THE OCCURRENCE OF CYANOBACTERIA AND THEIR TOXINS IN WATER USED FOR DOMESTIC PURPOSES IN RURAL AREAS

Dissertation submitted by

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in fulfilment of the requirements for the Degree

MAGISTER TECHNOLOGIAE

in

BIOTECHNOLOGY

in the

Faculty of Science

of the

University of Johannesburg

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JOHANNESBURG
January 2007

DECLARATION OF INDEPENDENT WORK

I, Elvis Fosso Kankeu, Identification number BRA 01776903; Passport number 647406 and Student Number 820411118, do hereby declare that this research project, submitted to the University of Johannesburg for the degree **MAGISTER TECHNOLOGIAE: BIOTECHNOLOGY,** is my own independent work.

This work has not been submitted before to any institution by myself, or, to the best of my knowledge, any other person in fulfillment of requirements for the attainment of any qualification.

SIGNATURE OF CANDIDATE **DATE**

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ACKNOWLEDGEMENTS

- Thanks to God
- To my family,
    - My parents (Mr Kankeu and Mrs nee Djoumte) thank you for your support and prayers
    - To my decease uncle Mr Luc Soh and aunt Mrs Wombiwo for their forever support, rest in peace
    - To my grand-mom Mrs nee Mafongang
    - To my elder brother Ndassi and his wife for their great support
    - To all my brothers and sisters
    - To my daughter Ms Kankeu M and her mother
- Prof Jagals, I am truly thankful for the great supervision
- Prof Du Preez, I am truly thankful for the great Co-supervision
- Thanks to Dr Barnard and Dr Safi for your time and comments
- Thanks to Dr S Du Plessis for kindly offering cyanobacteria growth media
- Thanks to Ms A Swanepoel, Ms L Van Baalen, Lucky, Mike, Olivier and to all my colleagues at Water and Health Research Unit
- My friends Georgy, Robert, Christophe, Francois, Tothy, Guy, Flavien, Clovis, Patrick, Joseph, Jehu, Remy, Teto, Beauvillier, J-bernard, Achille and Sandra
- Thanks to Mr L Alagiozoglou, Ms J Jacinda, Mr N De Villiers and Mr M Mbida
- Big thank you to Mr Eric Van-Zyl (Head of Department: Biotechnology) for your support
- Thanks to the National Research Foundation for their financial support.

Summary

Many people in the Vhembe district (Limpopo Province-South Africa) are compelled to use untreated water sources such as river water for drinking and other domestic purposes. Living at some distances to the source, they have to collect, transport and store their water in containers for later use. The consumption of untreated water exposed the population to pathogenic microorganisms including cyanobacteria.

Cyanobacteria are photo-autotrophic bacteria, which naturally occur in water and have the ability to attach to surface and form biofilm. Their occurrence in water is controlled by environmental factors such as temperature, light intensity, nutrients (phