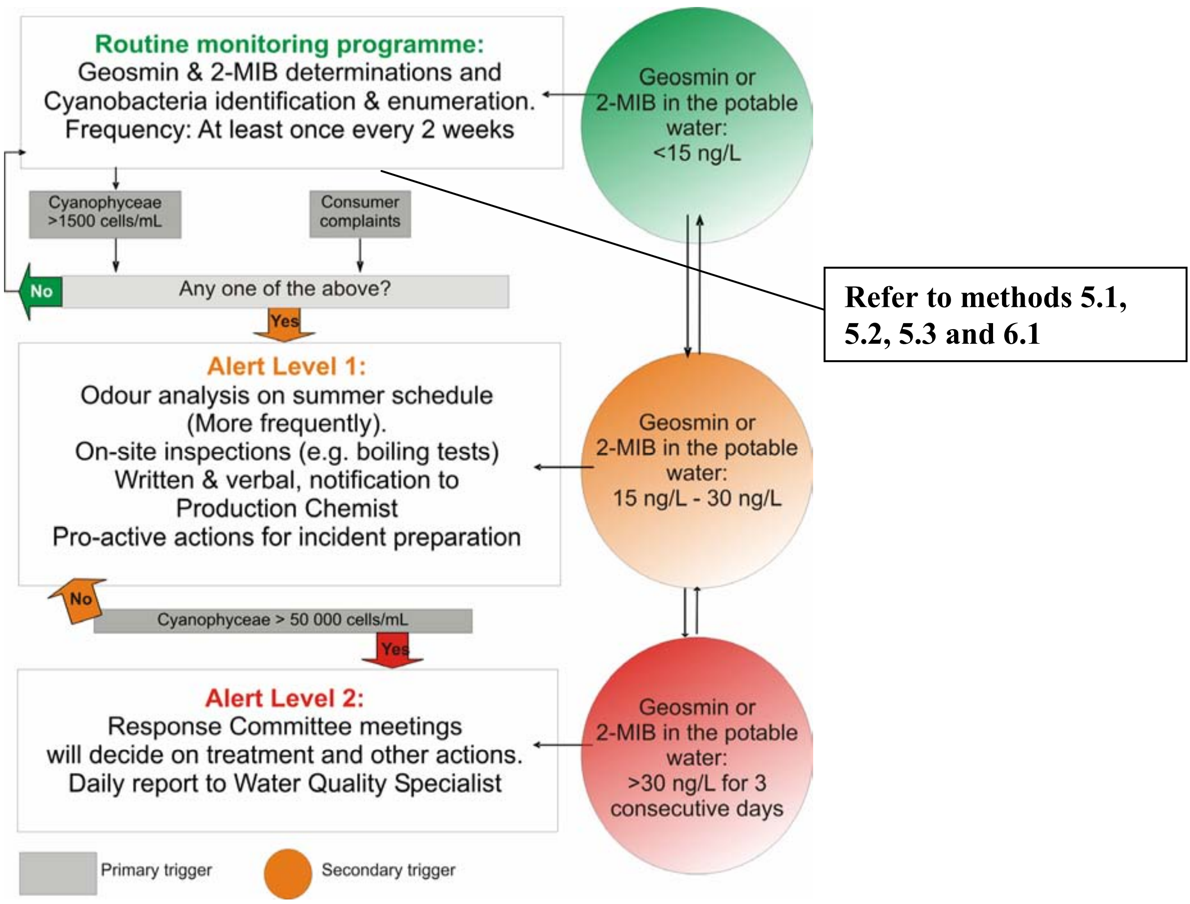

Figure 1.3 Cyanobacteria Incident Management Framework (CIMF) for the reaction to taste and odourous substances (as used in Rand Water).

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 waterbody 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 AIMS OF THE PROJECT

- The documentation of current methods for chlorophyll determinations, phytoplankton identification and enumeration, geosmin and 2-MIB analyses and cyanobacterial toxin analyses, internationally and nationally.
- The compilation of a condensed methods manual for the analysis of phytoplankton, geosmin and 2-MIB and cyanobacterial toxins in South African freshwaters.
- The compilation of a comprehensive document that includes all aspects of the above mentioned methods, including validations for reference purposes.

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CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL

This literature review focuses on methods used by accredited and internationally-recognized organizations and laboratories. The methods themselves are not described in this chapter.

2.2 CHOOSING THE SUITABLE METHOD

The choice of methods requires consideration of sources of variability and error at each stage of the process. Individual laboratories must identify the techniques that are suitable for their analytical requirements taking into account their own expertise and available technology.

2.3 ANALYSES OF DIFFERENT VARIABLES RELATED TO PHYTOPLANKTON

Depending on the aims of a study there are a few techniques available to determine analyse variables associated with phytoplankton and especially associated with cyanophyceae (cyanobacteria or blue-green algae). The most common approach would be to establish the phytoplankton concentration (whether it is by chlorophyll analysis or by individual species identification and enumeration). The second step would be to analyse for the organic compounds produced by these phytoplankton cells.

2.3.1 ESTIMATION OF PHYTOPLANKTON BIOMASS

Quantification of phytoplankton in water resources usually forms the principal component of phytoplankton monitoring programmes. There are different ways to establish the biomass of phytoplankton, each of which has its own accuracy, precision and applicability under different circumstances.

2.3.1.1 Chlorophyll analyses

An indirect method to establish the quantity of phytoplankton in water is to determine the total concentration of chlorophyll. This method, however, does not distinguish between different kinds of phytoplankton (e.g. green algae, diatoms or cyanobacteria). Thus, in the presence of a mixed population of phytoplankton, the data cannot be used to quantify a certain species or group of phytoplankton. In the case of water blooms of a single group of organisms (e.g. cyanobacteria), the measurement of chlorophyll is more informative of the biomass of the dominant group (Falconer, 2005). In this case the content of chlorophyll will be a good guide to

biomass and potential toxin concentration, as biomass is directly related to chlorophyll content and the effects of different sizes (biovolumes) of organisms are eliminated. However, it must be stressed that chlorophyll alone cannot be used to indicate the potential hazard to consumers of drinking water, because some strains of a species may be toxic while others are not.

Chlorophyll analysis requires commonly-available laboratory equipment, principally filtration apparatus, centrifuge and spectrophotometer. It is considerably less time-consuming than microscopic biomass determination (although less specific and less precise; Chorus & Bartram, 1999).

Two other methods are also used which make use of fluorometric or HPLC techniques to determine chlorophyll-*a* concentrations (APHA, 2001). Fluorometry is more sensitive than spectrophotometry, it requires less sample and can be used for *in-vivo* measurements. Fluorometry and spectrophotometry can significantly over- or under-estimate chlorophyll-*a* concentrations, in part because of the overlap of the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products. By contrast, HPLC provides a precise method for resolving and quantifying all photosynthetic pigments. A disadvantage is that the analysis is more expensive to carry out than that employing spectrophotometry.

Chlorophyll-665

Chlorophyll-665 analysis is not widely used outside the potable water purification industry. The chlorophyll-665 method analyses for total pigment concentrations (not only the chlorophylls, but the phaeophytins – chlorophyll breakdown products as well). Due to a very fast turn-around time (approximately 1 hour), results can be available quickly for decisions regarding the water purification process (Steynberg, 1986). Another advantage of the chlorophyll-665 analysis is that it is more sensitive than the chlorophyll-*a* method for the detection of very low concentrations (e.g. those which might occur in potable water).

Chlorophyll-*a*

The chlorophyll-*a* pigment generally comprises 0.5 - 1 per cent of the ash-free dry weight of phytoplankton organisms present in a sample (APHA, 2001; Chorus & Bartram, 1999). Although the pigment content may vary according to the physiological state of the organisms (e.g. it increases if light availability is low and may also differ from species to species), chlorophyll-*a* is a widely used and commonly-accepted measure of phytoplankton biomass. It is an especially useful measure during phytoplankton blooms, when the phytoplankton often consists of only one species. However, when chlorophyll-*a* determination is used with mixed phytoplankton populations, it may give an overestimation of certain problem-causing species (such as cyanobacteria, Chorus & Bartram, 1999). Standard protocols have been described (e.g. ISO, 1992) but preferred methods vary between laboratories. However, the main method steps

are essentially the same: solvent extraction of chlorophyll-*a*, determination of the concentration of the pigment by spectrophotometry, and adjustments to the result, to reduce the interference of phaeophytin-*a* (APHA, 2001).

2.3.1.2 **Identification and enumeration of phytoplankton**

The microscopic identification and enumeration of phytoplankton cells, filaments or colonies has the advantage of directly assessing the presence and numerical density of potentially toxic organisms. The basic analytical tool is the microscope. This method of biomass determination may, however, be rather time consuming, ranging from a few minutes to several hours per sample depending on the experience and proficiency of the analyst, the accuracy required and the number of species to be differentiated and counted.

It is widely accepted that only living cells (no dead or broken diatom frustules) should be counted.

Methods of phytoplankton enumeration have been systematized, but are not uniform worldwide (Falconer, 2005). Counting phytoplankton involves the nature of the 'units' to be counted. Some phytoplankton occur as filamentous (refer to **Figure 2.1**) or colonial forms (refer to **Figure 2.2**), consisting of a large number of cells that are often masked by each other and difficult to distinguish.

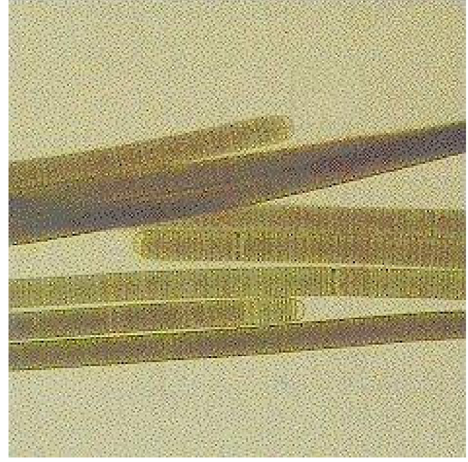

Figure 2.1 An example of a filamentous alga (*Oscillatoria simplcissimia*)

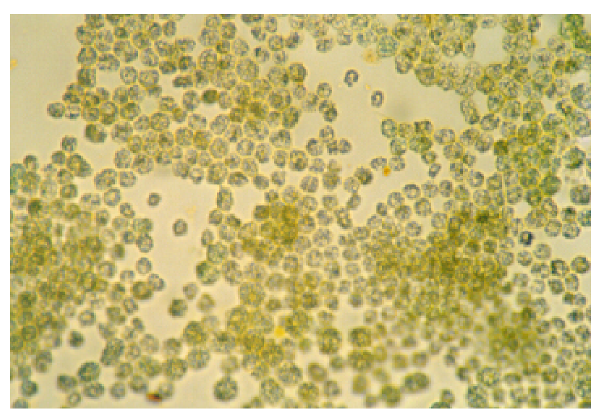

Figure 2.2 An example of a colonial alga (*Microcystis aeruginosa*)

The accuracy of the quantitative determination depends on the number of counted objects (e.g. individual cells or colonies); the relative error is approximately indirectly proportional to the square root of the number of objects counted (Chorus & Bartram, 1999). Both filaments and colonies can differ greatly in the number of cells present, hence results given as number of colonies (e.g. stating that 2.47 colonies of *Microcystis toxica* occurs in 1 mL of sample) provides little information on the quantity (biovolume) of phytoplankton present (Chorus &

Bartram, 1999). Typically, unicellular species are counted as cells per mL and filamentous species can be counted as number of filaments, and quoting an average number of cells per filament. Often, the first 30 filaments encountered are used to establish the average "cells per filament". Thereafter, the filaments are counted and converted to cells per mL. Alternatively, the total filament length per mL may be assessed (in samples where a high degree of fragmentation has occurred) as the sum of the length of each filament within a counting grid placed in the ocular of the microscope. The latter is more precise when the filament length is highly variable. For colonial species, disruption or disintegration of the colonies and subsequent counting of the individual cells is preferable to counting colonies and estimating colony size (Box, 1981).

The magnification of the light microscope is of importance because, although 100× and 200× magnification may be useful for counting large organisms or large colonies, a higher magnification is often required (APHA, 2001). The magnification mostly used is 400×.

To obtain an accurate estimate of the number of phytoplankton cells, colonies, or filaments, transects of the area, or randomly selected sub-areas of the sedimentation chamber or filter, must be counted. This procedure requires a counting grid. It usually is a simple cross-hatched grid (a grid marked with parallel lines), or a grid with random circular fields (Lawton & Marsalet, 1999), that is placed in one of the oculars of the microscope. In both these cases, the area that is to be counted must be known, as well as the area over which the phytoplankton are spread and the volume of the original sample. The area of the grid that is to be used for counting can be determined by the use of a commercially available calibrated slide, from which the length, and hence area of the grid, can be measured (Lawton & Marsalet, 1999).

As filaments and colonies will project from the area of the grid to be counted, an arbitrary but accurate method for correcting the count is to disregard the filaments projecting to the left of the grid and count those projecting from the right (Falconer, 2005). Some analysts use the method whereby colonies or filaments are counted if more than half of the colony or filament lies inside the grid, and to disregard those where more than half lies outside the grid. The same method may be used for large cells that lie on the edge of the grid. It is important that a standardized approach is adopted and adhered to.

Certain cyanobacterial species form globular colonies and therefore may be almost impossible to count without disrupting the colony. This can be achieved by vigorous mixing of the sample (by means of an electric stirrer), judicious ultra-sonification, mild alkaline hydrolysis or storage and strong shaking in Lugol's iodine solution (Falconer, 2005). The best available technique requires the use of an Ultra-Turrax-type laboratory blender.

Sedimentation technique

The most common method for the identification and enumeration of phytoplankton is the sedimentation technique described by Utermöhl (1931, 1958) and which was modified by Lund et al. (1958), making use of a sedimentation chamber and an inverted light microscope. The Utermöhl method is widely used by prominent cyanobacterial specialists in Europe (Olrik et al., 1998), and may be adapted or changed to suit the needs and specifications of individual laboratories (e.g. most laboratories in Australia; Hötzel & Croome, 1998). This method is well suited for assessment of a large variation of cell types and is widely accepted as one of the most reliable. Counting chambers and sedimentation chambers or tubes are commercially available, or can easily be constructed by the investigator. The most commonly used chambers have a diameter of 2.5 cm and a height of about 0.5 - 2 cm and thus can contain 2 - 10 mL of sample. These chambers can fit easily onto a custom-made stage of an inverted light microscope (Lawton & Marsalet, 1999). If larger volumes of water need to be analysed, as is the case when cell density is low (e.g. at the onset of population development or the analysis of drinking water), then the height of the tube can be increased (Chorus & Bartram, 1999). The problem of low phytoplankton density can also be overcome by using a tube in two sections, which allows the supernatant to be removed (after settling the sample) without disturbing the sediment cells on the bottom glass and adding an additional volume of sample to be settled. This, however, may be time-consuming and in laboratories where turn-around time is of the essence, other methods of concentration may be used. An example of such a concentration technique is centrifuging the sample, discarding the supernatant, re-suspending it in a smaller volume of distilled water and then allowing it to settle in the counting chamber.

Filtration technique

An approach with a faster turn-around time than the sedimentation technique is to filter a measured quantity of water through a suitable filter paper or membrane. Colonies, filaments, and individual cells can be counted directly on the filter. Use of a 10 - or 20 mL hypodermic syringe with a membrane filter of 0.45 μm pore size will enable easy preparation (Falconer, 2005). After the water is slowly pressed through the filter, the filter holder is disassembled and the membrane, with the colonies uppermost, air-dried on a microscope slide. When it is dry, a few drops of microscope immersion oil are added to the membrane, which is covered with a cover slip. The phytoplankton is then visible on a transparent membrane (Falconer, 2005). The single over-riding large disadvantage of this method is that some species may become squashed and therefore not be recognizable to the untrained eye. Analysts using this method regularly have learned to distinguish between species.

Bio-volume estimation

Phytoplankton data expressed as volume-per-volume are often more useful than numbers per milliliter (APHA, 2001).

The bio-volume of phytoplankton can be obtained from cell counts by determining the average cell volume for each species or unit counted and then multiplying this value by the cell number present in the sample (Chorus & Bartram, 1999). Cell sizes of an organism may, however, differ substantially under different circumstances (such as at different times of the year or with different types of water); therefore, average measurements from at least 20 individuals of each species for each sampling period should be made (APHA, 2001). Given a specific weight of almost 1 $mg.mm^{-3}$ for phytoplankton cells, this bio-volume corresponds quite closely to its biomass. Average volumes are determined by assuming idealized geometric bodies for each species (e.g. spheres for *Microcystis* or *Chlorella* cells, cylinders for filaments like *Spirogyra* sp.), measuring the relevant geometric dimensions of 10 to 30 cells (depending upon variability) of each species, and calculating the corresponding mean volume of the respective geometric body (Chorus & Bartram, 1999).

For example, if analysing cell and filament numbers only, the number of *Oscillatoria* filaments would be at least a 100-fold less than that of *Microcystis* cells, because an *Oscillatoria* filament is about 100 times larger than a single *Microcystis* cell. Therefore bio-volumes would give a much better indication of phytoplankton biomass. Both these species (*Microcystis* and *Oscillatoria*) often contain cyanotoxins and it is possible to compare the relative toxin content per bio-volume or biomass, whereas there is little point in comparing toxin content in relation to the respective cell and or filament numbers (Chorus & Bartram, 1999).

This approach, however, is not as easy with other phytoplankton species, especially orders of the Volvocales and Chlorococclaes (Blomqvist & Herlitz, 1998), because of their complicated shape and form that requires long-term personal experience of the organism in question. Some species like *Pediastrum* and *Botryococcus* which are common and important species, may also be relatively hard to quantify in terms of volumes.

Other techniques used

The use of an inverted light microscope with counting chambers is generally the best approach and most widely used for determining phytoplankton numbers (Chorus & Bartram, 1999). However, a standard microscope is sufficient for pre-concentrated samples or for naturally dense samples from mass developments, provided the size of the water drop enumerated can be defined (e.g. by using a micropipette).

Other counting chambers (e.g. Sedgwick-Rafter, Lund cell or haemocytometer) are available for use with a standard microscope. The technique using such chambers is especially widely used in Australia (Hötzel & Croome, 1998).

2.3.2 ANALYSES OF TASTE AND ODOUR RELATED SUBSTANCES (GEOSMIN AND 2-METHYLISOBORNEOL)

With the development of closed-loop stripping (CLSA), organic compounds of intermediate volatility and intermediate molecular weight can be extracted from water and concentrated to allow quantitative and semi-quantitative analysis at nanograms per liter concentrations. This extract can be analysed on a GC (gas chromatograph) connected to one of several detectors. The purge and trap is a valuable method of concentrating the volatile organic compounds. The compounds are concentrated by bubbling an inert gas through the sample, followed by collection in and desorption from a sorbent trap. This extract may then be analysed by GC or by GC/MS methods.

The closed-loop-stripping-analysis - gas chromatographic/mass spectrometry (CLSA-GC/MS), procedure is suitable for the quantification of both geosmin and 2-MIB (2-Methyl-isoborneol). In closed-loop stripping, volatile organic compounds of intermediate molecular weight are stripped from water by a re-circulating stream of air. The organics are removed from the gas phase by an activated carbon filter and are then extracted from the filter with carbon disulfide (CS_2). A portion of the extract is injected into a capillary-column GC/MS for identification of the organic compounds by retention time and spectrum matching; quantification is performed by single-ion current integration (APHA, 2001)

Trace organics can be detected at 0.1 to 100 nanograms-per liter levels, if obtained with an elevated stripping temperature/salting-out technique (APHA, 2001).

The use of the reagent-grade solvents or better and the purest standards available, is very important (APHA, 2001). The identification of a compound is done by matching both retention time and spectra of the sample analysed to that of a standard. Different computer packages are available to analyse the data. If possible, both a reverse-search computer programme with a target-compound library and a forward-search programme with the National Institute of Standards and Technology library for tentative identification of other compounds present, should be used. To determine the concentration of a compound the peak areas of specific ions are compared (APHA, 2001).

2.3.3 ANALYSES FOR CYANOTOXIN CONCENTRATIONS

Presently upwards of 40 cyanobacterial species are known to produce toxins (Carmichael, 2001). The toxin most widely known, studied and analysed is Microcystin, produced most commonly but not exclusively by *Microcystis* sp. This cyanobacteria is common in most eutrophic water bodies all over the world. Well-documented cases of its toxic effects on livestock, wild animals and humans are widely available. Microcystin, Nodularin are hepatotoxins, while Anatoxin-a

and saxitoxin are neurotoxins which could cause death within a few minutes or hours. Cylindrospermopsin is a cytotoxic guanidine alkaloid, although initially described as a hepatotoxin (affecting the liver), it was also found to affect the kidneys, thymus and heart (Meriluoto et al., 2005).

2.3.3.1 **Immunological detection (ELISA–methods)**

The Enzyme-Linked Immuno Sorbent Assay (ELISA) technique is currently the most common method for rapid sample screening for microcystins because of its sensitivity, specificity and ease of operation. Monoclonal antibodies raised against microcystin-LA were initially developed by Kfir et al. (1986) and offered a simple approach to a general immunoassay for microcystins. An ELISA technique was subsequently developed by Chu et al. (2001) as a practical method. This assay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR. The antisera showed good cross-reactivity with most microcystins, but not all (e.g. microcystin–LY and –LA; Chorus & Bartram, 1999). The sensitivity of the assay showed approximately 50 per cent binding at a toxin concentration of 1 $ng.mL^{-1}$, which is appropriate for normal water quality testing. This method has been successfully employed for quantitation of cyanobacterial hepatotoxins in domestic water supplies and biomass extracts with detection limits of 0.2 $\mu g.L^{-1}$ and 0.25 $\mu g.g^{-1}$ of water and biomass samples, respectively (Chu et al., 1990).

Commercially available polyclonal ELISA kits are available for microcystins. The most commonly used kit is the 96-well microtitre plate Enviro Guard (Coring-System, Gernsheim, Germany; Chorus, 2001). The antibodies are fixed to the walls of the wells of a microtitre plate. The first step involves binding of the calibrators (a non-toxic microcystin-LR surrogate at 0.16 $\mu g.l^{-1}$, 0.6 $\mu g.L^{-1}$ and 2.5 $\mu g.L^{-1}$), a negative control, and the samples to the antibodies in the wells. This is followed by addition of a microcystin-enzyme conjugate which binds to the remaining antibodies. After thorough rinsing, the concentration of bound enzyme is measured colorimetrically in an ELISA plate reader. The microcystin concentration is then inversely proportional to the colour intensity (Chorus & Bartram, 1999).

The sensitivity range is 0.5 to 10.0 $ng.mL^{-1}$, with a detection limit of 10 pg in 50 µL per assay. This detection limit (of 0.2 µg/L) is sufficient to detect microcystin in unconcentrated lake water (Falconer, 2005).

Recently developed ELISA kits for the determination of Cylindrospermopsin and Saxitoxin concentrations are currently not widely available yet. It is envisaged that these kits would become more common in the analysis of cyanotoxins, due to the lack of available cyanotoxin standards for HPLC analyses.

Biological bioassays

There are biological detection methods for cyanotoxins that use the bioactivity of the toxins, such as potent hepatotoxicity, neurotoxicity, cytotoxicity, enzymatic activity and immunological interactions. However, for many years, the mouse bioassay alone has been used to determine bloom toxicity. Although this bioassay provides a measure of the total toxicity within a few hours, it is generally not very sensitive (other than at high concentrations) or specific. Considerable research efforts have been made to find suitable alternative methods to the mouse bioassay as a routine monitoring assay for cyanotoxins and many innovative and sensitive methods have become available in recent years (e.g. invertebrate bioassays, algal bioassays and bacterial bioassays). However, no single method is currently available to replace the mouse for the detection of all cyanotoxins using a single assay and further validation and comparison of methods is needed before general recommendations on their application can be given (Chorus & Bartram, 1999).

Analytical methods

Due to the fact that organic compounds consist of certain functional groups, the analytical methods to determine microcystin concentrations make use of their physico-chemical properties such as molecular weight, chromophores and reactivity to other compounds. Physico-chemical methods used for microcystin detection are summarised in **Table 2.1**.

Table 2.1: Physicochemical methods for the detection of microcystins (Chorus & Bartram, 1999)

Method	Cost*			Comments	Reference(s)
	Cap*	Con*	Pers*		
HPLC-PDA	H	M	L	UV Spectra can give tentative identification	Lawton et al., 1994b
LC/MS	VH	M	M/L	A number of different interfaces; mass confirmation; can have PDA	Kondo et al., 1992
TLC	L	L	M	Qualitative, requires standards and further confirmation of toxins.	Harada, 1996.
MMPB	H/VH	M	M	Detection by GC-MS or LC-MS detects total microcystins.	Harada et al., 1996
MALDI	VH	L	M/L	Initially poor but recent developments have improved accuracy.	Erhardt et al., 1997
CE-MS	H	L	M	Requires further development but has future promise.	
NMR	VH	M	M/H	Can characterize cyanotoxins; needs mg quantities and expert interpretation.	Harada, 1996

* Cap = Capital; Con = Consumables; Pers = Personnel; PDA = photodiode array
L = Low; M = Medium; H = High; VH = Very High.

HPLC

The most commonly used analytical system for the analysis of cyanotoxins is HPLC. It has been the most reliable method for the past two decades and remains the reference method against which newer and more sensitive methods are judged (Falconer, 2005). Combined with UV detection, HPLC has been used extensively for the detection of microcystins. However, because this method relies on retention (elution) time for identification, cyanotoxin standards are required [a](Harada, 1996). Detection by UV can be made more specific by using a photodiode array (PDA) UC detector (Lawton et al., 1994a), but it has very limited ability to identify individual microcystins because most microcystins show a similar UV spectrum. Recent advances in detection hardware can now provide high resolution spectra that detect slight variations in chemical composition and can be used in conjunction with advanced spectral matching software. These developments may assist in the identification of microcystins by spectral match data in conjunction with retention times. However, a fundamental problem still exists in the availability of standards. With over 60 microcystins known, it is currently impossible to create a definitive spectral library - a limiting factor when using this method to identify unknown microcystins. Other cyanotoxins like Nodularin, Anatoxin-a, Saxiton and Cylindrospermopsin are even less widely available than Microcystins, especially outside the USA.

The advantage of using HPLC is that there are well-standardized procedures available, and many laboratories undertaking analytical work already possess most of the requisite equipment (Falconer, 2005).

Typical HPLC analysis uses a reverse-phase C18 silica column with separation achieved over a gradient of water and acetonitrile, with both containing 1.05 per cent trifluoroacetic acid (TFA). The gradient has to cover a sufficient range of polarities (e.g. 30 - 70 per cent acetonitrile) to allow the analysis of all microcystins which are known to vary considerably in their polarities. Data is gathered at 238 nm and, where PDA is used, spectral information is collected between 200 and 300 nm. Use of HPLC-PDA can allow tentative identification of microcystins and this method was found to perform very well when over 20 samples were assessed by HPLC and compared with mouse bioassay data. No false negatives were observed and only one false positive was reported, the latter being attributed to a relatively low level of microcystin which failed to cause death in the mouse bioassay (Lawton et al., 1994b).

Other analytical methods

When further confirmation and identification of cyanotoxins is required, more advanced methodology may be necessary.

Liquid chromatography/mass spectrometry (LC/MS) is a very promising method because it enables the simultaneous separation and identification of microcystins in a mixture (Kondo et al., 1992; Edwards et al., 1993; Poon et al., 1993).

For identification of cyanotoxins from very small sample volumes (less than 1 mm^3 freeze-dried material), Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) has been recently developed (Erhardt et al., 1997). This method provides the molecular mass of all the peptides in a sample and thus gives strong indications of the microcystin variants present. Post Source Decay (PSD) spectra may be obtained, these being characteristic of different cyanotoxins. A library of the characteristics of different microcystins is currently being established (Chorus & Bartram, 1999). For rapid qualitative assessment of microcystins and other cyanobacterial peptides, this method is highly promising although quantitative assessment is not yet possible (Chorus & Bartram, 1999).

Different methods provide different and often complementary information, therefore combined use of suitable methods is recommended according to the purpose and type of data required. This is necessary because none of the methods currently available provides all the information that may be required (Chorus & Bartram, 1999).

A newly developed method and the most innovative thus far is the use of molecularly-imprinted polymers for both extraction and concentration of microcystins from dilute solution and use in piezoelectric sensors. The principal is that a synthetic polymer can be formed that has been imprinted with the molecular configuration of the molecule extracted. Thus, molecules of the imprinted shape will adhere in their selective pockets in the membrane, from which they can later be eluted. As a solid-phase extraction technique a concentration of 1000-fold has been achieved. As adherence of the test molecule to the membrane alters the electrical properties, a cell can be constructed with a piezoelectric sensor recording concentration (Chianella et al., 2002; Chianella et al., 2003). Such a technique has obvious potential for monitoring microcystin concentrations online during water treatment once the required sensitivity has been achieved. At present, pre-concentration is needed prior to the sensor for measurement of cyanotoxins in bulk water.

2.3.3.2 **Protein phosphatase inhibition assay (PPI)**

The ability of microcystins to inhibit specifically the catalytic subunits of the serine/threonine phosphatase 1 and 2A is used as basis for the PPI method of detecting microcystins (Mackintosh et al., 1990; Runnegar et al., 1981; Falconer & Yeung, 1992).

The protein phosphatase method employs a target protein phosphatase enzyme, either purified from biological tissue or produced by recombinant *E. coli* carrying the gene for the enzyme. The latest method uses a phosphorylated substrate that directly or indirectly develops colour when the phosphate is split off by the enzyme. The reduction in the liberated phosphate (or resulting colour) in the presence of microcystin is used as the measure for the enzyme inhibition (Heresztyn & Nicholson, 2001).

Questions still arise over inaccuracy in protein phosphatase inhibition assays at low toxin concentrations due to the interference from organic material present in certain samples (Falconer, 2005). This method will not be explored further in the current methods manual, since more research and development thereof is necessary to provide a useful tool in establishing microcystin concentrations in water samples.

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CHAPTER 3

GUIDANCE FOR SAMPLING OF PHYTOPLANKTON AND CYANOBACTERIA

3.1 DESIGN OF PHYTOPLANKTON AND CYANOBACTERIA SAMPLING PROGRAMMES

3.1.1 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 analysed (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).

The following publications are recommended for detailed information on designing sampling programmes:

- Water Quality - Sampling – Part 1: Guidance on the design of sampling programs (SANS 5667-1:1980).
- *Design of networks for monitoring water quality* (Sanders et al., 1987).
- Water Quality - Sampling – Part 4: Guidance on the design of sampling lakes, natural and man-made (SANS 5667-4:1987).
- Water Quality - Sampling – Part 6: Guidance on the design of sampling rivers and streams (SABS ISO 5667-6:1990).
- *Standard Methods for the examination of water and waste water* (APHA, 2001).
- Methods for quantitative assessment of phytoplankton in freshwater, Part 1 (Olrik et al., 1998).
- *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management* (Chorus & Bartram, 1999).
- National eutrophication monitoring program, implementation manual (DWAF, 2002).

3.1.2 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). The objectives of phytoplankton and cyanobacteria monitoring programmes are not generic and may include some of the following:

- Detecting and monitoring (long and short term trends) of algae, cyanobacteria, cyanotoxins and specific taste and odour compounds in water to determine its suitability for drinking water, live stock watering, irrigation and recreational use. Algae and cyanobacteria produce taste and odour compounds (for example: geosmin: trans-1,10-dimethyl-trans-9-decalol; 2-MIB: 2-methylisoborneol) and can clog filters or irrigation equipment. Cyanobacteria can also produce cyanotoxins (for example: anatoxins, saxitoxins, microcystins and nodularin).
- Monitoring the abundance and composition of phytoplankton and cyanobacteria to assess the eutrophication status of water bodies.
- Monitoring the effect of anthropogenic impacts, for example, inter-basin transfers, regulation of river flows and water abstractions.
- Providing ecological data on particular phytoplankton and cyanobacteria groups (Hötzel & Croome, 1998).

3.1.3 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).

3.1.4 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).

3.1.4.1 **Sample site selection: Streams and rivers**

When selecting a sample site the following two aspects are usually considered:

- Selecting the site along the stream or river and within the river basin.
- Selecting the precise location of sampling at the specific sample site (SABS ISO 5667-6:1990; Hötzel & Croome, 1998).

Sampling sites are often selected at a location:

- Upstream and down stream of point source, for example, a treatment plant, weir or tributary.
- Upstream and downstream of a source considered to have an ecological impact, for example, a dam (reservoir) or weir pool.

- At selected intervals along the river or stream to investigate longitudinal distribution of phytoplankton and cyanobacteria (SABS ISO 5667-6:1990; Hötzel & Croome, 1998).

If a site is located downstream of a specific point (pollution) source or river or stream tributary, the site should be selected where complete mixing has occurred (that is vertically, laterally and longitudinally). If the river is well mixed a mid-stream surface sample (0.5 m below the surface can be collected. If the lateral or longitudinal mixing is incomplete, samples at two or more locations across the width of the river should be taken. If vertical mixing is incomplete then depth integrated or depth discrete samples should be taken. This may also be advisable during some low flow conditions or when the weir pools are stratified (SABS ISO 5667-6:1990; Hötzel & Croome, 1998).

3.1.4.2 Sample site selection: Dams (storage reservoirs) and lakes

In dams and lakes phytoplankton and cyanobacteria can show significant horizontal and vertical spatial variation (SANS 5667-4:1987; Olrik et al., 1998). Samples sites should thus be distributed horizontally (see **Figure 3.1**) while in some cases it would be advisable to discrete vertical samples and not necessarily integrated samples. The positioning of sampling sites should be based on a basic hydrodynamic appraisal of the waterbody. In the sections below a basic approach is described.

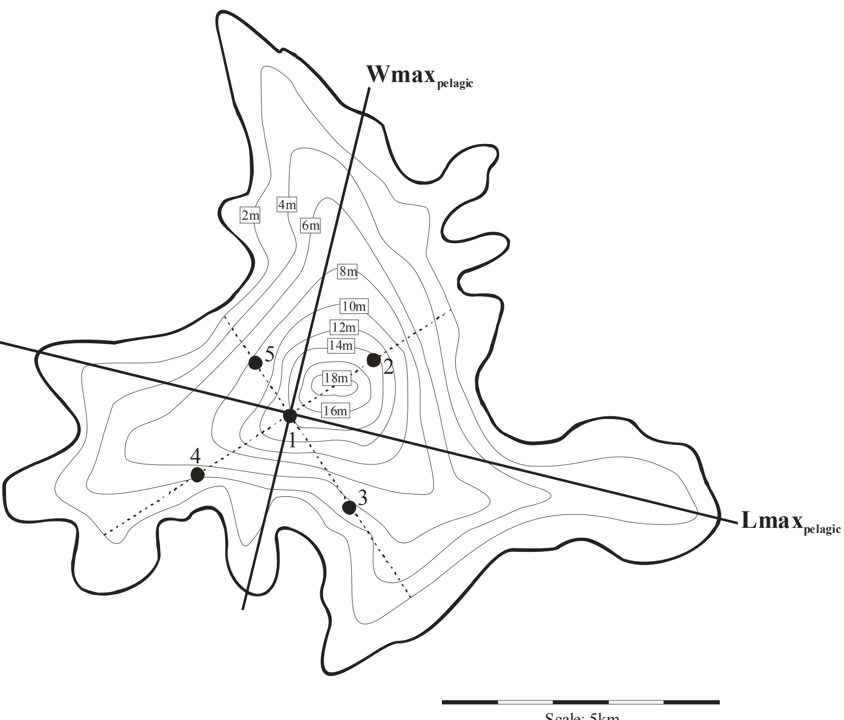

Figure 3.1: Depth profile map of a hypothetical lake showing $Lmax_{pelagic}$ and $Wmax_{pelagic}$ lines and the position of the horizontally located samples sites in relation to these lines (adapted from Olrik et al., 1998).

Horizontal distribution of sample sites

The sampling sites are horizontally distributed across the lake (Olrik et al., 1998).

Example:

Figure 1 presents a hypothetical lake with the following data:

Mean depth	5.5 m
Maximum depth	18.3 m
Total volume	617300 m^3

Step 1: Obtain a depth map with basic morphometric data.

Step 2: Distribute the sites within the area that is surrounded by the two-meter depth curve.

Step 3: Determine the maximum length ($Lmax_{pelagic}$) between the two points on the two-meter depth curve and record it on the depth profile map.

Step 4: Determine the maximum width ($Wmax_{pelagic}$) perpendicular to the maximum length ($Lmax_{pelagic}$).

Step 5: The first sampling site is located at the intersection of the $Lmax_{pelagic}$ and the $Wmax_{pelagic}$ lines. The next four sample sites are placed in the middle of the four sub areas created by the $Lmax_{pelagic}$ and $Wmax_{pelagic}$ lines. Five sampling sites have subsequently been distributed (**Figure 3.1**).

Step 6: If more than 5 sampling sited have to be distributed, the $Wmax_{pelagic}$ line is first placed on the map and the longer of the two subsections of the $Lmax_{pelagic}$ is then divided into two equal lengths divided by the second line parallel to the $Wmax_{pelagic}$ line. No sampling site is located at the intersection of this line and the $Lmax_{pelagic}$ Line. After all the sub areas have been divided, the sampling stations can be distributed (**Figure 3.1**). If the number of sampling sites is an uneven number, a sampling site is not allocated to the smallest area.

Step 7: If a water body has several large sub-basins, each basin can be treated as a single sub-unit and a sampling programme can be designed for each. Alternatively, if the water body is considered to be a single unit, the $Lmax_{pelagic}$ could only be determined for the largest sub-basin.

Step 8: If a water body has several small sub-basins one sampling site should be located in the centre of each (Olrik et al., 1998).

Vertical distribution of samples

In many instances where the phytoplankton and cyanobacteria are not homogeneously distributed throughout the water column, it is advisable to take integrated samples or discrete vertical samples (i.e. individual samples from different depths). If discrete vertical samples are collected, the depth interval (interval thickness) must be determined (see equation one). Further, if it is not possible to collect samples at all the vertical sample sites, the sample sites should cover at least 90% of the volume of water in the lake. The number of samples sites at each depth interval can be determined by correlating the volume of water at each depth interval. These sites are then distributed randomly, for example, using a lottery procedure (Olrik et al., 1998).

Example:

Step 1: Determine the depth interval thickness

Interval thickness = mean depth * empirical factor 0.4
= 5.5 * 0.4
= 2.2

Equation 1

Step 2: Round off interval thickness using the maximum depth of the lake according to **Table 3.1**.

The depth intervals that have been determined thus represent a specific depth sampling site. The maximum depth (Dmax) for the lake (Dmax = 18.2 m) is between 10 and 20 m. The interval thickness value (2.2 m) is rounded off to 2 meter.

If it is not possible to collect samples at all the vertical sample sites proceed to **Step 3.**

Table 3.1: Rounding-off factors for sampling interval thickness for discrete vertical sampling (adapted from Olrik et al., 1998).

Maximum depth of lake or dam (m)	Round-off depth interval thickness to
0-5	Even half-meter intervals
5-10	Even meter intervals
10-20	Even two meter intervals
20-40	Even four meter intervals

Step 3: Determine the volume of water in the different two-meter intervals in the lake and their relative contribution to the total volume of the water in the dam.

Step 4: Determine the volume of water in the different two-meter intervals in the lake and their relative contribution to the total volume of the water in the lake.

Step 5: Determine the depth interval to be covered by the sampling volume.

The sampling programme should include all depth intervals to cover at least 90% of the total lake volume. This is determined by calculating the cumulative relative contribution to the total volume of each depth interval by starting from the surface layer. In the present example more than 90% of the volume is covered by all depth intervals up to and including the 8- 10 m interval. The 8-10 m interval is therefore the deepest interval that will be covered by the sampling programme (**Table 3.2**). Each depth interval thus represents a specific sample site.

Step 6: Determine the distribution of samples sites between the depth intervals.

The volume of the deepest sampling depth interval is given a proportional factor of 1 (in the example the volume of the 8-10 m depth interval – 45062 m^3). The volume of each depth interval above is then divided by the volume at the 8-10 m depth interval (**Table 3.2**). For example, the number of samples in the 2-4 m depth interval is calculated to 3 (that is 137657/45062 = 3.055 = 3).

Step 7: Determine the distribution of sample sites within the lake.

The number of sample sites at the surface layer is determined by the vertical distribution of samples. The sampling sites in the other layers are distributed randomly, for example, making use of a lottery (**Table 3.3**).

Table 3.2: The volume of water in the different two-meter intervals in the lake and their relative contribution to the total volume of the water in the dam (adapted from Olrik et al., 1998).

Depth interval (m)	Volume (m^3)	Relative contribution to the total volume (%)	Cumulative relative contribution (%)	Number of samples in the layer
0-2	239512	38.8	36.8	5
2-4	137657	22.3	59.1	3
4-6	93829	15.2	74.3	2
6-8	60495	9.8	84.1	1
8-10	45062	7.3	93.4	1
10-12	14815	2.4	95.8	-
12-14	12346	2	97.8	-
14-16	7408	1.2	99.0	-
16-18.5	6173	1.0	100	-

Table 3.3: Sampling site distribution for the horizontally and vertically integrated sampling in the hypothetical lake (adapted from Olrik et al., 1998).

Depth Station	Depth Interval Sampling Stations				
	0-2	2-4	4-6	6-8	8-10
1	X	X	X		
2	X		X		
3	X	X			X
4	X				
5	X	X			

3.1.4.3 **Sample site selection: Draw-off points at lakes and rivers**

Sampling sites should cover all the draw-off points (including the draw-off points at various depths) for drinking water purification, irrigation or other agricultural use (SANS 5667-1: 1980; Du Preez and Van Baalen, 2006). This entails taking integrated samples, but preferably collecting discrete vertical samples at discrete vertical sample sites. If the source water is transported for some distance (via a canal or pipe system) from the point of abstraction, or transported and then stored in a holding reservoir (dam), then a sampling site must be located in the reservoir as well as at a point just before the water (inlet water) enters the drinking water treatment works. The same should be considered when water is used for irrigation.

3.1.4.4 **Sample site selection: At drinking water purification plants**

Sample sites should be located at the intakes (see section 3.1.4.3) and where the source water (inlet water) enters the purification plant (that is before purification) as well as where the purified

drinking water leaves the purification plant (a sampling point connected directly to a pumping main) after chlorination or other final disinfection process(es).

3.1.5 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 is it 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 3.2** (DWAF 2002; Du Preez & Van Baalen, 2006).

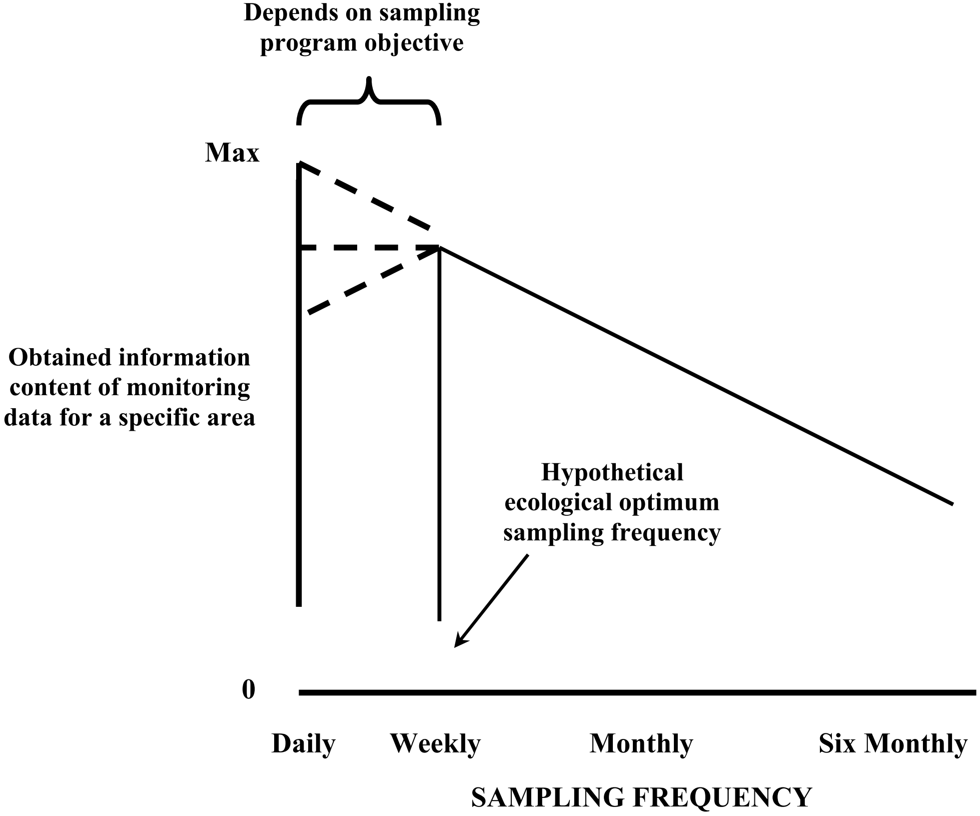

Figure 3.2: The relationship between the obtained information content of the monitoring data (hypothetical) and the sampling frequency (adapted from DWAF, 2002.).

3.1.5.1 **Sampling frequency: Streams and rivers**

To detect rapid changes in the phytoplankton and cyanobacterial composition and abundance, a sampling frequency of one week is generally recommended. If only cyanobacterial bloom formation is monitored, a frequency of days or in some cases daily sampling, is recommended (Hötzel & Croome, 1998). The sampling frequency of a specific monitoring programme(for example, for cyanobacteria) can change as concentrations of the cyanobacteria change in the water column (frequency of sampling: routine monitoring programme < vigilance level monitoring programme < alert level monitoring level programme: Du Preez and & Van Baalen, 2006). This will, however, be governed by factors such as current cell concentrations, river flow, weather conditions and possible upstream releases. In some rivers it is advisable not to reduce the sampling frequency during the winter months as blooms have been observed in low flow conditions and lower turbidity conditions (Hötzel & Croome, 1998).

Cyanotoxin monitoring may be less frequent than the assessment of cyanobacteria numbers, but the frequency will change as concentrations of the toxic cyanobacteria change or the cells start to undergo lyses (frequency of sampling: Vigilance level monitoring programme < Alert level 1 monitoring level programme < Alert level 2 monitoring programme < Alert level 3 monitoring programme: Chorus & Bartram, 1999; Du Preez and & Van Baalen, 2006).

3.1.5.2 **Sampling frequency: Dams (storage reservoirs) and lakes**

To detect the changes in phytoplankton and cyanobacteria composition and abundance, and to provide data that enable the monitoring of fluctuations, a sampling frequency of at least every two weeks is recommended (DWAF, 2002). The frequency of a specific monitoring programme (for example, for cyanobacteria) can change as concentrations of the cyanobacteria change in the water column (frequency of sampling: routine monitoring programme < vigilance level monitoring programme < alert level monitoring level programme: Du Preez & Van Baalen, 2006).

Cyanotoxin monitoring may be less frequent than the assessment of cyanobacteria numbers, but the frequency will change as concentrations of the toxic cyanobacteria change or the cells start to undergo lyses (frequency of sampling: Vigilance level monitoring programme < Alert level 1 monitoring level programme < Alert level 2 monitoring programme < Alert level 3 monitoring programme: Chorus & Bartram, 1999; Du Preez & Van Baalen, 2006).

3.1.5.3 **Sampling frequency: Draw-off points at lakes and rivers**

To detect the changes in the phytoplankton and cyanobacteria composition and abundance, and to provide data that enable the monitoring of fluctuations, a sampling frequency of at least every two weeks is recommended. The frequency of a specific monitoring programme (for example, for cyanobacteria) the sampling frequency can change as concentrations of the cyanobacteria change in the water column (frequency of sampling: Routine monitoring programme - every second week < Vigilance level monitoring programme – once a week frequency of sampling < Alert level 1 monitoring level programme - once a day < Alert level 2 monitoring programme - once a day < Alert level 3 monitoring programme – twice a day: Du Preez and & Van Baalen, 2006).

Cyanotoxin monitoring may be less frequent than the assessment of cyanobacteria numbers, but the frequency will change as concentrations of the toxic cyanobacteria change or the cells start to undergo lyses (frequency of sampling: Vigilance level monitoring programme - no samples < Alert level 1 monitoring level programme - once a week < Alert level 2 monitoring programme – once a day < Alert level 3 monitoring programme - twice a day: Du Preez and & Van Baalen, 2006).

3.1.5.4 **Sampling frequency: At drinking water purification plants**

Sample sites should be located at the intakes (see section 3.1.4.3) and where the source water (inlet water) enters the purification plant (namely, before purification) as well as when the purified drinking water leaves the purification plant, that is at a sampling point (tap connected directly to a pumping main) after chlorination or other final disinfections' process.

To detect the changes in the phytoplankton and cyanobacteria composition and abundance, and to provide data that enable the monitoring (at intakes and at the inlet to the purification plant) of fluctuations, a sampling frequency of at least every two weeks is recommended. The frequency of a specific monitoring programme for cyanobacteria must be changed as the concentrations of the cyanobacteria change in the source water at intakes and the inlet to the purification plant (frequency of sampling: Routine monitoring programme - every second week < Vigilance level monitoring programme – once a week frequency of sampling < Alert level 1 monitoring level programme - once a day < Alert level 2 monitoring programme - once a day < Alert level 3 monitoring programme – twice a day: Du Preez and & Van Baalen, 2006). It is also recommended that the purified water be monitored at the same frequency in order to monitor the possible inefficient removal of cyanobacteria cells.

Cyanotoxin monitoring may be less frequent than the assessment of cyanobacteria numbers, but the frequency will change as concentrations of the toxic cyanobacteria change or the cells start to undergo lyses. The frequency of sampling of source water (at abstraction point and at the inlet to the purification plant) and of the purified drinking water (after chlorination) is recommended. Vigilance level monitoring programme - no samples < Alert level 1 monitoring level programme - once a week < Alert level 2 monitoring programme – once a day < Alert level 3 monitoring programme - twice a day: Du Preez and & Van Baalen, 2006).

3.2 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.

3.3 SAMPLING EQUIPMENT

3.3.1 APPARATUS

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) caring 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).

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 (Olrik 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).

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.

Large plastic ladle or equivalent device

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

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.

Cooler box and ice bricks

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

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).

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.

3.4 **PRESERVATIVES**

3.4.1 SAMPLES FOR 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 analysed 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).

3.4.1.1 **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 5.1.6.1, 5.1.6.2 and 5.1.6.3)

Preparation

Dissolve 100 g potassium iodide (IK) in 1000 mL distilled water. Then dissolve 50 g pure iodine (I_2) in this solution. A few days before use add 100 g glacial acetic acid (96 – 100% CH_3COOH) 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.

Advantages

- Flagellated spp. retain their flagella.

- The Acid Lugol's solution preserves the cells and at the same time increases their specific weight, thereby facilitating sedimentation.

Disadvantages

- Identification of cell may be difficult if they are overstained.
- Silica (diatom frustules) dissolves during long-term storage.
- Samples must be kept in the dark during transportation and storage.
- Samples need to be topped-up with preservatives annually.
- Inhalation of iodine vapours may be harmful to human health. Refer to Material Safety Data Sheets from supplier for updated information.

3.4.1.2 **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 place in the sample bottle cap.

Advantages

- Samples can be stored for several years.
- Samples do not discolour during preservation.

Disadvantages

- The cell shape of flagellates may be distorted and the flagella thrown off and the cell contents bleached out.
- Silica (diatom frustules) dissolves during long-term storage.
- Formalin is a toxic chemical and a health hazard. Preventative measure must be taken to avoid direct contact or exposure to vapours. Refer to Material Safety Data Sheets from supplier for updated information.

3.4.1.3 **Neutralised Glutaraldehyde**

Preparation

Use glutaraldehade (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.

Advantages

- Preserves samples that will be examined under the electron microscope.
- Samples do not discolour during preservation.

Disadvantages

- Only used as a fixative if samples are to be examined under the electron microscope.
- Glutaraldehyde is a toxic chemical and a health hazard. Preventative measure must be taken to avoid direct contact or exposure to vapours. Refer to Material Safety Data Sheets from supplier for updated information.

3.4.2 SAMPLES FOR CHLOROPHYLL DETERMINATION

Samples are usually only cooled and stored in the dark where after they are analysed with 8 h of sampling. If the samples cannot be analysed 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.

3.4.2.1 **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.

Advantages

- Preserved samples that can be kept for a month if sampling conditions do not allow for immediate analysis.

Disadvantages

- Filtration under field conditions may be cumbersome.

3.5 SAMPLING PROCEDURE

3.5.1 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.

3.5.2 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.

3.5.3 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.

3.5.4 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.

3.5.5 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.

3.5.6 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.

3.5.7 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.

3.6 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 summarised in **Table 3.4**.

Table 3.4: Techniques generally suitable for the preservation, storage and transporting of samples

Parameter to be analysed	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) Brown polyethylene bottles (100 mL or 2 L for drinking water.) if not preserved with Lugol's iodine	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. Keep in the dark if clear sample bottles are used.
		Add 0.5 ml Acid Lugol's and 3 mL buffered per 100 mL sample	More than a year stored in the dark		
		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.

<table>
<tr><td rowspan="2">Chlorophyll</td><td rowspan="2">Brown glass bottle
(1 L to 2 L)

Brown polyethylene bottles
(1 L or 2 L)</td><td>Cool to < 8°C and keep in the dark</td><td>8h after sampling and store in dark</td><td>Cool to < 8°C and keep in the dark</td><td rowspan="2">Perform analysis as soon as possible.

Keep in the dark if clear sample bottles are used.</td></tr>
<tr><td>Filtered residue stored in 10 ml analytical grade (95%) ethanol and if possible frozen</td><td>1 month stored in the dark</td><td>Cool to < 8°C, sample bottle wrapped in tinfoil and keep in the dark</td></tr>
<tr><td>Geosmin and 2-MIB</td><td>Brown glass bottle (1 L)

Brown polyethylene bottles(1 L)</td><td>Cool to < 8°C and keep in the dark</td><td>24h after sampling and store in dark</td><td>Cool to < 8°C and keep in the dark</td><td></td></tr>
<tr><td rowspan="2">Cyanotoxins</td><td rowspan="2">Brown glass bottle (1 L)

Brown polyethylene bottles (1 L)</td><td>Cool to < 8°C and keep in the dark</td><td>24h after sampling and store in dark</td><td>Cool to < 8°C and keep in the dark</td><td>Different fractions can be analysed (Total or cell bound or dissolved).</td></tr>
<tr><td>Samples frozen in liquid nitrogen stored in 50 mL polypropylene tubes</td><td>1 year</td><td>Frozen</td><td>Different fractions must be separated before freezing.</td></tr>
</table>

3.7 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 (**Table 3.5**) and the chain of custody sheet (**Table 3.6**) Ensure that the samples that cannot be analysed quickly are stabilized by cooling to below 8°C or preserved as indicated in **Table 3.4**. The quality assurance samples used for transportation, stabilization and storage should be processed in the same way as the samples to be analysed (ISO 5667-14: 1998).

Table 3.5: An example of a field sampling form for samples

<table>
<tr><th colspan="2">FIELD SAMPLING RECORD</th></tr>
<tr><td colspan="2">Project/ Survey Number:</td></tr>
<tr><td colspan="2">Water body name:</td></tr>
<tr><td colspan="2">Water body type: River Lake Tap Other- specify</td></tr>
<tr><td colspan="2">Site Name: Site Code: Site Coordinate:</td></tr>
<tr><td colspan="2">Site Description:</td></tr>
<tr><td>Sampling Date:</td><td>Sampling time:</td></tr>
<tr><td colspan="2">LOCAL CONDITIONS</td></tr>
<tr><td colspan="2">Weather: Raining Overcast Sunshine
General comments:</td></tr>
<tr><td colspan="2">Wind speed: Very strong Strong Mild Calm</td></tr>
<tr><td colspan="2">Wind direction (N, S, W, NW, etc):</td></tr>
<tr><td colspan="2">Algal growth causing discoloration of water:
No Yes If Yes: Floating
Submerge
Scums</td></tr>
<tr><td colspan="2">Water smelling: No Yes Describe</td></tr>
<tr><td colspan="2">FIELD MEASUREMENTS TAKEN</td></tr>
<tr><td colspan="2">Temperature pH Conductivity Dissolved Oxygen
Secchi Transparency Velocity Water Dept (m) Gauge height</td></tr>
<tr><td colspan="2">PARTICULARS OF WATER SAMPLE</td></tr>
<tr><td>Dept taken at (m):</td><td>Method of sampling:</td></tr>
<tr><td colspan="2">Type of sample: Surface Integrated (0 to m)
Discrete Dept sample Discrete Dept sample, intervals (m)</td></tr>
<tr><td colspan="2">Location sample if river: North/South t bank East/Right bank
Mid-stream Not applicable</td></tr>
<tr><td colspan="2">Water samples taken for: Phytoplankton and cyanobacteria ID and enumeration
Chlorophyll Cyanotoxins Geosmin and 2-MIB
Macro/Micro chemical variables Turbidity</td></tr>
<tr><td colspan="2">Additional Comments:</td></tr>
<tr><td colspan="2">Collectors Name (print and sign):</td></tr>
<tr><td>Agency name:</td><td>Phone:</td></tr>
<tr><td colspan="2">Agency address:</td></tr>
</table>

Table 3.6: An example of a chain-of-custody record form for samples received by the laboratory

CHAIN OF CUSTODY RECORD						
Projector/ Survey Number:						
Collecting Agency: Name **Tel Number**			**Address**			
Sampler (print and sign)						
Sample ID Name/Number	**Sample date**	**Type of analysis and number of sample containers**				
		Phytoplankton	**Chlorophyll**	**Cyanotoxins**	**Geosmin & 2-MIB**	**Other-Specify**
DELIVERY SHIPMENT RECORD						
Deliver/Posted to: Name **Tel Number**			**Address**			
Delivery method: Hand Carry			**Mail**	**Courier**		
Relinquished by: Name (print and sign)				**Date:**		**Time:**
Received for the laboratory by: Name (print and sign)				**Date:**		**Time:**

3.8 QUALITY ASSURANCE OF WATER SAMPLING AND HANDLING

Quality control procedures must be implemented that will 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).

3.8.1 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-ionised 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-ionised water samples. This technique can be applied to a sampling programme taking samples for geosmin, 2-MIB and cyanotoxin analysis.

3.8.2 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. An example of a Shewhart control chart is presented in **Figure 7.13**.

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CHAPTER 4

CHLOROPHYLL DETERMINATION IN WATER

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.

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

Table 4.1: 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-*a***
Analysis of total pigment	Analysis of chlorophyll-*a*
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

4.1 CHLOROPHYLL-665 (TOTAL PIGMENTS)

4.1.1 INTRODUCTION

4.1.1.1 **Scope**

The amount of chlorophyll in water is used as an indirect quantitative measure of the phytoplankton biomass.

4.1.1.2 **Definition**

Chlorophyll, in various forms, is bound within the living cells of phytoplankton and other phytoplankton found in surface water. Chlorophyll enables plants and other chlorophyll containing organisms to perform photosynthesis. Total chlorophyll pigment includes the chlorophyll-*a* pigment, phaeophytin-*a* (a degradation product) as well as other pigments in algal particles. Chlorophyll-665 (total pigment) is defined as all pigments in algal particles extracted with methanol with maximum absorbance at 665 nm.

4.1.1.3 **Field of application**

This method is suitable for all types of water, such as tap, rivers, dams, industrial and sewage effluents.

4.1.1.4 **Interferences**

- Inorganic turbidity (>100 NTU) may block the glass fiber filter (GF/C) through which the water containing phytoplankton is filtered. This results in small volumes of water containing low concentrations of phytoplankton being filtered with consequent low absorbance values.
- Multi-cellular phytoplankton in the form of colonies, filaments or flocs is usually not uniformly distributed through a sample even after proper stirring. This may result in larger than expected variance (>10%) between replicates.

4.1.1.5 **Method range**

It is not possible to determine the lowest quantifiable concentration (LQC) for this method due to the lack of formal standards. The method limit of detection (MLD) for this method is 0.0005. The MLD should be determined for individual laboratories, as laboratories vary in capacity and competency (see Section 4.2.11.3 for recommended procedures). In the source water that Rand Water monitors, this method has been used to determine chlorophyll-665 (total pigment) values in the range of 0 (not detectable) to 300 µg/L.

4.1.2 PRINCIPLE

A measured volume of sample is filtered, aided by gentle suction, thereby concentrating the phytoplankton onto a filter paper. Chlorophyll-655 (total pigment) is extracted from the concentrated phytoplankton in a known volume of methanol. After 1 hour extraction in a waterbath, the extract is clarified by centrifugation. The absorbance of the extract at 665 nm (corrected for "background" interferences using the absorbance at 750 nm) is undertaken by using a spectrophotometer. The concentration of the total pigment (µg/L) is then calculated using a formula derived from Sartory (1982) and Steynberg (1986).

4.1.3 WATER QUALITY

4.1.3.1 **Significance of the chlorophyll-655 (total pigment) analysis**

Phytoplankton related water purification and source water problems include:

- Phytoplankton in source water is known to have an effect on the production of potable water. Phytoplankton and their extra-cellular products interfere with the physico-chemical water purification processes.
- Phytoplankton passes through purification systems resulting in the production of aesthetically unacceptable water quality (taste, odour and colour).
- Phytoplankton not only produce neuro- and hepatotoxins that could be detrimental to consumer's health but also algal products which may act as trihalomethane precursors and a source of carbon for microbiological and other heterotrophic growth.

- Algal blooms in source water have a range of negative impacts on the aquatic environment, for example tastes, odours, deoxygenated bottom waters with associated chemical effects, fish mortality, loss of biodiversity and loss of property values, etc.

To be able to manage phytoplankton related water quality problems, routine monitoring of phytoplankton concentrations in source water is necessary. Furthermore, the routine monitoring of chlorophyll-665 (total pigment) is a good tool to access the effectiveness of the treatment process and to ensure compliance with set potable water guidelines.

4.1.3.2 **Water quality guideline**

Guidelines as set out in **Table 4.2** can be used for source and potable water (Rand Water guidelines for chlorophyll-665).

Table 4.2: Water quality guidelines for chlorophyll-665 in source and drinking water (Rand Water guidelines).

Quality variable	Measuring units	Source Water		Potable Water		
		Recommended maximum limit	Maximum allowable limit	Recommended limit	Maximum allowable limit	Crisis limit
Chlorophyll-665	µg/L	0 – 15	>30	1	5	7

4.1.4 APPARATUS, MATERIALS AND REAGENTS

4.1.4.1 **Instruments and equipment**

- Centrifuge
- Filtering apparatus (Refer to **Figure 4.1**)
- Printer (optional)
- Spectrophotometer
- Uninterrupted power supply
- Vacuum pump
- Bottle top dispenser or 10 mL pipette
- Balance
- Vortex shaker
- Refrigerator
- Waterbath that maintains a stable temperature at 60ºC. Temperature is checked against a certified thermometer or one of equivalent accuracy.
- Thermostat

Figure 4.1: Filtering apparatus.

4.1.4.2 **Glassware**

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

4.1.4.3 **Other materials**

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

4.1.4.4 **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.

4.1.5 DISPOSAL OF SAMPLES AND REAGENTS

Samples are disposed via the drainage system except for toxic effluents that are disposed as hazardous waste according to appropriate procedures.

4.1.6 PROCEDURE

4.1.6.1 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.

4.1.6.2 Remove the filter and the entrapped phytoplankton without disturbing the phytoplankton or tearing the filter. Gently roll the filter without applying pressure.

4.1.6.3 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.

4.1.6.4 Place the test tubes in a waterbath at 60ºC for ± 1 hour.

4.1.6.5 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.

4.1.6.6 Centrifuge the extract for ± 5 minutes at ± 4800 rpm (to clarify the extract). Ensure the test tubes in the baskets are balanced.

4.1.6.7 Read the absorbance, using the spectrophotometer at 665 nm and 750 nm wavelengths.

4.1.6.8 The absorbance reading taken at 750 nm is subtracted from the absorbance reading taken at 665 nm and the result is multiplied by a factor (see Section 4.1.8).

4.1.7 SAFETY PRECAUTIONS

4.1.7.1 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.

4.1.7.2 Clothing

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

4.1.7.2 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.

4.1.8 CALCULATIONS AND EXPRESSION OF RESULTS

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

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

Where E = Chlorophyll (phaeophytin)
A = Absorption coefficient of 0.0133
A_{665} = Absorbance at 665 nm
A_{750} = Absorbance at 750 nm
Ve = Volume of solvent (mL)
Vm = Volume of sample (mL)
R = Path length of cuvette (cm)
× = 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.

4.1.9 RECORDS AND DATA KEEPING

According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

4.1.10 QUALITY ASSURANCE

4.1.10.1 **General**

- Ensure that the "blank" reading of the spectrophotometer is within an acceptable range *i.e.* 0.0000 ± 0.0005 before performing any analytical work.
- Ensure that the reagent water does not contain detectable concentrations of chlorophyll by conducting a monthly test using the chlorophyll-665 (total pigment) method.
- Ensure all instruments and equipment used is in working condition and serviced and calibrated regularly.
- Ensure that analysts performing analyses have a proven competency record.

4.1.10.2 **Precision and accuracy**

- The percentage coefficient of variance between duplicate samples should not exceed 5% except for conditions described in section 4.2.11.1.
- Participate in the chlorophyll-655 (total pigment) proficiency testing scheme.

4.1.10.3 **Maintenance and service**

All instruments (vacuum pump, vacuum gauge, spectrophotometer, printer, centrifuge, balance) used should be serviced regularly as appropriate (e.g. centrifuge serviced once a year, vacuum pump serviced monthly).

4.1.10.4 **Calibration**

- **Spectrophotometer**

  The spectrophotometer should be calibrated daily (use the instrument manual) to ensure that it is in proper working condition every time a sample is analysed.

  Contact the supplier of the spectrophotometer immediately should any of the calibration tests fail to request a service.

- **Balance**

  The balance should be calibrated regularly (confirm an appropriate interval with the instrument supplier).

  Perform a quality check on the balance, before use, by weighing a set of reference weights.

4.1.10.5 **Verification**

The purpose of this verification is twofold:

- To verify the repeatability and difference during dispensing by different analysts
- To detect a change in the volume dispensed

Bottle top dispenser/Suitable alternative pipette

- Calibrate the balance according to manual specifications.
- Use the 5 decimal range.
- Weigh a poly-top bottle, or equivalent, and then zero the balance.
- Add 10 mL methanol to the bottle using the dispenser and write down the weight.
- Repeat the measurement of 10 mL of ethanol ten times.
- Calculate the statistics on these measurements.
- The measurements' mean should be ± 7.9 g (10 mL methanol).
- The coefficient of variance should not exceed 1%.
- Ensure verification is carried out on a regular pre-determined schedule (e.g. monthly).

4.1.11 TYPICAL VALIDATIONS FOR THE METHOD

(Refer to section 4.2.11 under Chlorophyll-*a* method)

4.2 **CHLOROPHYLL-*a***

4.2.1 INTRODUCTION

4.2.1.1 **Scope**

The chlorophyll-*a* method is used as an indirect quantitative indication of algal biomass in water.

4.2.1.2 **Definition**

Chlorophyll-*a* is the pigment that gives phytoplankton their green colour and is the major agent in the process of photosynthesis.

4.2.1.3 **Field of application**

This method is suitable for all types of water, such as tap, rivers, dams, industrial and sewage effluents.

4.2.1.4 **Interferences**

- Inorganic turbidity (>100 NTU) may block the glass fiber filter (GF/C) through which the water containing phytoplankton is filtered. This results in small volumes of water, containing low concentrations of phytoplankton being filtered, with consequent low absorbance values.
- Multi-cellular phytoplankton in the form of colonies, filaments or flocs is usually not uniformly distributed through a sample even after proper stirring. This may result in a larger than expected variance (>10%) between replicates (Du Preez & Guglielmi, 1998).
- Dissolved substances absorbing at the same wavelength.

4.2.1.5 **Method range**

The Lowest Quantifiable concentration (LQC) for the chlorophyll-*a* method is 2 µg/L and any results below are less accurate. The Method Limit of Detection (MLD) for this method is set at 0.0005 (for chlorophyll total pigment). The volume of water filtered is dependent on the algal density. In the source water this method can be used to determine chlorophyll-*a* values in the range of 0 (not detectable) to greater than 1023 µg/L as dilutions dependant.

4.2.2 PRINCIPLE

A measured volume of sample is filtered, aided by gentle suction, thereby concentrating the phytoplankton. Chlorophyll-*a* is extracted from the concentrated phytoplankton in a known volume of ethanol and the test tube is placed in a waterbath at 78 ºC for 5 minutes

and then in the dark. After 24 ± 7 hours extraction in the dark, the extract is clarified by centrifugation. The absorbance of the extract at 665 nm (corrected for "background" interferences using the absorbance at 750 nm) is undertaken by using a spectrophotometer. The concentration of chlorophyll-*a* (µg/l) is then calculated using a formula (Sartory, 1982).

4.2.3 WATER QUALITY

4.2.3.1 Significance of the chlorophyll-*a* analysis

Phytoplankton related water purification and source water problems include:

- Phytoplankton in source water is known to have an effect on the production of potable water. Phytoplankton and their extra-cellular products interfere with physico-chemical water purification processes.
- Phytoplankton passes through purification systems resulting in water of aesthetically unacceptable quality being produced (taste, odour and colour).
- Phytoplankton not only produce neuro- and hepatotoxins that could be detrimental to the consumer's health, but algal products may also act as trihalomethane precursors and a source of carbon for microbiological and other heterotrophic growth.
- Algal blooms in source water has a range of negative impacts on the aquatic environment for example tastes, odours, deoxygenated bottom waters with associated chemical effects, fish mortality, loss of biodiversity, loss of property values, etc.)

To be able to manage phytoplankton-related water quality problems, routine monitoring of phytoplankton concentrations in source water is necessary. Furthermore, the routine monitoring of chlorophyll-665 (total pigment) is a good tool to monitor the effectiveness of the treatment process and to ensure compliance to set potable water guidelines.

4.2.3.2 Water quality guideline

Rand Water adopted the Department of Water Affairs and Forestry's (DWAF, 1996 a & b) guidelines for recreational and domestic use for the compliance monitoring of source water. The DWAF (1996 a) guidelines are tabled below:

Table 4.3: DWAF (1996a) guidelines for recreational and domestic water quality

Chlorophyll-*a* (µg/L)	**Target water quality range**	**Crisis limit**
Recreational	0 – 15	>30
Domestic	0 – 1	>10

4.2.4 APPARATUS, MATERIALS AND REAGENTS

4.2.4.1 Instruments and equipment

- Centrifuge
- Filtering apparatus (refer to **Figure 4.1**)
- Micropipette
- Tecnomara pipetboy (or equivalent)
- Printer
- Spectrophotometer
- Uninterruptible power supply
- Vacuum pump
- Bottle top dispenser or equivalent pipette
- Balance
- Vortex shaker
- Refrigerator
- Waterbath

4.2.4.2 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

4.2.4.3 Other materials

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

4.2.4.4 Reagents

- Ethanol (95%) - AnalR grade - pro analisi
- 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.

4.2.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

Samples are disposed via the drainage system except for toxic effluents that are disposed as hazardous waste according to appropriate procedures.

4.2.6 PROCEDURE

4.2.6.1 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.

4.2.6.2 Remove the filter and the entrapped phytoplankton without disturbing the phytoplankton or tearing the filter. Gently roll the filter without applying pressure.

4.2.6.3 Place 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.

4.2.6.4 Place test tubes in the waterbath at 78 ± 2 ºC for 5 minutes prior to placing in the dark at room temperature for 24 ± 7 hours.

4.2.6.5 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.

4.2.6.6 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.

4.2.6.7 Carefully decant the supernatant into marked test tubes.

4.2.6.8 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.

4.2.6.9 Read the absorbency of the remaining supernatant, using the spectrophotometer at 665 nm and 750 nm wavelengths.

4.2.6.10 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*.

4.2.6.11 Read the acidified sample as for point 4.2.6.9.

4.2.6.12 The absorbency values obtained in 4.2.6.9 and 4.2.6.11 are used to calculate the chlorophyll-*a* concentration (see 4.2.6.10).

4.2.7 SAFETY PRECAUTIONS

4.2.7.1 **Hazard warning**

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

4.2.7.2 **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.

4.2.7.3 **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.

4.2.7.4 **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 of the acid, increasing the contact surface and thus increasing the severity of the reaction.
- Wear acid-proof gloves when handling acids.
- Wear protective shoes.

4.2.8 CALCULATION AND EXPRESSION OF RESULTS

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

$$\text{Chl}a\ (\mu g/L) = \frac{[(A_{665} - A_{750}) - (A_{665a} - A_{750a})] \times 28.66 \times Ve}{Vm}$$

Where A_{665} = Absorbance at 665 nm before acidification

A_{750} = Absorbance at 750 nm before acidification

A_{665a} = Absorbance at 665 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)

Ve = Volume of ethanol used for extraction in mL (usually 10 mL)

Vm	=	Volume of sample filtered in mL.
×	=	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.

4.2.9 RECORDS AND DATA KEEPING

According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

4.2.10 QUALITY ASSURANCE

4.2.10.1 **General**

- Ensure the "blank" reading of the spectrophotometer is within an acceptable range *i.e.* 0.0000 ± 0.0005 before performing any analytical work.
- Ensure the reagent water does not contain detectable concentrations of chlorophyll by conducting a monthly test using the chlorophyll-665 (total pigment) method.
- All instruments and equipment used are in working condition and serviced and calibrated regularly.
- Analysts performing analysis have a proven competency record.

4.2.10.2 **Precision and accuracy**

- The coefficient of variance between duplicate samples should not exceed 5% except for conditions described in 4.2.11.1.
- Participate in chlorophyll-655 (total pigment) proficiency testing scheme.

4.2.10.3 **Maintenance and service**

All instruments (vacuum pump, vacuum gauge, spectrophotometer, printer, centrifuge, balance) used should be serviced regularly as appropriate (e.g. centrifuge serviced once a year, vacuum pump serviced monthly).

4.2.10.4 **Calibration**

- **Spectrophotometer**
  Spectrophotometer should be calibrated regularly (using instrument manual) to ensure that it is in proper working condition every time a sample is analysed.
  Contact the supplier of the spectrophotometer immediately should any of the calibration tests fail to request a service.

- **Balance**
  The balance should be calibrated regularly (confirm an appropriate interval with instrument supplier).
  Perform a quality check on the balance before use by weighing a set of reference weights.

4.2.10.5 **Verification**

The purpose of the verification is twofold:

- To verify the repeatability and difference during dispensing by different analysts
- To detect change in volume dispensed.
- Bottle top dispenser/suitable alternative pipette verification:
- Calibrate balance according to manual specifications.
- Use the 5 decimal range.
- Weigh a poly-top bottle or equivalent and then zero the balance.
- Add 10 mL methanol to the bottle using the dispenser and write down the weight.
- Repeat weighing of 10 mL of methanol ten times.
- Calculate the statistics on these measurements.
- The measurements mean should be ± 7.9 g (10 mL methanol).
- The coefficient of variance should not exceed 1%.
- Verification carried out on a regular pre-determined schedule (e.g. monthly).

4.2.11 TYPICAL VALIDATIONS FOR CHLOROPHYLL METHODS

To establish the chlorophyll methods in a laboratory it is necessary to perform some validations. Validation of certain aspects of a method is necessary for individual laboratories as different instruments and pipettes are used, different analysts are performing analysis and laboratories have different environments. Therefore analyst sensitive, instrument sensitive and environment sensitive aspects of methods should be validated in individual laboratories. These aspects may include:

- the percentage uncertainty of a result
- tolerance limits for pipettes used
- limits for variance between duplicate samples

- limits for variance between different analysts
- method limit of detection
- acceptable range for a blank result
- calibration criteria for a spectrophotometer

Should any section of a method be validated it is important to have a documented procedure that will be followed, traceability of all results, documented report on the validation that sets out the procedures and details the results obtained with proper and appropriate statistical analysis.

The following sections will provide guidance on procedures that can be followed when validating different aspects of the chlorophyll methods.

4.2.11.1 **Permissible difference between chlorophyll-*a* duplicate results**

AIM
Determining the permissible difference between duplicate chlorophyll-*a* results.

PROCEDURE
Samples (ranging in chlorophyll concentration from 3 to 150 µg/L) from various sampling sites (list sites) for the period 01 January to 30 July were analysed in duplicate with the documented chlorophyll-*a* method used in the laboratory by one (or more than one) analyst.
The results were grouped by concentration as follows: 3-5; 6-10; 11-20; 21-30; 31-40; 41-50; >50 µg/L. Thereafter the difference between the duplicates was calculated.

Basic statistical analysis and graphical presentation of the data was used to indicate the differences between duplicate results.

RESULTS AND DISCUSSION
In this section details should be given about the results, the statistics and how much the duplicates varied at the different chlorophyll-*a* concentrations. Results should be portrayed in a table and graphically illustrated in a appropriate graph. All raw data should be included in an appendix.

Table 4.4: Example of a results table containing results and statistics

Mean chl-*a* groupings	Number of duplicate differences	Min	Max	95 percentile	Algal genera	Growth form
6 - 10	213	0.01	5.20	2.10	*Chlamydomonas* sp Centric diatoms *Aulacoseira* sp	Single Single Filament
21 – 30	368	0.06	7.20	3.81	*Microcystis* sp *Chlamydomonas* sp Centric diatoms *Aulacoseira* sp	Colony Single Single Filament

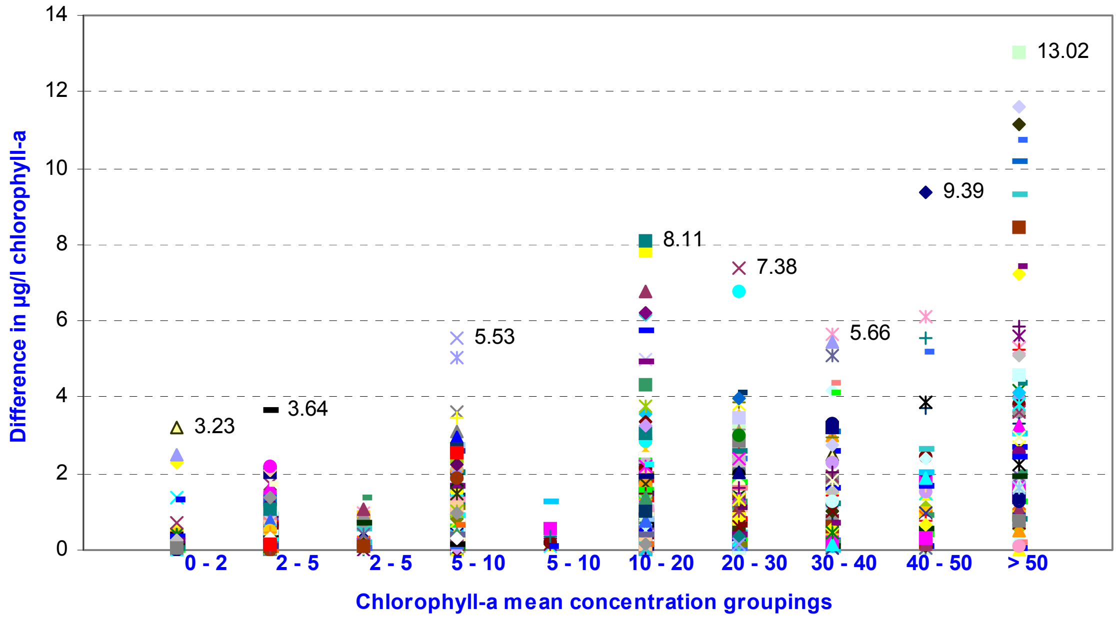

Figure: 4.2: Example of a graph to illustrate duplicate result differences of the different concentration groupings from various sampling sites.

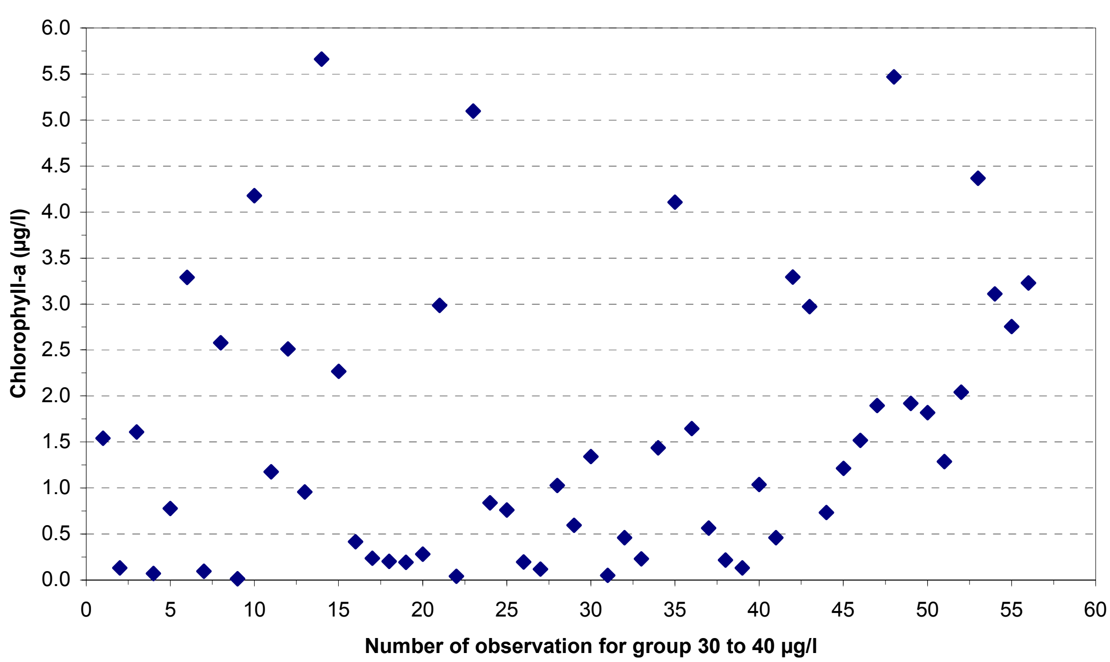

Figure 4.3: Example of a graph illustrating number of observations for the group 30 to 40 µg/L chlorophyll-*a*.

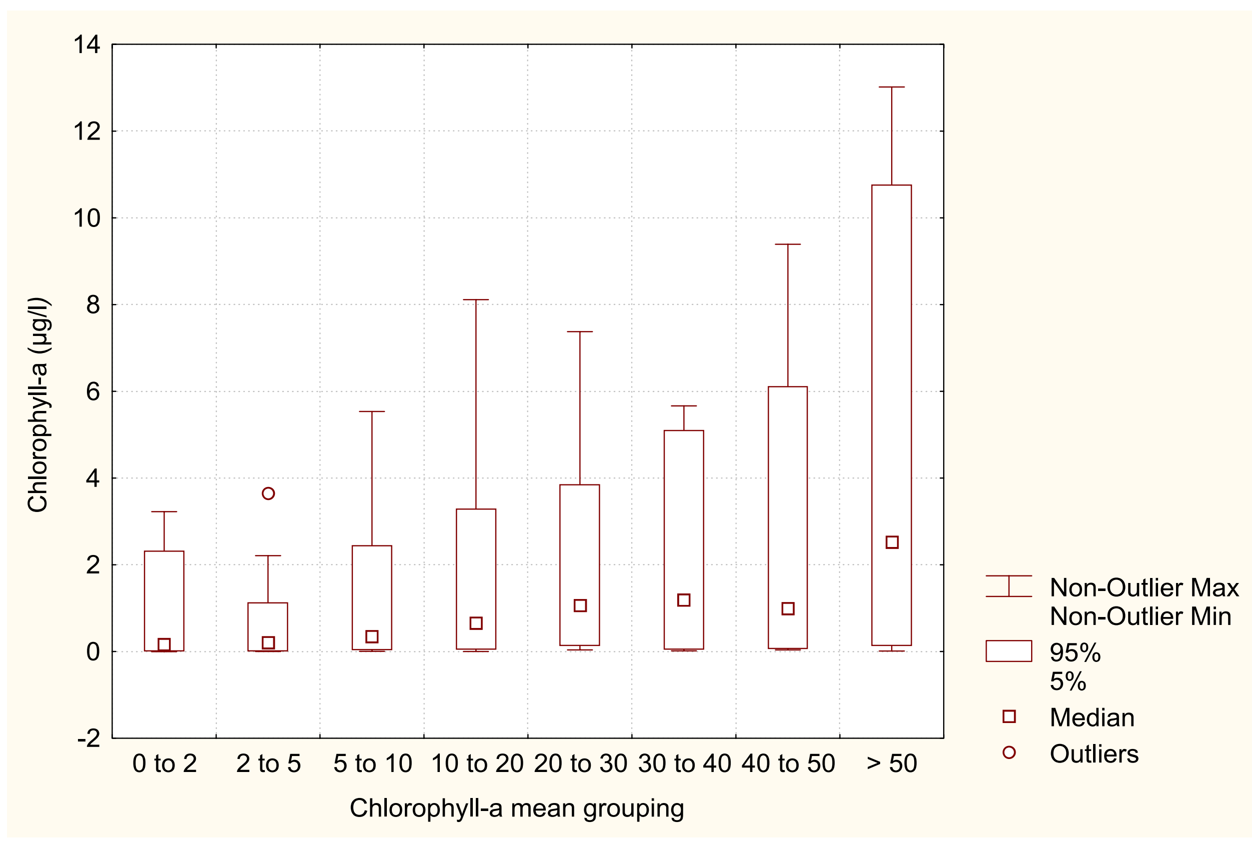

Figure 4.4: Example of a Box and Whiskers plot for all the different chlorophyll groups

CONCLUSION

The conclusion should summarise the experiment findings on the variance between duplicate analyses.

RECOMMENDATION

The recommendation should stipulate very clearly what the acceptable variance between duplicate chlorophyll-*a* concentrations is permissible when analysing samples in the laboratory. This recommendation must then be included in the method.

4.2.11.2 Determination of the tolerance limits for the dispenser pipette used in chlorophyll analysis.

AIM

To determine the tolerance limits for the dispenser pipette that is used in the chlorophyll methods for 10 mL ethanol and methanol dispensing.

PROCEDURE

Ethanol and methanol (10 mL) was respectively weighed 10 times by three analysts on ten different days described in the methods above. The mean, standard deviation and percentage coefficient of variance were calculated for every analyst on each day of weighing. The overall mean, standard deviation and percentage coefficient of variance were also calculated.

RESULTS AND DISCUSSION

In this section details should be given about the results, the statistics and variation there was between analysts and for the pipette. Results should be portrayed in a Table and graphically illustrated in appropriate graphs. A control chart must also be made which shows the upper and lower tolerance limit for the pipette. All raw data should be included in an appendix.

Table 4.5: Example of a table for results and statistics

Number	Mean	SD	% COV	Analyst
10	9.98	0.00108	1.20	AB
10	9.25	0.00109	1.21	SD
10	9.61	0.00021	0.67	LA
10	9.26	0.00042	0.52	AB
10	9.81	0.00101	1.11	SD
10	9.98	0.00062	0.68	LA

Table 4.6: Example of overall statistics

Number	Mean	SD	%COV
280	9.76	0.00087	0.75

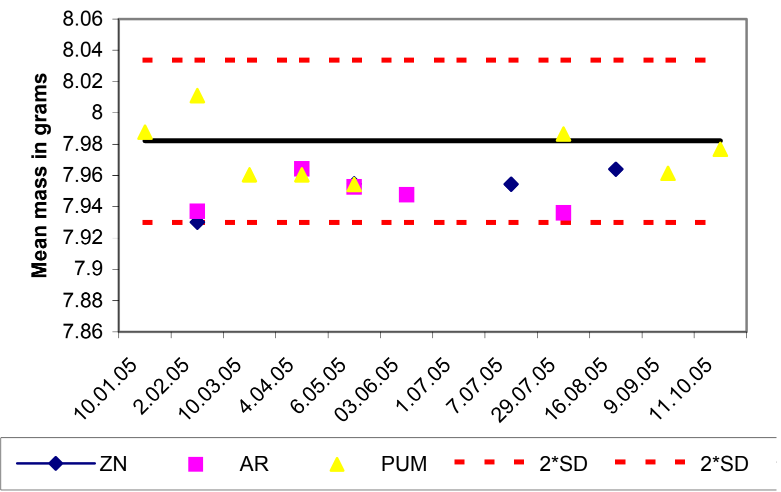

Figure 4.5: Example of a control chart illustrating tolerance limits for the dispenser pipette used for methanol dispensing.

CONCLUSION

The conclusion should summarise the experiment findings.

RECOMMENDATION

The recommendation should stipulate very clearly what the acceptable tolerance limits are for the pipette at 10 mL

4.2.11.3 **Estimation of uncertainty and method limit of detection (MLD) for the chlorophyll method**

AIM

To calculate the uncertainty of measurement and the MLD for the chlorophyll method

PROCEDURE

Chlorophyll data from 100 samples (ranging from low to high concentrations) analysed from 1 June 2005 to 21 May 2006 was used to calculate the uncertainty of measurement. The following formula was used:

Uncertainty	=	(2 x std dev of sample data) / mean of data) x 100
	=	x %

To determine the MLD blank results were used from all the samples analysed above. The MLD was calculated as follows:

MLD	=	mean value of blank sample + ($n \times$ std dev of replicates)

Document the Results, Discussion, Conclusion and Recommendation as stipulated in the above sections.

4.2.11.4 **Inter-analyst comparisons**

Analyst competency should be evaluated quarterly to ensure the reliability of results. New analysts should also be proven competent before allowed to analyse routine samples or samples for clients.

Analyst competency can be evaluated by instructing analysts (at least two) to analyse five replicates each of the same source water sample by using the documented chlorophyll method.

Calculate the chlorophyll concentration as stipulated in the method. Use any computer package to calculate the statistics (number of analysts, average, SD, % COV, minimum, maximum, minimum-maximum, difference between average results of the different analysts).

Analysts are deemed competent if all comply with the following:

- Each analyst has a less than five percent coefficient of variance.
- The difference in the average chlorophyll results between the analysts does not exceed 3 µg/L

Should an analyst be deemed incompetent by the results obtained, the analyst should not be used for routine analysis until he/she is deemed competent.

Chlorophyll-665 (Total Pigment) Inter-analyst Competency

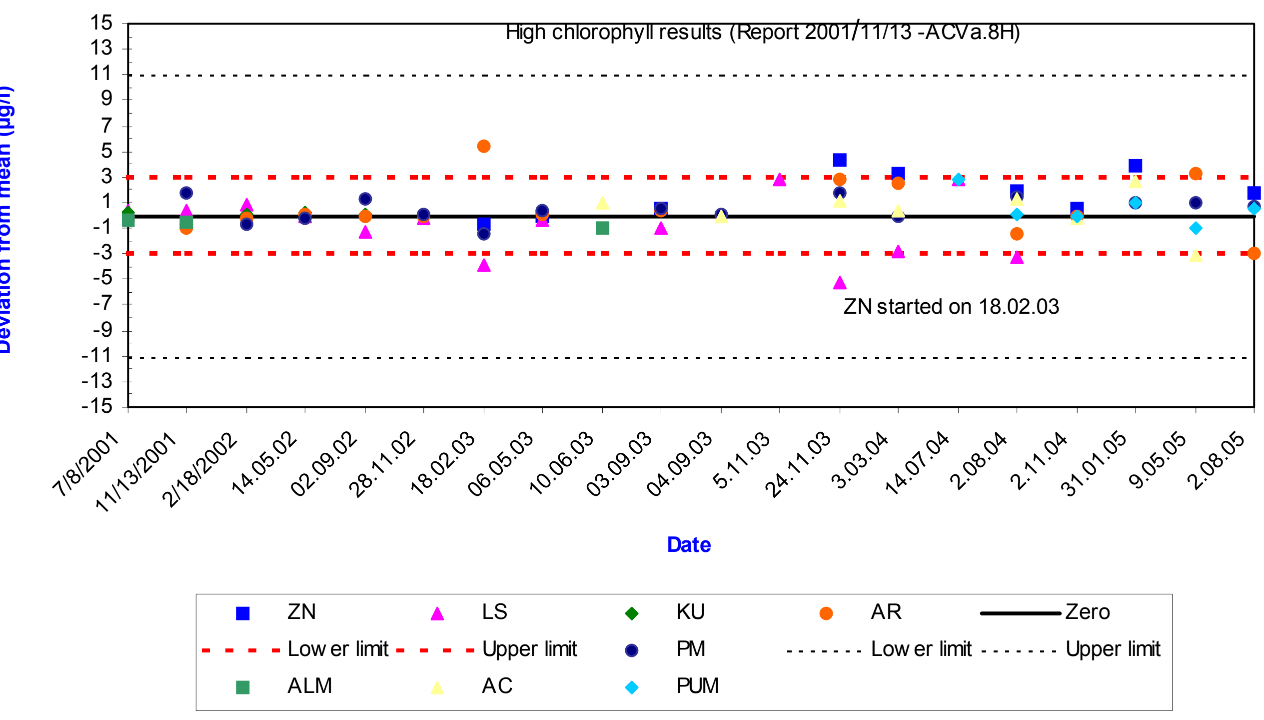

Figure 4.6: Example of a control chart for inter-analyst comparisons

4.3 SUMMARY

The determination of chlorophylls (either chlorophyll-*a* or chlorophyll-665), are easy and relatively reliable methods to determine total phytoplankton biomass in either raw or potable water. They can be used as tools to determine the effectiveness of certain unit processes in water purification. The apparatus needed for chlorophyll determinations is relatively cheap and easy to operate.

The determination of chlorophyll-*a* is a very common method for the indication of total phytoplankton biomass in raw water samples. 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.

4.4 REFERENCES

Du Preez, H.H., & Guglielmi, M.M., 1998. *Quality control of duplicate chlorophyll-a data.* Internal Report: 98/11/17-ACVa.2 (H) to Rand Water.

DWAF, 1996a. *South African Water Quality Guidelines Volume 2 – Recreational Use* (2nd Edition).

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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.

CHAPTER 5

PHYTOPLANKTON AND CYANOBACTERIA IDENTIFICATION AND ENUMERATION

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. haemacytometer. The sample is then analysed by using a compound microscope and usually by making use of phase contrast.

DECISION WHETHER TO USE THE SEDIMENTATION OR MEMBRANE FILTRATION TECHNIQUES

Table 5.1: 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 sedimentation chamber) / compound when using haemacytometer	Inverted microscope (using sedimentation chamber) / compound when using haemacytometer	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

Table 5.1 (cont): Characteristics of the sedimentation and membrane filtration techniques

Sedimentation technique - gravity -	Sedimentation technique - centrifugation -	Membrane filtration technique
Algal cells not deformed	Algal cells not deformed	If filtering not done properly, algae can be difficult to recognise
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

5.1 PHYTOPLANKTON IDENTIFICATION AND ENUMERATION METHOD 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 5.2), 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.

5.1.1 INTRODUCTION

5.1.1.1 Scope

Together with benthic phytoplankton and macrophytes, phytoplankton constitutes the primary producers in aquatic ecosystems. The species composition of phytoplankton can be used as an indicator of water quality. Changes in species composition may indicate changes in water quality. The study of the phytoplankton composition in samples taken from different stages of the water purification process may indicate inefficiencies in the unit processes.

5.1.1.2 Definition

The term phytoplankton encompasses all suspended and free-living micro algae in a waterbody belonging to all taxonomic algal groups and including the cyanobacteria.

5.1.1.3 **Field of application**

This method is suitable for all types of waters with oligotrophic, mesotrophic or eutrophic nutrient status and any morphological character (river, dam, lake, underground water and potable water).

5.1.1.4 **Interferences**

- The presence of colloidal, suspended and organic matter in the water may obscure phytoplankton from view by sedimenting on top of phytoplankton.
- High phytoplankton biomass concentrations in the sample can lead to cells obscuring one another.
- Phytoplankton may have a non-random distribution on the chamber floor due to natural "patchy" distribution of phytoplankton in the water column.
- Multi-cellular phytoplankton in the form of colonies, filaments or loosely aggregated "flocs" is usually not uniformly distributed through a sample even after proper stirring. This may result in larger than expected variance between replicates.
- A low phytoplankton biomass (<1000 cells/mL) may enhance the variability between replicate analyses of the same sample, as species distributions are rare and non-uniform.

5.1.1.5 **Method range**

It is not possible to determine the method limit of detection (MLD) for this method, as the phytoplankton community structure and biomass are part of a dynamic system and cannot be limited.

The lowest quantifiable concentration (LQC) for this method is 34 cells/mL if 60 fields are analysed and 3 mL sedimented. The LQC will be lower when more than 60 fields are analysed.

5.1.2 PRINCIPLE

A water sample is fixed to immobilize all phytoplankton species and to preserve the sample from decomposition and algal growth. The sample is then pressurised to deflate the gas vacuoles of some cyanobacterial species. A known volume of sample is pipetted into a marked sedimentation chamber, in which all of the phytoplankton present is sedimented by centrifugation of the water sample. The phytoplankton species are identified and enumerated using an inverted light microscope and standard phytoplankton keys. Concentrations of phytoplankton are expressed as cells/mL.

5.1.3 WATER QUALITY

Wherever conditions of temperature, light and nutrient status are conducive, surface water may host increased growth of phytoplankton. The occurrence of high concentrations of phytoplankton is usually associated with nutrient enrichment of aquatic systems (a process called eutrophication). Several of South Africa's fresh water impoundments, used for potable water production, are rated as eutrophic – causing large-scale, algal-related problems in the South African water purification industry.

5.1.3.1 Significance of phytoplankton identification and enumeration

Phytoplankton in source water, known to be sensitive indicators of water quality, have an effect on the production of potable water, the aesthetic aspect of recreational waters and consumer health, because:

- Phytoplankton and their cellular products interfere with the physical and/or chemical water purification processes.
- Phytoplankton is able to pass through purification processes resulting in water of aesthetically unacceptable quality (taste, odour and colour).
- Some phytoplankton species have the ability to produce substances that can be detrimental to the health of consumers and can be a source of carbon for bacteriological growth.
- Excessive growth of phytoplankton in source water (water bloom) can create aesthetically unacceptable recreational and potable water and may pose a health risk to consumers (taste, odour, scum and toxin).

Advantages of phytoplankton monitoring include:

- Detecting the presence of and examining the short-term trends in the growth of phytoplankton species in order to determine the suitability of a particular water for drinking and recreational use.
- Providing data to determine long-term trends in phytoplankton composition within a particular waterbody. This information can be used to assess, for example, the effect of sewage discharge and agricultural run-off containing fertilizers and harmful chemicals.
- Monitoring the effects of management measures such as river regulation, inter-basin transfers and water abstractions within a particular system.

5.1.4 APPARATUS, MATERIALS AND REAGENTS

5.1.4.1 **Instrument and equipment**

- Homogeniser, 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 homogeniser is not used, it is important to count more fields or strips.
- Inverted light microscope (when using a sedimentation chamber; refer to **Figure 5.2**); compound light microscope (when using counting chambers such as a haemacytometer, refer to **Figure 5.3**).

Figure 5.1 Homogeniser with variable speed

Figure 5.2: Inverted light microscope.

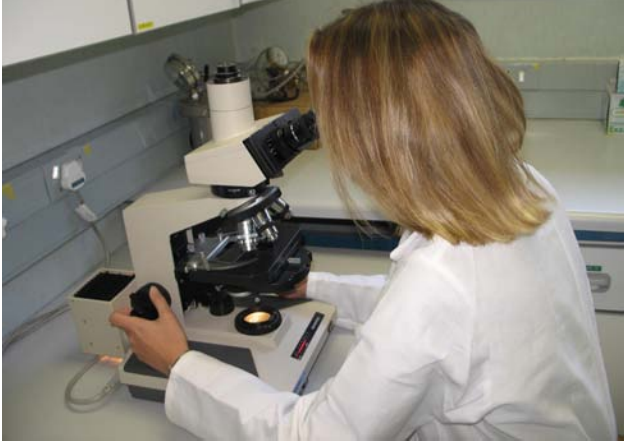


Figure 5.3: Compound light microscope.

- Centrifuge where the buckets can swing out 90º
- 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 5.4(a)**)

Figure 5.4(a): Deflation instrument (mechanical hammer), used to disrupt the gas vacuoles of the cyanobacteria, in order for cells to settle to the bottom of the counting chamber.

5.1.4.2 **Glassware**

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

5.1.4.3 **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

5.1.4.4 **Reagents**

- Formaldehyde solution / Lugol's acidic iodine solution (refer to Chapter 3 for the preparation of solutions).
- Ethanol (95%).
- Reagent water - water that has been filtered by reverse osmosis.

5.1.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

- All samples fixed with formaldehyde or with Lugol's solution, should be disposed of via the waste disposal system, where waste is picked up by a certified waste disposal company. It is important not to mix samples fixed with formaldehyde and samples fixed with Lugol's.
- All contaminated material such as latex gloves, pipette tips and used paper towels, should be disposed of via the waste disposal system.

5.1.6 PROCEDURE

5.1.6.1 **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 5.2: 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 × dilution factor) and then use the made up dilution to dilute further, using **Table 5.2** above. Example: dilute sample two times and then select from Table e.g. 10 times dilution i.e. (2 × dilution factor) × (10 × dilution factor) = 50 × 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 (± 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 homogeniser 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.

5.1.6.2 **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 haemacytometer).
- Ensure that the 40x (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 analysed per sample (see section 1.11). Alternatively, analysis can be stopped when at least 100 cells (of the dominant species) have been counted before 60 fields have been analysed.
- 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 in section 5.2.6.2)

5.1.7 SAFETY PRECAUTIONS

5.1.7.1 **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.

5.1.7.2 **Clothing**

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

5.1.7.3 **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.

5.1.7.4 **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, CO_2, foam or powder may be used to extinguish the fire.
- In case of spillage, ethanol may be washed to drain with plenty of water.

5.1.8 CALCULATIONS AND EXPRESSION OF RESULTS

5.1.8.1 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:**

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

NOTE: Round the final conversion factor to 3 decimal places, but round the final algal concentration (cells / mL) to the nearest integer.

Example:

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

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

Where π = 3.14
r = 8000 µm

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

- **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.

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.

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

$\therefore$ Area of a field = Length × Length
$= 180\ \mu m \times 180\ \mu m$
$= \underline{32\ 400\ \mu m^2}$

5.1.8.2 **Calculating the percentage composition of a species**

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

5.1.8.3 **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 for the details on how to calculate the biovolumes of different species.

5.1.9 RECORDS AND DATA KEEPING

- Data should be recorded on a form that is kept with the results. The details must include the number of fields counted, dilution / concentration factors, objective used as well as the conversion factor.
- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

5.1.10 QUALITY ASSURANCE

Quality assurance of the phytoplankton identification and enumeration method consists of two aspects, internal quality control and external quality control.

The purpose of verification and quality control in this method is to ensure that results are continuously reliable and meet the set precision and accuracy guideline. It also ensures continued analyst competency.

5.1.10.1 **General**

- Ensure that counting chambers are cleaned properly when re-used so that no Ensure that counting chambers are cleaned properly when re-used so that no contamination from previous samples influences the results. Chambers should be washed according to a specific working instruction, where they are soaked in tap water, washed in 95% ethanol, washed in tap water again and finally rinsed in distilled water. A quality control procedure should be put in place where, for example, 5% of all chambers used are inspected under the microscope before use and, if contamination is observed, the whole batch of chambers should be re-washed and inspected again before use.
- Ensure that pipette tips are clean when used. Preferably use new pipette tips for each new sample.
- Ensure that reagent water (used in the rinsing of the apparatus, and in diluting very concentrated samples) does not contain any algal cells. All reagent water bottles should be covered with foil so that no light penetration can occur, that may cause algal growth.
- Ensure that all instruments and equipment used are in working condition and serviced and calibrated regularly.
- Dispenser pipette verification should be performed monthly. It is important that each verification procedure should include the appropriate volumes used in this method.

- Ensure that analysts performing the analyses have proven competency records. Analyst competency should be evaluated quarterly by analysing five replicates of the same sample and then using the criteria stipulated in section 5.1.10.2. The identification should also be verified by a specialist.
- Duplicate analyses should be performed on 5% of all samples analysed. The results should be evaluated using the criteria stipulated in section 5.1.10.2.
- A phytoplankton identification and enumeration proficiency testing scheme should be performed quarterly.

5.1.10.2 **Precision and accuracy**

- Total biomass concentrations of duplicate / replicate phytoplankton analyses of the same sample should be within the 2 standard deviation (tolerance limits) as derived from the validation report done within a laboratory (see section 5.1.11).
- There should be an 80 percent similarity between duplicate / replicate analyses of the phytoplankton genera identified with the highest percentage composition in a sample except for conditions described in section 5.1.11 of this method.
- The % coefficient of variance between duplicate / replicate analyses of the same sample should be less than 40% (see section 5.1.11).

5.1.10.3 **Maintenance and service**

- Inverted light microscope - request annual service from supplier.
- Centrifuge - request annual service from supplier.
- Dispenser pipette - request service, if necessary, from supplier.

5.1.10.4 **Calibration**

- Balance – calibrate the balance as per instructions in the manual.
- Stage micrometer – calibrate once every three years by SABS or equivalent supplier.

5.1.11 TYPICAL VALIDATIONS FOR THE METHOD

5.1.11.1 **Estimation of uncertainty of measurement for the phytoplankton identification and enumeration sedimentation method, using centrifugation.**

AIM

To calculate the uncertainty of measurement for the algal identification and enumeration method.

PROCEDURE

Fourteen samples with differing phytoplankton concentrations were used for the identification and enumeration of phytoplankton present. The data were used for the

estimation of uncertainty and basic statistics, such as mean and standard deviation, were included.

To determine the uncertainty the following formula was used:

Uncertainty	=	(2 × std dev of sample data) / mean of data) × 100
	=	x%

RESULTS AND DISCUSSION

Table 5.3 lists the results that were used to calculate uncertainty for the phytoplankton identification and enumeration method.

Table 5.3: Phytoplankton total biomass and estimation of uncertainty

Sample	Mean	SD	2*SD	Uncertainty	"Total" uncertainty
1	1134	435	870	77	Uncertainty including all samples: **67**
2	39223	7747	15494	40	
3	6616	2175	4350	66	
4	551	255	510	93	
5	1106	428	856	77	
6	1326	409	818	62	
7	1134	348	695	61	
8	1057	834	1667	158	When removing outlier (158) **60**
9	41399	6482	12963	31	
10	36464	4363	8726	24	
11	46043	2845	5690	12	
12	5541	2318	4635	84	
13	6487	2312	4625	71	
14	3949	1557	3114	79	

Fourteen samples with differing concentrations were used to give a realistic indication of uncertainty from samples with low to high concentrations. From **Table 5.3** it is evident that the uncertainty per sample ranged from 12 – 158. The calculated total uncertainty, obtained by averaging the uncertainties of the different samples, was 67. The uncertainty of sample 8 (158) was determined to be an outlier, and, when excluding it from the analysis, the total uncertainty is 60.

CONCLUSION

The results from samples used indicated an uncertainty of 60%. Some factors that can affect the uncertainty of a sample analysed are the following:

- human factors
- environmental conditions

- equipment
- sampling
- handling of test items.

5.1.11.2 **Verification of the dispenser pipette precision**

AIM

Verification of the dispenser pipette (500 µL – 5000 µL) precision that will be used for the phytoplankton identification and enumeration method.

PROCEDURE

Three volumes of reagent water (0.5 mL, 3 mL, 5 mL) were pipetted and weighed ten times by two analysts on the same day. Eight verifications were carried out.

RESULTS AND DISCUSSIONS

The results and statistical analysis are represented in **Tables 5.4** and **5.5, Tables 5.6** and **5.7** and **Tables 5.7** and **5.8**. Variations between the analysts were recorded. The coefficient of variance between the analysts was less than 2% at 0.5 mL and 3 mL, while it was less than 1% at 5 mL (**Tables 1, 2 and 3**). The tolerance of the Dispenser pipette was set between 1×SD for the three volumes analysed at 0.0 ± 0.08, 0.0 ± 0.04, 0.0 ± 0.05 respectively for 0.5 mL, 3 mL and 5 mL (**Figures 5.5-5.7**). The control charts indicate the tolerances of the dispenser pipette at the three volumes analysed (**Figure 5.4(b)**). Using all of the data obtained from the eight verification experiments, the one time standard deviation was calculated for the three volumes analysed and is presented in **Table 5.7** and **5.8**.

Table 5.4: Statistics of the verification results by two analysts at 0.5 mL.

Experiment number	Number	Mean (±0.49 g)	SD	% COV (<2%)	Analyst
1	10	0.48917	0.00161	0.33013	AM
	10	0.49542	0.00893	1.80176	LB
2	10	0.48751	0.01900	3.89695	AM
	10	0.49719	0.00240	0.48296	LB
3	10	0.49714	0.00514	1.03479	AM
	10	0.49902	0.00252	0.50449	LB
4	10	0.49809	0.00283	0.56791	AM
	10	0.49803	0.00125	0.25032	LB
5	10	0.48811	0.00630	1.28988	AM
	10	0.49861	0.00443	0.88908	LB
6	10	0.49643	0.00332	0.66838	AM
	10	0.49709	0.00154	0.31055	LB
7	10	0.48285	0.00906	1.87617	LB
	10	0.48284	0.01086	2.24877	LE
8	10	0.49939	0.00382	0.76500	LB
	10	0.49616	0.00180	0.36245	LE

Table 5.5: **Basic statistics calculation of the 0.5 mL results.**

Number	Mean (±0.49 g)	SD	% COV (<2%)
160	0.49383	0.00847	1.71525

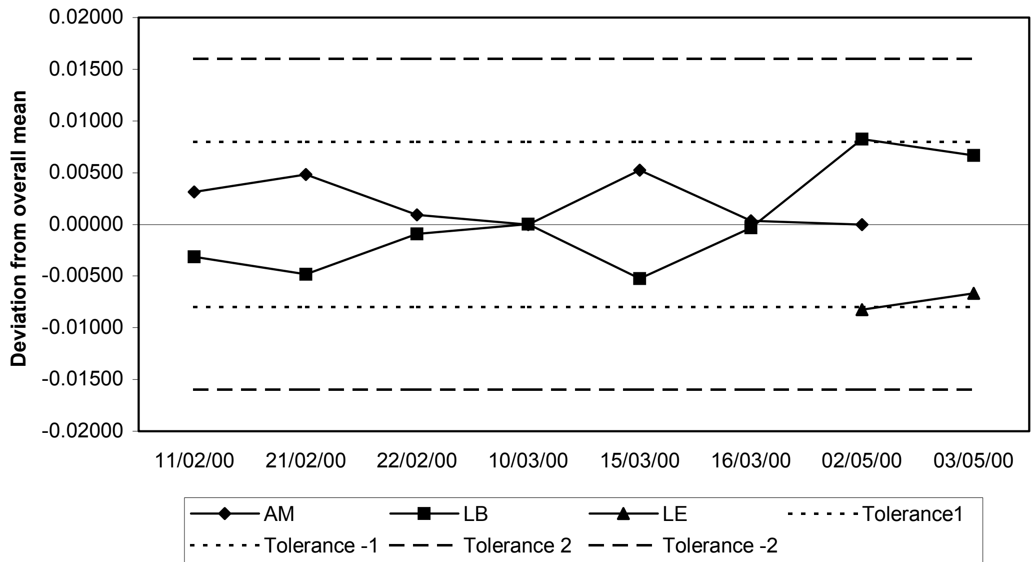


Figure 5.4(b): The deviations from zero for the Dispenser pipette at 0.5 mL control chart.

Table 5.6: Statistics of the verification results by two analysts at 3.0 mL.

Experiment number	Number	Mean (±3.0 g)	SD	% COV (<1%)	Analyst
1	10	2.99552	0.00851	0.2841	AM
	10	2.97049	0.00788	0.2651	LB
2	10	2.95939	0.00914	0.3088	AM
	10	2.96198	0.07264	0.2453	LB
3	10	2.99077	0.01133	0.3789	AM
	10	2.98929	0.00813	0.2719	LB
4	10	2.98687	0.00809	0.2709	AM
	10	2.98055	0.00516	0.1730	LB
5	10	2.97941	0.00927	0.3112	AM
	10	2.91546	0.12527	4.2966	LB
6	10	2.95364	0.00621	0.2104	AM
	10	2.94743	0.00450	0.1525	LB
7	10	2.95663	0.10140	0.3429	LB
	10	2.95235	0.01405	0.4760	LE
8	10	2.98168	0.01239	0.4157	LB
	10	2.98998	0.00372	0.1245	LE

Table 5.7(a): Basic statistics calculation of the 3.0 mL results.

Number	Mean (±3.0 g)	SD	% COV (<2%)
160	2.96946	0.04100	1.3817

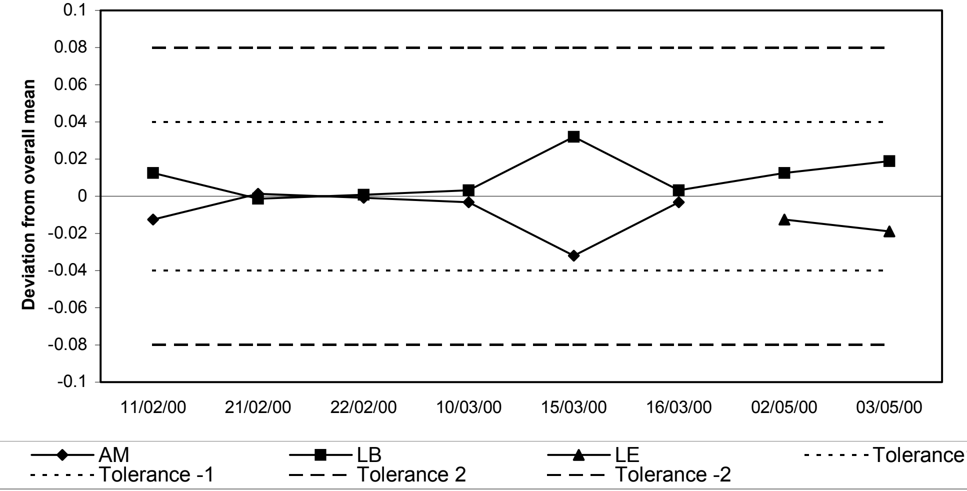


Figure 5.6: The deviations from zero for the Dispenser pipette at 3.0 mL control chart.

Table 5.7(b): Statistics of the verification results by two analysts at 5.0 mL

Experiment number	Number	Mean (±4.9 g)	SD	% COV (<1%)	Analyst
1	10	4.99289	0.00398	0.0797	AM
	10	4.97233	0.00610	0.1226	LB
2	10	4.96097	0.00365	0.0737	AM
	10	4.98664	0.00907	0.1818	LB
3	10	4.93471	0.01769	0.3585	AM
	10	4.92726	0.01911	0.3877	LB
4	10	4.91772	0.00908	0.1847	AM
	10	4.99274	0.00512	0.1026	LB
5	10	4.98320	0.00319	0.0640	AM
	10	4.88207	0.01229	0.2518	LB
6	10	4.88710	0.04117	0.8424	AM
	10	4.93679	0.00651	0.1318	LB
7	10	4.96633	0.01089	0.2192	LB
	10	4.88405	0.09682	1.9824	LE
8	10	4.88714	0.03741	0.7655	LB
	10	4.96770	0.00747	0.1504	LE

Table 5.8: Basic statistics calculation of the 5.0 mL results.

Number	Mean (±4.9 g)	SD	% COV (<1%)
160	4.94248	0.04866	0.9845

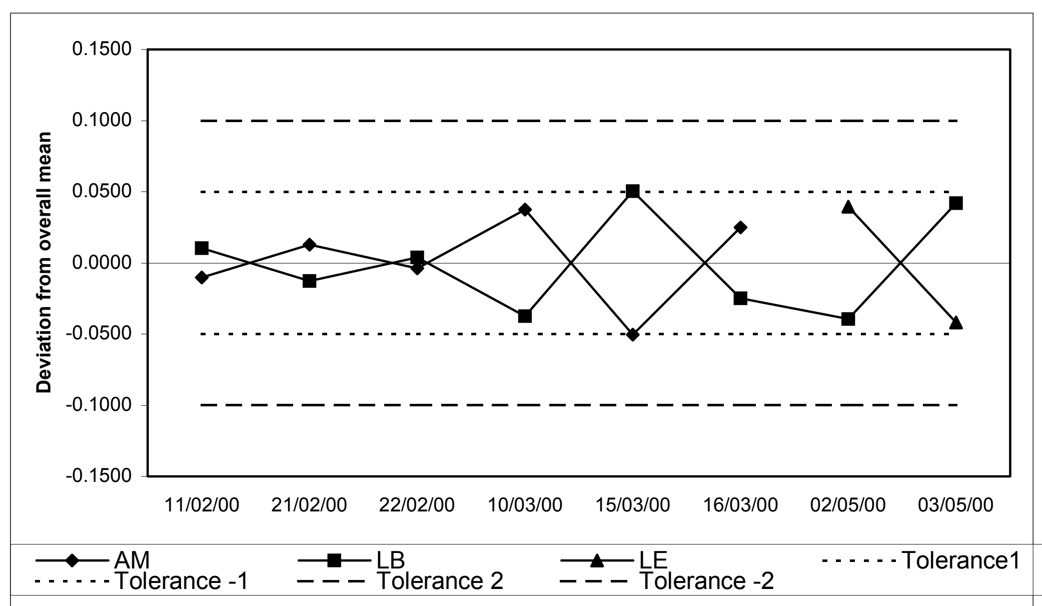


Figure 5.7: The deviations from zero for the Dispenser pipette at 5.0 mL control chart.

CONCLUSION

Even though three different analysts executed the experiments the results were within the ± 1 standard deviation tolerance. Some variations between analysts were recorded but it was within the ± 1 standard deviation tolerance. These results indicate that the dispenser pipette (500 µL - 5000 µL) is precise enough to be used in this method.

RECOMMENDATION

Use tolerance as 0 ± 0.008 for 0.5 mL, 0 ± 0.004 for 3.0 mL and 0 ± 0.005 for 5.0 mL for the dispenser pipette.

If the tolerance is out of control (above or below control chart specification) repeat the verification. If it is still out of control, take it out of use by initiating a corrective action report and have it calibrated. Verify the new dispenser pipette before use. Verification checks should be carried out every month.

5.1.11.3 **Verification of replicate for the phytoplankton identification and enumeration, sedimentation method, using centrifugation.**

AIM

Verification of replicate phytoplankton analyses to determine acceptable variation limits that will ensure competency and accuracy of results.

PROCEDURE

Source water samples (used for routine analysis) and samples made from an *Euglena* sp. culture were used for this investigation. The *Euglena* sp. culture was made by adding a known volume of culture to one liter of reagent water and mixing it thoroughly. Phytoplankton identification and enumeration were performed on ten replicates of fourteen different samples.

The total number of dominant species per sample and the percentage detected per replicate were determined for every sample analysed. Mean concentrations, standard deviations and percent coefficient of variance were also determined.

Two control charts have been constructed from this data. The first is to control results obtained by different analysts for the same sample (inter-analyst). It was developed in the following way:

1. Total biomass concentrations of the ten replicates analysed per sample were converted to natural logarithms.
2. The average of these ten natural log transformed replicates was determined.
3. Each of the ten log transformed replicate values was then subtracted from the calculated average.
4. Two times standard deviation was calculated on these "subtracted" values.
5. These "subtracted" values were then non-selectively grouped into duplicates and plotted onto a line chart.
6. The calculated two times standard deviation was also plotted on the same chart to indicate tolerance limits.

The second control chart was developed to control variation between duplicate analyses of the same sample. The same principle described in the points above (1-6) was followed except that averages were calculated on all possible combinations of the ten replicates (instead of only one average for the ten replicates) as duplicates. One of the duplicate natural log transformed values was then subtracted from the average obtained for the two values that represent one duplicate analysis and this "subtracted" value was then plotted on the control chart. Two times standard deviation was calculated on all of the

"subtracted" values and then plotted on the same control chart to serve as a tolerance limit.

RESULTS AND DISCUSSIONS

Results and statistical analysis are represented in **Tables 5.9** and **5.10**. The percentage coefficient of variance (% COV) between replicate analysis of all the samples analysed varied considerably, ranging from 0 - 71 (**Table 5.9**). When ten replicates of one sample were analysed by one analyst the average % COV ranged from 6 to 79 (**Table 5.9**). When the ten replicates were randomly grouped as duplicates, the average % COV ranged from 5 to 38 (**Table 5.9**). In replicate analysis performed with all three regimes, it was noted that a % coefficient of variance greater than 40 was almost always associated the presence of algal colonies in the sample that resulted in larger variations (e.g. **Table 5.10**). This is due to the fact that colonies and algae in general are not randomly distributed in a sample. The control charts indicate that the tolerances of the various replicate analyses were between the ±2SD for most of the samples analysed (**Figures 5.7, 5.9** and **5.10**). The ± two times standard deviation can thus be used as a limit to control variation between replicate analyses.

The number of dominant species between replicate analysis of all the samples analysed varied from 1 - 4 (**Table 5.9**). The percentage of these dominant phytoplankton species identified between duplicate samples varied from 50 – 100%. It was found that multi-cellular species can overshadow the "real" dominant species in a sample. One colony or loosely aggregated floc can consist of many cells all aggregated on one spot, e.g. colonies of *Microcystis* sp. can easily consist of 500 cells in one colony, and if only one colony is detected in a sample, it may overshadow the other algal species that were dominant in the sample and detected in most of the fields analysed. Nevertheless, the species composition data from these experiments indicate that the detection/non-detection of dominant species in replicate/duplicate samples can be used to evaluate competency with species identification.

Table 5.9: Statistics of replicate analysis (total biomass) performed by one analyst.

Sample Number	Total Biomass Replicate 1	Total Biomass Replicate 2	Average	SD	% COV	Total number of dominant species	% dominant species identified
Sample 1 (± 1000 cells/mL)	1270	1211	1240	42	3	4	50*
	1595	620	1108	689	62	3	100
	1506	1654	1580	104	7	3	100
	1447	502	975	668	69	3	100
	886	650	768	167	22	1	100
	% COV all	38			Average 33		
Sample 2 (± 40000 cells/mL)	29121	44626	36873	10963	30	*Euglena* sp. culture	
	45723	48170	46947	1730	4		
	40759	44591	42675	2709	6		
	25923	41645	33784	11117	33		
	30849	40826	35838	7055	20		
	% COV all	20			Average 19		
Sample 3 (± 6500 cells/mL)	7340	5375	6358	1389	22	2	100
	8063	10337	9200	1608	17	2	50*
	5065	7650	6358	1827	29	2	50*
	7133	3308	5220	2705	52	1	100
	3825	8063	5944	2997	50	2	50*
	% COV all	33			Average 34		
Sample 4 (± 500 cells/mL)	1172	413	793	536	68	3	100
	482	482	482	0	0	2	100
	448	586	517	97	19	2	100
	758	413	586	244	42	3	65
	517	241	379	195	51	1	100
	% COV all	46			Average 36		

* The presence of various multi-cellular algae overshadows the “true” dominant algal species present as one colony can consist of many cells.

Table 5.9 (cont): Statistics of replicate analysis (total biomass) performed by one analyst.

Sample Number	Total Biomass Replicate 1	Total Biomass Replicate 2	Average	SD	% COV	Total number of dominant species	% dominant species identified
Sample 5 (± 1100 cells/mL)	2102	999	1551	780	50	3	65
	1103	793	948	219	23	2	100
	965	758	861	146	17	2	100
	758	1551	1154	560	49	1	100
	827	1206	1017	268	26	3	100
	% COV all	39			Average 33		
Sample 6 (± 1200 cells/mL)	1890	1506	1698	272	16	2	100
	945	1536	1240	418	34	2	100
	709	945	827	167	20	2	100
	1949	1181	1565	543	35	2	50 Act hantz
	1181	1418	1300	167	13	3	65 Scene
	% COV all	31			Average 24		
Sample 7 (± 1000 cells/mL)	1181	709	945	334	35	2	100
	679	1684	1181	710	60	2	100
	1536	945	1240	418	34	2	100
	1270	1270	1270	0	0	4	75
	768	1300	1034	376	36	3	65 Micract
	% COV all	31			Average 33		
Sample 8 (± 1000 cells/mL)	709	3338	2023	1859	92	2	50 Pedias
	975	1181	1078	146	14	2	100
	620	620	620	0	0	2	50 Actin h
	975	679	827	209	25	3	65 Micract
	443	1034	738	418	57	3	65 Eudor
	% COV all	79			Average 38		
Sample 9 (± 40000 cells/mL)	31095	33105	32100	1421	4	*Euglena* sp culture	
	44762	42131	43447	1860	4		
	49938	43068	46503	4858	10		
	34335	41503	37919	5068	13		
	45944	48109	47027	1531	3		
	% COV all	16			Average 7		

* The presence of various multi-cellular algae overshadows the "true" dominant algal species present as one colony can consist of many cells.

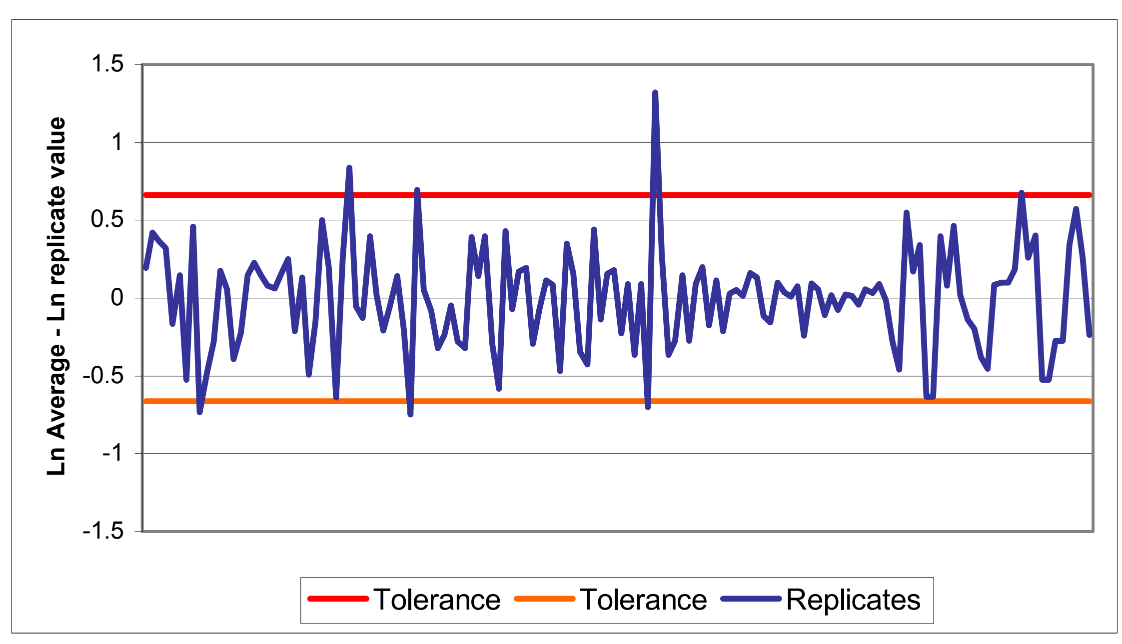


Figure 5.8: **Natural Log transformed total biomass concentrations of all the samples analysed.**

Table 5.10: Probable species responsible for % COV > 40 in Table 5.9.

Sample number	Replicate Number with % COV > 40	Probable species responsible	Type
1	2	*Micractinium pusillum*	Colony
		Micractinium pusillum	Colony
	4	*Scenedesmus acuminatus*	Colony
3	4	*Dictyosphaerium elegans*	Colony
		Pandorina morum	Colony
		Scenedesmus acuminatus	Colony
	5	*Microcystis aeruginosa*	Colony
4	1	*Microcystis aeruginosa*	Colony
	4	*Scenedesmus opoliensis*	Colony
	5	*Characium limneticum*	Single cell
5	1	*Microcystis aeruginosa*	Colony
		Crusigenia lauterbornii	Colony
	4	*Actinastrum hantzhii*	Colony
7	2	*Micractinium pusillum*	Colony
8	1	*Actinastrum hantzchii*	Colony
		Micractinium pussilum	Colony
		Pediastrum simplex	Colony
	5	*Eudorina elegans*	Colony

Table 5.10 (cont): Probable species responsible for % COV > 40 in Table 5.9.

Sample number	Replicate Number with % COV > 40	Probable species responsible	Type
13	5	*Aulacoseira granulata*	Filament
		Phacotus lenticularis	Single cell
14	3	*Scenedesmus acuminatus*	Colony
		Aulacoseira granulata	Filament
	4	*Chlamydomonas incerta*	Single cell
		Aulacoseira granulata	Filament
		Scenedesmus acuminatus	Colony

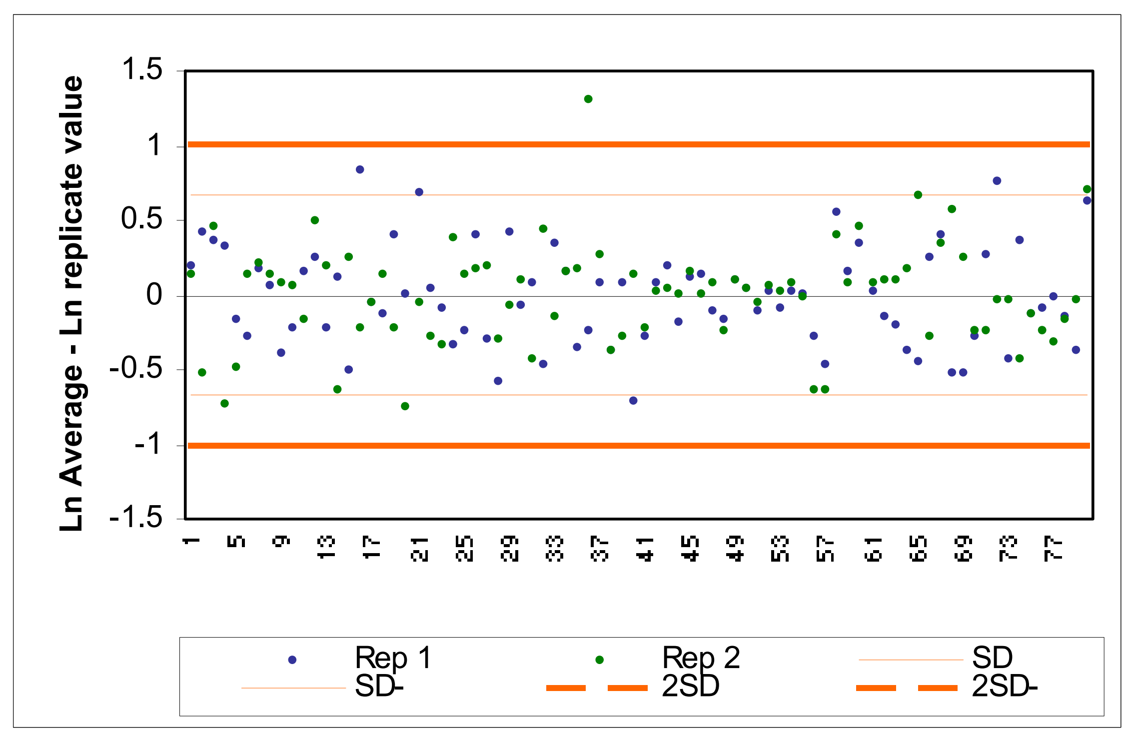


Figure 5.9: Control chart for inter-analyst comparisons.

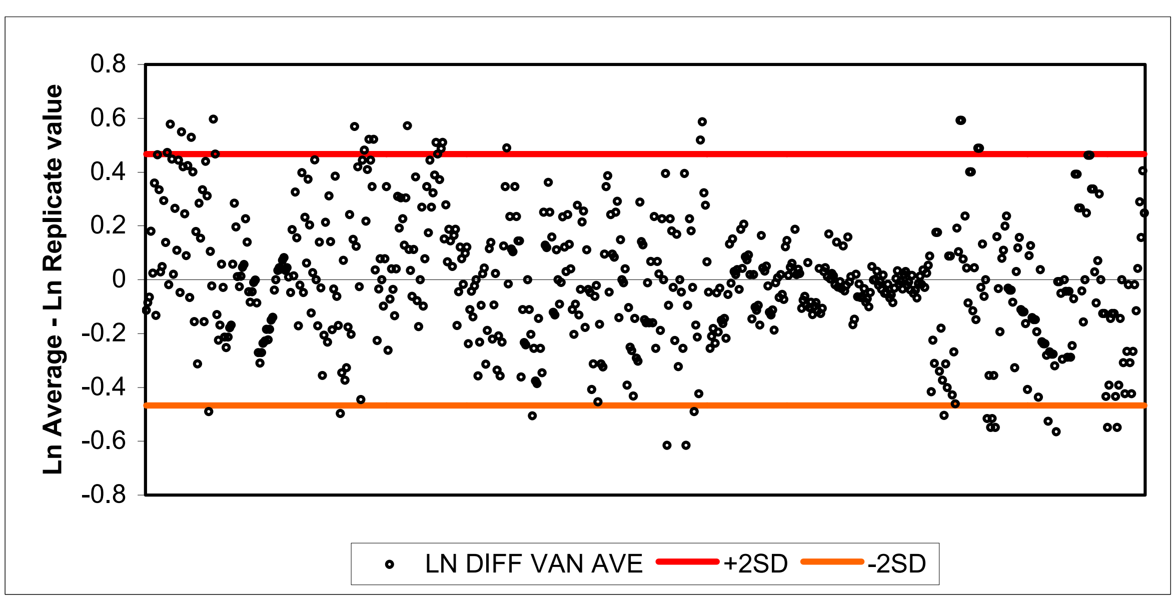


Figure 5.10: Control chart for duplicate analysis.

CONCLUSIONS

The coefficient of variance between replicate samples can vary from 0 – 71, which is almost always associated with the presence of multi-cellular/colony algal forms. A coefficient of variance of less than 40% between replicates was determined between most of the replicates, except when multi-cellular forms were present. The tolerance between replicate samples is between the ± 2 standard deviation. At least 80% of the dominant algal species in a sample has been detected, except for situations where multi-cellular algae may overshadow "real" dominant species.

RECOMMENDATIONS

Total biomass concentrations between replicates of one analyst must not exceed the 40% coefficient of variance accept when multi-cellular forms are the cause of greater variation (40 – 100%). The ± 2 standard deviation on the total biomass concentration between replicates, should be used as the tolerance limits for replicate counts.

If the variation between replicates is out of the set tolerance, investigate if the cause is due to multi-cellular algae that can cause % COV of up to 100%. If the cause is not due to multi-cellular algae, or the % COV is greater than 100% if multi-cellular algae are present, repeat the analysis. If it remains out of specification, the analyst must be deemed incompetent until further training proves competency.

Eighty percent (80%) of all dominant species in a sample must be identified in replicates. If this is not achieved (and it is not due to multi-cellular algal presence) repeat the

analysis. If it is still out of specification, the analyst must be deemed incompetent until further training proves competency.

Verification of replicate results between analysts must be carried out every three months as indicated per annual schedule in the logbook and 5% of all samples analysed must be performed in duplicate.

5.1.11.4 Verification of the cycle speed for the phytoplankton identification and enumeration sedimentation method, using centrifugation.

AIM

Verification of the cycle speed at which to centrifuge samples for accurate phytoplankton analysis.

PROCEDURE

Replicate phytoplankton analyses were carried out on four samples of different concentration and were centrifuged at three different cycle speeds, namely: 3500 rpm, 2500 rpm and 1000 rpm. Standard Methods (APHA, 2001) recommends that a sample be centrifuged at 1000 rpm for 20 minutes. Alternative speeds of 2500 rpm and 3500 rpm for 10 minutes were also evaluated. A control sample, in which the phytoplankton were allowed to settle under gravity at 24 hours for every 1 cm depth (as described by the Lund, 1958), was included in the analysis. Phytoplankton identification and enumeration were performed on all samples. Basic statistical analyses and Tukey's multiple comparison tests were performed on the Total Biomass concentrations of all samples to indicate significant differences between the different cycle speeds and control.

RESULTS AND DISCUSSIONS

The results and statistical analyses are presented in **Tables 5.11** and **5.12**. The Tukey's multiple comparison tests indicted that there was a significant difference between the total biomass concentration of the control and 1000 rpm and 3500 rpm treatments. The control had a significantly lower average total biomass concentrations compared to all of the other treatments (**Figure 5.11**). This is attributed to not all of the phytoplankton sedimenting under gravity within the time frame recommended by Lund (1958). The control was therefore excluded from further statistical analyses - where after the Tukey's test indicated no significant difference between the different treatments. However, from the mean biomass calculated, it is evident that centrifuging at a cycle speed of 3500 gave the greatest consistency of results (**Figure 5.11**).

Table 5.11: Basic statistics of total biomass concentrations of samples centrifuged at 2500 rpm, 3500 rpm, 1000 rpm and control speeds.

Sample number	Cycle speed (rpm)	Number	Average	SD	% COV	Min	Max	Max-Min
1	1000	10	6487	2312	36	3928	12150	8222
	2500	10	5541	2318	42	2688	8787	6099
	3500	10	6616	2175	33	3308	10340	7032
	Control	10	3949	1557	39	2171	6513	4342
2	1000	10	7178	2795	39	4527	11710	7183
	2500	10	5516	895	16	4430	7029	2599
	3500	10	6429	1919	30	4527	11280	6753
	Control	10	4603	921	20	2940	5743	2803
3	1000	10	1134	348	31	679	1684	1005
	2500	10	1326	409	31	709	1949	1240
	3500	10	1134	435	38	502	1654	1152
	Control	10	1057	834	79	443	3338	2895
4	1000	10	36460	4363	12	28470	41380	12910
	2500	10	41400	6482	16	31100	49940	18840
	3500	10	39220	7747	20	25920	48170	22250
	Control	10	45950	2801	6	41150	50300	9150

Table 5.12: Tukey's multiple comparison test at centrifugation speeds: 2500 rpm, 3500 rpm, 1000 rpm and control.

Sample No.	Cycle speed	Mean Total Biomass (cells/mL)	Tukey's multiple comparison test			
				p-value	Mean difference	95% Cl of diff
1	1000	6487	3500 *vs* 1000	> 0.05	129	-2421 to 2679
	2500	5541	2500 *vs* 3500	> 0.05	-1075	-3625 to 1475
	3500	6616	2500 *vs* 1000	> 0.05	-946	-3496 to 1604
	Control	3949				
2	1000	7178	3500 *vs* 1000	> 0.05	-749	-2891 to 1394
	2500	5516	2500 *vs* 3500	> 0.05	-913	-3055 to 1230
	3500	6429	2500 *vs* 1000	> 0.05	-1661	-3804 to 481
	Control	4603				
3	1000	1134	3500 *vs* 1000	> 0.05	-0.10	-488 to 488
	2500	1326	2500 *vs* 3500	> 0.05	192	-296 to 680
	3500	1134	2500 *vs* 1000	> 0.05	192	-297 to 680
	Control	1057				

Table 5.12 (cont.): Tukey's multiple comparison test at centrifugation speeds: 2500 rpm, 3500 rpm, 1000 rpm and control.

Sample No.	Cycle speed	Mean Total Biomass (cells/mL)	Tukey's multiple comparison test			
4	1000	36460	3500 *vs* 1000	> 0.05	2760	-4833 to 10350
	2500	41400	2500 *vs* 3500	> 0.05	2176	-5417 to 9769
	3500	39220	2500 *vs* 1000	> 0.05	4936	-2658 to 12530
	Control	45950				

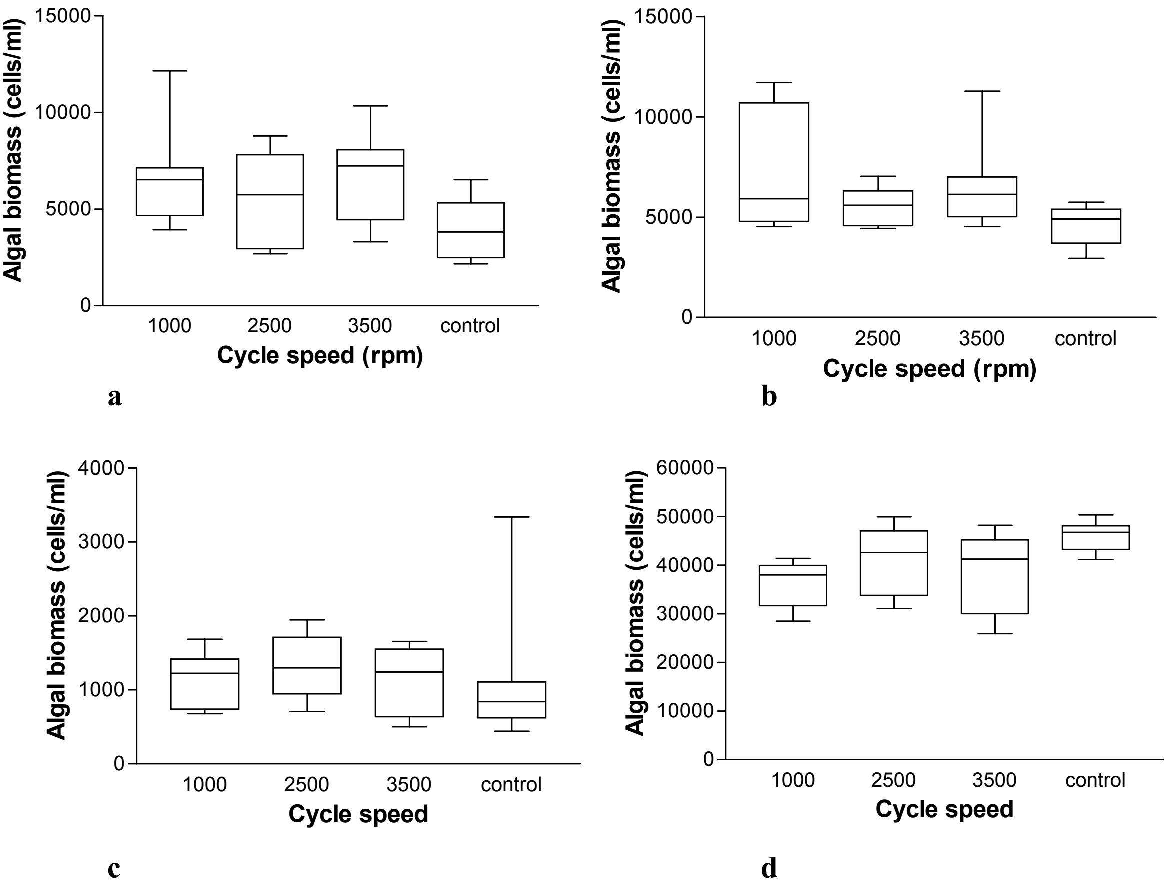


Figure 5.11: Total algal biomass concentrations at the different cycle speeds for the four samples analysed (sample 1: a; sample 2: b; sample 3: c; sample 4: d)

CONCLUSIONS

Centrifugation may be used to successfully sediment phytoplankton. Not all algae were sedimented by gravity in the control sample. If the control is excluded from statistical analysis, no significant difference exists between the three cycle speeds evaluated. However, a cycle speed of 3500 appears to yield the highest level of reproducibility.

RECOMMENDATIONS

A cycle speed of 3500 rpm should be used to sediment phytoplankton for analysis.

5.1.11.5 Verification of the number of fields to be analysed for the phytoplankton identification and enumeration sedimentation method, using centrifugation.

INTRODUCTION

In Standard Methods (APHA, 2001) it is recommended that for a counting precision of 20% per phytoplankton species in a sample, a hundred cells of that species must be counted. Due to different types of samples with different concentrations and compositions, seasonality and time constraints, the goal of a 100 cells cannot always be reached even if the whole sample is analysed. The use of "number of cells per phytoplankton species", as an indicator of when a sample is analysed sufficiently, was considered impractical for a bulk potable water supplier that require accurate same day results. For this reason Rand Water decided to rather evaluate the "number of fields to be analysed per sample" that will be sufficient to give an accurate representation of the phytoplankton species composition and (numerical/biomass) concentration in a sample.

AIM

Verification of the number of fields to be analysed for phytoplankton that will inform accurate determinations of the species representation and biomass concentrations in a sample.

PROCEDURE

Six samples were used for this verification experiment. On each sample two scenarios were tested, i.e. identification and enumeration using, respectively, 60 and 120 fields. Using 120 fields is representative of ∀ 10% of the total area of the sedimentation chamber. The alternatively use of 60 fields was evaluated as a means of reducing the time spent on a single analysis. Six verifications (repetitions) were carried out.

RESULTS AND DISCUSSIONS

The results and statistical analysis are represented in **Tables 5.13, 5.14** and **5.15**. Applying the unpaired T-test indicated that no significant differences (P>0.05) in total algal biomass determinations were found if determinations were done at 60 or 120 fields (**Table 5.14**). An exception here was the result for Sample 5. However, the non-parametric test, namely the Tukey's test (distribution free test), revealed no significant difference between the total biomass concentrations determined from counts of 60 or 120 fields (**Table 5.15**).

Table 5.13: Basic statistics of the total phytoplankton biomass concentrations at 60 and 120 fields.

Sample number	Number of fields	Number	Average	SD	% COV	Min	Max	Max-Min
1	60	10	5541	2318	42	2688	8787	6099
	120	10	5516	895	16	4430	7029	2599
2	60	10	6616	2175	33	3308	10340	7032
	120	10	6429	1919	30	4527	11280	6753
3	60	10	6487	2312	36	3928	12150	8222
	120	10	7177	2795	39	4527	11710	7183
4	60	10	3949	1557	39	2171	6513	4342
	120	10	4603	921	20	2940	5743	2803
5	60	10	1151	473	41	758	2102	1344
	120	10	2063	917	44	1340	3882	2542
6	60	10	551	255	46	241	1172	931
	120	10	544	200	37	347	928	581

Table 5.14: T-test for the independent number of fields: 60 *versus* 120

Sample No.	Mean total biomass (cells/mL)		T-test for independent samples		
	60 fields	120 fields	t-value	Df	P
1	5541	5516	0.03	18	0.98
2	6616	6429	0.20	18	0.84
3	6487	7177	0.60	18	0.55
4	3949	4603	1.14	18	0.27
5	1151	2063	2.80	18	0.01
6	551	544	0.94	18	0.24

Table 5.15: Tukey's multiple comparison test of the number of fields compared.

Sample No.	Number of fields	Mean Total Biomass (cells/mP)	Tukey's multiple comparison test			
				p-value	Mean difference	95% Cl of diff
1	60	5541	60 *vs* >120	> 0.05	24.78	-2303 to 2353
	120	5516				
2	60	6616	60 *vs* >120	> 0.05	187	-2141 to 2515
	120	6429				
3	60	6487	60 *vs* >120	> 0.05	-691	-3019 to 1637
	120	7177				
4	60	3949	60 *vs* >120	> 0.05	-654	-2982 to 1674
	120	4603				
5	60	1151	60 *vs* >120	> 0.05	-912	-3240 to 1416
	120	2063				
6	60	551	60 *vs* >120	> 0.05	7.7	-2320 to 2336
	120	544				

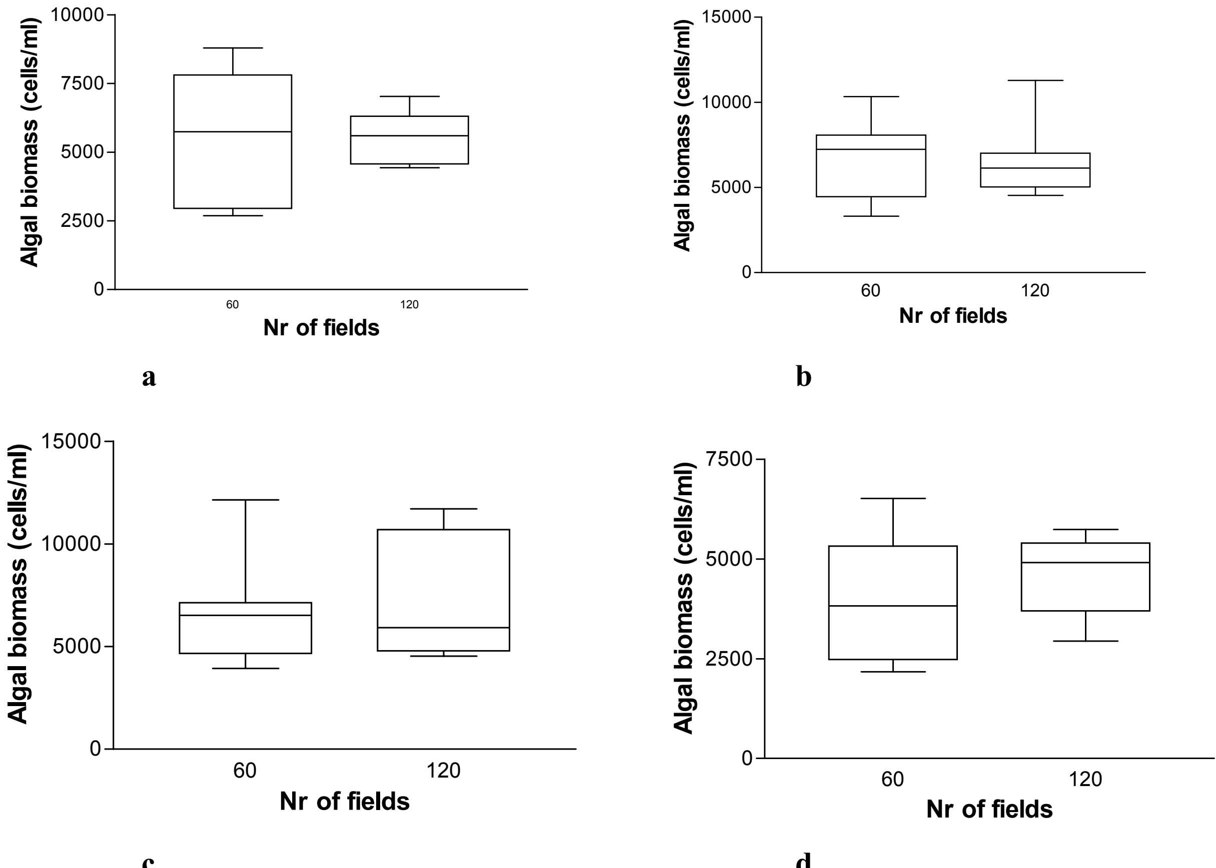
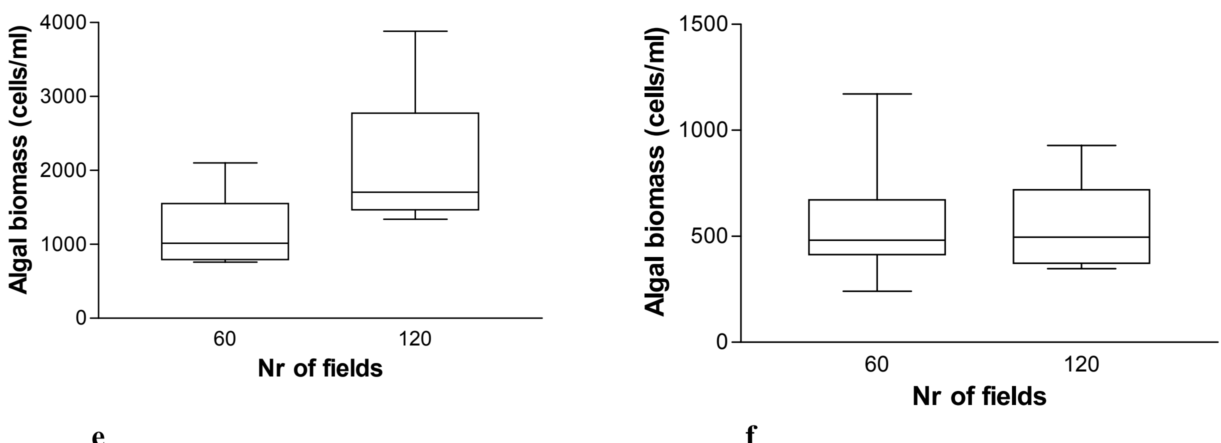


Figure 5.12: Total algal biomass concentrations at 60 fields and 120 fields for the samples analysed (sample 1: a; sample 2: b; sample 3: c; sample 4: d)



e **f**

Figure 5.12 (cont.): Total algal biomass concentrations at 60 fields and 120 fields for the samples analysed (sample 5: e; samples 6: f)

CONCLUSIONS

No significant differences were detected between total biomass concentrations determined from counts of 60 or 120 fields comparisons (Tukey's test). The T-test did detect one significant difference between the 60 and 120 field comparison for Sample 5. From the results it is concluded that no significant differences arise from analysing 60 or 120 counting fields.

RECOMMENDATIONS

If one hundred algal cells have not been counted before 60 fields have been examined, sufficient fields are deemed to have been examined to provide an accurate representation of the sample.

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

5.2.1 INTRODUCTION

5.2.1.1 Scope

The scope of this method is the identification and enumeration of phytoplankton. The composition of a phytoplankton community is the result of a combination of numerous variables influencing the aquatic environment, including nutrient concentration and availability, light, temperature, pH, etc. Since certain types of phytoplankton have certain requirements with regards to environmental variables, determination of the dominant taxa in a water resource can provide important information about the water quality and health of the aquatic ecosystem. Using this method, an analyst can generate data on the composition of the phytoplankton community and in so doing, aid the management of water resources.

5.2.1.2 Definition

Phytoplankton refers to all algal taxa suspended in the water column.

5.2.1.3 Field of application

This method is suitable for the analysis of any water resource containing phytoplankton. This includes reservoirs such as dams, lakes and rivers spanning all trophic states.

5.2.1.4 Interferences

- Phytoplankton samples often contain a large amount of detritus and fine inorganic material that obscure phytoplankton and hamper identification.
- When the sample has a high algal biomass some cells may obscure others.
- Low algal biomass leads to a greater variation in results between replicate samples.
- Phytoplankton is not always distributed uniformly on the bottom of the sedimentation chamber. Patchy distribution is often aggravated by the presence of colonies and filaments, resulting in a concentration of cells on certain parts of the bottom of the sedimentation chamber.

5.2.1.5 Method range

In this method, a minimum of one lane (refer to **Figure 5.9**) is analysed when the biomass is high. When the sample contains a low biomass, a maximum of 5 lanes are analysed. The lowest quantifiable concentration for this method is, 6 cells/mL when 5 lanes and 6 mL of a sample are analysed. A lane runs through the centre of the circular

sedimentation chamber. At 400× magnification, a lane has a width of 176 µm, a length equal to the diameter of the sedimentation chamber (in this case 16 300 µm) and it consists of ca 90 square counting blocks (or Whipple grids) next to each other.

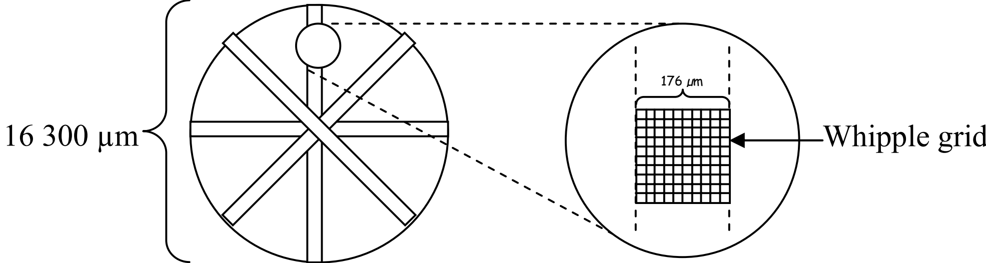


Figure 5.13: Line diagram showing the orientation of lanes and the Whipple grid.

5.2.2 PRINCIPLE

Phytoplankton samples are fixed using a suitable fixative. The sample is then pressurised to rupture gas vacuoles present in cyanobacteria (blue-green algae), after which a sub-sample of known and appropriate size (1-6 mL) is transferred to a sedimentation chamber. The sample is left to settle for a certain period of time (refer to section 5.2.6.1 to determine the appropriate time applicable to the chamber that is being used). After this period of time, phytoplankton taxa are identified, as far as possible, to species level and enumerated simultaneously. The results of the enumeration are expressed as a concentration of cells per volume of water (cells/mL).

5.2.3 WATER QUALITY

5.2.3.1 **Significance phytoplankton identification and enumeration**

Phytoplankton in source water, known to be sensitive indicators of water quality, have an effect on the production of potable water, the aesthetic aspect of recreational waters and consumer health, because:

- Phytoplankton and their cellular products interfere with the physical and/or chemical water purification processes.
- Phytoplankton is able to pass through purification processes resulting in water of aesthetically unacceptable quality (taste, odour and colour).
- Some phytoplankton species have the ability to produce substances that can be detrimental to the health of consumers and can be a source of carbon for bacteriological growth.
- Excessive growth of phytoplankton in source water (water bloom) can create aesthetically unacceptable recreational and potable water and may pose a health risk to consumers (taste, odour, scum and toxin).

Advantages of phytoplankton monitoring include:

- Detecting the presence of and examining the short-term trends in the growth of phytoplankton species in order to determine the suitability of particular water for drinking and recreational use.
- Providing data to determine long-term trends in phytoplankton composition within a particular waterbody. This information can be used to assess, for example, the effect of sewage discharge and agricultural run-off containing fertilizers and harmful chemicals.
- Monitoring the effects of management measures such as river regulation, inter-basin transfers and water abstractions within a particular system.

5.2.4 APPARATUS, MATERIALS AND REAGENTS

5.2.4.1 **Instruments and equipment**

- Inverted light microscope with a 40 × 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

5.2.4.2 **Glassware**

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

5.2.4.3 **Other materials**

- Lens cleaning tissue
- Lens cleaning liquid

5.2.4.4 **Reagents**

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

5.2.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

Samples and hazardous reagents are disposed of using a hazardous waste disposal system.

5.2.6 PROCEDURE

5.2.6.1 **Sample preparation**

- Note, that before any work is undertaken, it is imperative that the analyst is familiar with the safety precautions found in section 5.2.7.1 - 5.2.7.3 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.

5.2.6.2 **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 5.13**). Continue counting in this manner until at least one lane is completed. Note that a minimum of 200 cells need to be identified.
- 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 analysed and continue as above, this time from right to left. Continue these steps until a total greater that 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 analysed 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 following literature is recommended for accurate identification of phytoplankton. Some other references not listed below, may also be useful.
  - Belcher, H. & Swale, E. 1976. A beginner's guide to Freshwater Algae. Her Majesty's Stationery Office (HMSO). ISBN 0 11 881393 5.
  - Belcher, H. & Swale, E. 1979. An illustrated guide to River Phytoplankton. Her Majesty's Stationery Office (HMSO). ISBN 0 11 886602 8.
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  - Entwisle, T.J., Sonneman, J.A. & Lewis, S.H. 1997. Freshwater Algae in Australia. Sainty and Associates Pty Ltd, NSW, Australia.
  - John, D.M., Whitton, B.A. & Brook, A.J. 2002. The Freshwater Algal Flora of the British Isles. An identification guide to freshwater and terrestrial algae. Cambridge: Cambridge University Press.
  - Janse van Vuuren, S., Taylor, J., Gerber, A. & van Ginkel, C. 2006. Easy identification of the most common freshwater algae. A guide for the identification of microscopic algae in South African freshwaters. North-West University, Private Bag X6001, Potchefstroom, South Africa and DWAF, Private Bag X313, Pretoria, South Africa.
  - Prescott, G.W. 1951. Algae of the western great lakes area. Wm. C. Brown Co. Publ., Dubuque, Iowa.
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  - Huber-Pestalozzi, G. 1950. Das Phytoplankton des Susswassers: Systematik und Biologie. Tl. 3. Cryptophyceen, Choromadinen, Peridineen. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.
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➢ Huber-Pestalozzi, G. 1962a. Das Phytoplankton des Susswassers: Systematik und Biologie. Tl. 1. Algemeiner Teil. Blaualgen. Bakterien, Pilze. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.
➢ Huber-Pestalozzi, G. 1962b. Das Phytoplankton des Susswassers: Systematik und Biologie. Tl. 2. Diatomeen. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.
➢ Huber-Pestalozzi, G. 1962c. Das Phytoplankton des Susswassers: Systematik und Biologie. Tl. 2. Hlf. 1. Chrysophyceen, Farblose Flagellaten, Heterokonten. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

5.2.7 SAFETY PRECAUTIONS

5.2.7.1 **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.

5.2.7.2 **Clothing**

- Laboratory coat
- Latex gloves
- Safety glasses

5.2.7.3 **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, CO_2, 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 5.1.7.3.

5.2.7.4 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 5.1.7.4.

5.2.8 CALCULATIONS AND EXPRESSION OF RESULTS

5.2.8.1 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:

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

- Calculate the area of one rectangular lane:

$$\text{Lane area} = \text{diameter of sedimentation chamber} \times \text{width of Whipple grid} = 16\ 300\ \mu m \times 176\ \mu m = 2\ 868\ 800\ \mu m^2$$

- 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 analysed.

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

At this stage it is important to remember the volume of the original sample that was sedimented as mentioned in 5.2.6.1. The conversion factor is divided by the volume (mL) of sample that was used.

$$\text{Final conversion factor} = \frac{\text{Conversion factor}}{\text{Volume of sample used}}$$

$$= \frac{72.739}{3\text{ mL}}$$

$$= 24.246$$

- 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.

$$\text{Biomass} = \text{Count} \times \text{Final conversion factor}$$

$$= 78 \times 24.246$$

$$= 1891.188$$

$$\approx 1891 \text{ cells/mL (rounded to the nearest integer)}$$

5.2.8.2 Calculating the percentage composition of a taxon

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

5.2.8.3 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 5.4.

5.2.9 RECORDS AND DATA KEEPING

- 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. Store the data sheets with the final results. It is imperative to have hard- and soft copies of data in a well organised filing system.

- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory).

5.2.10 QUALITY ASSURANCE

Quality assurance of the phytoplankton identification and enumeration method consists of two aspects: internal and external quality control.

The purpose of verification and quality control in this method is to ensure that results are continuously reliable and meet the set precision and accuracy guideline. It also ensures continued analyst competency.

5.2.10.1 **General**

- Ensure that sedimentation chambers are cleaned properly before re-use so that no contamination from previous samples influences the results. Chambers should be washed according to a specific working instruction, whereby they are soaked in tap water, washed in 95% ethanol, washed in tap water again and lastly rinsed in distilled water. Where perspex chambers are used, the use of ethanol should be avoided, as this may lead to the formation of fine cracks in the perspex. A quality control procedure should be put in place where, for example, 5% of all chambers used are inspected under the microscope before use and, if contamination occurs, the whole batch of chambers should be re-washed and inspected again before use.
- Ensure that pipette tips are clean before used. Preferably use new pipette tips for each sample.
- Ensure that distilled water (used in diluting very concentrated samples) does not contain any algal cells. All distilled water bottles should be covered with foil so that no light penetration can occur and result in algal growth. Bottles should be acid washed every 3 months.
- Ensure that all instruments and equipment are in working condition and serviced and calibrated regularly.
- Dispenser pipette verifications should be performed monthly, and it is important that each verification procedure should include the appropriate volumes used in this method.
- Ensure that analysts performing analysis have a proven, documented competency record. Analyst competency should be evaluated quarterly by analysing five replicates of the same sample and then using the criteria in section 5.2.10.2. The identification should also be verified by a specialist.
- Duplicate analyses should be performed on 5% of all samples analysed. The results should be evaluated by using the criteria in section 5.2.10.2.

- Phytoplankton identification and enumeration proficiency testing scheme should be performed quarterly.

5.2.10.2 **Precision and accuracy**

- Total biomass concentrations of duplicate/replicate phytoplankton analyses of the same sample should be within 2 Standard Deviation (tolerance limits) as derived from the validation report done within a laboratory (see section 5.2.11).
- There should be an 80% similarity between duplicate/replicate analyses of the phytoplankton genera identified with the highest percentage composition in a sample except for conditions described in section 5.2.11 of this method.
- The % Coefficient of Variance (% COV) between duplicate/replicate analyses of the same sample should be less than 40% (see section 5.2.11).

5.2.10.3 **Maintenance and service**

- Inverted light microscope - request annual service from supplier.
- Dispenser pipette - request service if necessary from supplier.

5.2.11 TYPICAL VALIDATIONS FOR THE METHOD

5.2.11.1 **Permissible differences in biomass between replicate samples**

AIM

The aim is to establish the permissible amount of variance between replicate results from sedimentation chambers prepared from the same sample.

PROCEDURE

Three sub-samples were drawn from the original sample, one by each analyst. Each analyst then proceeded to prepare 10 replicates in sedimentation chambers for analysis from his/her sub-sample (as discussed in section 5.2.6.1), thereby creating 10 replicates of the same sub-sample. Each replicate was then analysed for phytoplankton and the result reported according to the method above. The mean, Standard Deviation (SD) and % COV were determined for the total biomass (cells/mL) in the replicates of the sub-sample. The % dominant species identified by each analyst was also reported.

RESULTS AND DISCUSSION

The results of the phytoplankton analyses are given in the figures and tables below.

Table 5.16: Summary of the phytoplankton biomass analyses for each analyst

Biomass (cells/mL)					
Analyst	Repetitions	Mean	SD	% COV	% Dominant species identified
1	10	39910	4244.9117	10.64	100
2	10	30606	6416.7487	20.97	100
3	10	33125	3050.2324	9.21	100

Table 5.17: Summary of the phytoplankton biomass analyses for all analysts

Total Biomass (cells/mL)			
Replicates	Mean	SD	% COV
30	34549	6103.2133	17.67

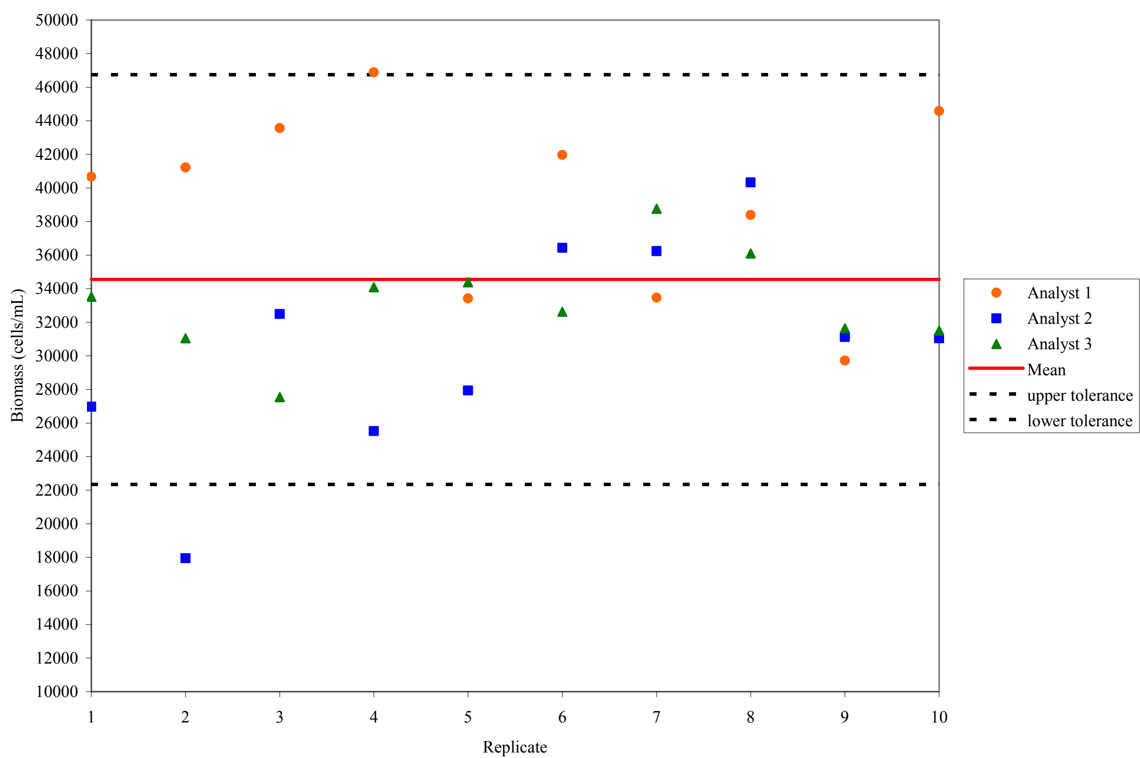


Figure 5.14: Total biomass concentrations for 10 replicates prepared from the same sample

Figure 5.14 indicates that, with the exception of two replicates, all values fell within the upper and lower tolerance limits. The high value for Analyst 1, falling outside upper tolerance limit, was due to the presence of a larger amount of colonial and filamentous cyanobacteria settling within the lane area. The opposite is true for the low value of Analyst 2, where fewer colonial and filamentous cyanobacteria settled in the lane area.

The fact that all other values fell well within the tolerance limits indicates the high measure of precision inherent of the method itself, despite the presence of colonial and filamentous organisms. The low overall % COV of 17.67% (**Table 5.17**) also reflects this precision, when taking into account that it only has to be lower than 40% for replicate phytoplankton analyses (Van Baalen & Du Preez, 2000b).

Table 5.17 shows that all the analysts displayed a % COV of less than 21%, which, too, is much less than the prescribed value of 40%. This indicates that each of the analysts was precise in their preparation and analyses of his/her own sub-sample sedimentation chambers. **Table 5.16** shows that all the analysts were able to identify the dominant taxa in the sample.

CONCLUSION

The analyses of replicate samples yielded precise results, indicating overall success in the application of the method and analyses. Only two out of 30 values fell outside the tolerance limits, these being ascribed to the presence of more/less than usual cyanobacterial colonies/filaments randomly settling in the lane area. All the analysts were able to identify the dominant taxa in the sample.

RECOMMENDATION

It is recommended that each analyst should analyse replicate samples on a regular basis (every 3 months) to ensure continued competency in terms of the preparation of samples for analyses. If the total biomass falls outside the tolerance limits for a particular analysis and the % COV exceeds 40%, the cause of this outlier value should be determined. Should the outlier be a result of a higher number of colonies/filaments in the sample, and the % COV is less than 100% then the result can be accepted (Van Baalen & Du Preez, 2000b). If the outlier is more than 100% and a result of colonial/filamentous phytoplankton, or more than 40% without colonial/filamentous phytoplankton, the analysis should be repeated. Should the results of the second analysis still not fulfil the criteria as described above, the analyst should be considered incompetent and receive further training.

5.2.11.2 Determination of tolerance limits for the dispenser pipette used in phytoplankton analysis

AIM

The aim was to establish the tolerance limits for the dispenser-pipette used in the analysis.

PROCEDURE

A calibrated balance was used to determine the mass of a volume of distilled water as set on the particular dispenser-pipette. The mass of 1 cm^3 (1 mL) of water is 1 g and can therefore be used as a measure to determine the volume of water dispensed by the pipette. For each volume typically used during this method, 20 repetitions were pipetted by each analyst into a beaker on a mass balance and the mass of each repetition measured and noted. The mean, standard deviation and percentage coefficient of variance (degree of variation) were calculated and tolerance limits were established for each volume.

RESULTS AND DISCUSSION

The results of the measurements are given in the figures and tables below.

Table 5.18: 1 mL Dispenser pipette verification values per analyst.

Analyst	Repetitions	Mean	SD	% COV
1	20	0.9960	0.0024	0.2441
2	20	0.9905	0.0082	0.8306
3	20	0.9985	0.0187	1.8731

Table 5.19: Overall 1 mL Dispenser pipette verification values.

Repetitions	Mean	SD	% COV
60	0.9950	0.0122	1.2218

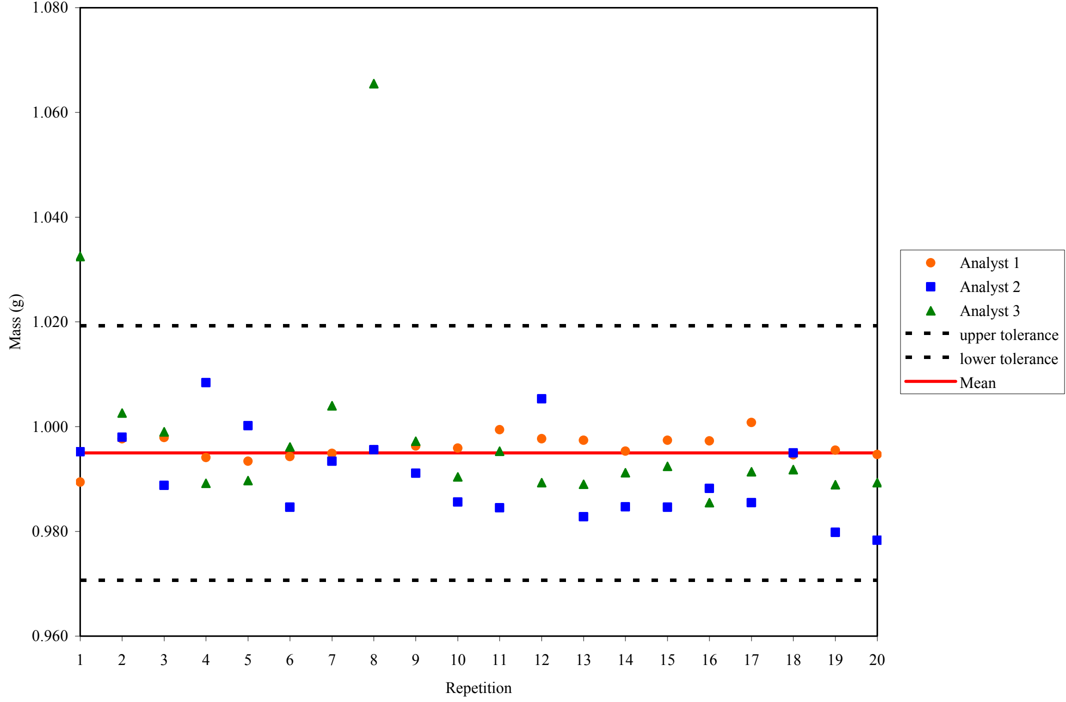


Figure 5.15: 1 mL dispenser pipette verification values for 3 analysts

Values for the 1 mL setting on the 1 mL pipette were within the tolerance limits 96.7% of the time. The only values outside the tolerance limits were 2 values of analyst 3. These outlier values could be ascribed to analyst error, as these values were well above the upper tolerance limit and all the other values occurred well within the tolerance limits. The percentage coefficient of variance (% COV) for each analyst needs to be less than 2% (Van Baalen & Du Preez, 2000a). None of the analysts exceeded this value. Analyst 3 had an elevated % COV (1.87%), but this could again be ascribed to the two outlier values. The overall % COV (1.22%) indicates that the pipette is relatively precise when set at 1 mL.

Table 5.20: 0.5 mL Dispenser pipette verification values per analyst.

Analyst	Repetitions	Mean	SD	% COV
1	20	0.4973	0.0012	0.2330
2	20	0.4898	0.0063	1.2859
3	20	0.4925	0.0069	1.4058

Table 5.21: Overall 0.5 mL Dispenser pipette verification values.

Repetitions	Mean	SD	% COV
60	0.4932	0.0062	1.2569

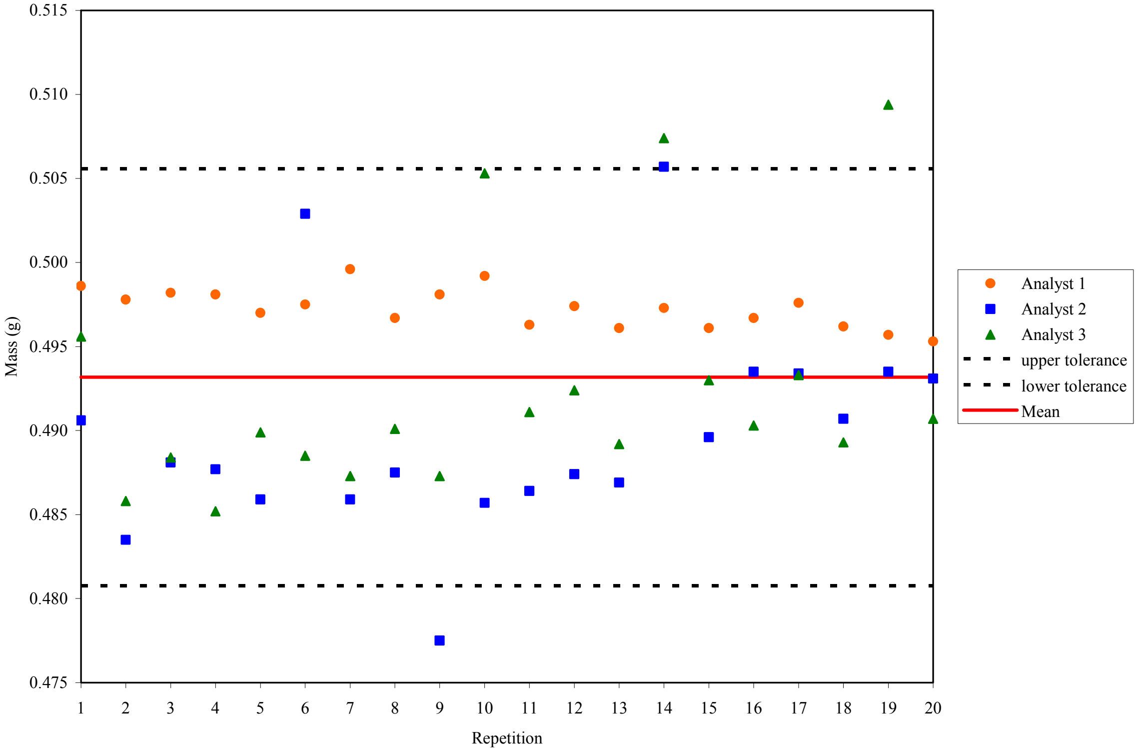


Figure 5.16: 0.5 mL dispenser pipette verification values for 3 analysts

For the 0.5 mL setting on the same Gilson P1000 pipette, the values were within the tolerance limits 93.3% of the time. Four values were outside the tolerance limits, one occurring just outside the upper tolerance. The values outside the tolerance limits could be ascribed to analyst error, as values of analyst 1 were all between the tolerance limits and these measurements were taken under the same conditions as set by the manufacturers. The % COV of all the analysts was less than 2%, suggesting a high degree of precision. The same could be said for the overall precision of the pipette when set at a volume of 0.5 mL (% COV < 2).

Table 5.22: 0.25 mL Dispenser pipette verification values per analyst.

Analyst	Repetitions	Mean	SD	% COV
1	20	0.2471	0.0010	0.4006
2	20	0.2459	0.0032	1.3074
3	20	0.2521	0.0032	1.2751

Table 5.23: Overall 0.25 mL Dispenser pipette verification values.

Repetitions	Mean	SD	% COV
60	0.2484	0.0038	1.5285

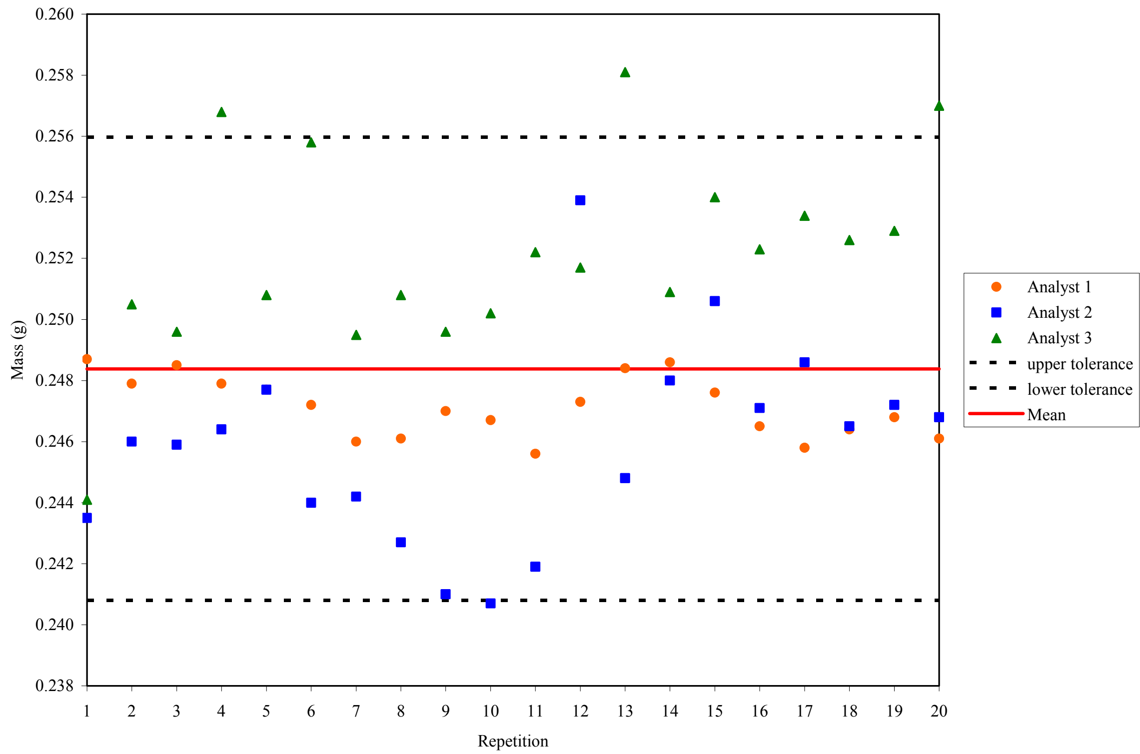


Figure 5.17: 0.25 mL dispenser pipette verification values for 3 analysts

Values for the 0.25 mL setting on the same Gilson P1000 pipette were between the tolerance limits 93.3% of the time. The 4 values outside the tolerance limits could be ascribed to analyst error for the same reasons as for the 0.5 mL setting above. There was, however, a greater variability in the measurements taken, as reflected by the higher % COV values of the individual analysts, as well as the overall values. This could be because of the greater chance of analyst error due to the smaller volume. In spite of the greater variability, the % COV of each analyst, as well the overall % COV was still less than 2%.

CONCLUSION

The pipette used for analysis is within acceptable limits at 1 mL, 0.5 mL and, and to a lesser extent, 0.25 mL. Variability in the measurements taken was a result of analyst error.

RECOMMENDATION

As shown in the verifications of the dispenser pipette above, analyst error could play a major part in accuracy and precision. The transferring of sample water with a dispenser pipette should be done slowly and care should be taken not to spill any of the sample in the process.

For smaller volumes (< 0.25 mL), a pipette of smaller volume should be used, for example a 0.25 mL pipette. This should increase the accuracy and precision with which the analyst works.

5.2.11.3 **Estimation of uncertainty and method limit of detection (MLD)**

AIM

The aim was to estimate the uncertainty arising in the analysis of phytoplankton samples using this method and to determine the lowest biomass concentration at which phytoplankton cells can be detected.

PROCEDURE

The results of the analyses of 30 samples were used to determine the uncertainty of this method. The following formula was used:

Uncertainty	=	[(2 × SD of sample data) / mean of data] × 100

The MLD for the analysis of phytoplankton samples is the lowest biomass concentration at which phytoplankton can be identified and enumerated. For this method the MLD is

calculated by determining the lowest value for the final conversion factor (the lowest value that a count of one would represent in terms of biomass).

RESULTS AND DISCUSSION

The calculation of the % uncertainty is given below.

$$\% \text{ Uncertainty} = [(2 \text{ x SD of sample data}) / \text{mean of data}] \times 100$$
$$= [(2 \text{ x } 6103.2133) / 34549] \times 100$$
$$= 35.33$$

The uncertainty value (35.33%) for this method is the result of the amount of variation that can be expected on both sides of the mean. 35.33% is the result of a 17.67% variation either side of the mean - a value still relatively small, considering that it should not exceed 40% (% COV) on both sides of the mean. The uncertainty inherent in this method is, by implication, relatively small.

The lowest value for the final conversion factor (MLD) is ca. 2 cells/mL when 5 lanes are analysed and 6 mL of sample is settled. This was calculated as follows:

$$\text{Sedimentation chamber floor area} = \pi r^2$$
$$= \pi \times (7\,962.5\ \mu m)^2$$
$$= 199\,181\,392\ \mu m^2$$

$$\text{Lane area} = \text{diameter of sedimentation chamber} \times \text{width of Whipple grid}$$
$$= 15\,925\ \mu m \times 176\ \mu m$$
$$= 2\,802\,800\ \mu m^2$$

The conversion factor is the total sedimentation chamber floor area divided by the total lane area. Note that the total lane area is the area of one lane multiplied by the amount of lanes analysed (maximum of 5 lanes).

$$\text{Conversion factor} = \frac{\text{Sedimentation chamber floor area}}{\text{Total lane area}}$$
$$= \frac{199\,181\,392\ \mu m^2}{(2\,802\,800\ \mu m^2 \times 5)}$$
$$= 14.213$$

Maximum sample volume settled = 6 mL.

Final conversion factor	=	$\frac{\text{Conversion factor}}{\text{Volume of sample used}}$

$$= \frac{14.213}{6 \text{ mL}}$$

$$= 2.368$$

Should only one phytoplankton cell be counted in this sample, the biomass would be 2 cells/mL (rounded to the nearest integer).

CONCLUSION

The uncertainty associated with this method is relatively low. Using this method analysts will be able to detect, with great accuracy, the biomass concentrations for each taxon identified.

RECOMMENDATION

It is recommended that the level of uncertainty be re-established every 3 months, at the same time as the analyst competency. This, too, will provide an accurate measure of the success with which the method is applied in the particular laboratory.

5.2.11.4 **Inter-analyst comparisons**

AIM

The aim was to determine the accuracy with which the individual analysts identify and enumerate phytoplankton.

PROCEDURE

One analyst prepared a set of 10 replicates for analyses in sedimentation chambers. Each replicate was then analysed for phytoplankton by all the analysts and the result reported according to the method above. The mean, standard deviation (SD) and % COV were determined for the total biomass (cells/mL) in the replicates of the sub-sample. The % dominant species identified by the analyst was also reported.

RESULTS AND DISCUSSION

The results of the phytoplankton analyses are given in the figures and tables below.

Table 5.24: Summary of the phytoplankton biomass analyses for each analyst

Analyst	Repetitions	Mean (cells/mL)	SD	% COV	% Dominant species identified
1	9	37928.6490	7055.4353	18.60	100
2	9	21929.8659	4904.5907	22.36	100
3	10	33124.7583	3050.2324	9.21	100

Table 5.25: Summary of the phytoplankton biomass analyses for all analysts

Repetitions	Mean (cells/mL)	SD	% COV
28	31070.5078	8370.7539	26.94

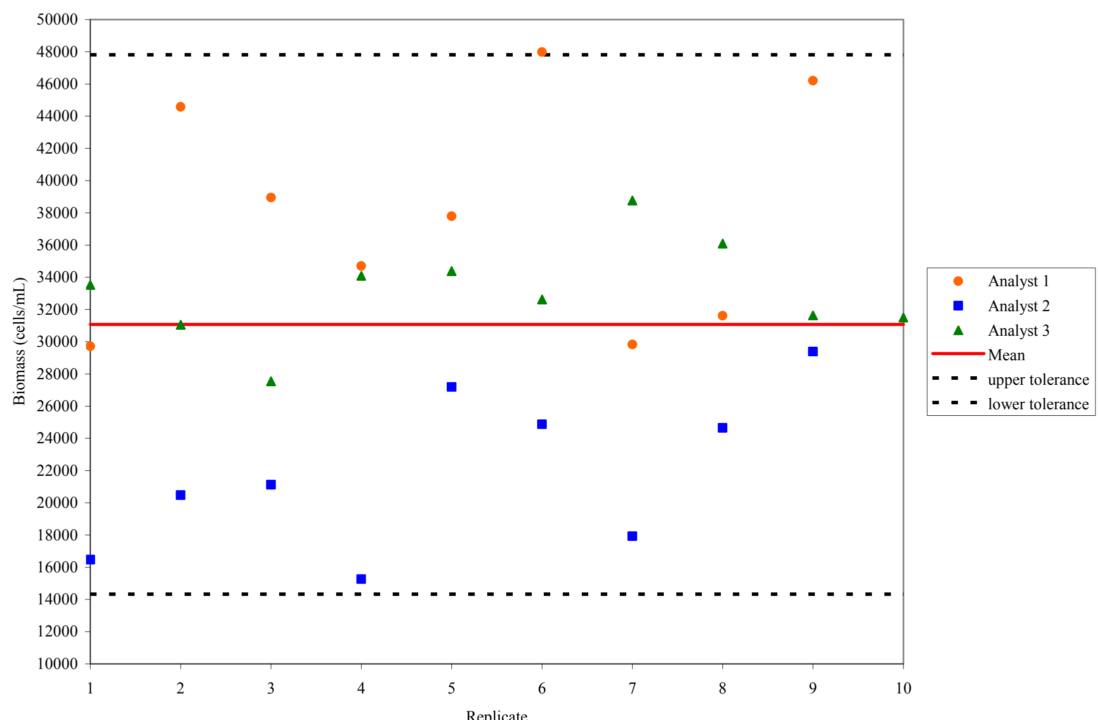


Figure 5.18: 6 Total biomass concentrations for 10 replicates analysed by 3 analysts

From **Figure 5.18** it can be seen that the total biomass concentration fell outside the tolerance limits (upper limit) on only one occasion (Analyst 1). This slightly higher value was due to the presence of a larger amount of cyanobacterial colonies/filaments. Individually, analysts had relatively low % COV values (**Table 5.24**) indicating a high degree of precision. Although one value for Analyst 1 fell outside the tolerance limit, this analyst still achieved a % COV lower than 40% (18.6%). Analysts 2 and 3, too, did not exceed the recommended 40% as mentioned in section 2.11.1 of this document. The overall % COV for the three analysts (26.94%, **Table 5.25**), and 100% identification of dominant species by all the analysts, indicates their satisfactory competence in the identification and enumeration of phytoplankton. All analysts achieved a high degree of accuracy (% COV < 40%). Mention should be made of the fact that all values for Analyst 2 were below the mean. This could be explained by the underestimation of the amount of colony/filamentous cyanobacteria in the sample.

CONCLUSION
Analyses of the same 10 replicate samples yielded precise and accurate results. The total biomass concentration fell outside the determined tolerance limits on only one occasion, but this outlier could be ascribed to the presence of a higher number of cyanobacterial colonies/filaments randomly settling in the lane area. The analysts were all able to identify the dominant taxa in the sample.

RECOMMENDATION
See the recommendation in section 5.2.11.1 of this document.

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

5.3.1 INTRODUCTION

5.3.1.1 Scope

This method is suitable for the quantification and identification of free-floating algae in water. The membrane filter method of determining algal numbers is used, as it is a rapid method capable of detecting algae at low concentrations.

5.3.1.2 Definition

"Algae" is a term referring collectively to a wide range of pigmented, oxygen-producing, photosynthetic organisms usually present in surface waters. Algae range from microscopically small unicellular forms, the size of bacteria, to larger filamentous forms that can be meters in length. Like other plants, algae are primary producers requiring light, carbon dioxide, water, nutrients such as nitrate and phosphate, and trace elements for growth. The classification of algae is extremely complex but can be broadly grouped as follows:

- Blue-green algae – typically dominate nutrient-enriched waters and are sometimes referred to as cyanobacteria, e.g. *Anabaena* and *Microcystis*.
- Green algae – common over summer in less enriched water bodies, e.g. *Chlorella* and *Crucigenia*.
- *Euglena* – flagellate unicellular algae, typical of organically-enriched water.
- Diatoms – flagellate unicellular algae surrounded by a silica coating and often dominate winter algal populations, e.g. *Cyclotella* and *Synedra*.

5.3.1.3 Field of application

This method is suitable for all types of freshwater including dams, rivers and treated drinking water.

5.3.1.4 Interferences

Excessive turbidity caused by suspended silt may prevent sufficient sample from being filtered and may obscure cells from view.

5.3.1.5 Method range

The method is capable of identifying and quantifying algae in very low or high concentrations where additional blending and/or dilution steps are included for very dense algae populations.

5.3.2 PRINCIPLE

The method is capable of identifying and quantifying algae in very low or high concentrations where additional blending and/or dilution steps are included for very dense algae populations.

5.3.3 WATER QUALITY

5.3.3.1 **Significance of phytoplankton identification and enumeration**

Phytoplankton in source water, known to be sensitive indicators of water quality, have an effect on the production of potable water, the aesthetic aspect of recreational waters and consumer health, because:

- Phytoplankton and their cellular products interfere with the physical and/or chemical water purification processes.
- Phytoplankton is able to pass through purification processes resulting in water of aesthetically unacceptable quality (taste, odour and colour).
- Some phytoplankton species have the ability to produce substances that can be detrimental to the health of consumers and can be a source of carbon for bacteriological growth.
- Excessive growth of phytoplankton in source water (water bloom) can create aesthetically unacceptable recreational and potable water and may pose a health risk to consumers (taste, odour, scum and toxin).

Advantages of phytoplankton monitoring include:

- Detecting the presence of and examining the short-term trends in the growth of phytoplankton species in order to determine the suitability of a particular water for drinking and recreational use.
- Providing data to determine long-term trends in phytoplankton composition within a particular waterbody. This information can be used to assess, for example, the effect of sewage discharge and agricultural run-off containing fertilizers and harmful chemicals.
- Monitoring the effects of management measures such as river regulation, inter-basin transfers and water abstractions within a particular system.

5.3.3.2 **Water quality guideline**

The guidelines in **Table 5.26** are used by Umgeni Water for water treatment purposes.

Table 5.26: Warning Limits for the different algal species

Description	Units	Limit	Sites	Potential problem
Total Algal count	cells per mL	3000	Raw/Abstraction	Dependant on species
Total Algal count	cells per mL	15000	Dam	Dependant on species
Anabaena	cells per mL	1000	Raw/Abstraction	Taste and odour and toxin producing
Microcystis	cells per mL	3000	Raw/Abstraction	Taste and odour and toxin producing
Oscillatoria	cells per mL	1000	Raw/Abstraction	Taste and odour and toxin producing
Chlorella	cells per mL	3000	Raw/Abstraction	Filter clogging
Cosmarium	cells per mL	3000	Raw/Abstraction	Taste and Odour
Crucigenia	cells per mL	3000	Raw/Abstraction	Filter penetration
Cyclotella	cells per mL	3000	Raw/Abstraction	Filter clogging
Diatoma	cells per mL	3000	Raw/Abstraction	Filter clogging
Dinobryon	cells per mL	1000	Raw/Abstraction	Taste and odour and filter clogging
Melosira	cells per mL	1000	Raw/Abstraction	Filter clogging

5.3.4 APPARATUS, MATERIALS AND REAGENTS

5.3.4.1 **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 5.3**).
- Vacuum manifold fitted with membrane filter holders capable of holding 47 mm diameter or other similar membrane filters (refer to **Figure 5.19**). Vacuum pump with a vacuum gauge and adjustable vacuum connected (via a collection vessel) to the vacuum manifold.
- Homogeniser, variable speed **(Figure 5.1)**

Figure 5.19: Vacuum manifold fitted with 47 mm membrane filter holders.

5.3.4.2 **Glassware**

25 mL, 50 mL and 2000 mL measuring cylinders

5.3.4.3 **Other materials**

- 0.45 µm filters of appropriate quality.

5.3.4.4 **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.

5.3.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

Samples are disposed via the drainage system except for toxic effluents that are disposed as hazardous waste according to appropriate procedures.

5.3.6 PROCEDURE

5.3.6.1 **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 80 kPa.

- 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, homogenise approximately 100 mL sample for approximately 10 seconds using the homogeniser 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. (See 3.10.4)

5.3.6.2 **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 (see section 5.2.6.1 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.

5.3.7 SAFETY PRECAUTIONS

5.3.7.1 **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 above substances.

5.3.7.2 **Clothing**

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

5.3.8 CALCULATION AND EXPRESSION OF RESULTS

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

$$\text{Conversion factor (CF)} = \frac{\text{Area of filter}}{\text{Area of view under microscope}}$$

$$\text{Algae number} = \frac{\text{CF} \times \text{no. of individuals counted}}{\text{No. of fields} \times \text{volume filtered (mL)}}$$

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.

5.3.9 RECORDS AND DATA KEEPING

According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory).

5.3.10 QUALITY ASSURANCE

5.3.10.1 General

A random sample that is selected by a technician is analysed in duplicate. The results for both the original and duplicate filters are captured onto the relevant form. The total count of algae for both the original and duplicate analysis should not differ by more than 30%, and nor should the counts of the dominant genera.

Regular (quarterly) participation in an appropriate, external Proficiency Testing Scheme (PTS). The PTS compares the technician's identification skills against other participants and the reference lab, and recommends corrective action to be taken by potential failures and in areas of improvement. If the results fail the PTS, an investigation and a report will be done on the possible causes, and will be attached to the PTS report.

5.3.10.2 **Calibration of instruments**

Balance

- The balance should be calibrated regularly (confirm an appropriate interval with instrument supplier).
- Perform a quality check on the balance before use by weighing a set of reference weights.

5.3.11 TYPICAL VALIDATIONS FOR THE METHOD

To establish this method in a laboratory it is necessary to perform validation. Validation of a method is necessary for individual laboratories as different analysts are performing analysis and laboratories have different environments. Therefore analyst sensitivity should be validated in individual laboratories.

Should any section of a method be validated it is important to have a documented procedure that will be followed, traceability of all results, documented report on the validation that sets out the procedures and details the results obtained with proper and appropriate statistical analysis.

The following sections will provide guidance on procedures that can be followed when validating this method.

5.3.11.1 **Repeatability**

A sample can be split and analysed 15 times by the same analyst. Summary statistics can then be done using any software package such as EXCEL. The results can be accepted if the covariance lies within the limits set up by the respective laboratory. Furthermore, the dominant genera quantified for each sample split should be comparable.

5.3.11.2 **Inter-analyst comparisons**

Analyst competency should be evaluated regularly (e.g. quarterly) to ensure the reliability of results. New analysts should also be proven competent before allowed to analyse routine samples or samples for clients.

Analysts are deemed competent if all comply with the following:

- The total algal counts for the same sample are within acceptable limits
- The total number of different genera observed are relatively close and the dominant genera are observed by both analysts

5.4 BIOVOLUME DETERMINATIONS

5.4.1 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 (μm^3/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.

5.4.2 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 × – 1000 ×) 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.

Other methods (automated and semi-automated) 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.

5.4.3 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 *Tetraedron* 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 5.27** 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 5.27: Shapes, formulas and genus list.

	Shape	Formula	Genus
1	**Cylinder** (Hillebrand et al., 1999)	$V = \pi/4 \cdot d^2 \cdot h$ **OR** $V = \pi \times radius^2 \times h$	*Arthrospira* *Aulacoseira* *Cyclostephanos* *Cyclotella* *Cylindrospermopsis* *Melosira* *Oscillatoria* *Spirogyra* *Spirulina* *Stephanodiscus*

Table 5.27 (cont): Shapes, formulas and genus list.

2	**Sphere** (Hillebrand et al., 1999)	$V = \pi/6 \cdot d^3$	*Anabaena* [1, 2, 14] *Aphanocapsa* *Carteria* [1, 3, 14] *Chlamydomonas* [3, 14] *Chlorella* *Chlorococcum* [3, 14] *Coelastrum* [3, 14] *Dictyosphaerium* [3, 14] *Eudorina* *Golenkinia* *Micractinium* [4] *Microcystis* *Sphaerocystis* *Tetrastrum* [14] *Volvox*
3	**Prolate spheroid** (Hillebrand et al., 1999)	$V = \pi/6 \cdot d^2 \cdot h$ Sub-spherical body with circular cross-section and elliptical apical section. (Hillebrand et al., 1999).	*Cryptomonas* [1, 14] *Dinobryon*[1, 13, 14] *Mallomonas* *Oocystis* *Pandorina* [14] *Scenedesmus* [14]

Table 5.27 (cont): Shapes, formulas and genus list.

4	**Ellipsoid** (Hillebrand et al., 1999)	$V = \pi/6 \cdot a \cdot b \cdot h$ This body is sub-spherical with three different dimensions, i.e. prolate spheroid with elliptical cross-sections (Hillebrand et al., 1999).	*Characium* [14] *Lagerheimia* *Peridinium* *Strombomonas* [13] *Trachelomonas* [13]
5	**Prism on parallelogram** (Hillebrand et al., 1999)	$V = ½ . a . b . c$ Rhombic diatom species belong for example to the genera *Pleurosigma* and *Gyrosigma*, the basic parallelogram is even-sided (Hillebrand et al., 1999).	*Gyrosigma* *Nitzschia* [10, 14] *Pleurosigma*

Table 5.27 (cont): Shapes, formulas and genus list.

6	**Elliptic prism** (Hillebrand et al., 1999) Prism on elliptic base apical valve view apical girdle view transapical view apical valve view Elliptic prism with trans-apical constriction	$V = \pi/4 \cdot a \cdot b \cdot c$ 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).	*Achnanthes* [4-7] *Cocconeis* *Diatoma* [6-7] *Fragilaria* [4, 6, 14] *Navicula* [4, 6] *Surirella* *Pediastrum* [8, 14] *Phacus* [9, 14]

Table 5.27 (cont): Shapes, formulas and genus list.

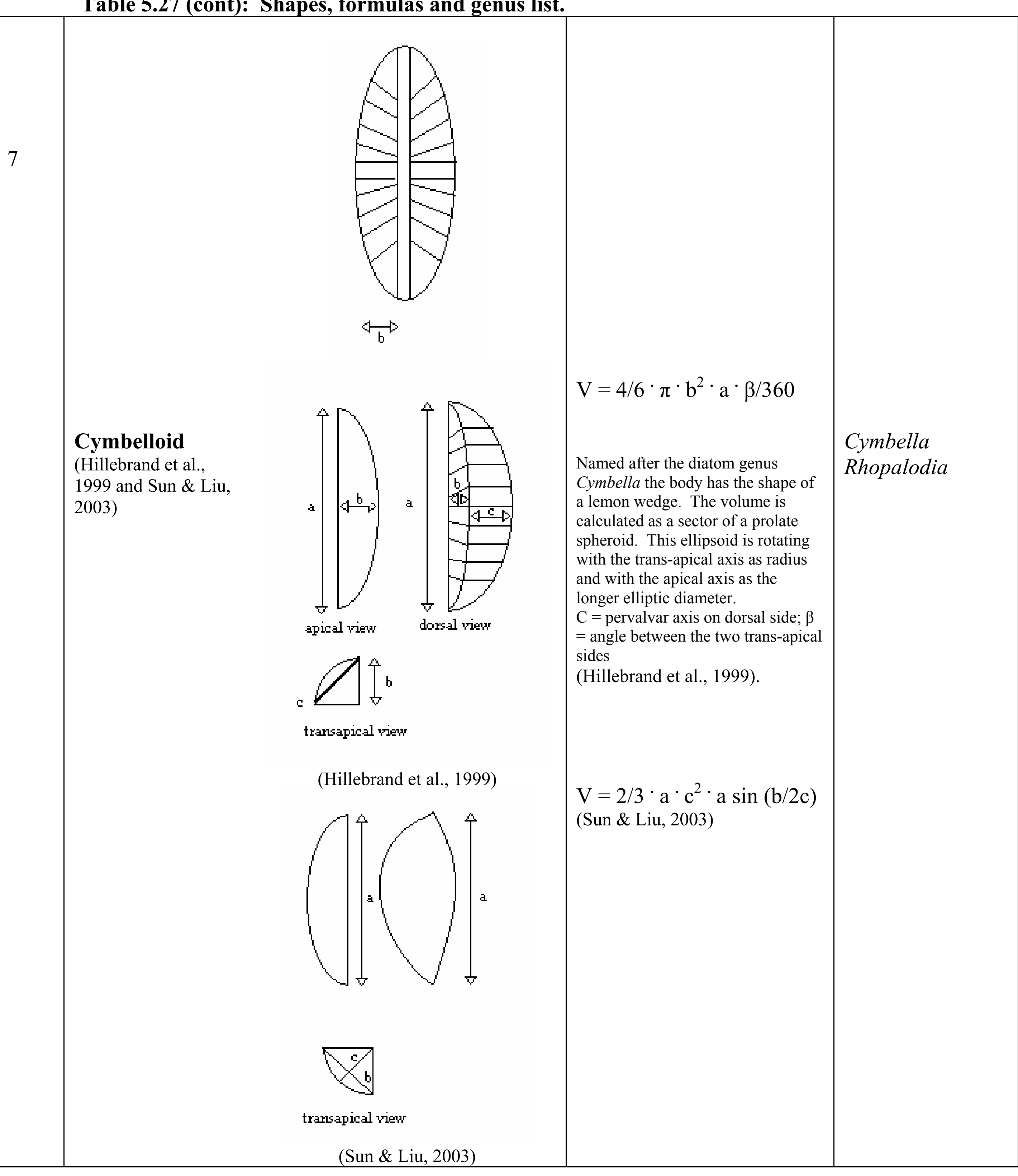
7	**Cymbelloid** (Hillebrand et al., 1999 and Sun & Liu, 2003)  (Hillebrand et al., 1999) (Sun & Liu, 2003)	$V = 4/6 \cdot \pi \cdot b^2 \cdot a \cdot \beta/360$ Named after the diatom genus *Cymbella* the body has the shape of a lemon wedge. The volume is calculated as a sector of a prolate spheroid.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

Table 5.27 (cont): Shapes, formulas and genus list.

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

Table 5.27 (cont): Shapes, formulas and genus list.

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

Table 5.27 (cont): Shapes, formulas and genus list.

12	**Sickle-shaped monoraphidioid** (Hillebrand et al., 1999) b2 a b apical view d cross view	$V = d^2/8 \cdot (2b - d + a) \cdot (\pi^2/6 + 1)$ A special case is lunate bodies which are circular in cross-section. The chlorophyte genera *Monoraphidium* and *Kirchneriella* are examples. The maximum diameter of the body is given as d (= $b - b_2$), all other abbreviations as given (Hillebrand et al., 1999).	*Kirchneriella* *Monoraphidium/ Selenastrum*
13	**Box** (Modified from Hillebrand et al., 1999) b c a a c apical girdle view a b apical valve view c b transapical view	$V = a \cdot b \cdot c$	*Tetraedron* [14]
14	**Cube** (Modified from Hillebrand et al., 1999) a	$V = a^3$ A cube is a special case of the shape in 3.13 where a=b=c, then $V=a^3$ (Hillebrand et al., 1999).	*Crucigenia* [14] *Merismopedia* [14]

Table 5.27 (cont): Shapes, formulas and genus list.

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

Table 5.27 (cont): Shapes, formulas and genus list.

17	**Half ellipsoid** + **cone on elliptic base** (Constructed from Hillebrand et al., 1999) For half ellipsoid see 9 Cone on elliptic base **OR** **OR** **Cone** + **half sphere** + **cylinder** (Sun & Liu, 2003)	See: 9 + $V = \pi/12 \cdot x \cdot z \cdot c$ (Constructed from Hillebrand et al., 1999) **OR** See: 11 + 1	*Euglena* [1, 9, 14]

Table 5.27 (cont): Shapes, formulas and genus list.

18	**2 truncated cones**	Truncated cone:	Formula for one truncated cone: $V = \pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2)$	*Staurastrum* [14] *Staurodesmus*
19	**Cylinder** + **ellipsoid** + **3 cones** (Modified from Hillebrand et al., 1999 and Sun & Liu, 2003)	cross view of ellipsoid	$V = \pi/4 \cdot h \cdot d^2$ + $\pi/6 \cdot a \cdot b \cdot c$ + $\pi/12 \cdot (e + f + g) \cdot b_2^2$.	*Ceratium* [1, 12]

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 × 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.

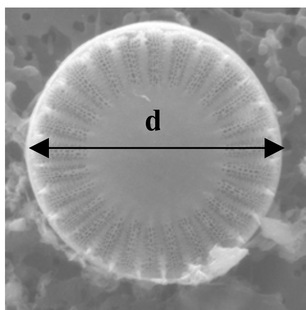
10. The genus *Nitzschia* is quite variable in its shape. The sigmoid and rhombic cells can be calculated as prism on a parallelogram as described. Elliptic species are to be calculated as elliptic prisms, linear species as boxes.
11. These genera include some species which are straight and others which are bent. The latter cells should be calculated as Monoraphidiods (see number 12 in **Table 5.27**.
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-thecal 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.

N.B. Most footnotes are modified from Hillebrand et al. (1999).

5.4.4 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.

5.4.4.1 Calculating the biovolume of *Cyclotella meneghiniana*



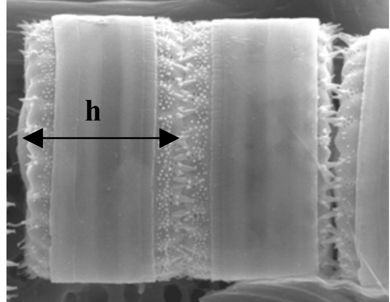


Figure 5.20: Scanning electron microscope photo demonstrating linear measurements of *Cyclotella meneghiniana*.

Table 5.28 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 5.27**.

Formula for calculating biovolume of a cylinder:

$$V\ (\mu m^3) = \pi/4 \cdot d^2 \cdot h$$
$$= \pi/4 \cdot 9.84^2 \cdot 6.27$$
$$= 476.81$$

5.4.4.2 **Calculating the biovolume of *Staurastrum tetracerum*:**

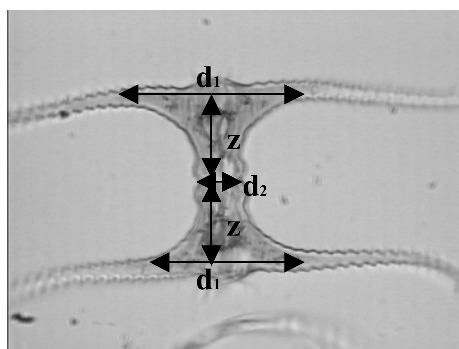


Figure 5.21: Light microscope photo demonstrating linear measurements of *Staurastrum tetracerum*.

Table 5.29Dimensions of *Staurastrum tetracerum*:

Repetition	First truncated cone			Second truncated cone	
	z	**d_1**	**d_2^{**}**	**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 5.27**.

Formula for calculating the biovolume of one truncated cone:

V_1 (μm^3) = $\pi/12 \cdot z \cdot (d_1^2 + (d_1 \cdot d_2) + d_2^2)$

Biovolume of first truncated cone:

V_1 (μm^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

Biovolume of second truncated cone:

V_2 (μm^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

Total biovolume:

V_{total} (μm^3) = $V_1 + V_2$

= 994.269 + 1043.653

= 2037.922

5.5 SUMMARY

Different methods exist to determine the biomass of phytoplankton of which the sedimentation- and membrane filtration techniques are the most common. Variations on both are described in this chapter.

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 μm^3/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 5.1**).

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CHAPTER 6

GEOSMIN AND 2-METHYLISOBORNEOL (2-MIB)

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).

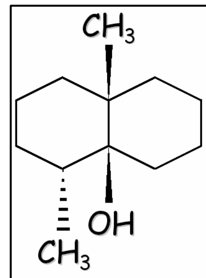

Figure 6.1: Biochemical structure of geosmin

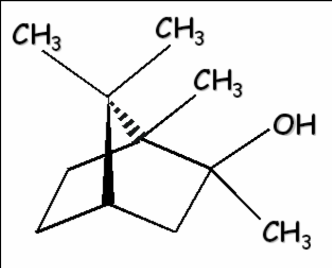

Figure 6.2: Biochemical structure of 2-MIB

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 easily 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.

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

6.1.1 INTRODUCTION

Geosmin and 2-methyl-isoborneol are secondary metabolites produced by certain cyanobacteria (blue-green algae) and actinomycetes. They have been reported as imparting an earthy or musty odour to drinking water.

Both compounds (geosmin and 2-MIB) are extracted using a Purge and Trap procedure to extract and concentrate the analytes, which are then analysed using Gas Chromatography-Mass Spectrometry.

6.1.1.1 Scope

This method is for the determination of geosmin and 2-methyl-isoborneol (2-MIB) in raw and potable waters.

Organic compounds that are extracted during this procedure may co-elute with the compounds of interest. The uniqueness of the mass spectrum of each target compound makes it possible to confirm compound identity with a high probability when co-eluting components are present. During the GC analysis, operation is performed in the single ion monitoring mode.

6.1.1.2 Definition

Chemical formulae for geosmin and 2-methyl-isoborneol

Geosmin – $C_{12}H_{22}O$

2-Methyl-isoborneol – $C_{11}H_{20}O$

Both geosmin and 2-methyl-isoborneol are saturated cyclic tertiary alcohols (see **Figures 6.1** and **6.2**).

6.1.1.3 Field of application

For the identification of geosmin and 2-MIB in raw and potable water samples.

6.1.1.4 Interferences

Samples with a high turbidity should be filtered first to prevent clogging of the column.

6.1.1.5 Method range

The method limit of detection for both geosmin and 2-MIB is summarised in **Table 6.1**.

Table 6.1: Method limits of detection

LIMITS	GEOSMIN	2-MIB
Linear Dynamic Range	0 ng/l – 40 ng/l	0 ng/l – 40 ng/l
Lowest Quantifiable Concentration	10 ng/l	10 ng/l

6.1.2 PRINCIPLE

Geosmin and 2-MIB are analysed using Purge and Trap coupled to Gas Chromatography-Mass Spectrometry. An inert gas is bubbled through a 25 mL water sample contained in a specially designed purging chamber at 70°C. The analytes are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent trap where the analytes are trapped. After purging is completed, the trap is heated and back-flushed with the inert gas to desorb the purgeables onto a gas chromatographic column. The gas chromatograph is temperature programmed to separate the analytes that are then detected with a mass spectrometer. The external standard method of quantification is used. The responses obtained are compared with those of standard solutions treated similarly and results are reported as ng/L.

6.1.3 WATER QUALITY

6.1.3.1 Significance of geosmin/2-MIB analysis

Geosmin and 2-MIB are two compounds most frequently responsible for musty or earthy taste and odour in drinking water and are virtually unaffected by chlorination.

6.1.3.2 Quality criteria / standards guidelines

There are currently no water quality guidelines (worldwide) associated with the occurrence of geosmin or 2-MIB, since they are not regarded as a health risk. Rand Water has an internal recommended geosmin limit of 30 ng/L in the final drinking water, but no recommended limit for 2-MIB.

6.1.4 APPARATUS, MATERIALS AND REAGENTS

All instruments are operated in accordance with the manufacturers instructions.

6.1.4.1 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 6.2: Parameters for GC Operating conditions

Parameter	Value
Oven temperature Program	70EC to 160EC at 50EC/min, 180EC at 4EC/min, 200EC at 10EC 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

6.1.4.2 **Glassware**

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

6.1.4.3 **Other materials**

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

6.1.4.4 **Reagents**

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

6.1.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

No special precautions other than standard laboratory procedures are necessary.

6.1.6 PROCEDURE

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

6.1.6.1 **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.

6.1.6.2 **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.

6.1.6.3 **Calibration procedure**

- A calibration curve is generated by analysing 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 6.3**, by spiking 1000 mL milli-Q water with 0.2 ng/µL.

Table 6.3: 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.

6.1.6.4 **Setting the instrument parameters**

Make sure the instrument parameters are set as indicated in **Tables 6.4** and **6.5.**

Table 6.4: 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 6.5: 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

6.1.6.5 **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.

6.1.7 SAFETY PRECAUTIONS

6.1.7.1 **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!**

6.1.7.2 **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.

6.1.7.3 **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.

6.1.8 CALCULATIONS AND EXPRESSION OF RESULTS

6.1.8.1 **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 6.1.6.3 of this method.

6.1.9 RECORDS AND DATA KEEPING

- Record the results of the sample into the geosmin/2-MIB final results file on an applicable form.
- The results are reported as ng/L. The results lower than the lowest quantifiable concentrations, are reported as <10 ng/L for both geosmin and 2-MIB.
- All the chromatograms and associated reports for each batch of samples must be retained.
- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

6.1.10 QUALITY ASSURANCE

6.1.10.1 **General**

- The handling of the GC and MS is specialized and complicated and analysts should be very familiar with the apparatus and proven competent to execute the method correctly.
- Interpretation and authorisation of results should be done by the senior / principle scientist.

6.1.10.2 **Precision and accuracy**

The accuracy and uncertainty for this method was validated and details are discussed in sections 6.1.11.3 and 6.1.11.4. The accuracy for the geosmin and 2-MIB methods was determined to be 20%.

6.1.10.3 **Maintenance and service**

Maintenance of instruments is dependent on the number of samples analysed and their physical characteristics. If many samples are analysed and physical properties of the samples include very turbid and contaminated samples, maintenance should be done more frequently.

- Instrument maintenance should be done as per manufacturer or supplier.
- Ion source should be cleaned at least once a year or as often as necessary.
- Autotune on the MS should be done weekly.
- When resolution or sensitivity of the column decreases, the column can be baked.

6.1.10.4 **Calibration**

Calibration of the instrument should be done prior to all analysis (refer to Section 6.1.6.3).

6.1.10.5 **Verification**

Every 20th sample run, should be a verification standard, to make sure the instrument is functioning properly. If the verification standard is out by more than 20%, the instrument should be re-calibrated and all samples analysed prior to the verification standard should be re-analysed.

6.1.11 TYPICAL VALIDATIONS FOR THE METHOD

6.1.11.1 **Geosmin validations**

6.1.11.1.1 ***Range of standards***

10 ng/L to 40 ng/L

6.1.11.1.2 ***Calibration data***

Concentration (ng/L)	Response Ratio
10	629.039
20	1326.035
30	1902.382
40	2384.045

K	4
sum(x)	100
sum(x)2	3000
SS(X)	500
sumY	6241.501
sum(y)2	11456787
SS(Y)	1717703
sumXY	185244.4
SP(XY)	29206.83
K	4
sum(x)	100

6.1.11.1.3 ***Regression Analysis***
Summary output

Regression Statistics	
Multiple R	0.996610147
R Square	0.993231785
Adjusted R Square	0.989847677
Standard Error	76.24232513
Observations	4

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	1706077.253	1706077.253	293.4989	0.003389853
Residual	2	11625.78428	5812.892142		
Total	3	1717703.037			

	Coefficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***	***Upper 95%***	***Lower 95.0%***	***Upper 95.0%***
Intercept	100.034	93.3773	1.0712	0.3961	-301.7367	501.8048	-301.7367	501.8047
X Variable 1	58.41365	3.4096	17.1318	0.0033	43.7430	73.0842	43.7430	73.0842

- Slope (b) 58.41365000
- Y-intercept (a) 100.03400000
- Linearity F_{calc} = 293

  F_{calc} large, therefore significant linearity

6.1.11.1.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 76.24232513
- Slope Uncertainty ($S_{b)}$ 3.40966044
- Y-intercept Uncertainty (S_a) 93.37739669

6.1.11.1.5 ***Method Limit of Detection***

- $Y_{LOD} = Y_B + 3S_B = bX_{LOD} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L

  3.92 ng/L

6.1.11.1.6 ***Method Limit of Quantitation***

- $Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$
- Assume Y_B=a and $S_B=S_{y/x}$
- Thus, $X_{LOQ}=10S_{y/x}/b$, ng/L

  13.05 ng/L

6.1.11.1.7 ***Accuracy***

A verification Standard, concentration of 20 ng/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	20.57	1301.603
#2	19.52	1240.268
#3	21.06	1330.225
#4	21.10	1332.526
#5	19.34	1229.754
#6	18.21	1163.747
#7	19.37	1231.506
Mean	19.8814	1261.3756
Std Deviation	1.0667	62.3028

- **RSD of concentration, (s × 100/mean), %**
  5.37
- **Mean Method Accuracy (% of true conc.), %**
  99.41

6.1.11.1.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0 + a$

For $x_0 = 19.8814$

$y_0 = 1261.38081$

ave.y = 1261.37557143

y_0-ave.y = 0.00523864

$(y_0\text{-ave.y})(y_0\text{-ave.y}) = 2.74434 \times 10^{-5}$

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
10	-15	225
20	-5	25
30	5	25
40	15	225

Sum = 500

Sum^2= 250000

$Sx_0 = S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2\text{sum}(x_i\text{-ave.x})^2\}^{1/2}$

Where m = determinations = 1; n = calibration points = 4

Sx_0 = 1.459273832

B Uncertainty of Repeatability

Std Dev.y/ave.y = 0.049392767

C Uncertainty of Purity

Purity = 100%+-0.5%
Assume rectangular distribution
$U(P)=0.5/3^{1/2}$= 0.29

D Uncertainty of Volume

i) 10 mL volumetric flask, S.N 0134
10 mL ± 0.04 mL, 20°C
Assume rectangular distribution
$U(V_1) = 0.04/3^{1/2} = 0.023094011$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00021
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.004849742
Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe
100 µL ± 0.12 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.12/3^{1/2} = 0.069282032$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.34410076
100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe
1000 µL ± 0.49 µL, 20°C
Assume rectangular distribution

$U(V_1) = 0.49/3^{1/2} = 0.282901632$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 3.441007604

1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$= 0.003452617 µL

iv) 1000 µL syringe

D Total Uncertainty

Total Uncertainty, at $x_0 = 19.8814$

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$

U(t) = 1.763201217 ng/µL

90% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$

$x_0 \pm \{3.18*U(t)\}$

19.8814 ng/l ± 5.148547554 ng/l

6.1.11.2 **2-MIB validations**

6.1.11.2.1 ***Range of standards***

10 to 40ng/l

6.1.11.2.2 ***Calibration data***

Concentration (ng/L)	Response Ratio
10	67.883
20	137.005
30	181.289
40	244.552

K	4
sum(x)	100
sum(x)2	3000
SS(X)	500
sumY	630.729
sum(y)2	116049.9
SS(Y)	16595.09
sumXY	18639.68
SP(XY)	2871.455
b	5.74291
a	14.1095

6.1.11.2.3 ***Regression Analysis***

Summary output

Regression Statistics	
Multiple R	0.996844134
R Square	0.993698228
Adjusted R Square	0.990547342
Standard Error	7.231128705
Observations	4

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	16490.50763	16490.50763	315.3711	0.003155866
Residual	2	104.5784447	52.28922235		
Total	3	16595.08608			

	Coefficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***	***Upper 95%***	***Lower 95.0%***	***Upper 95.0%***
Intercept	14.1095	8.8562	1.5931	0.2521	-23.9960	52.2150	-23.9960	52.2150
X Variable 1	5.7429	0.3233	17.7586	0.0031	4.3514	7.13438	4.3514	7.1343

- Slope (b) 5.74291000
- Y-intercept (a) 14.10950000
- Linearity F_{calc} = 315

  F_{calc} large, therefore significant linearity

6.1.11.2.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 7.23112871
- Slope Uncertainty ($S_{b)}$ 0.32338591
- Y-intercept Uncertainty (S_a) 8.85628780

6.1.11.2.5 ***Method Limit of Detection***

- $Y_{LOD} = Y_B + 3S_B = bX_{LOD} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L

3.78 ng/L

6.1.11.2.6 ***Method Limit of Quantitation***

- $Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$
- Assume Y_B=a and $S_B=S_{y/x}$
- Thus, $X_{LOQ}=10S_{y/x}/b$, ng/L

12.59 ng/L

6.1.11.2.7 ***Accuracy***

A verification Standard, concentration of 20 ng/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	19.86	128.637
#2	18.77	121.904
#3	17.82	116.448
#4	20.72	133.102
#5	20.02	129.082
#6	19.38	125.407
#7	19.31	125.005
Mean	19.4114	125.6550
Std Deviation	0.9345	5.4071

- **RSD of concentration, (s × 100/mean), %**

  4.81

- **Mean Method Accuracy (% of true conc.), %**

  97.06

6.1.11.2.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0 + a$
For $x_0 = 19.4114$
$y_0 = 125.5875873$
ave.y = 125.65500000
y_0-ave.y = -0.06741274
(y_0-ave.y)(y_0-ave.y) = 0.004544478

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
10	-15	225
20	-5	25
30	5	25
40	15	225
	Sum =	500
	Sum^2=	250000

$$Sx_0 = S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$$

Where m = determinations = 1; n = calibration points = 4

Sx_0 = 1.407761513

B Uncertainty of Repeatability

Std Dev.y/ave.y = 0.04303094

C Uncertainty of Purity

Purity = 100%+-0.5%
Assume rectangular distribution
U(P)=0.5/$3^{1/2}$= 0.29

D Uncertainty of Volume

i) 10 mL volumetric flask, S.N 0134

10 mL ± 0.04 mL, 20°C
Assume rectangular distribution
$U(V_1) = 0.04/3^{1/2} = 0.023094011$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00021

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.004849742

Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe

100 µL ± 0.12 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.12/3^{1/2} = 0.069282032$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.34410076

100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe

1000 µL ± 0.49 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.49/3^{1/2} = 0.282901632$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 3.441007604

1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$= 0.003452617 µL

iv) 1000 µL syringe

D Total Uncertainty

Total Uncertainty, at x_0 = 19.4114

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$

U(t) = 1.641303638 **ng/µL**

90% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$

$x_0 \pm \{3.18*U(t)\}$

19.4114 ng/l ± 4.792606623 ng/l

6.2 SUMMARY

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.

6.3 REFERENCES

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CHAPTER 7

CYANOBACTERIAL TOXIN ANALYSES

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 waterbodies. 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, (effecting 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 5).
- 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).

MICROCYSTINS AND NODULARINS

Most documented cases of cyanobacterial toxicosis were caused by the structurally-similar microcystins and nodularins (Carmichael, 1977). *Microcystis* spp are the main worldwide offenders, compared to the other toxin-producing genera. A study performed in the late 1990's, revealed that 80% of a set of source water samples taken from all over the USA and Canada

tested positive for microcystins of which 4.3% of the levels were higher than the WHO drinking water guideline level of 1 µg/L (Carmichael, 2001).

Because of the wide global distribution of *Microcystis* spp and the well-documented cases of their toxic effects on livestock, wild animals and humans, the determination of microcystin concentrations is well-researched. Different techniques exist for the determination of microcystin concentrations, two of which are taken up in this methods manual, namely the ELISA (enzyme linked immuno sorbent assay) as well as the HPLC (high pressure liquid chromatography) methods.

Figure 7.1: Microcystin-LR biochemical structure

Figure 7.2: Nodularin biochemical structure

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.

DECISION WHETHER TO USE ELISA OR HPLC FOR MICROCYSTIN ANALYSIS

Table 7.1: 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**
Analysis cost per sample:	**High**	**Relatively low**

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

Currently there is a range of different ELISA kits available for the determination of microcystin concentrations on the market. A very common kit used is the Envirologix Quantiplate™ Kit for Microcystins (as described in the method below). Another common (and widely preferred) plate used is the ABRAXIS microcystin test kit, which is

congener-independent due to the antibody being developed against the Adda region (the toxic region) of the microcystin molecule.

7.1.1 INTRODUCTION

7.1.1.1 **Scope**

Microcystins are a group of toxins that are produced by cyanobacteria (blue-green algae). These toxins can be contained in the cells (intracellular) or be released into the surrounding environment (extra-cellular). Microcystin toxins in potable and recreational water pose a health risk to both humans and animals. The quantification of this toxin is essential in activating management protocols to safeguard all living organisms from lethal poisoning (WHO, 1999). Use of the term 'toxin' in this method refers to the microcystin group of cyanobacterial toxins.

7.1.1.2 **Definition**

Microcystins are classified as hepatotoxic cyclic peptides containing seven amino acids. The two terminal amino acids of the linear peptide are condensed to form a cyclic compound (WHO, 1999).

The microcystin plate kit used to detect microcystins in water samples is a competitive Enzyme-Linked Immuno Sorbent Assay (ELISA) where the microcystins in the sample and the enzyme compete for binding sites on the walls of the micro wells.

7.1.1.3 **Field of application**

This method is suitable for water or water-based samples.

7.1.1.4 **Interferences**

- Presence of humic substances.
- Presence of chlorine.

7.1.1.5 **Method range**

The method limit of detection is 0.15 µg/L and the method limit of quantification is 0.18 µg/L. These limits were not validated but used as stated in the pamphlet received with the kit.

7.1.2 PRINCIPLE

Microcystin toxin in the sample competes with enzyme (horseradish peroxidase)-labeled microcystin for a limited number of antibody binding sites on the inside surface of the test wells. After a wash step, the outcome of the competition is visualized with a colour

development step and quantified with a microtiter plate reader. Microcystin concentration is inversely proportional to colour development (darker colour = lower concentration and *vise versa*).

7.1.3 WATER QUALITY

7.1.3.1 **Significance of microcystin analysis**

The presence of the microcystin in source and potable water poses a health risk to clients such as allergenic reactions, gastro-enteritis and liver damage. Advantages of microcystin monitoring include:

- Pro-active warning to drinking water supplier and catchment management departments regarding possible health risk to clients upon consumption of the water.
- Pro-active actions to avoid possible health related illnesses arising from consumption of source or potable water containing the toxin.
- Pro-active warning to recreational users and farmers regarding the possible health risk upon consumption of the water.

7.1.3.2 **Water quality guideline**

No SANS standard currently exists for microcystins in *potable* water. The World Health Organisation has set a guideline for cyanobacterial toxins at 1 µg/L of microcystin equivalents; therefore Rand Water has set an internal guideline of 1 µg/L of total microcystin in the *potable* water. According to Rand Water's Incident Management Framework (Du Preez & Van Baalen, 2006) a number of management options will be put into place when cyanobacteria concentrations in the *raw* water exceeds a certain level (2000 cell/mL and 100 000 cells/mL respectively) and microcystin concentrations in the *potable* water exceeds a certain level (0.2 µg/L, 0.7 µg/L and 2.5 µg/L respectively). Microcystins can easily be treated by the addition of sufficient amounts of free chlorine to the final drinking water (Acero et al., 2005; Knappe et al., 2004; Chorus & Bartram 1999). The chlorine oxidizes the microcystins, rendering it non-toxic. Note however, that other cyanobacterial toxins may not be treated as easily as microcystins.

7.1.4 APPARATUS, MATERIALS AND REAGENTS

7.1.4.1 **Instruments and equipment**

- Air displacement pipette (or otherwise called a dispenser pipette) with disposable tips (able to measure 20 µL – 125 µL) 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.

7.1.4.2 **Glassware**

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

7.1.4.3 **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 mm) 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.

7.1.4.4 **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.
- 0.16 ppb (µg/L) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 0.6 ppb (µg/L) microcystin-LR calibrator – as supplied by Envirologix or equivalent supplier as part of the standard ELISA screening test kit for microcystins.
- 2.5 ppb (µg/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.

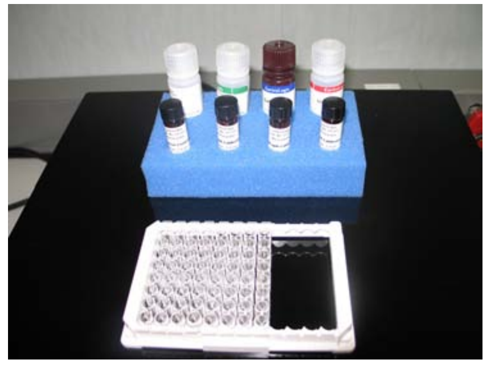


Figure 7.3: Example of an ELISA screening test kit (e.g. Envirologix).

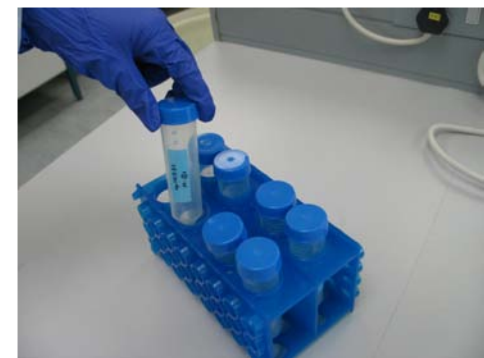


Figure 7.4: Polypropylene tubes used for sample preparation.

7.1.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

7.1.5.1 Solutions from micro wells are disposed via the waste disposal system:

- Dispose all solid wastes and used ELISA strips (antibody coated micro wells), together with the solutions from the micro wells, into designated containers for biological hazard removal, like Sanumed boxes.
- Full Sanumed boxes must not exceed a weight of 15 kilograms. Close the box and seal the lid with the bio-hazardous tape supplied by Sanumed (or equivalent supplier).
- Store boxes in a waste disposal room until collection.
- The staff member of the waste disposal company must deliver a document called "Waste Manifest" when collecting full boxes. This document states the number of full boxes and Sharps containers that have been collected at the time, as well as a document on the number of containers and boxes that have been delivered.
- File the copies of the "Waste Manifest" document in the Waste Tech (Sanumed) file.

7.1.5.2 Used wash solution in the waste bottle should be bleached before discarded by adding between 5 mL - 10 mL of commercial bleach (JIK) to the waste bottle before disposing *via* the drainage system.

7.1.6 PROCEDURE

7.1.6.1 **Sample preparation**

- Determine the presence of total chlorine.
- Should total chlorine be present (>0.1 mg/L) add sodium thiosulphate to sample in the ratio 800 µL to 1 L of sample before analysis and shake the sample to ensure uniform distribution.

Prepare polypropylene tubes (refer to **Figure 7.4**) for every sample to be analysed 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:

Table 7.2: 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 polytpropylene 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 polytpropylene 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.

7.1.6.2 **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 analysing 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 accompanying diagram).

Removable strip 1 : Normally used for the four calibrators (standards) in duplicate.

Removable strip 2 : Normally used for the first set of four samples (in duplicate).

Removable strip 3 : Used for the second set of four samples (in duplicate), depending on the amount of samples that need to be analysed.

Figure 7.5: 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 <u>calibrators</u> (standards) to be placed in duplicate below one another. The second strip may be used for the first four <u>samples</u> to be placed in duplicate below one another etc. During the analysis captured in the picture, 12 samples were analysed - 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. 7.5**. *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.*

- Complete the analysis details on a form as the test proceeds.
- Mix all the reagents on a vortex shaker fro 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 7.5**).
- 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 µL 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 µL 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).

7.1.7 SAFETY PRECAUTIONS

7.1.7.1 **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.

7.1.7.2 **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.

7.1.7.3 **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!

7.1.7.4 **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.

7.1.8 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 µg/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-analysed or the concentration reported as >2.5 µg/L.

- If the microcystin concentration of a sample is lower than 0.18 µg/L the results should be reported as <0.18 µg/L or when it is higher than that of the highest calibrator it should be reported as >2.5 µg/L.

7.1.9 RECORDS AND DATA KEEPING

- Data should be recorded on a form that is kept with the results. The details must include sample name and number, date of analysis, chlorine concentration of sample, adjusted chlorine concentration (if applicable), dilution factors (if applicable), sample order of arrangement in strips, kit lot number, batch numbers of individual calibrators and reagents as well as times at which each reagent is added as the analysis progresses.
- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

7.1.10 QUALITY ASSURANCE

7.1.10.1 **General**

- Do not use the kit components after the expiry date.
- Do not use the reagents or test well strips from one plate kit with reagents or test well strips from a different plate kit (unless they have the same batch number).
- Quality assurance of the microcystin screening test method consists of a set of guidelines, which will produce analytical results of known accuracy and precision. It consists of two aspects, internal quality control and external quality control.

7.1.10.2 **Precision and accuracy**

- Percentage coefficient of variance (% COV) between replicate samples (applicable to inter-analyst competency) should not exceed the two times standard deviation as determined by validation.
- The optical density (OD) of duplicate samples and percentage BO (which is the [(average OD of calibrators or sample / average OD of negative control)*100]) of controls should not exceed 20% coefficient of variance.

Note: Should 20% COV be exceeded by calibrators, the results may be accepted if the correlation coefficient (r) is >0.95 and the average concentration of the calibrator is in close proximity to the actual concentration of the calibrator. Should 20% COV be exceeded by a sample with concentration <0.18 or >2.5 µg/L the results may be accepted.

- Percentage BO of each calibrator should fall within these ranges (or as per instruction received per kit):

Note: Should any of the calibrators exceed these ranges, results may be accepted if the correlation coefficient obtained from the equation is >0.95. If all of the calibrators exceed these ranges, the test should be repeated.

Table 7.3: Percentage BO ranges

Calibrator	Recommended % BO	% BO (2% above and below limit)
0.16 µg/L	78 – 91%	76 – 94%
0.6 µg/L	45 – 65%	48 – 61%
2.5 µg/Ld	13 – 30%	12 – 31%

7.1.10.3 Maintenance and service

- Microplate readers and incubators should be checked/serviced once a year by the manufacturing company.
- The microplate washer should be serviced at least once a year, or if analysis frequency is very high, every six months. Special attention should be given to connecting pipes and washing tips.

7.1.10.4 Calibration

- Before each analysis it is important to test and calibrate the system.
- Depending on the software package, a system test should be executed first. Print the outcome of the system test and file the report in the applicable file.
- If the system test is passed, proceed to the calibration test with the Universal Calibration Test Plate. The Universal Calibration Test Plate is a reference plate for calibrating instruments and is purchased with the microplate reader. The Universal Calibration Test Plate should be sent for calibration/verification once a year.
- Make sure the calibration test is set up to run at 405 nm, 450 nm, 490 nm, 492 nm, 630 nm and 670 nm.
- Print the Calibration Plate Analysis report and file the report in the applicable file.
- Replace the calibration plate in its container and always handle it with care.

7.1.10.5 Verification

The purpose of verification in this method is to ensure that results are continuously reliable. It also ensures continued analyst competency.

- Dispenser pipette verification should be performed monthly at 20 µL, 100 µL and 125 µL.

- Inter-analyst comparisons should be performed annually by using a sample with a positive microcystin concentration.
  - Each analyst must use the microcystin screening method to analyse four replicates of the raw sample.
  - Use any computer package to work out the average, standard deviation and percentage coefficient of variance (% COV).
  - The % COV of the replicates should be within the two times standard deviation as derived from the initial validation of the method.
  - The % COV should be less than 20 percent.
  - If the above criteria are not met, the inter-analyst comparison should be repeated. If the above criteria are still not met analysts should be retrained and deemed incompetent until proven competent.

7.1.11 TYPICAL VALIDATIONS FOR THE METHOD

7.1.11.1 **Verification of the dispenser pipette precision (indicate the serial number of the pipette)**

AIM

Verification of the dispenser pipette (20 µL – 200 µL) precision which will be used for the microcystin screening test method.

METHOD

Three volumes of reagent water (20 µL, 100 µL, 125 µL) were pipetted and weighed ten times by four analysts on different days. Thirty-two verifications were carried out.

RESULTS AND DISCUSSIONS

The results and statistical analysis are represented in **Tables 7.4 – 7.7**. Variations between the analysts were recorded. The coefficient of variance between the analysts was less than 1.5% for the three volumes evaluated (**Tables 7.4, 7.5** and **7.6**). The tolerance of the dispenser pipette was set between 2 × SD for the three volumes analysed at 0.00023, 0.00064 and 0.00150 from the average respectively for 20 µL, 100 µL and 125 µL (**Figures 7.6 - 7.8**). The control charts indicate the tolerances of the dispenser pipette at the three volumes analysed (**Figure 7.6 - 7.8**). From the three control charts it can be seen that the 1 ± SD is exceeded at times and it was decided to set the tolerance of the pipette at 2 ± SD. Using all the data of the thirty-two verification experiments the two times standard deviation was calculated for the three volumes analysed and is presented in **Table 7.7**.

Table 7.4: Statistics of the verification results by two analysts at 20 μL.

Number	Mean (g)	SD	% COV (<2%)	Analyst
10	0.01933	0.00016	0.83	1
10	0.01968	0.00006	0.32	1
10	0.01963	0.00020	1.0	1
10	0.01999	0.00009	0.44	1
10	0.01942	0.00007	0.37	1
10	0.01948	0.00012	0.59	2
10	0.01962	0.00013	0.68	2
10	0.01960	0.00012	0.62	2
10	0.01967	0.00007	0.33	3
10	0.01962	0.00005	0.25	3
10	0.01970	0.00009	0.47	3
10	0.01952	0.00024	1.2	4
10	0.01958	0.00025	1.3	4
10	0.01962	0.00029	1.5	4
10	0.01961	0.00009	0.44	1
10	0.01962	0.00009	0.45	1
10	0.01967	0.00013	0.65	1
10	0.01929	0.00011	0.59	1
10	0.01933	0.00013	0.65	1
10	0.01939	0.00009	0.45	1
10	0.01946	0.00021	1.1	4
10	0.01962	0.00022	1.1	4
10	0.01945	0.00024	1.2	4
10	0.01946	0.00011	0.55	2
10	0.01943	0.00014	0.71	2
10	0.01937	0.00006	0.28	2
10	0.01969	0.00006	0.30	3
10	0.01947	0.00028	1.4	3
10	0.01932	0.00014	0.74	3
10	0.01917	0.00010	0.50	3
10	0.01923	0.00010	0.52	3
10	0.01917	0.00013	0.66	3

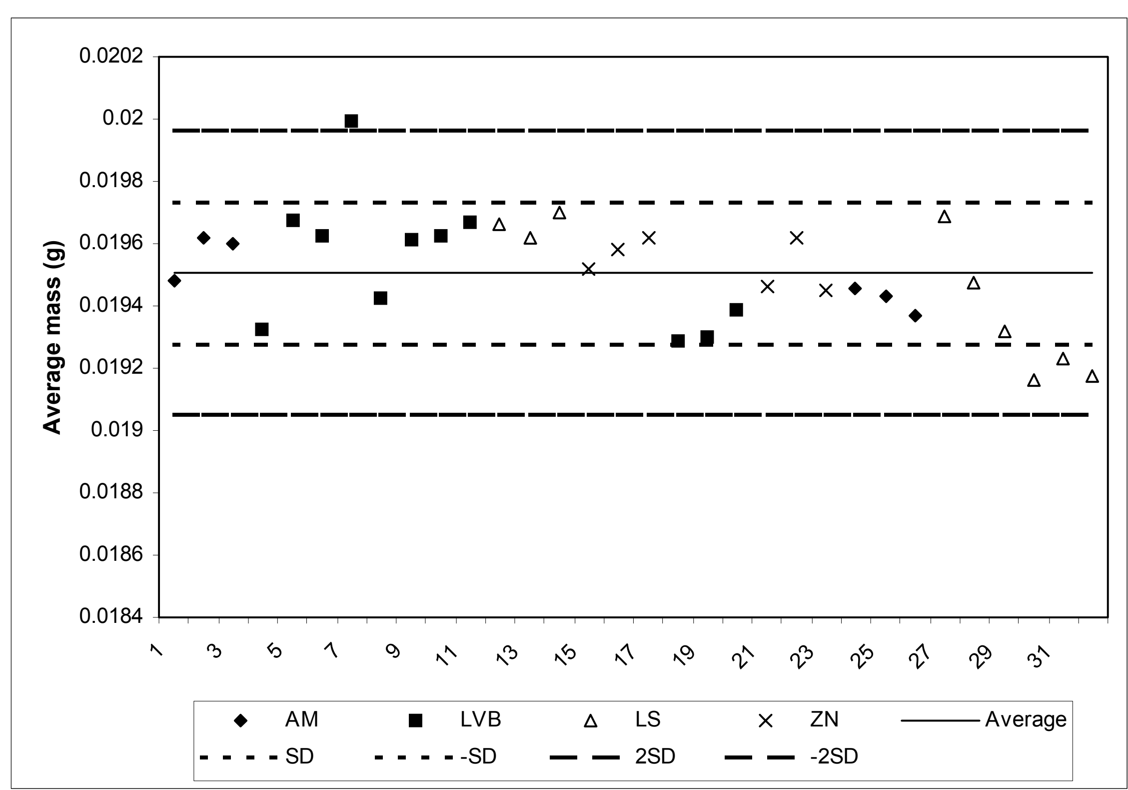


Figure 7.6: The deviations from zero for the Dispenser pipette at 20 μL control chart.

Table 7.5: Statistics of the verification results by two analysts at 100 μL.

Number	Mean (g)	SD	% COV (<0.5%)	Analyst
10	0.09853	0.00065	0.66	1
10	0.09889	0.00019	0.19	1
10	0.09896	0.00031	0.31	1
10	0.09866	0.00030	0.30	1
10	0.09832	0.00023	0.24	1
10	0.09833	0.00018	0.19	2
10	0.09853	0.00012	0.12	2
10	0.09856	0.00018	0.18	2
10	0.09864	0.00016	0.16	3
10	0.09859	0.00019	0.19	3
10	0.09856	0.00014	0.14	3
10	0.09921	0.00043	0.43	4
10	0.09916	0.00049	0.49	4
10	0.09907	0.00070	0.71	4
10	0.09830	0.00028	0.28	1
10	0.09831	0.00011	0.12	1

Table 7.5: Statistics of the verification results by two analysts at 100 μL (cont.).

Number	Mean (g)	SD	% COV (<0.5%)	Analyst
10	0.09822	0.00014	0.14	1
10	0.09810	0.00025	0.26	1
10	0.09795	0.00030	0.31	1
10	0.09804	0.00012	0.12	1
10	0.09862	0.00016	0.16	4
10	0.09881	0.00025	0.25	4
10	0.09874	0.00029	0.30	4
10	0.09751	0.00023	0.24	2
10	0.09716	0.00025	0.26	2
10	0.09740	0.00018	0.19	2
10	0.09760	0.00055	0.56	3
10	0.09738	0.00018	0.18	3
10	0.09766	0.00015	0.15	3
10	0.09779	0.00037	0.38	3
10	0.09751	0.00025	0.26	3
10	0.09749	0.00017	0.18	LS

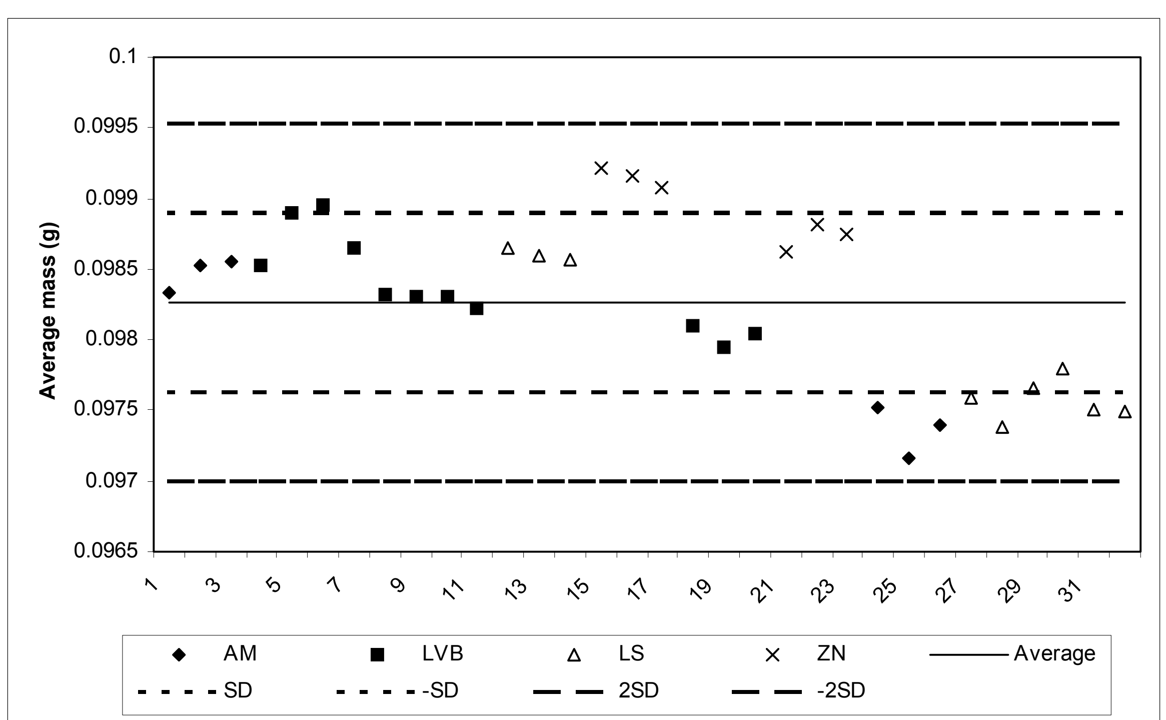


Figure 7.7: The deviations from zero for the Dispenser pipette at 100 μL control chart.

Table 7.6: Statistics of the verification results by two analysts at 125 μL.

Number	Mean (g)	SD	% COV (<0.5%)	Analyst
10	0.12337	0.00023	0.19	1
10	0.12347	0.00055	0.44	1
10	0.12362	0.00110	0.89	1
10	0.12351	0.00024	0.19	1
10	0.12309	0.00021	0.17	1
10	0.12337	0.00022	0.17	2
10	0.12309	0.00014	0.11	2
10	0.12335	0.00014	0.11	2
10	0.12316	0.00045	0.37	3
10	0.12278	0.00041	0.33	3
10	0.12339	0.00031	0.25	3
10	0.12375	0.00033	0.27	4
10	0.12405	0.00073	0.59	4
10	0.12421	0.00061	0.49	4
10	0.12329	0.00042	0.34	1
10	0.12323	0.00014	0.12	1
10	0.12325	0.00022	0.18	1
10	0.12254	0.00019	0.15	1
10	0.12250	0.00010	0.08	1
10	0.12257	0.00014	0.11	1
10	0.12365	0.00048	0.39	4
10	0.12390	0.00017	0.14	4
10	0.12371	0.00019	0.15	4
10	0.12190	0.00028	0.23	2
10	0.12214	0.00015	0.12	2
10	0.12175	0.00034	0.28	2
10	0.12254	0.00023	0.18	3
10	0.12314	0.00158	1.3	3
10	0.122803	0.00011	0.09	3
10	0.12891	0.03240	25	3
10	0.12022	0.00332	2.8	3
10	0.119305	0.00039	0.32	3

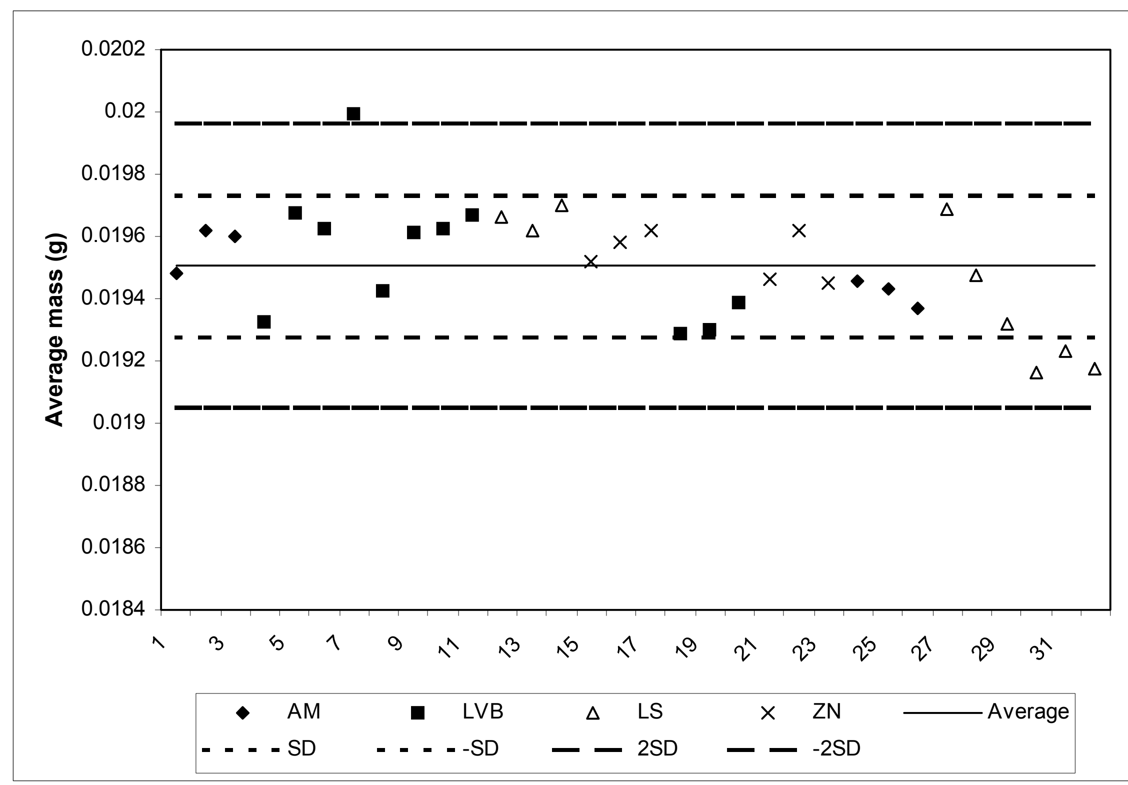


Figure 7.8: The deviations from zero for the Dispenser pipette at 125 µL control chart.

Table 7.7: Basic statistical calculation of the three volumes verified.

Volume	Number	Mean (g)	SD	Upper tolerance	Lower tolerance	% COV
20 µL	320	0.01951	0.00023	0.01996	0.01905	1.2
100 µL	320	0.09827	0.00064	0.09954	0.09699	0.65
125 µL	320	0.12280	0.00150	0.12580	0.11979	1.2

CONCLUSIONS

Some variations between analysts were recorded but they were mostly within the ± 2 standard deviations of tolerance. These results indicate that the Dispenser pipette (20 µL - 200 µL) can be used in this method but that analyst precision should be verified regularly.

RECOMMENDATIONS

Verification analysis needs to be carried out monthly by an analyst on a staff rotation basis and at least once annually by all analysts as indicated on the annual schedule.

The tolerance limits for the three volumes (20 µL, 100 µL and 125 µL) are stipulated in **Table 7.10**. If the lower or upper limits are exceeded then the verification should be repeated. If the limits are still exceeded then a corrective action should be initiated.

The tolerance limits for the three volumes (20 μL, 100 μL and 125 μL) are stipulated in **Table 7.7**. If the lower or upper limits are exceeded then the verification should be repeated. If the limits are still exceeded then a corrective action should be initiated.

7.1.11.2 Validation of the time a sample can be kept for the determination of total microcystin concentration

BACKGROUND

Some blue-green algal species have the potential to produce microcystin toxins. These toxins are released into the surrounding media when cells lyse or die (WHO, 1999). The lifetime of these toxins is important as it determines how long a sample can be kept before analysis commences and that is going to be addressed in this report.

AIM

Verification of the time a sample can be kept before determining total microcystin concentration.

METHOD

Analyses were performed on one sample from the Hartbeespoort Dam with high microcystin concentration. Analysis was performed when the sample was received in the laboratory (Day 1), the day after receipt in the laboratory (Day 2) and five days from receipt of the sample in the laboratory (Day 5). All samples analysed were sub-sampled from one "main" sample. Samples were stored in the refrigerator at 5 ± 3 ºC during the time when analysis was not performed (EPA, 1991). On day one fourteen replicates were analysed while on day 2 and 5 only ten replicates were analysed per day.

Mean optical densities, standard deviations and percent coefficient of variance were determined. A non-parametric Kreskas-Wallis test (suitable for small data sets) was performed on the optical densities to determine significant differences between the total toxin concentrations from day one to day five.

RESULTS AND DISCUSSIONS

The results and statistical analysis are represented in **Table 7.8** and **Table 7.9**. **Figure 7.9** illustrates the optical densities measured from the day of arrival to five days after arrival. From this figure it can be seen that the optical densities of the sample analysed on the day of arrival were lower compared to the densities measured on day 2 and day 5 which indicate that the total microcystin concentration decreased from day of arrival in the laboratory to five days after arrival. This could be explained by toxin degradation over time due to bacteriological activity in the sample. According to WHO (1999), a sample should be analysed within 24 hours from sampling and kept in the dark to prevent toxin

degradation. If not possible, the sample should be frozen. Samples from which algal cells have been removed can be refrigerated for up to five days before analyses commence (WHO, 1999). Applying the non-parametric Kruskal-Wallis test, a significant difference ($P<0.05$) between the optical densities of samples analysed on the day of arrival (day of sampling) to the samples analysed two days and five days after sampling was detected (**Table 7.9**).

Table 7.8: Basic statistics of the optical densities from day one to day five.

	DAY ONE	**DAY TWO**	**DAY FIVE**
Optical densities measured over time	0.014	0.435	6.350
	0.017	0.550	5.850
	0.024	0.455	7.350
	0.025	0.385	6.700
	0.012	0.405	5.600
	0.016	0.265	3.450
	0.018	0.305	2.900
	0.014	0.350	3.950
	0.016	0.355	3.200
	0.017	0.360	3.150
	0.015		
	0.016		
	0.015		
	0.015		
Average	0.017	0.387	4.850
Standard deviation	0.0036	0.081	1.689
% COV	22	21	35
Min	0.012	0.265	3.150
Max	0.025	0.550	7.350
Max-Min	0.013	0.285	4.200

Table 7.9: Non-parametric Kreskas-Wallis test to determine significant differences over time regarding to toxin concentration

	Kruskal-Wallis statistic	30.01	
	Difference in rank sum	**P value**	**Summary**
Day 1 *vs* Day 2	12.00	<0.05	*
Day 1 *vs* Day 5	22.00	<0.001	***
Day 2 *vs* Day 5	10.00	>0.05	Ns

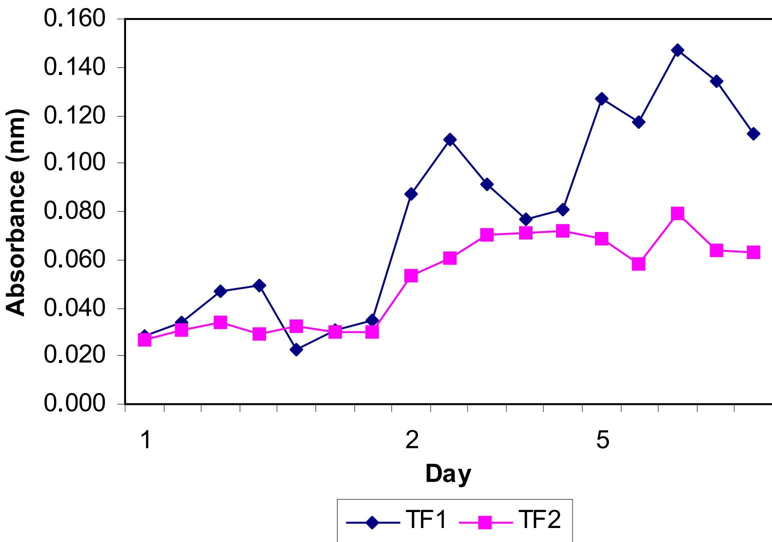


Figure 7.9: Absorbance values of the two freeze thawed treatments (TF1 = Freeze thawed once; TF2 = Freeze thawed twice) on a sample analysed on the day of arrival (day 1), the day after arrival (day 2) and five days after arrival (day 5).

CONCLUSIONS

Toxin concentrations in a sample decrease from the day of sampling to five days after sampling was done. A significant difference between the optical densities of a sample analysed on the day of arrival (sampling) to the optical densities obtained when analysed two days and five days after sampling was detected with a non-parametric Kruskal-Wallis test that is suitable for small data sets.

RECOMMENDATIONS

The following is recommended:

- Source water samples should be analysed the same day as sampled.
- If not possible, samples should be frozen until analysis can commence (only total toxin concentration can be determined after sample has been frozen).
- Samples from which all the algal cells have been removed can be refrigerated for up to five days (WHO, 1999).

7.1.11.3 Comparison of optical density readings between two different instruments (this would only be applicable if one instrument is replaced with another after validations of the method).

Note: whenever any instrument or part of an instrument is replaced, it is of utmost importance to validate the new instrument or part of the instrument, by either re-doing all the validations of the method applicable to the instrument or by comparing the results from the new instrument with that of the old instrument.

AIM

To determine if there is a significant difference between the optical density readings of the ELX800 and the Powerwave XS.

METHOD

Various samples ranging from drinking water samples to source water samples were filtered and spiked with the algal culture *Selenastrum capricornutum.* In some cases laboratory reference standards spiked with the alga mentioned were also used. These samples were pipetted into 24-well microplates and read with both the ELX800 and the Powerwave XS to determine the optical densities of each well. Microplates were left on a light cabinet and read again after 3 days with both the instruments. Each well was read four times on day 0 and day 3.

Mean optical densities, standard deviations and percentage coefficient of variance were determined. A paired t-test was also performed on the generated data (optical densities) to evaluate significant differences between the two instrument readings.

RESULTS AND DISCUSSIONS

The results and statistical analysis are represented in **Table 7.10** and **Table 7.11**. **Table 7.10** shows the average, standard deviation and % COV between replicate readings of each sample of the ELX800 and Powerwave XS. Even though apparent major differences in optical density readings are not apparent there is a tendency for the ELX 800 to record slightly higher % COV between replicate samples. Applying the paired T-test readings indicated a significant difference ($P<0.0001$) between the optical densities of the ELX800 and the Powerwave XS.

A discussion with the supplier of both instruments regarding the significant difference in the reading of samples revealed the following:

- The Powerwave XS is a spectrophotometer (using monochromator) while the ELX800 (using filters) is not.

- The Powerwave XS reads from the bottom of the wells while the ELX800 reads from the top of the well. When the liquid in the wells "slosh" around while the plate is being read there is more scattering of the light path when reading from the top compared to the bottom. Therefore the ELX800 will have higher % COV between readings compared to the Powerwave XS.
- The Powerwave XS has a PMT detector while the ELX800 has a photodiode detector.
- Generally the spectrophotometers are more sensitive than the readers.

Table 7.10: Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/06/29	Sample	Down	ELX 800	0.075	0.004	4.682
	Day 0		POWERWAVE	0.072	0.002	2.678
2004/06/29		Down-read	ELX 800	0.076	0.003	3.722
			POWERWAVE	0.072	0.002	3.208
2004/06/29		Upstream	ELX 800	0.073	0.003	3.948
			POWERWAVE	0.069	0.001	1.393
2004/06/29		Effluent	ELX 800	0.072	0.001	2.076
			POWERWAVE	0.069	0.001	0.843
2004/06/29		Sludge	ELX 800	0.230	0.005	2.079
			POWERWAVE	0.219	0.002	1.011
2004/06/07		271941	ELX 800	0.074	0.004	5.686
			POWERWAVE	0.070	0.004	5.447
2004/06/07		271942	ELX 800	0.080	0.003	3.602
			POWERWAVE	0.071	0.001	1.344
2004/06/07		271943	ELX 800	0.140	0.001	0.583
			POWERWAVE	0.134	0.001	0.749
2004/07/13		Canal	ELX 800	0.142	0.002	1.725
			POWERWAVE	0.138	0.002	1.260
2004/07/13		S2	ELX 800	0.109	0.001	0.876
			POWERWAVE	0.114	0.002	1.834
2004/07/13		K19	ELX 800	0.078	0.002	2.183
			POWERWAVE	0.075	0.003	4.173
2004/07/13		264345	ELX 800	0.081	0.002	2.586
			POWERWAVE	0.074	0.002	2.300
2004/07/13		274346	ELX 800	0.077	0.001	1.307
			POWERWAVE	0.070	0.001	1.166
2004/07/13		274347	ELX 800	0.135	0.002	1.263
			POWERWAVE	0.127	0.001	0.909
2004/03/06		Cd 0.05	ELX 800	0.061	0.002	2.811
			POWERWAVE	0.059	0.001	1.616
2004/03/06		Cd 0.10	ELX 800	0.060	0.001	1.602
			POWERWAVE	0.058	0.001	1.004
2004/03/06		Cd 0.15	ELX 800	0.061	0.002	2.469
			POWERWAVE	0.057	0.001	2.217
2004/03/06		Cd 0.20	ELX 800	0.060	0.001	2.088
			POWERWAVE	0.058	0.001	1.658

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/03/06		Cd 0.25	ELX 800	0.059	0.001	1.957
			POWERWAVE	0.057	0.001	0.873
2004/04/06		Cd 0.05	ELX 800	0.068	0.002	3.663
			POWERWAVE	0.059	0.002	2.961
2004/04/06		Cd 0.10	ELX 800	0.065	0.002	2.512
			POWERWAVE	0.059	0.001	0.987
2004/04/06		Cd 0.15	ELX 800	0.063	0.003	4.673
			POWERWAVE	0.056	0.002	3.942
2004/04/06		Cd 0.20	ELX 800	0.065	0.001	0.895
			POWERWAVE	0.059	0.001	1.384
2004/04/06		Cd 0.25	ELX 800	0.065	0.001	1.550
			POWERWAVE	0.058	0.003	4.329
2004/04/06		Cd 0.30	ELX 800	0.064	0.001	2.033
			POWERWAVE	0.058	0.000	0.858
2004/09/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.069	0.002	2.466
			POWERWAVE	0.063	0.001	2.372
2004/09/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.070	0.002	2.431
			POWERWAVE	0.064	0.001	1.502
2004/09/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.075	0.001	1.886
			POWERWAVE	0.069	0.001	1.183
2004/09/06		$K_2Cr_2O_7$ 25	ELX 800	0.084	0.002	2.572
			POWERWAVE	0.079	0.001	1.598
2004/09/06		$K_2Cr_2O_7$ 50	ELX 800	0.110	0.004	3.765
			POWERWAVE	0.100	0.002	2.160
2004/09/06		$K_2Cr_2O_7$ 100	ELX 800	0.157	0.004	2.725
			POWERWAVE	0.155	0.005	3.279
2004/11/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.096	0.015	16.143
			POWERWAVE	0.067	0.001	1.434
2004/11/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.074	0.003	4.273
			POWERWAVE	0.062	0.001	1.626
2004/11/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.073	0.001	1.937
			POWERWAVE	0.060	0.001	1.602
2004/11/06		$K_2Cr_2O_7$ 25	ELX 800	0.083	0.001	0.700
			POWERWAVE	0.075	0.000	0.669
2004/11/06		$K_2Cr_2O_7$ 50	ELX 800	0.102	0.002	1.790
			POWERWAVE	0.100	0.001	1.005
2004/11/06		$K_2Cr_2O_7$ 100	ELX 800	0.152	0.001	0.985
			POWERWAVE	0.151	0.001	0.384
2004/02/06		266523	ELX 800	0.081	0.003	4.120
			POWERWAVE	0.078	0.001	1.813
2004/02/06		266524	ELX 800	0.082	0.002	2.076
			POWERWAVE	0.078	0.000	0.643
2004/02/06		266525	ELX 800	0.149	0.012	7.751
			POWERWAVE	0.145	0.010	6.778

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/06/29	Sample	Down	ELX 800	0.116	0.023	20.043
	Day 3		POWERWAVE	0.114	0.013	11.016
2004/06/29		Down-read	ELX 800	0.109	0.010	8.823
			POWERWAVE	0.121	0.013	11.032
2004/06/29		Upstream	ELX 800	0.307	0.057	18.496
			POWERWAVE	0.378	0.008	2.222
2004/06/29		Effluent	ELX 800	0.124	0.013	10.273
			POWERWAVE	0.110	0.003	2.917
2004/06/29		Sludge	ELX 800	0.376	0.064	17.010
			POWERWAVE	0.415	0.030	7.245
2004/06/07		271941	ELX 800	0.383	0.093	24.366
			POWERWAVE	0.369	0.092	24.820
2004/06/07		271942	ELX 800	0.235	0.008	3.596
			POWERWAVE	0.240	0.018	7.627
2004/06/07		271943	ELX 800	0.298	0.019	6.392
			POWERWAVE	0.296	0.011	3.728
2004/07/13		Canal	ELX 800	0.267	0.007	2.470
			POWERWAVE	0.265	0.047	17.856
2004/07/13		S2	ELX 800	0.407	0.075	18.335
			POWERWAVE	0.364	0.020	5.608
2004/07/13		K19	ELX 800	0.229	0.018	7.658
			POWERWAVE	0.241	0.020	8.368
2004/07/13		264345	ELX 800	0.204	0.028	13.626
			POWERWAVE	0.217	0.035	16.004
2004/07/13		274346	ELX 800	0.224	0.053	23.821
			POWERWAVE	0.230	0.041	18.006
2004/07/13		274347	ELX 800	0.284	0.070	24.625
			POWERWAVE	0.275	0.046	16.883
2004/03/06		Cd 0.05	ELX 800	0.441	0.014	3.158
			POWERWAVE	0.534	0.045	8.365
2004/03/06		Cd 0.10	ELX 800	0.408	0.014	3.349
			POWERWAVE	0.495	0.011	2.279
2004/03/06		Cd 0.15	ELX 800	0.425	0.025	5.876
			POWERWAVE	0.499	0.019	3.749
2004/03/06		Cd 0.20	ELX 800	0.447	0.017	3.714
			POWERWAVE	0.535	0.035	6.554
2004/03/06		Cd 0.25	ELX 800	0.439	0.019	4.318
			POWERWAVE	0.521	0.026	5.085
2004/04/06		Cd 0.05	ELX 800	0.442	0.018	4.004
			POWERWAVE	0.475	0.016	3.412
2004/04/06		Cd 0.10	ELX 800	0.443	0.029	6.572
			POWERWAVE	0.541	0.045	8.264
2004/04/06		Cd 0.15	ELX 800	0.432	0.039	9.015
			POWERWAVE	0.543	0.053	9.764
2004/04/06		Cd 0.20	ELX 800	0.458	0.007	1.431
			POWERWAVE	0.570	0.017	3.015

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/04/06		Cd 0.25	ELX 800	0.466	0.034	7.368
			POWERWAVE	0.574	0.021	3.708
2004/04/06		Cd 0.30	ELX 800	0.520	0.023	4.357
			POWERWAVE	0.552	0.072	13.105
2004/09/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.206	0.009	4.213
			POWERWAVE	0.325	0.008	2.426
2004/09/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.083	0.008	10.020
			POWERWAVE	0.215	0.015	7.045
2004/09/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.077	0.003	3.922
			POWERWAVE	0.184	0.012	6.712
2004/09/06		$K_2Cr_2O_7$ 25	ELX 800	0.091	0.006	6.348
			POWERWAVE	0.212	0.010	4.569
2004/09/06		$K_2Cr_2O_7$ 50	ELX 800	0.103	0.004	3.607
			POWERWAVE	0.217	0.014	6.516
2004/09/06		$K_2Cr_2O_7$ 100	ELX 800	0.151	0.007	4.561
			POWERWAVE	0.249	0.006	2.381
2004/11/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.233	0.007	3.005
			POWERWAVE	0.339	0.020	5.997
2004/11/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.089	0.008	8.679
			POWERWAVE	0.227	0.014	6.237
2004/11/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.069	0.002	3.039
			POWERWAVE	0.182	0.014	7.574
2004/11/06		$K_2Cr_2O_7$ 25	ELX 800	0.082	0.008	9.394
			POWERWAVE	0.189	0.011	5.899
2004/11/06		$K_2Cr_2O_7$ 50	ELX 800	0.104	0.005	4.915
			POWERWAVE	0.205	0.011	5.357
2004/11/06		$K_2Cr_2O_7$ 100	ELX 800	0.149	0.003	2.280
			POWERWAVE	0.226	0.009	3.975
2004/02/06		266523	ELX 800	0.210	0.026	12.550
			POWERWAVE	0.257	0.028	10.795
2004/02/06		266524	ELX 800	0.239	0.037	15.536
			POWERWAVE	0.313	0.033	10.616
2004/02/06		266525	ELX 800	0.441	0.147	33.234
			POWERWAVE	0.452	0.047	10.304
2004/06/29	Control	Down	ELX 800	0.061	0.002	3.620
	Day 0		POWERWAVE	0.056	0.000	0.889
2004/06/29		Down-read	ELX 800	0.061	0.002	3.935
			POWERWAVE	0.056	0.001	1.717
2004/06/29		Upstream	ELX 800	0.061	0.003	4.373
			POWERWAVE	0.056	0.000	0.889
2004/06/29		Effluent	ELX 800	0.061	0.003	4.915
			POWERWAVE	0.056	0.000	0.000
2004/06/29		Sludge	ELX 800	0.062	0.003	4.836
			POWERWAVE	0.057	0.001	2.198

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/06/07		271941	ELX 800	0.058	0.002	3.620
			POWERWAVE	0.055	0.000	0.000
2004/06/07		271942	ELX 800	0.060	0.003	4.447
			POWERWAVE	0.055	0.001	0.913
2004/06/07		271943	ELX 800	0.056	0.001	1.717
			POWERWAVE	0.054	0.002	4.449
2004/07/13		Canal	ELX 800	0.062	0.002	3.312
			POWERWAVE	0.059	0.001	0.851
2004/07/13		S2	ELX 800	0.061	0.002	2.788
			POWERWAVE	0.058	0.001	0.858
2004/07/13		K19	ELX 800	0.060	0.004	6.930
			POWERWAVE	0.057	0.002	3.066
2004/07/13		264345	ELX 800	0.064	0.002	3.375
			POWERWAVE	0.060	0.001	2.088
2004/07/13		274346	ELX 800	0.063	0.003	4.673
			POWERWAVE	0.058	0.002	2.597
2004/07/13		274347	ELX 800	0.064	0.002	3.827
			POWERWAVE	0.057	0.001	2.198
2004/03/06		Cd 0.05	ELX 800	0.059	0.003	5.576
			POWERWAVE	0.058	0.002	3.012
2004/03/06		Cd 0.10	ELX 800	0.059	0.003	5.536
			POWERWAVE	0.057	0.001	2.481
2004/03/06		Cd 0.15	ELX 800	0.060	0.003	5.181
			POWERWAVE	0.057	0.002	3.873
2004/03/06		Cd 0.20	ELX 800	0.060	0.002	2.911
			POWERWAVE	0.057	0.000	0.881
2004/03/06		Cd 0.25	ELX 800	0.063	0.010	15.883
			POWERWAVE	0.057	0.001	1.022
2004/04/06		Cd 0.05	ELX 800	0.068	0.003	4.650
			POWERWAVE	0.058	0.001	2.245
2004/04/06		Cd 0.10	ELX 800	0.063	0.004	5.939
			POWERWAVE	0.056	0.002	3.942
2004/04/06		Cd 0.15	ELX 800	0.064	0.003	3.922
			POWERWAVE	0.057	0.001	1.432
2004/04/06		Cd 0.20	ELX 800	0.063	0.003	4.233
			POWERWAVE	0.058	0.001	1.408
2004/04/06		Cd 0.25	ELX 800	0.071	0.009	12.309
			POWERWAVE	0.057	0.001	0.873
2004/04/06		Cd 0.30	ELX 800	0.061	0.001	2.054
			POWERWAVE	0.057	0.001	2.285
2004/09/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.067	0.003	3.911
			POWERWAVE	0.061	0.001	1.653
2004/09/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.066	0.002	3.802
			POWERWAVE	0.059	0.000	0.844
2004/09/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.065	0.002	3.398
			POWERWAVE	0.060	0.000	0.837

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/09/06		$K_2Cr_2O_7$ 25	ELX 800	0.069	0.009	13.718
			POWERWAVE	0.059	0.001	1.384
2004/09/06		$K_2Cr_2O_7$ 50	ELX 800	0.071	0.005	6.419
			POWERWAVE	0.058	0.001	1.644
2004/09/06		$K_2Cr_2O_7$ 100	ELX 800	0.066	0.002	3.802
			POWERWAVE	0.059	0.000	0.844
2004/11/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.104	0.028	27.034
			POWERWAVE	0.056	0.002	3.571
2004/11/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.068	0.004	5.759
			POWERWAVE	0.057	0.001	1.432
2004/11/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.062	0.001	2.099
			POWERWAVE	0.056	0.000	0.000
2004/11/06		$K_2Cr_2O_7$ 25	ELX 800	0.066	0.004	6.170
			POWERWAVE	0.057	0.001	1.022
2004/11/06		$K_2Cr_2O_7$ 50	ELX 800	0.061	0.003	4.875
			POWERWAVE	0.057	0.001	1.770
2004/11/06		$K_2Cr_2O_7$ 100	ELX 800	0.066	0.011	16.978
			POWERWAVE	0.056	0.001	0.889
2004/02/06		266523	ELX 800	0.067	0.002	2.605
			POWERWAVE	0.055	0.001	1.835
2004/02/06		266524	ELX 800	0.067	0.004	6.321
			POWERWAVE	0.056	0.002	3.751
2004/02/06		266525	ELX 800	0.068	0.005	7.304
			POWERWAVE	0.056	0.003	4.767
2004/06/29	Control	Down	ELX 800	0.364	0.003	0.723
	Day 3		POWERWAVE	0.351	0.007	2.048
2004/06/29		Down-read	ELX 800	0.364	0.021	5.888
			POWERWAVE	0.352	0.019	5.420
2004/06/29		Upstream	ELX 800	0.387	0.011	2.885
			POWERWAVE	0.378	0.008	2.222
2004/06/29		Effluent	ELX 800	0.383	0.015	3.848
			POWERWAVE	0.375	0.016	4.321
2004/06/29		Sludge	ELX 800	0.373	0.061	16.412
			POWERWAVE	0.371	0.063	16.880
2004/06/07		271941	ELX 800	0.485	0.030	6.100
			POWERWAVE	0.478	0.025	5.139
2004/06/07		271942	ELX 800	0.509	0.032	6.342
			POWERWAVE	0.472	0.026	5.512
2004/06/07		271943	ELX 800	0.528	0.013	2.536
			POWERWAVE	0.476	0.028	5.920
2004/07/13		Canal	ELX 800	0.527	0.046	8.761
			POWERWAVE	0.463	0.053	11.357
2004/07/13		S2	ELX 800	0.521	0.011	2.108
			POWERWAVE	0.482	0.011	2.320

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/07/13		K19	ELX 800	0.509	0.041	8.025
			POWERWAVE	0.491	0.019	3.871
2004/07/13		264345	ELX 800	0.390	0.012	3.193
			POWERWAVE	0.380	0.022	5.721
2004/07/13		274346	ELX 800	0.370	0.015	4.158
			POWERWAVE	0.368	0.012	3.177
2004/07/13		274347	ELX 800	0.361	0.012	3.316
			POWERWAVE	0.357	0.016	4.498
2004/03/06		Cd 0.05	ELX 800	0.378	0.026	6.858
			POWERWAVE	0.402	0.032	7.930
2004/03/06		Cd 0.10	ELX 800	0.420	0.018	4.328
			POWERWAVE	0.459	0.010	2.082
2004/03/06		Cd 0.15	ELX 800	0.447	0.018	4.047
			POWERWAVE	0.490	0.014	2.784
2004/03/06		Cd 0.20	ELX 800	0.462	0.015	3.210
			POWERWAVE	0.503	0.009	1.784
2004/03/06		Cd 0.25	ELX 800	0.464	0.007	1.466
			POWERWAVE	0.506	0.021	4.158
2004/04/06		Cd 0.05	ELX 800	0.403	0.045	11.236
			POWERWAVE	0.394	0.045	11.480
2004/04/06		Cd 0.10	ELX 800	0.429	0.008	1.860
			POWERWAVE	0.439	0.016	3.577
2004/04/06		Cd 0.15	ELX 800	0.460	0.010	2.266
			POWERWAVE	0.483	0.007	1.359
2004/04/06		Cd 0.20	ELX 800	0.475	0.012	2.508
			POWERWAVE	0.500	0.007	1.351
2004/04/06		Cd 0.25	ELX 800	0.476	0.012	2.579
			POWERWAVE	0.519	0.009	1.700
2004/04/06		Cd 0.30	ELX 800	0.475	0.020	4.224
			POWERWAVE	0.523	0.040	7.567
2004/09/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.432	0.057	13.190
			POWERWAVE	0.532	0.085	15.929
2004/09/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.515	0.034	6.527
			POWERWAVE	0.597	0.058	9.763
2004/09/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.499	0.011	2.300
			POWERWAVE	0.537	0.014	2.558
2004/09/06		$K_2Cr_2O_7$ 25	ELX 800	0.513	0.034	6.650
			POWERWAVE	0.584	0.031	5.381
2004/09/06		$K_2Cr_2O_7$ 50	ELX 800	0.493	0.010	1.993
			POWERWAVE	0.547	0.006	1.083
2004/09/06		$K_2Cr_2O_7$ 100	ELX 800	0.488	0.023	4.666
			POWERWAVE	0.513	0.026	5.074
2004/11/06		$K_2Cr_2O_7$ 3.125	ELX 800	0.449	0.010	2.128
			POWERWAVE	0.532	0.037	6.919

Table 7.10 (cont.): Samples average, standard deviation and % COV data generated between the ELX 800 and the Powerwave XS.

Date		Sample	Instrument	Average OD	Stdev	% COV
2004/11/06		$K_2Cr_2O_7$ 6.25	ELX 800	0.470	0.026	5.473
			POWERWAVE	0.534	0.017	3.274
2004/11/06		$K_2Cr_2O_7$ 12.5	ELX 800	0.454	0.014	3.141
			POWERWAVE	0.511	0.042	8.246
2004/11/06		$K_2Cr_2O_7$ 25	ELX 800	0.446	0.015	3.285
			POWERWAVE	0.498	0.023	4.600
2004/11/06		$K_2Cr_2O_7$ 50	ELX 800	0.430	0.007	1.539
			POWERWAVE	0.473	0.012	2.546
2004/11/06		$K_2Cr_2O_7$ 100	ELX 800	0.428	0.009	2.079
			POWERWAVE	0.461	0.009	1.895
2004/02/06		266523	ELX 800	0.433	0.030	7.019
			POWERWAVE	0.447	0.015	3.426
2004/02/06		266524	ELX 800	0.467	0.010	2.245
			POWERWAVE	0.485	0.019	3.966
2004/02/06		266525	ELX 800	0.449	0.025	5.514
			POWERWAVE	0.479	0.012	2.534

Table 7.11: Paired t-test results comparing the optical density readings between the ELX 800 and Powerwave XS.

	t	df	Nr of pairs	P	R^2	Significant difference?
ELX 800 Vs Powerwave XS	9.424	639	640	<0.0001	0.1220	Yes

CONCLUSIONS

A significant difference between the optical densities measured with the ELX800 and Powerwave XS was detected with a paired t-test. It was also noted that the % COV between replicate optical density readings of the ELX 800 was slightly higher compared to the Powerwave XS. Even though there is a significant difference between the two instruments it is accepted that the Powerwave XS should be more accurate than the ELX800, being a spectrophotometer with less variance between replicate readings.

RECOMMENDATIONS

It is recommended that the Powerwave XS be used to analyse samples in microplate format and that the ELX800 only be used as the back-up instrument.

7.1.11.4 Validation of replicate microcystin screening analysis

AIM

Verification of replicate microcystin screening analysis to determine acceptable variation limits that will ensure competency and accuracy of results.

METHOD

Analyses were performed on fourteen samples that differed in microcystin toxin concentration. The first five samples with toxin concentrations of 0.16 μg/L, 0.5 μg/L and 1.6 μg/L were made up standards and cover the detection range of the test. The remaining were source water samples from the Hartbeespoort Dam with high microcystin concentration. Screening for microcystins with the Microcystin Kit provided by Envirologix was performed over three days with various replicates per sample. Number of replicates per sample was not constant as only two kits were available for validation (due to cost implications) and the analysis was optimized accordingly. Historical data of routine raw and potable water samples taken from Rand Water's catchment was also included in this validation report to derive tolerance limits for the percentage coefficient of variance.

Mean concentrations, standard deviations and percentage coefficient of variance were determined.

RESULTS AND DISCUSSIONS

Results and statistical analyses (not performed on historical data) are represented in **Table 7.12**. The percentage coefficient of variance (% COV) between replicate analysis of all the samples analysed (experimental and historical) varied considerably, ranging from 0 - 137 (**Table 7.12**). In replicate analyses performed with all the samples (data generated in experiment described in Section two and historical data) with varying concentrations it was noted that the % COV were below 20% for 93% of the analysis performed (**Figures 7.10, 7.11** and **7.12**). The 20% COV is slightly higher than the supplier's recommendation of 15% COV between replicates. This could be attributed to the variety of samples analysed during this verification that include the reality of samples that has to be analysed by Rand Water. The average % COV of data obtained from the experiment was 12% and from the historical data was 6.9%. A control chart (**Figure 7.13**) generated from % COV data of the experiment and historical data illustrate that most of the variation is within the two times standard deviation.

Table 7.12: Summary statistics on optical density values

	OD1	OD2	AVE REP	AVE ALL	SD REP	SD ALL	% COV REP	%COV ALL
Sample 1	1.762	1.809	1.786		0.033		1.861	
	1.880	1.862	1.871		0.013		0.680	
	1.970	1.851	1.911		0.084		4.404	
	1.729	1.740	1.735		0.008		0.448	
	1.798	1.661	1.730		0.097		5.601	
	1.690	1.518	1.554		0.051		3.276	
	1.519	1.493	1.506		0.018		1.221	
	1.425	1.581	1.503	1.699	0.110	0.162	7.339	9.541
			AVE % COV REP				**3.104**	
Sample 2	1.388	1.551	1.470		0.115		7.843	
	1.572	1.601	1.587		0.021		1.293	
	1.513	1.443	1.478		0.049		3.349	
	1.286	1.361	1.324		0.053		4.007	
	1.470	1.280	1.375		0.134		9.771	
	1.237	1.228	1.233		0.006		0.516	
	1.138	1.087	1.113		0.036		3.242	
	1.151	1.166	1.159	1.342	0.011	0.169	0.916	12.573
			AVE % COV REP				**3.867**	
Sample 3	0.742	0.657	0.700		0.060		8.592	
	0.647	0.669	0.658		0.016		2.364	
	0.855	0.661	0.758		0.137		18.097	
	0.695	0.587	0.641		0.076		11.914	
	0.608	0.644	0.626		0.025		4.066	
	0.544	0.089	0.317		0.322		101.654	
	0.425	0.511	0.468	0.595	0.061	0.178	12.994	29.935
			AVE % COV REP				**22.812**	
Sample 4	0.384	0.289	0.337		0.067		19.963	
	0.381	0.376	0.379		0.004		0.934	
	0.435	0.488	0.462		0.037		8.121	
	0.386	0.374	0.380		0.008		2.233	
	0.334	0.292	0.313	0.374	0.030	0.060	9.488	16.087
			AVE % COV REP				**7.813**	
Sample 5	0.341	0.340	0.341		0.001		0.208	
	0.399	0.353	0.376		0.033		8.651	
	0.288	0.412	0.350		0.088		25.052	
	0.392	0.338	0.365		0.038		10.461	
	0.434	0.399	0.417	0.370	0.025	0.044	5.942	12.033
			AVE % COV REP				**10.063**	
Sample 6	0.009	0.019	0.014		0.007		50.508	
	0.021	0.013	0.017		0.006		33.276	
	0.014	0.033	0.024		0.013		57.170	
	0.021	0.028	0.025		0.005		20.203	
	0.011	0.012	0.012		0.001		6.149	
	0.014	0.017	0.016		0.002		13.686	
	0.019	0.016	0.018	0.018	0.002	0.007	12.122	37.572
			AVE % COV REP				**27.588**	

Table 7.12: Summary statistics on optical density values (cont.)

	OD1	OD2	AVE REP	AVE ALL	SD REP	SD ALL	% COV REP	%COV ALL
Sample 7	0.410	0.460	0.435		0.035		8.128	
	0.730	0.370	0.550		0.255		46.283	
	0.450	0.460	0.455		0.007		1.554	
	0.410	0.360	0.385		0.035		9.183	
	0.380	0.430	0.405	0.446	0.035	0.106	8.730	23.803
			AVE % COV REP				**14.776**	
Sample 8	6.100	6.600	6.350		0.354		5.568	
	5.800	5.900	5.850		0.071		1.209	
	6.900	7.800	7.350		0.636		8.658	
	5.900	7.500	6.700		1.131		16.886	
	5.700	5.500	5.600	6.370	0.141	0.796	2.525	12.494
			AVE % COV REP				**6.969**	
Sample 9	0.011	0.016	0.014		0.004		26.189	
	0.018	0.013	0.016		0.004		22.810	
	0.013	0.021	0.017		0.006		33.276	
	0.013	0.016	0.015		0.002		14.630	
	0.013	0.019	0.016		0.004		26.517	
	0.015	0.015	0.015		0.000		0.000	
	0.016	0.014	0.015	0.015	0.001	0.003	9.428	17.894
			AVE % COV REP				**18.978**	
Sample 10	0.250	0.280	0.265		0.021		8.005	
	0.320	0.290	0.305		0.021		6.955	
	0.350	0.350	0.350		0.000		0.000	
	0.350	0.360	0.355		0.007		1.992	
	0.340	0.380	0.360	0.327	0.028	0.041	7.857	12.572
Sample 11			**AVE % COV REP**				**4.962**	
	3.100	3.800	3.450		0.495		14.347	
	2.900	2.900	2.900		0.000		0.000	
	4.300	3.600	3.950		0.495		12.531	
	3.100	3.300	3.200		0.141		4.419	
	2.900	3.400	3.150	3.330	0.354	0.460	11.224	13.801
			AVE % COV REP				**8.504**	
Sample 12	1.133	1.110	1.122		0.016		1.450	
	0.990	1.233	1.112		0.172		15.459	
	1.013	0.786	0.900		0.161		17.845	
	0.957	1.964	1.461		0.712		48.754	
	0.999	0.864	0.932		0.095		10.248	
	0.446	0.690	0.568		0.173		30.376	
	0.754	0.622	0.688	0.969	0.093	0.359	13.567	37.029
			AVE % COV REP				**19.671**	
Sample 13	0.393	0.283	0.338		0.078		23.012	
	0.317	0.291	0.304		0.018		6.048	
	0.288	0.314	0.301		0.018		6.108	
	0.367	0.402	0.385		0.025		6.437	
	0.359	0.365	0.362	0.338	0.004	0.045	1.172	13.192
			AVE % COV REP				**8.555**	
Sample 14	0.070	0.072	0.071		0.001		1.992	
	0.052	0.058	0.055		0.004		7.714	
	0.080	0.067	0.074		0.009		12.507	
	0.064	0.061	0.063		0.002		3.394	
	0.074	0.071	0.073	0.067	0.002	0.008	2.926	12.395
			AVE % COV REP				**5.706**	

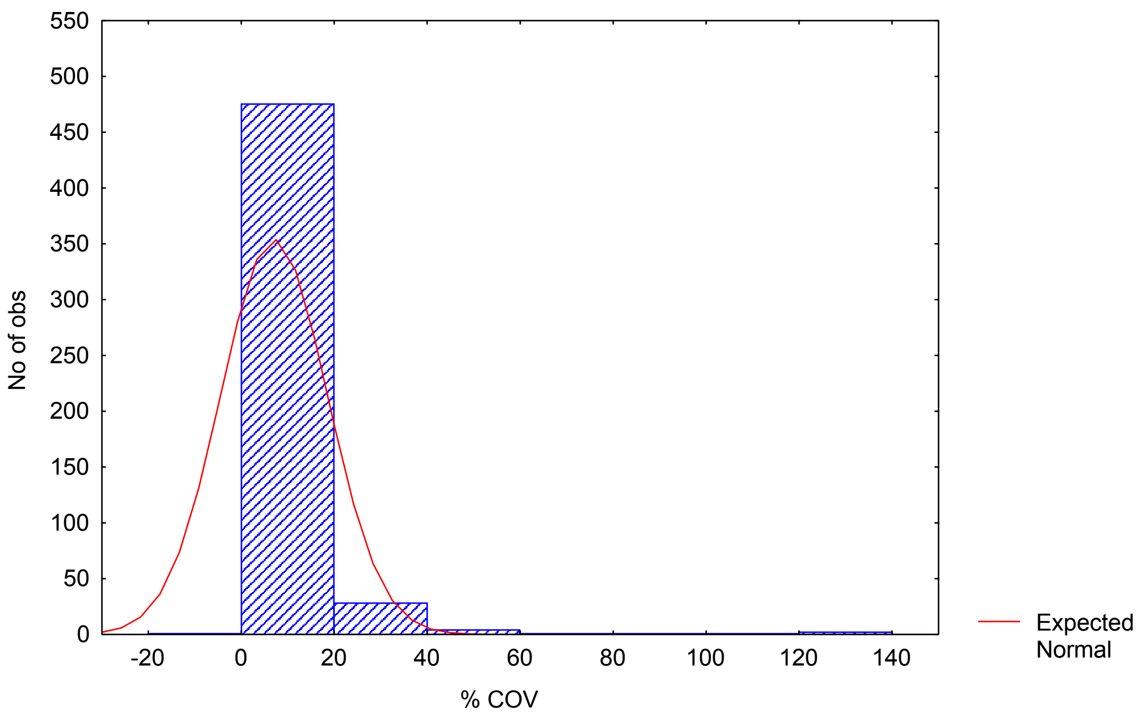


Figure 7.10: **Frequency distribution of the % COV obtained between replicate analyses.**

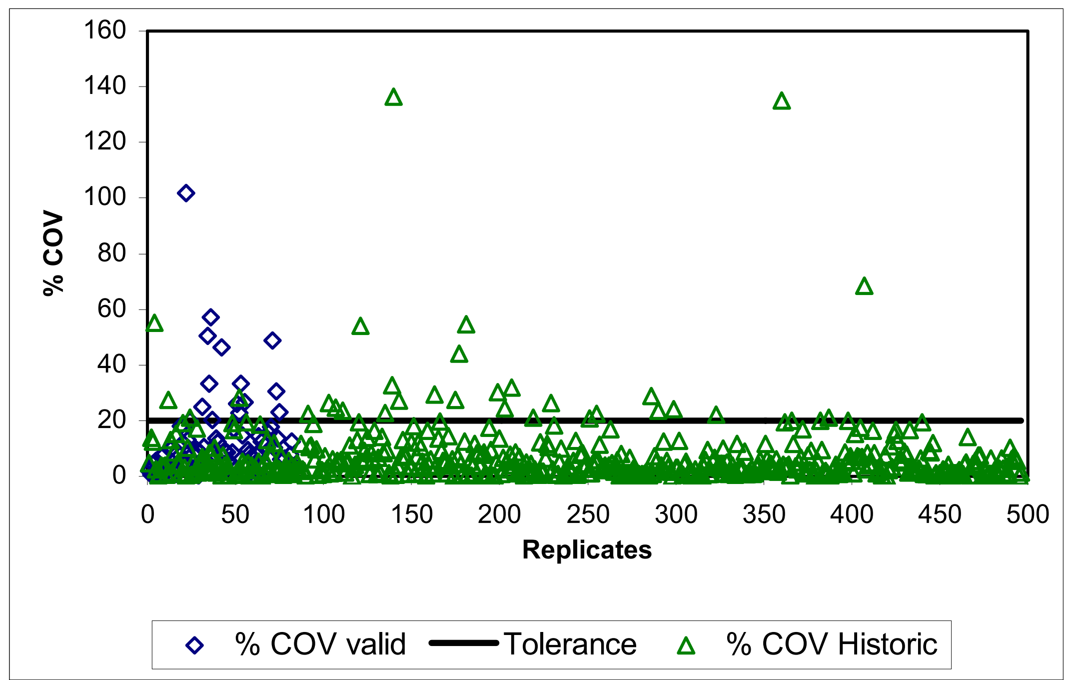


Figure 7.11: Scatter plot of the % COV of all the replicates analysed (including historical data).

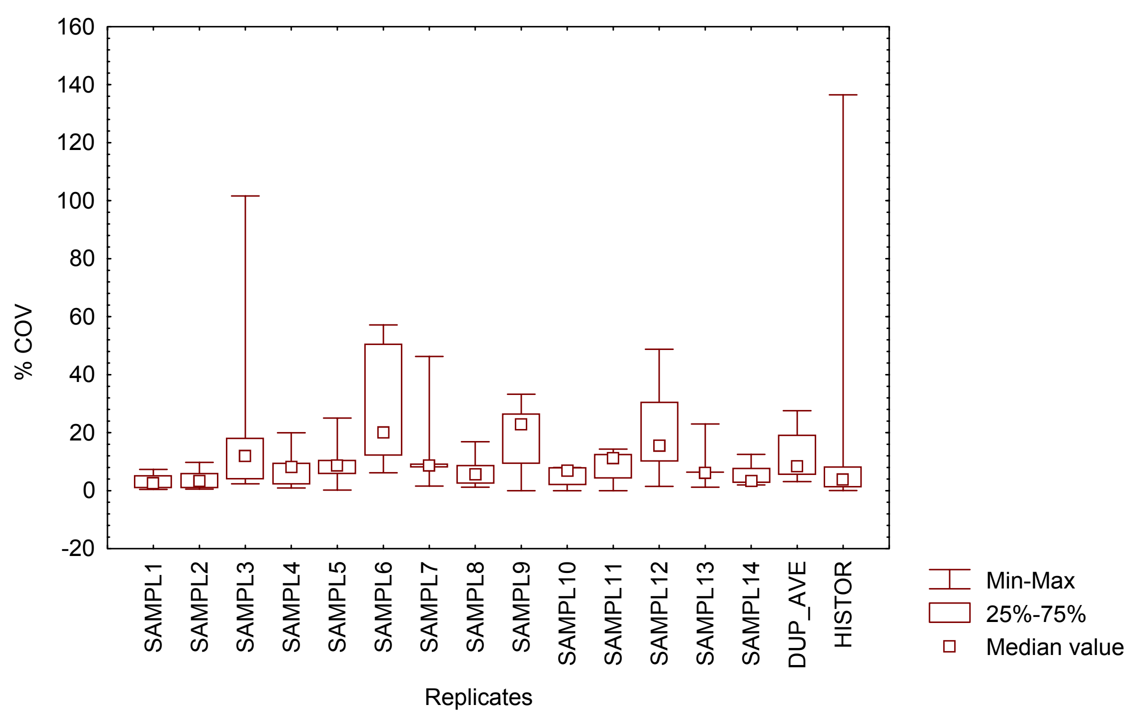


Figure 7.12: Box and whisker plot of the % COV of the 14 samples analysed and the average % COV of all the samples.

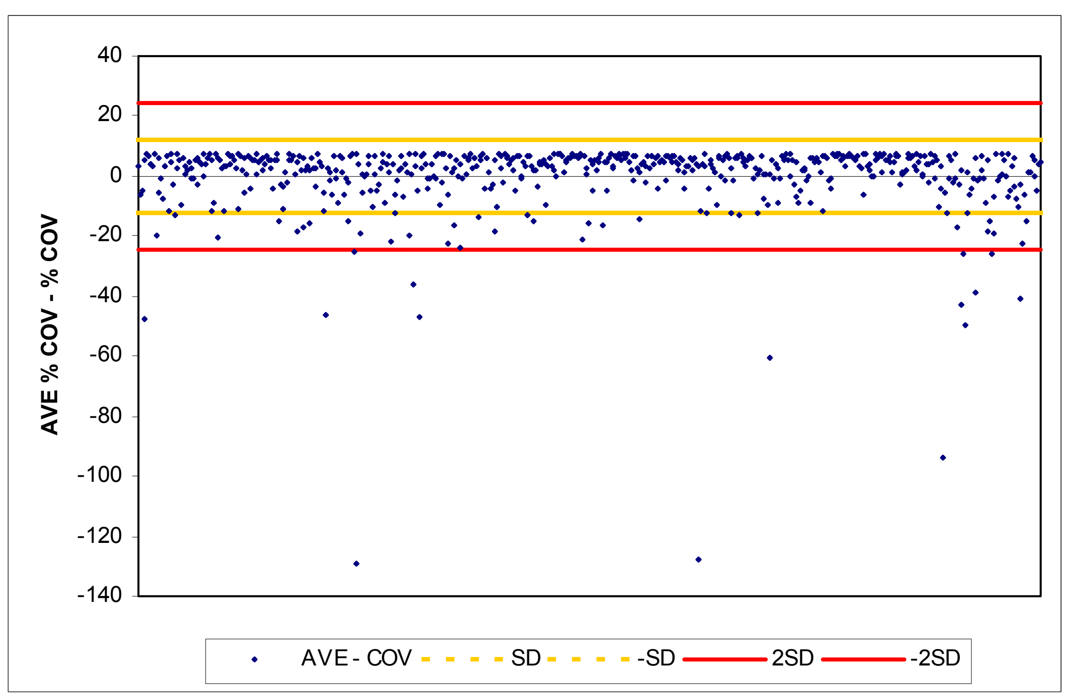


Figure 7.13: Control chart of the % COV of the experimental data and historical data.

CONCLUSIONS

The coefficient of variance between replicate samples can vary from 0 – 137 but for 93% of the samples analysed the % COV of variance is below 20%. Variation in % COV of the experimental data and historical data is mostly within the limits defined by two standard deviations (+-2SD).

RECOMMENDATIONS

The following recommendations are made regarding replicate analysis:

- Optical densities between replicate samples should not exceed the 20% coefficient of variance.
- Variation of % COV of replicate analyses should be within the two times standard deviation tolerance limit as derived from this report.
- These tolerance limits can be updated should the need arise.
- Verification of replicate results between analysts must be carried out as indicated on annual Hydrobiology schedule.

7.1.11.5 Validation of variation in "split sample" microcystin screening analysis to cover extraction procedure

Note: The use of the term "split sample" is used if a duplicate is taken from the same sample and (for the duration of both the preparation and analysis phases), treated as different samples.

AIM

Verification of "split sample" microcystin screening analysis to determine acceptable variation limits that will ensure accuracy of results.

METHOD

Analyses were performed on twenty seven samples differing in microcystin concentration. Samples were taken from various sources that included the Vaal Dam, Roodeplaat Dam, Hartbeespoort Dam and Rand Water's abstraction points. Screening for microcystins with the Microcystin Kit provided by Envirologix were performed with the following steps added to the sample preparation: a) analysing a "split sample" from the initial sample bottle, b) mixing sample with a blender before sub-sampling and c) freeze thawing for at least 10 minutes. All samples used for validation were analysed as "split samples" from the initial sub-sampling from the bottle the samples were received in.

Mean concentrations, standard deviations and percentage coefficient of variance were determined. Outliers were identified using procedures described in Standard Methods (2001) and excluded from the analysis.

RESULTS AND DISCUSSIONS

Results and statistical analysis are represented in **Table 7.13** contains the result printouts (optical densities) of the analyses described in the method.

Table 7.13: Summary statistics on optical density values

	OD1	OD2	Average	SD	%COV
Sample 1	0.354	0.948	0.651	0.420	64.5
Sample 2	1.130	1.197	1.164	0.047	4.1
Sample 3	1.420	1.002	1.211	0.296	24.4
Sample 4	1.347	1.252	1.300	0.067	5.2
Sample 5	1.278	1.234	1.256	0.031	2.5
Sample 6	0.975	0.531	0.753	0.314	41.7
Sample 7	0.897	0.848	0.873	0.035	4.0
Sample 8	1.142	1.141	1.142	0.001	0.1
Sample 9	1.126	1.111	1.119	0.011	0.9
Sample 10	1.597	1.289	1.443	0.218	15.1
Sample 11	1.296	1.504	1.400	0.147	10.5
Sample 12	0.798	0.657	0.728	0.100	13.7
Sample 13	1.433	1.129	1.281	0.215	16.8
Sample 14	0.665	0.632	0.649	0.023	3.6
Sample 15	0.488	0.648	0.568	0.113	19.9
Sample 16	0.541	0.509	0.525	0.023	4.3
Sample 17	0.699	0.577	0.638	0.086	13.5
Sample 18	0.858	0.810	0.834	0.034	4.1
Sample 19	1.053	1.081	1.067	0.020	1.9
Sample 20	0.885	0.802	0.844	0.059	7.0
Sample 21	0.662	0.735	0.699	0.052	7.4
Sample 22	0.595	0.593	0.594	0.001	0.2
Sample 23	0.567	0.689	0.628	0.086	13.7
Sample 24	0.568	0.491	0.530	0.054	10.3
Sample 25	0.496	0.487	0.492	0.006	1.3
Sample 26	0.409	0.279	0.344	0.092	26.7
Sample 27	0.551	0.443	0.497	0.076	15.4
Outliers excluded from analysis				Average % COV:	**9.1**

The percentage coefficient of variance (% COV) between split samples of all the different samples varied between 0 - 27 (**Table 7.13**). In split sample analysis performed with all the samples with varying concentrations it was noted that the % COV were below 5% COV for 44% of the analyses performed and below 20% COV for 92% of the analyses performed (**Figures 7.14, 7.15** and **7.16**). The average % COV of data obtained from the experiment was 9.1 (**Table 13**). A control chart (**Figure 7.16**) generated from % COV data of the experiment illustrates that most of the variation is within the two times standard deviation (17%).

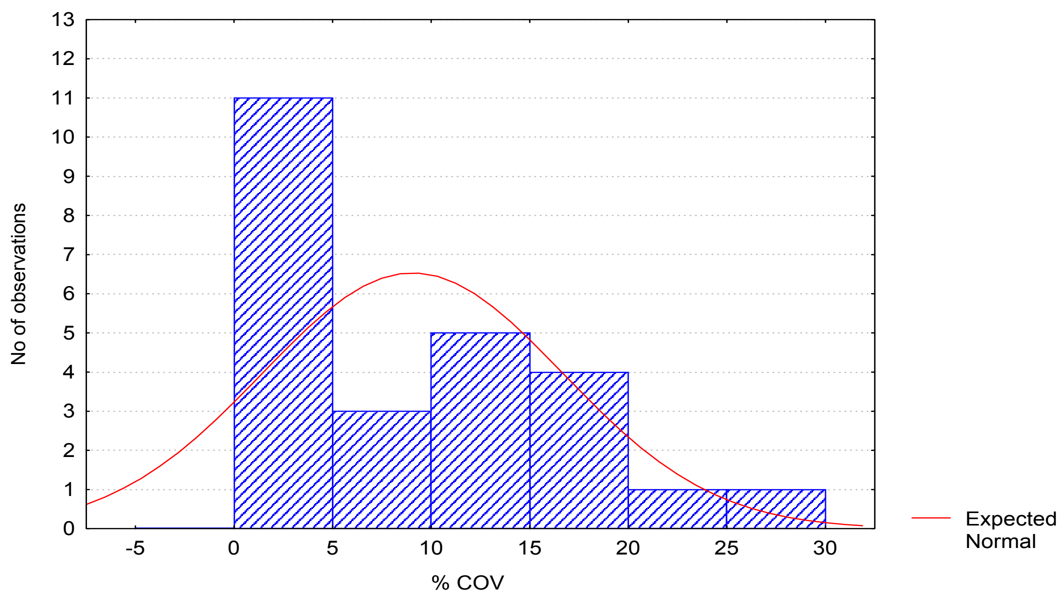


Figure 7.14: Frequency distribution of the % COV obtained between split sample analyses.

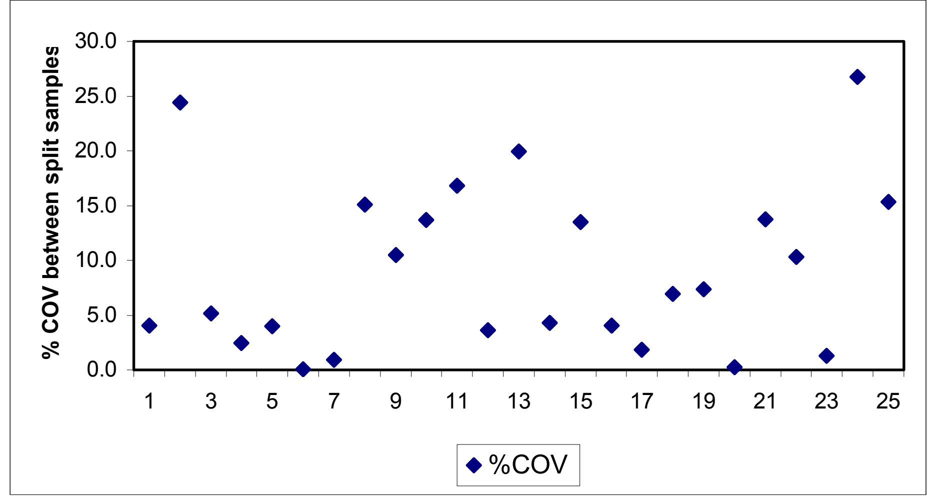


Figure 7.15: Scatter plot of the % COV of all the split samples analysed.

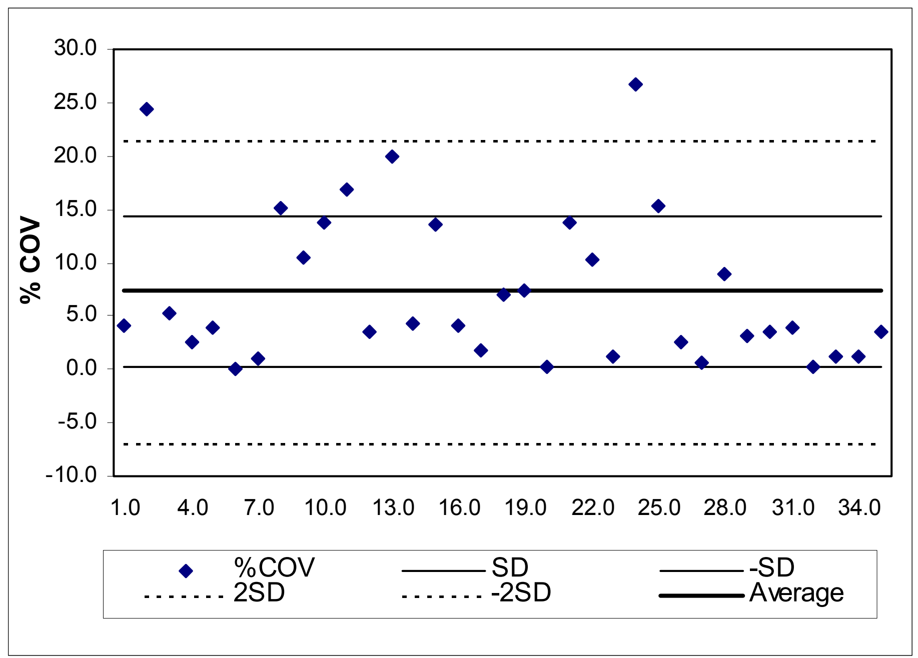


Figure 7.16: Control chart of the % COV between split samples.

CONCLUSIONS

The coefficient of variance between replicate samples can vary from 0 – 27 but for 92% of the samples analysed the % COV of variance was below 20. Variation in % COV of the data was mostly between the two times standard deviation. The variation of 20% COV between split samples was marginally higher than the recommendation of 15% COV by the suppliers, but is acceptable for laboratory conditions.

RECOMMENDATIONS

The following are recommended for split sample analyses:

- Variation of % COV of split sample analyses should be within the two times standard deviation tolerance limit as derived from this report.
- These tolerance limits should be updated after approximately one year to include more samples.
- Verification of split sample variation must be carried out on every 20th scheduled sample analysed.
- Method amendment should include split sample analysis procedures as derived from this report and thereafter the method is fit for purpose in the laboratory.

7.1.11.6 **Validation of variation in "split sample" microcystin screening analysis to cover extraction procedure (a year after the first validations were done)**

Note: The use of the term "split sample" is used if a duplicate is taken from the same sample and (for the duration of the whole preparation and analysis phases), treated as different samples.

AIM

To re-evaluate the tolerance limits determined under 1.11.5 to include data generated since the report.

METHOD

Analyses were performed on thirty seven samples that differed in microcystin toxin concentration. Samples were taken from various sources that included the Vaal Dam, Roodeplaat Dam, Hartbeespoort Dam and Rand Water's abstraction points. Screening for microcystins with the Microcystin Kit provided by Envirologix were performed with the following steps added to the sample preparation: a) analysing a "split sample" from the initial sample bottle, b) mixing sample with a blender before sub-sampling and c) freeze thawing for at least 10 minutes. All samples used for validation were analysed as "split samples" from the initial sub-sampling from the bottle the samples were received in.

Mean concentrations, standard deviations and percent coefficient of variance were determined. Outliers were identified using procedures described in Standard Methods (2001) and excluded from the analysis.

RESULTS AND DISCUSSIONS

Results and statistical analysis are represented in **Table 7.14** contains the result printouts (optical densities) of the analyses described in the method. The percentage coefficient of variance (% COV) between split samples of all the different samples varied between 0 - 27 (**Table 7.14**). In split sample analysis performed with all the samples with varying concentrations it was noted that the % COV were below 5% COV for 57% of the analyses performed and below 20% COV for 94% of the analyses performed (**Figures 7.17, 7.18** and **7.19**). The average % COV of data obtained from the experiment was 7.3 (**Table 7.14**). A control chart (**Figure 7.19**) generated from % COV data of the experiment illustrates that most of the variation is within the two times standard deviation (17%).

Table 7.14: Summary statistics on optical density values

	OD1	OD2	Average	SD	%COV
Sample 1	0.354	0.948	0.651	0.420	64.5
Sample 2	1.130	1.197	1.164	0.047	4.1
Sample 3	1.420	1.002	1.211	0.296	24.4
Sample 4	1.347	1.252	1.300	0.067	5.2
Sample 5	1.278	1.234	1.256	0.031	2.5
Sample 6	0.975	0.531	0.753	0.314	41.7
Sample 7	0.897	0.848	0.873	0.035	4.0
Sample 8	1.142	1.141	1.142	0.001	0.1
Sample 9	1.126	1.111	1.119	0.011	0.9
Sample 10	1.597	1.289	1.443	0.218	15.1
Sample 11	1.296	1.504	1.400	0.147	10.5
Sample 12	0.798	0.657	0.728	0.100	13.7
Sample 13	1.433	1.129	1.281	0.215	16.8
Sample 14	0.665	0.632	0.649	0.023	3.6
Sample 15	0.488	0.648	0.568	0.113	19.9
Sample 16	0.541	0.509	0.525	0.023	4.3
Sample 17	0.699	0.577	0.638	0.086	13.5
Sample 18	0.858	0.810	0.834	0.034	4.1
Sample 19	1.053	1.081	1.067	0.020	1.9
Sample 20	0.885	0.802	0.844	0.059	7.0
Sample 21	0.662	0.735	0.699	0.052	7.4
Sample 22	0.595	0.593	0.594	0.001	0.2
Sample 23	0.567	0.689	0.628	0.086	13.7
Sample 24	0.568	0.491	0.530	0.054	10.3
Sample 25	0.496	0.487	0.492	0.006	1.3
Sample 26	0.409	0.279	0.344	0.092	26.7
Sample 27	0.551	0.443	0.497	0.076	15.4
Sample 28	1.357	1.308	1.333	0.035	2.6
Sample 29	1.206	1.194	1.200	0.008	0.7
Sample 30	0.022	0.025	0.024	0.002	9.0
Sample 31	0.385	0.368	0.377	0.012	3.2
Sample 32	0.762	0.801	0.782	0.028	3.5
Sample 33	1.242	1.173	1.208	0.049	4.0
Sample 34	1.149	1.152	1.151	0.002	0.2
Sample 35	1.249	1.226	1.238	0.016	1.3
Sample 36	1.213	1.235	1.224	0.016	1.3
Sample 37	1.233	1.172	1.203	0.043	3.6
Outliers excluded from analysis				Avg % COV:	**7.3**

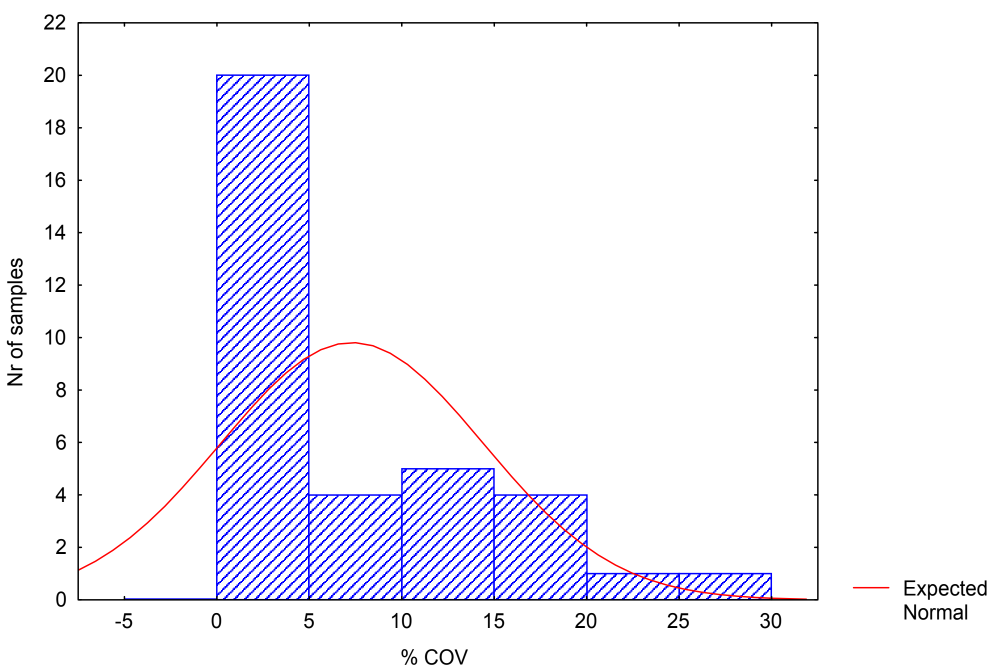


Figure 7.17: Frequency distribution of the % COV obtained between split sample analyses.

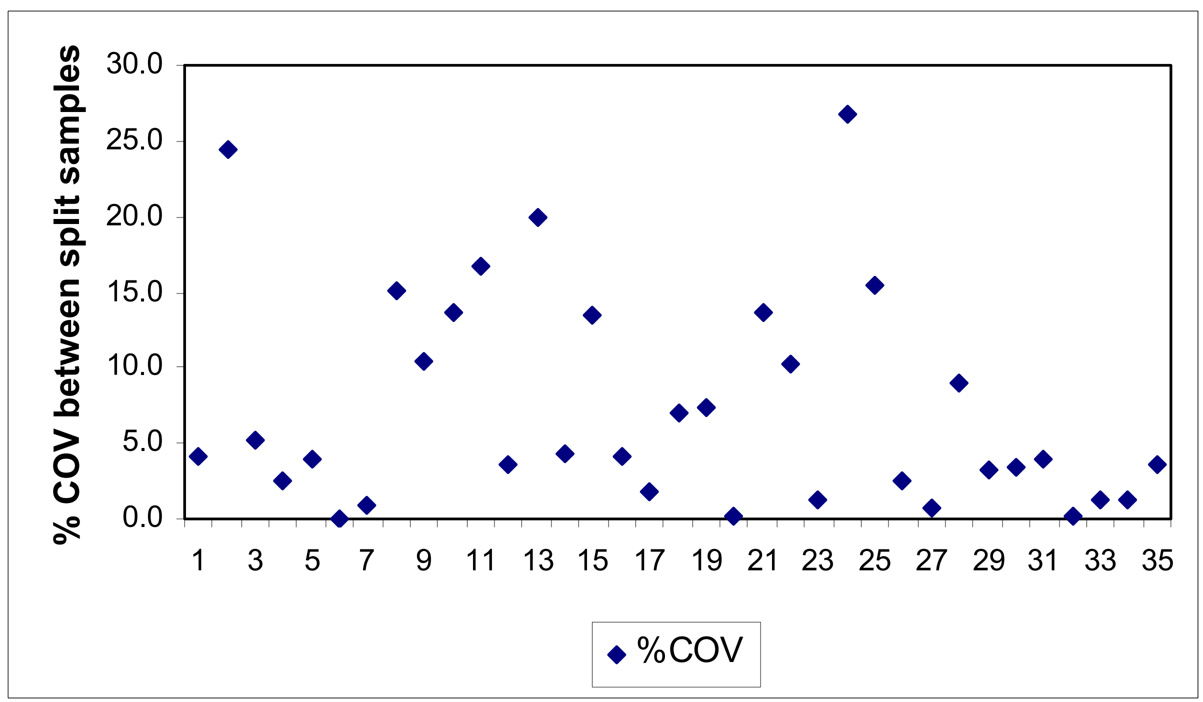


Figure 7.18: Scatter plot of the % COV of all the split samples analysed.

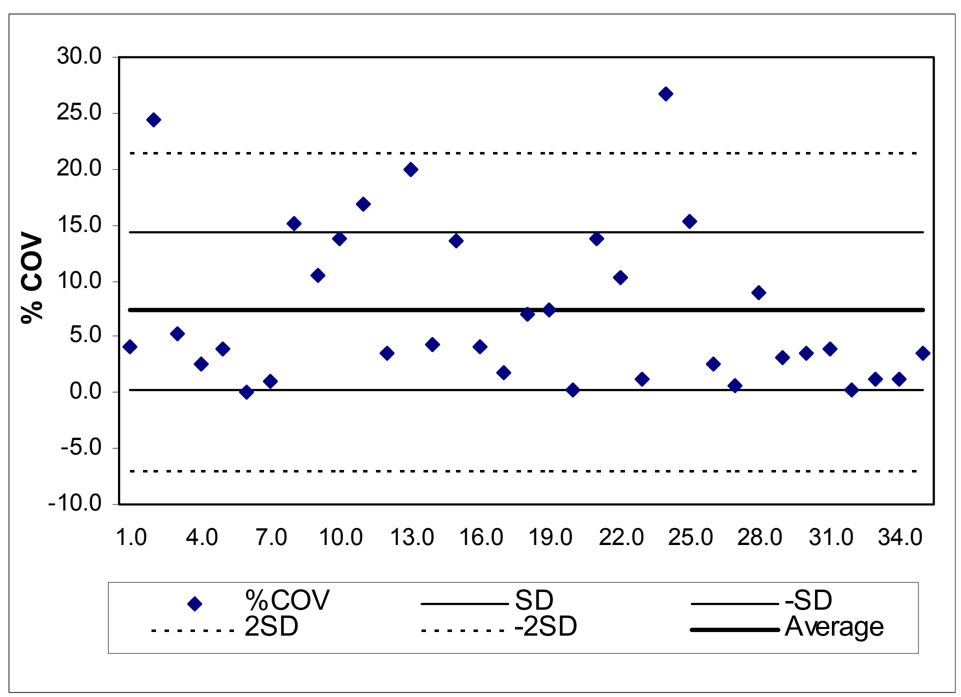


Figure 7.19: Control chart of the % COV between split samples.

CONCLUSIONS

The coefficient of variance between replicate samples can vary from 0 – 27 but for 94% of the samples analysed the % COV of variance was below 20. Variation in % COV of the data was mostly between the two times standard deviation. The variation of 20% COV between split samples was marginally higher than the recommendation of 15% COV by the suppliers, but is acceptable for laboratory conditions.

RECOMMENDATIONS

The following is recommended for split sample analyses:

- Variation of % COV of split sample analyses should be within the two times standard deviation tolerance limit as derived from this report.
- Verification of split sample variation must be carried out once a month on a sample identified by the senior scientist or laboratory supervisor.
- Method amendment should include split sample analysis procedures as derived from this report and thereafter the method is fit for use in the laboratory.

7.1.11.7 Estimation of uncertainty of measurement for the Microcystin screening ELISA test

AIM

To calculate the uncertainty of measurement for the microcystin screening ELISA test.

METHOD

The data used for the validation of the replicate analyses (section 7.1.11.4) were used for the estimation of uncertainty and basic statistics such as mean and standard deviation were included.

To determine the uncertainty the following formula was used:

Uncertainty	=	(2 × std dev of sample data) / mean of data) × 100
	=	x %

RESULTS

Table 7.15 lists the results from section 7.1.11.4 that was used to calculate uncertainty for the microcystin screening ELISA method. Fourteen samples were used with differing absorbance to give a realistic indication of uncertainty from samples with low to high concentrations. From **Table 7.15** it can be seen that the uncertainty per sample ranged from 19 - 75. The calculated "total" uncertainty by averaging the uncertainties of the different samples was 37.

Table 7.15. Microcystin absorbance and estimation of uncertainty (data from section 1.11.4)

Sample	Mean	SD	2*SD	Uncertainty	"Total" uncertainty
1	1.699	0.162	0.324	19	**37**
2	1.342	0.169	0.337	25	
3	0.595	0.178	0.356	60	
4	0.374	0.060	0.120	32	
5	0.370	0.044	0.089	24	
6	0.018	0.007	0.013	75	
7	0.446	0.106	0.212	48	
8	6.370	0.796	1.592	25	
9	0.015	0.003	0.005	36	
10	0.327	0.041	0.082	25	
11	3.330	0.460	0.919	28	
12	0.969	0.359	0.717	74	
13	0.338	0.045	0.089	26	
14	0.067	0.008	0.017	25	

CONCLUSIONS

The results from samples used indicated an uncertainty of 37%. Some factors that can affect the uncertainty of a sample analysed are the following:

- human factors
- environmental conditions
- equipment
- sampling
- handling test items.

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

7.2.1 INTRODUCTION

7.2.1.1 Scope

A method for the determination and quantification of microcystins in raw and treated waters is described. The method is validated for Microcystin-RR (MCYST-RR), Microcystin-LR (MCYST-LR) and Nodularin.

7.3.1.2 Definition

Microcystins are classified as hepatotoxic cyclic peptides with seven amino acids (hepatopeptides). The two terminal amino acids of the linear peptide are condensed to form a cyclic compound (WHO, 1999). Nodularin, while also a cyclic molecule, is a pentapeptide.

7.2.1.3 Field of application

This method is suitable for water-based samples. If "total" microcystin concentrations need to be determined, it is important to freeze-thaw the sample in liquid nitrogen for at least 20 minutes, where after the temperature is increased rapidly in a waterbath to reach ambient temperature. This is to release the intracellular microcystins into the water.

7.2.1.4 Known Interferences

- Presence of humic substances.
- Presence of chlorine.
- Phytoplankton cells.

7.2.1.5 Method range and statistics

Table 7.16: Method range and statistics associated with Microcystins and Nodularin.

	Range (µg/L)	LOD (µg/L)	LOQ (µg/L)	RSD (%)	Accuracy (%)	F_{Calc}
Microcystin-RR	**0.517 to 2.065**	**0.311**	**10.36**	**8.95**	**101.98**	**124**
Microcystin-LR	**0.506 to 2.022**	**0.164**	**0.547**	**5.45**	**101.04**	**427**
Nodularin	**0.400 to 1.600**	**0.076**	**0.253**	**3.09**	**100.85**	**1248**

The large F_{Calc} values indicate linearity of the calibration curves.

If samples are suspected to the calibration range they should be diluted (preferably prior to extraction).

7.2.2 PRINCIPLE

Filtered and purified water samples are extracted and enriched using Solid Phase Extraction (SPE). Water samples containing cyanobacterial material (biomass) are filtered. The biomass is extracted with a solvent and interfering compounds removed using Solid Phase Extraction. Microcystins and Nodularin are quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection (APHA, 2001; ISO/CD, 2003).

7.2.3 WATER QUALITY

7.2.3.1 Significance of the analysis

The presence of the microcystin in source and potable water poses a health risk to clients such as allergenic reactions, gastro-enteritis and liver damage. Advantages of microcystin monitoring include:

- Pro-active warning to drinking water supplier and catchment management departments regarding possible health risk to clients upon consumption of the water.
- Pro-active actions to avoid possible health related illnesses arising from consumption of source or potable water containing the toxin.
- Pro-active warning to recreational users and farmers regarding the possible health risk upon consumption of the water.

7.2.3.2 Water quality guideline

No SANS standard currently exists for microcystins in *potable* water. The World Health Organization has set a guideline for cyanobacterial toxins at 1 µg/L of microcystin equivalents; therefore Rand Water has set an internal guideline of 1 µg/L of microcystin in the *potable* water. According to Rand Water's Incident Management Framework (Du Preez & Van Baalen, 2006) a number of management options will be put into place when cyanobacteria concentrations in the *raw* water exceeds a certain level (2000 cell/mL and 100 000 cells/mL respectively) and microcystin concentrations in the *potable* water exceeds a certain level (0.2 µg/L, 0.7 µg/L and 2.5 µg/L respectively). Microcystins can easily be treated by the addition of extra chlorine to the final drinking water. The chlorine oxidises the microcystins, rendering it non-toxic (Acero et al., 2005; Knappe et al., 2004; Chorus & Bartram 1999). Note however, that other cyanobacterial toxins may not be treated as easily as microcystins.

7.2.4 APPARATUS, MATERIALS AND REAGENTS

7.2.4.1 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.

7.2.4.2 **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.

7.2.4.3 **Other materials**

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

7.2.4.4 **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, $C_2HF_3O_2$, 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 ($Na_2S_2O_3$) 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 Phenomanex 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 analysed as extraction methodologies may vary for different water types.

Table 7.17: 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

7.2.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

- Dispose all solid wastes into specially designated biohazard waste disposal containers like Sanumed boxes.
- Full Sanumed boxes must not exceed a weight of 15 kilograms. Close the box and seal the lid with the bio-hazardous tape supplied by Sanumed or equivalent supplier.
- Store boxes in the waste disposal room until collection.
- The staff member of waste disposal company must deliver a document called "Waste Manifest" when collecting full boxes. This document states the number of full boxes and Sharps containers that have been collected at the time, as well as a document on the number of containers and boxes that have been delivered.
- File the copies of the "Waste Manifest" document in the Waste Tech (Sanumed) file.

7.2.6 PROCEDURE

7.2.6.1 **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 7.2.4.4) and store at 4°C.

7.2.6.2 **Sample preparation**

- **Treated water / tap water** Extract and concentrate microcystins and nodularin in water samples using solid phase extraction (section 7.2.4.4).
- **Raw water containing phytoplankton:** Filter the sample [recommended volume: 50 mL to 100 mL, V_{Sample}(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 7.2.4.4). Extract the biomass on the filter separately (section 7.2.6.3) followed by clean-up (section 7.2.6.4) of the extract prior to HPLC analysis (section 7.2.6.6).

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.

7.2.6.3 **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, $V_{supernatant}$) 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*)].

7.2.6.4 **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 analyse using HPLC.
- It is necessary that a method blank (an unspiked water sample) be analysed to ensure that interferences from reagents do not compromise the integrity of the results.

7.2.6.5 **SPE for microcystin clean-up**

- Apply the extract from Step 7.2.6.3 to the conditioned cartridge (section 7.2.6.4) 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 [80-10 (*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 analyse on the HPLC.
- 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).

7.2.6.6 **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.

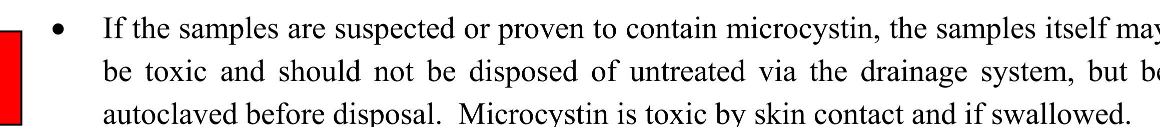
Table 7.18: HPLC mobile phase gradient

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

7.2.7 SAFETY PRECAUTIONS

7.2.7.1 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).

7.2.7.2 Clothing

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

7.2.7.3 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!

7.2.7.4 **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.

7.2.7.5 **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.

7.2.7.6 **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.

7.2.8 CALCULATIONS AND EXPRESSION OF RESULTS

7.2.8.1 **Calibration curve and calculations**

The spiked microcystin calibration standards (**Table 7.17**) 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.

7.2.8.2 **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.

7.2.8.3 **Microcystin concentration calculations**

Assume that the concentration determined from the calibration curve is *y* µg for the 0.5 mL extract (from 1 mL of the supernatant).

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

7.2.8.4 **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 µg/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)

7.2.9 RECORDS AND DATA KEEPING

- Data should be recorded on a form that is kept with the results.
- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

7.2.10 QUALITY ASSURANCE

7.2.10.1 **General**

- The HPLC method is quite involved in the sense that analysts should be very familiar with the apparatus and proven competent to execute the method correctly.
- Interpretation and authorisation of results should be done by the senior / principle scientist.

7.2.10.2 **Precision and accuracy**

- Percentage coefficient of variance (% COV) between replicate samples (applicable to inter-analyst competency) should not exceed the two times standard deviation as determined by validation.

7.2.10.3 **Maintenance and service**

- The vacuum pumps used should be serviced once a year by the supplier or equivalent manufacturer.
- If HPLC column back pressures are raised 20% above normal values, the guard column should be replaced. The column should also be rinsed with acetonitrile after analyses have been completed. The HPLC system serviced by the Supplier at least once a year.

7.2.10.4 **Calibration**

- Before each analysis commences, it is important to test the system. Standards should be injected to ensure that chromatography and absorbances are within 20% of the usual values.
- Depending on the software package, a system test should be executed first. Print the outcome of the system test and file the report in the applicable file.

7.2.10.5 **Verification**

The purpose of verification in this method is to ensure that results are continuously reliable. It also ensures continued analyst competency.

- Syringe verification should be performed monthly.
- Inter-analyst comparisons should be performed annually by using a sample with a positive microcystin concentration.
  - Each analyst must analyse four replicates of the raw sample.
  - Use any computer package to work out the average, standard deviation and percentage coefficient of variance (% COV).
  - The % COV of the replicates should be within the two times standard deviation as derived from the initial validation of the method.
  - The % COV should be less than 20 percent.

- If the above criteria are not met, the inter-analyst comparison should be repeated. If the above criteria are still not met analysts should be retrained and deemed incompetent until proven competent.

7.2.11 TYPICAL VALIDATIONS FOR THE METHOD

7.2.11.1 **Nodularin validation**

7.2.11.1.1 ***Range of standards***

0.400 µg/L to 1.600 µg/L

7.2.11.1.2 ***Calibration data***

Concentration (µg/L)	**Peak Height**
0.4000	14350
0.8000	27466
1.2000	43166
1.6000	55898

K	4
sum(x)	4.0000
sum(x)2	4.8
SS(X)	0.8
sumY	140880
sum(y)2	5.95×10^{9}
SS(Y)	9.86×10^{8}
sumXY	168948.8
SP(XY)	28068.8
B (SLOPE)	35086

7.2.11.1.3 ***Regression Analysis***

Summary output

Regression Statistics	
Multiple R	0.999199751
R Square	0.998400143
Adjusted R Square	0.997600214
Standard Error	888.2846391
Observations	4

ANOVA	Df	SS	MS	F	Significance F
Regression	1	984821916.8	984821916.8	1248.112	0.000800249
Residual	2	1578099.2	789049.6		
Total	3	986400016			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	134	1087.9220	0.1231	0.9132	-4546.9508	4814.951	-4546.9508	4814.9508
X Variable 1	35086	993.1324	35.3286	0.0008	30812.8960	39359.1	30812.8961	39359.1039

- Slope (b) 35086.00000000
- Y-intercept (a) 134.00000000
- Linearity F_{calc} = 1248
  F_{calc} large, therefore significant linearity

7.2.11.1.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 888.28463907
- Slope Uncertainty (S_{b}) 993.13241816
- Y-intercept Uncertainty (S_a) 1087.92205603

7.2.11.1.5 ***Method Limit of Detection***

- $Y_{LOD} = Y_B + 3S_B = bX_{LOD} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L
  0.076 µg/L

7.2.11.1.6 ***Method Limit of Quantitation***

- $Y_{LOQ} = Y_B + 10S_B = bX_{LOQ} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOQ} = 10S_{y/x}/b$, ng/L
  0.253 µg/L

7.2.11.1.7 ***Accuracy***

A spiked sample, concentration of 0.800 µg/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	0.819	28884
#2	0.828	29198
#3	0.808	28494
#4	0.832	29325
#5	0.784	27645
#6	0.770	28452
Mean	**0.8068**	**28666.3333**
Std Deviation	**0.0249**	**613.6203**

- RSD of concentration, (s x100/mean), %

  3.09

- Mean Method Accuracy (% of true conc.), %

  100.85

7.2.11.1.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0+a$

For x_0 = 0.8068

y_0 = 28442.55433

ave.y = 28666.33333333

y_0-ave.y = -223.7790

(y_0-ave.y)(y_0-ave.y) = 50077.04084

x_i	x_i-ave.x	(x_i-ave.x)*(x_i-ave.x)
0.4000	-0.600	0.36
0.8000	-0.200	0.04
1.2000	0.200	0.04
1.6000	0.600	0.36
	Sum =	0.8
	Sum^2=	0.64

$$Sx_0 = Sy/_{x/b}\{1/m+1/n+(y_0\text{-ave.y})^2/b^2\text{sum}(x_i\text{-ave.x})^2\}^{1/2}$$

(Where m = determinations = 1; n = calibration points = 4)

Sx_0 = 0.028306381

B Uncertainty of repeatability

Std Dev.y/ave.y = 0.021405608

C Uncertainty of purity

Purity = 100% ± 0.5%
Assume rectangular distribution
$U(P) = 0.5/3^{1/2} = 0.29$

D Uncertainty of volume

i) 10 mL volumetric flask, S.N 0134
10 mL ± 0.04 mL, 20°C
Assume rectangular distribution
$U(V_1) = 0.04/3^{1/2} = 0.023094011$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00021
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume * temp. Var.*coefficient of volume expansion)/$3^{1/2}$
= 0.004849742
Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe
100 µL ± 0.12 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.12/3^{1/2} = 0.069282032$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ =Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.34410076
100 µL Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe
1000 µL ± 0.49 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.49/3^{1/2} = 0.282901632$

Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 3.441007604
1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$ = 0.003452617 µL

E Total Uncertainty

Total Uncertainty, at x_0 = 0.8068

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$

U(t) = 0.033531486 µg/L

95% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$

$x_0 \pm \{3.18*U(t)\}$

0.8068 µg/L ± 0.106630126 µg/L

7.2.11.2 Microcystin-LR validations

7.2.11.2.1 *Range of standards*

0.506 µg/L to 2.022 µg/L

7.2.11.2.2 *Calibration data*

Concentration (µg/L)	Peak Height
0.5060	14135
1.0110	23531
1.5160	37249
2.0220	48016

K	4
sum(x)	5.0550
sum(x)2	7.664897
SS(X)	1.276641
sumY	122931
sum(y)2	4.45×10^9
SS(Y)	6.69×10^8
sumXY	184 500

SP(XY)	29 145.94
B (SLOPE)	22 830.18

7.2.11.2.3 ***Regression Analysis***
Summary output

Regression Statistics	
Multiple R	0.997666881
R Square	0.995339205
Adjusted R Square	0.993008808
Standard Error	1248.168131
Observations	4

ANOVA	***df***	***SS***	***MS***	***F***	***Significance F***
Regression	1	665406905.4	665406905.4	427.1114	0.002333119
Residual	2	3115847.367	1557923.684		
Total	3	668522752.8			

	Coefficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***	***Upper 95%***	***Lower 95.0%***	***Upper 95.0%***
Intercept	1881.1123	1529.1916	1.2301	0.3437	-4698.4680	8460.693	-4698.46805	8460.692789
X Variable 1	22830.1781	1104.6857	20.6666	0.0023	18077.0991	27583.26	18077.0991	27583.25716

- Slope (b) 22830.17814526
- Y-intercept (a) 1881.11236893
- Linearity F_{calc} = 427
  F_{calc} large, therefore significant linearity

7.2.11.2.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 1248.16813113
- Slope Uncertainty (S_b) 1104.68571567
- Y-intercept Uncertainty (S_a) 1529.19160192

7.2.11.2.5 ***Method Limit of Detection***

- $Y_{LOD} = Y_B+3S_B = bX_{LOD}+a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L
  0.164 µg/L

7.2.11.2.6 ***Method Limit of Quantitation***

- $Y_{LOQ} = Y_B + 10S_B = bX_{LOQ} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOQ} = 10S_{y/x}/b$, ng/l

0.547 µg/L

7.2.11.2.7 ***Accuracy***

A spiked sample, concentration of 1.011 µg/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	1.087	26686
#2	1.052	25888
#3	0.977	24176
#4	1.049	25643
#5	1.029	25193
#6	0.935	23097
Mean	**1.0215**	**25113.8333**
Std Deviation	**0.0557**	**1288.7571**

- RSD of concentration, (s × 100/mean), %

5.45

- Mean Method Accuracy (% of true conc.), %

101.04

7.2.11.2.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0 + a$

For x_0 =	1.0215
y_0 =	25202.13934
ave.y =	25113.83333333
y_0-ave.y =	88.3060
$(y_0\text{-ave.y})(y_0\text{-ave.y})$ =	7797.951575

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.5060	-0.758	0.574185063
1.0110	-0.253	0.063882563
1.5160	0.252	0.063630063
2.0220	0.758	0.574943063
	Sum =	1.27664075
	Sum^2=	1.629811605

$Sx_0 = S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2\text{sum}(x_i\text{-ave.x})^2\}^{1/2}$

(Where m = determinations = 1; n = calibration points = 4)

Sx_0 = 0.061125214

B Uncertainty of Repeatability

Std Dev.y/ave.y = 0.051316624

C Uncertainty of Purity

Purity = 100% ± 0.5%

Assume rectangular distribution

$U(P) = 0.5/3^{1/2} = 0.29$

D Uncertainty of Volume

i) 10 mL volumetric flask, S.N 0134

10 ml ± 0.04 mL, 20°C

Assume rectangular distribution

$U(V_1) = 0.04/3^{1/2} = 0.023094011$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00021

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.004849742

Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe

100 µL ± 0.12 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.12/3^{1/2} = 0.069282032$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.34410076
100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe
1000 µL ± 0.49 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.49/3^{1/2} = 0.282901632$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 3.441007604
1000 µL Syringe Uncertainty = $U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_C)/V_C$ = 0.003452617 µL

E Total uncertainty

Total Uncertainty, at x_0 = 1.0215
$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$
U(t) = 0.080770921 µg/L

95% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$
$x_0 \pm \{3.18*U(t)\}$
1.0215 µg/L ± 0.256851529 µg/L

7.2.11.3 **Microcystin-RR validation**

7.2.11.3.1 ***Range of standards***
0.517 µg/L to 2.065 µg/L

7.2.11.3.2 ***Calibration data***

Concentration (µg/L)	Peak Height
0.5170	4505
1.0330	8100
1.5490	13428
2.0650	19947

K	4
sum(x)	5.1640
sum(x)2	7.998004
SS(X)	1.33128
sumY	45980
sum(y)2	6.64×10^8
SS(Y)	1.36×10^8
sumXY	72686.91
SP(XY)	13326.73
B (SLOPE)	10010.47

7.2.11.3.3 *Regression Analysis*

Summary output

Regression Statistics	
Multiple R	0.992030246
R Square	0.984124009
Adjusted R Square	0.976186013
Standard Error	1037.336059
Observations	4

ANOVA	***df***	***SS***	***MS***	***F***	***Significance F***
Regression	1	133406785.8	133406785.8	123.9764	0.007969754
Residual	2	2152132.2	1076066.1		
Total	3	135558918			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-1428.5104	1271.2927	-1.1236	0.3779	-6898.4418	4041.421	-6898.4418	4041.4209
X Variable 1	10010.4651	899.0519	11.1344	0.0079	6142.1569	13878.77	6142.1569	13878.7733

- Slope (b) 10010.46511628
- Y-intercept (a) -1428.51046512
- Linearity F_{calc} = 124

  F_{calc} large, therefore significant linearity

7.2.11.3.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 1037.33605934
- Slope Uncertainty ($S_{b)}$ 899.05191635
- Y-intercept Uncertainty (S_a) 1271.29278992

7.2.11.3.5 ***Method limit of Detection***

- $Y_{LOD} = Y_B + 3S_B = bX_{LOD} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L

0.311 µg/L

7.2.11.3.6 ***Method limit of Quantitation***

- $Y_{LOQ} = Y_B + 10S_B = bX_{LOQ} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOQ} = 10S_{y/x}/b$, ng/l

1.036 µg/L

7.2.11.3.7 ***Accuracy***

A spiked sample, concentration of 1.033 µg/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	1.079	9387
#2	1.224	10839
#3	1.054	9141
#4	0.953	8146
#5	1.004	8838
#6	1.007	8864
Mean	**1.0535**	**9202.5000**
Std Deviation	**0.0943**	**903.2817**

- RSD of concentration, (sx100/mean), %

8.95

- Mean Method Accuracy (% of true conc.), %

101.98

7.2.11.3.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0 + a$

For x_0 = 1.0535

y_0 = 9117.514535

ave.y = 9202.50000000

y_0-ave.y = -84.9855

(y_0-ave.y)(y_0-ave.y) = 7222.529281

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.5170	-0.774	0.599076
1.0330	-0.258	0.066564
1.5490	0.258	0.066564
2.0650	0.774	0.599076
	Sum =	1.33128
	Sum^2=	1.772306438

$Sx_0 = S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 \text{sum}(x_i\text{-ave.x})^2\}^{1/2}$

(Where m = determinations = 1; n = calibration points = 4)

Sx_0 = 0.115858337

B Uncertainty of repeatability

Std Dev.y/ave.y = 0.098156125

C Uncertainty of purity

Purity = 100% ± 0.5%

Assume rectangular distribution

U(P) = $0.5/3^{1/2}$ = 0.29

D Uncertainty of volume

(i) 10 mL volumetric flask, S.N 0134

10 mL ± 0.04 mL, 20°C

Assume rectangular distribution

$U(V_1) = 0.04/3^{1/2} = 0.023094011$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00021

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.004849742

Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe

100 µL ± 0.12 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.12/3^{1/2} = 0.069282032$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.34410076

100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe

1000 µL ± 0.49 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.49/3^{1/2} = 0.282901632$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 3.441007604

1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$ = 0.003452617 µL

E Total uncertainty

Total Uncertainty, at x_0 = 1.0535

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$

U(t) = 0.155430351 µg/L

95% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$

$x_0 \pm \{3.18*U(t)\}$

1.0535 µg/L ± 0.494268516 µg/L

ANATOXIN-A AND CYLINDROSPERMOPSIN

The alkaloid neurotoxin (anatoxin-a) is produced by strains of *Anabaena* and *Oscillatoria* spp. It is a potent post-synaptic neuromuscular blocking agent that can cause death in a few minutes to a few hours, depending on the organism ingesting it, the amount of toxin ingested, and the amount of food in the stomach (Carmichael, 2001). *Anabaena* and *Oscillatoria* spp. are quite common in the summer months in the upper Vaal River region studied on a continuous basis by Rand Water.

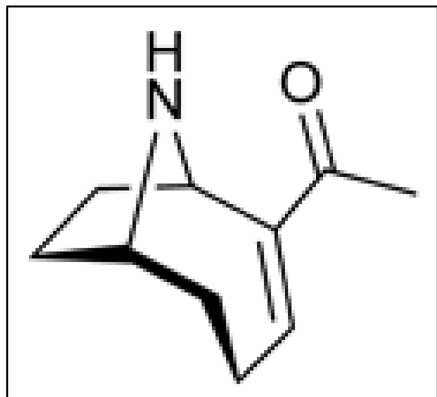


Figure 7.20: Anatoxin-a biochemical structure

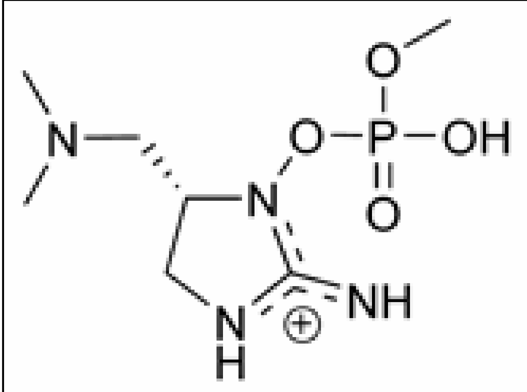


Figure 7.21: Anatoxin-a-S biochemical structure

Cylindrospermopsin, also an alkaloid toxin, is produced by *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, as well as *Umezakia natans* (found in Japan). *Cylindrospermopsis* and *Aphanizomenon* spp. have not been identified in the upper Vaal River regions (Rand Water's catchment area) up to date, but sporadic blooms of *Cylindrospermopsis raciborskii* are becoming more frequent in the warmer Orange River system, causing large scale purification problems in the smaller municipal water treatment plants along the Orange River. This species has also recently been detected in Lake St Lucia.

Figure 7.22: Cylindrospermopsin biochemical structure.

7.3 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 analysed 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.

7.3.1 INTRODUCTION

7.3.1.1 **Scope**

A method for the determination and quantification of Anatoxin-a and Cylindrospermopsin in raw and treated waters, is described. The method is validated for Anatoxin-a and Cylindrospermopsin.

7.3.1.2 **Definition**

Anatoxin-a is an alkaloid neurotoxin that is a potent post-synaptic neuromuscular blocking agent that can cause death in a few minutes.

Cylindrospermopsin is a hepatotoxin that poses a public health problem especially in more tropical regions. Hepatotoxicity is the main toxic manifestation but lesions involving the kidney, heart and thymus has also occurred in animals that were tested. It has been shown that chlorination, ozonation and the use of UV photocatalysis have the ability to degrade cylindrospermopsin although the formation of byproducts may be of concern (Kuiper-Goodman et al., 1999).

7.3.1.3 **Field of application**

This method is suitable for water-based samples. If "total" toxin concentrations need to be determined, it is important to freeze-thaw the sample in liquid nitrogen for at least 20 minutes, where after the temperature is increased rapidly in a waterbath to reach ambient temperature. This is to release the intracellular toxins into the water.

7.3.1.4 **Interferences**

- Presence of humic substances.
- Presence of chlorine.
- Phytoplankton cells.

7.3.1.5 **Method range and statistics**

Table 7.19: Method range and statistics regarding Anatoxin-a and Cylindrospermopsin.

	Range (µg/L)	**LOD (µg/L)**	**LOQ (µg/L)**	**RSD (%)**	**Accuracy (%)**	**F_{Calc}**
Anatoxin-a	0.590 to 2.340	0.270	0.900	4.72	69.8	210
Cylindrospermopsin	1.00 to 4.00	0.399	1.132	9.42	116.78	390

The large F_{Calc} values indicate linearity of the calibration curves.
If samples are suspected to the calibration range they should be diluted (preferably prior to extraction).

7.3.2 PRINCIPLE

Purified water samples are extracted and enriched using Solid Phase Extraction (SPE). Anatoxin-A and Cylindrospermopsin are quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection.

7.3.3 WATER QUALITY

7.3.3.1 **Significance of the analysis**

The presence of the cyanotoxins such as anatoxin-a and cylindrospermopsin, in source and potable water, poses a health risk to consumers. Advantages of cyanotoxin monitoring include:

- Pro-active warning to drinking water supplier and catchment management departments regarding possible health risk to clients upon consumption of the water.
- Pro-active actions to avoid possible health related illnesses arising from consumption of source or potable water containing the toxin.

- Pro-active warning to recreational users and farmers regarding the possible health risk upon consumption of the water.

7.3.3.2 **Water quality guideline**

No SANS standard currently exists for anatoxins or cylindrospermopsin in *potable* water. The World Health Organization has set a guideline for cyanobacterial toxins at 1 µg/L of microcystin equivalents.

7.3.4 APPARATUS, MATERIALS AND REAGENTS

7.3.4.1 **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.

7.3.4.2 **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.

7.3.4.3 **Reagents**

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

- Methanol: CH_3OH, HPLC grade.

- Acetonitrile: CH_3CN, HPLC grade.
- Water: H_2O, HPLC grade.
- Trifluoroacetic acid: $C_2HF_3O_2$, analytical grade.
- Re-dissolving solvent: Methanol/water [50/50 (*V/V*)].
- Sodium thiosulphate solution: Dissolve 1 g of sodium thiosulphate, $Na_2S_2O_3$, in 100 mL of water.
- SPE elution solution: Methanol containing 0.1% (*V/V*) TFA.
- Acetic acid: CH_3COOH, HPLC grade. 1% in HPLC grade H_2O (*V/V*).
- Ammonium hydroxide solution: Dissolve 2 g of ammonium hydroxide, NH_4OH, in 100 mL water.
- **Solid phase extraction cartridges (SPE) for anatoxin-a and cylindrospermopsin enrichment:** Reversed phase C_{18} SPE cartridges are used to extract and concentrate Anatoxin-a and Cylindrospermopsin. Any suitable C_{18} 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 Phenomanex 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 analysed 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 7.20: 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

7.3.5 DISPOSAL OF SAMPLES AND HAZARDOUS REAGENTS

- Dispose all solid wastes into Sanumed boxes.
- Full Sanumed boxes must not exceed a weight of 15 kilograms. Close the box and seal the lid with the bio-hazardous tape supplied by Sanumed.
- Store boxes in the waste disposal room until collection.
- The staff member of Sanumed must deliver a document called "Waste Manifest" when collecting full boxes. This document states the number of full boxes and Sharps containers that have been collected at the time, as well as a document on the number of containers and boxes that have been delivered.
- File the copies of the "Waste Manifest" document in the Waste Tech (Sanumed) file.

7.3.6 PROCEDURE

7.3.6.1 **Sampling and preservation**

Collect water samples in 1 L amber coloured glass bottles, add 1000 µL sodium thiosulphate solution (section 7.3.4.4) and store at 4°C.

7.3.6.2 **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 analyse on the HPLC (8).

- It is necessary that a method blank (an unspiked water) be analysed to ensure that interferences from reagents don't compromise the integrity of the results.

7.3.6.3 **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 7.21: 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

7.3.7 SAFETY PRECAUTIONS

7.3.7.1 **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).

7.3.7.2 **Clothing**

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

7.3.7.3 **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!

7.3.7.4 **Safety instruction 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.

7.3.7.5 **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.

7.3.7.6 **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.

7.3.8 CALCULATIONS AND EXPRESSION OF RESULTS

7.3.8.1 **Calibration curve and calculations**

The spiked Anatoxin-a and Cylindrospermopsin calibration standards (**Table 7.20**) are used to prepare the calibration curve. These standards cover the range of 0.59 µ/L to 2.34 µg/L for the Anatoxin-a and 1.0 µg/L to 4.0 µg/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.

7.3.8.2 **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.

7.3.9 RECORDS AND DATA KEEPING

- Data should be recorded on a form that is kept with the results.
- According to the ISO and SANAS guidelines all records should be kept as long as is necessary for an audit trail (as determined by each individual laboratory). (At Rand Water, hard copies of all data are kept for 3 years after which only electronic copies of the data, with back-ups, are kept.)

7.3.10 QUALITY ASSURANCE

7.3.10.1 **General**

- The HPLC method is quite involved in the sense that analysts should be very familiar with the apparatus and proven competent to execute the method correctly.
- Interpretation and authorisation of results should be done by the senior / principle scientist.

7.3.10.2 **Precision and accuracy**

- Percentage coefficient of variance (% COV) between replicate samples (applicable to inter-analyst competency) should not exceed the two times standard deviation as determined by validation.

7.3.10.3 **Maintenance and service**

- The vacuum pumps should be serviced annually by the supplier or equivalent manufacturer.
- If HPLC column back pressures are raised 20% above normal values, the guard column should be replaced. The column should also be rinsed with acetonitrile after analyses have been completed. The HPLC system serviced by the Supplier at least once a year.

7.3.10.4 **Calibration**

- Before each analysis commences, it is important to test the system. Standards should be injected to ensure that chromatography and absorbances are within 20% of the usual values.
- Depending on the software package, a system test should be executed first. Print the outcome of the system test and file the report in the applicable file.

7.3.10.5 **Verification**

The purpose of verification in this method is to ensure that results are continuously reliable. It also ensures continued analyst competency.

- Syringe verification should be performed monthly.
- Inter-analyst comparisons should be performed annually by using a sample with a positive Anatoxin-a and Cylindrospermopsin concentration.
  - Each analyst must analyse four replicates of the raw sample.
  - Use any computer package to work out the average, standard deviation and percentage coefficient of variance (% COV).
  - The % COV of the replicates should be within the two times standard deviation as derived from the initial validation of the method.
  - The % COV should be less than 20 percent.
  - If the above criteria are not met, the inter-analyst comparison should be repeated. If the above criteria are still not met analysts should be retrained and deemed incompetent until proven competent.

7.3.11 TYPICAL VALIDATIONS FOR THE METHOD

7.3.11.1 **Anatoxin-a validation**

7.3.11.1.1 ***Range of standards***

1.00 µg/L to 4.00 µg/L

7.3.11.1.2 ***Calibration data***

Concentration (µg/L)	Peak Height
0.5900	2035
1.1700	4215
1.7600	5915
2.3400	7336

K	4
sum(x)	5.8600
sum(x)2	10.2902
SS(X)	1.7053
sumY	19501
sum(y)2	1.11×10^8
SS(Y)	15639301
sumXY	33708.14
SP(XY)	5139.905
B (SLOPE)	3014.076

7.3.11.1.3 ***Regression Analysis***

Summary output

Regression Statistics	
Multiple R	0.995281619
R Square	0.990585502
Adjusted R Square	0.985878253
Standard Error	271.3265287
Observations	4

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	15492064.9	15492064.9	210.4383	0.004718381
Residual	2	147236.1703	73618.08517		
Total	3	15639301.07			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	459.5030	333.2527	1.3788	0.3019	-974.3679	1893.374	-974.3679	1893.3740
X Variable 1	3014.0764	207.7743	14.5064	0.0047	2120.0956	3908.057	2120.0956	3908.0571

- Slope (b) 3014.07640884
- Y-intercept (a) 459.50306104
- Linearity F_{calc} = 210

  F_{calc} large, therefore significant linearity

7.3.11.1.4 ***Regression Uncertainties***

- Random Uncertainty ($S_{y/x}$) 271.32652869
- Slope Uncertainty (S_{b}) 207.77431660
- Y-intercept Uncertainty (S_a) 333.25277522

7.3.11.1.5 ***Method Limit of Detection***

- $Y_{LOD} = Y_B+3S_B = bX_{LOD}+a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L

0.270 µg/L

7.3.11.1.6 ***Method Limit of Quantitation***

- $Y_{LOQ} = Y_B+10S_B = bX_{LOQ}+a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOQ} = 10S_{y/x}/b$, ng/L

0.900 µg/L

7.3.11.1.7 ***Accuracy***

A spiked sample, concentration of 0.59 ug/L was used to determine accuracy.

Determination	**Concentration**	**Peak height**
#1	0.393	1645
#2	0.418	1719
#3	0.448	1809
#4	0.405	1681
#5	0.403	1675
#6	0.404	1676
Mean	**0.4118**	**1700.8333**
Std Deviation	**0.0194**	**58.0083**

- RSD of concentration, (s × 100/mean), %

4.72

- Mean Method Accuracy (% of true conc.), %

69.80

7.3.11.1.8 ***Uncertainty***

A Uncertainty of regression

$y_0 = bx_0+a$

For $x_0 =$ 0.4118

$y_0 =$ 1700.800195

ave.y = 1700.83333333

y_0-ave.y = -0.0331

(y_0-ave.y)(y_0-ave.y) 0.001098121

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.5900	-0.875	0.765625
1.1700	-0.295	0.087025
1.7600	0.295	0.087025
2.3400	0.875	0.765625
	Sum =	1.7053
	Sum^2=	2.90804809

$$Sx_0= S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$$

(Where m = determinations = 1; n = calibration points = 4)

Sx_0 = 0.100645186

B Uncertainty of Repeatability

Std Dev.y/ave.y = 0.03410583

C Uncertainty of Purity

Purity = 100%+-0.5%

Assume rectangular distribution

U(P) = $0.5/3^{1/2}$ = 0.29

D Uncertainty of Volume

(i) 10 mL volumetric flask, S.N 0134

10 mL ± 0.04 mL, 20°C

Assume rectangular distribution

$U(V_1) = 0.04/3^{1/2} = 0.023094011$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00021

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.004849742
Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe
100 µL ± 0.12 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.12/3^{1/2} = 0.069282032$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 0.34410076
100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe
1000 µL ± 0.49 µL, 20°C
Assume rectangular distribution
$U(V_1) = 0.49/3^{1/2} = 0.282901632$
Lab temperature = 20°C ± 4
Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 3.441007604
1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_C)/V_C$ = 0.003452617 µL

E Total uncertainty

Total Uncertainty, at $x_0 = 0.4118$
$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$
U(t) = **0.101652401 µg/L**

95% CL of x_0: $x_0 \pm \{t3_{;0.05}*U(t)\}$
$x_0 \pm \{3.18*U(t)\}$
0.4118 µg/L ± 0.323254635 µg/L

7.3.11.2 **Cylindrospermopsin validation**

7.3.11.2.1 ***Range of standards***

1.00 μg/L to 4.00 μg/L

7.3.11.2.2 ***Calibration data***

Concentration (μg/L)	**Peak height**
1.0000	1259
2.0000	2589
3.0000	4029
4.0000	5032

K	4
sum(x)	10.0000
sum(x)2	30
SS(X)	5
sumY	12909
sum(y)2	49841867
SS(Y)	8181297
sumXY	38652
SP(XY)	6379.5
B (SLOPE)	1275.9

7.3.11.2.3 ***Regression Analysis***

Summary output

Regression Statistics	
Multiple R	0.997448696
R Square	0.994903901
Adjusted R Square	0.992355851
Standard Error	144.3826513
Observations	4

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	1	8139604.05	8139604.05	390.457	0.002551304
Residual	2	41692.7	20846.35		
Total	3	8181296.75			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	37.5	176.8319	0.2120	0.8517	-723.3463	798.3463	-723.3463	798.3463
X Variable 1	1275.9	64.5698	19.7599	0.0025	998.0782	1553.722	998.0782	1553.7217

- Slope (b) 1275.90000000
- Y-intercept (a) 37.50000000
- Linearity F_{calc} = 390
  F_{calc} large, therefore significant linearity

7.3.11.2.4 *Regression Uncertainties*

- Random Uncertainty ($S_{y/x}$) 144.38265131
- Slope Uncertainty ($S_{b)}$ 64.56988462
- Y-intercept Uncertainty (S_a) 176.83191171

7.3.11.2.5 *Method Limit of Detection*

- $Y_{LOD} = Y_B + 3S_B = bX_{LOD} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOD} = 3S_{y/x}/b$, ng/L
  0.339 µg/L

7.3.11.2.6 *Method Limit of Quantitation*

- $Y_{LOQ} = Y_B + 10S_B = bX_{LOQ} + a$
- Assume $Y_B = a$ and $S_B = S_{y/x}$
- Thus, $X_{LOQ} = 10S_{y/x}/b$, ng/L
  1.132 µg/L

7.3.11.2.7 *Accuracy*

A spiked sample, concentration of 2.0 µg/L was used to determine accuracy.

Determination	Concentration	Peak height
#1	2.028	2625
#2	2.461	3177
#3	2.091	2705
#4	2.391	3088
#5	2.526	3261
#6	2.516	3248
Mean	**2.3355**	**3017.3333**
Std Deviation	**0.2200**	**280.9076**

- RSD of concentration, (s × 100/mean), %

  9.42

- Mean Method Accuracy (% of true conc.), %

  116.78

7.3.11.2.8 ***Uncertainty***

A Uncertainty of Regression

$y_0 = bx_0+a$

For x_0 =	2.3355
y_0 =	3017.36445
ave.y =	3017.33333333
y_0-ave.y =	0.0311
(y_0-ave.y)(y_0-ave.y) =	0.000968247

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
1.0000	-1.500	2.25
2.0000	-0.500	0.25
3.0000	0.500	0.25
4.0000	1.500	2.25
	Sum =	5
	Sum^2 =	25

$$Sx_0 = S_{y/x}/b\{1/m+1/n+(y_0-ave.y)^2/b^2 sum(x_i-ave.x)^2\}^{1/2}$$

(Where m = determinations = 1; n = calibration points = 4)

Sx_0 = 0.12651831

B Uncertainty of Repeatability

Std Dev.y/ave.y = 0.09309796

C Uncertainty of Purity

Purity = 100% ± 0.5%

Assume rectangular distribution

U(P) = $0.5/3^{1/2}$ = 0.29

D Uncertainty of Volume

(i) 10 mL volumetric flask, S.N 0134

10 mL ± 0.04 mL, 20°C

Assume rectangular distribution

$U(V_1) = 0.04/3^{1/2} = 0.023094011$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00021

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.004849742

Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$ = 0.002359774 mL

ii) 100 µL syringe

100 µL ± 0.12 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.12/3^{1/2} = 0.069282032$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149

Assume rectangular distribution

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.34410076

100 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$ = 0.003510062 µL

iii) 1000 µL syringe

1000 µL ± 0.49 µL, 20°C

Assume rectangular distribution

$U(V_1) = 0.49/3^{1/2} = 0.282901632$

Lab temperature = 20°C ± 4

Coefficient of volume expansion = 0.00149
Assume rectangular distribution
$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$
= 3.441007604
1000 µL Syringe Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$
$U(V_C)/V_C$ = 0.003452617 µL

E Total uncertainty

Total Uncertainty, at $x_0 = 2.3355$

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2\}^{1/2}$
U(t) = **0.251973936 µg/L**

95% CL of x_0 : $x_0 \pm \{t3_{;0.05}*U(t)\}$
$x_0 \pm \{3.18*U(t)\}$
2.3355 µg/L ± 0.801277117 µg/L

7.4 SUMMARY

Many freshwater cyanobacteria (blue-green algae) are capable of producing toxins that are known to cause death in humans and animals. Therefore, it is very important to be able to test for cyanotoxins, especially in the potable water industry.

The cyanotoxin most frequently encountered is microcystin (produced by many genera of cyanobacteria). The method most frequently used to test for microcystins is the ELISA (enzyme-linked immuno sorbent assay) method, which can be implemented easily, with relatively low initial cost equipment in any laboratory. The execution of the ELISA method 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 analysed at a fairly low cost in comparison to the ELISA method (refer to **Table 7.1**).

Another advantage of having the HPLC equipment is that it allows for the analyses of almost all of the other important cyanotoxins like, Nodularin, Anatoxin-a, and Cylindrospermopsin (all methods described in this chapter).

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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 (± 5 – 50 mL)	Merck	011 345-9000 www.merck.co.za
Glass tube (± 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 µg/L) with disposable tips	Merck	011 345-9000 www.merck.co.za
Pipette dispenser (500 – 5000 µL) 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 µm)	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
Waterbath	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)				✓				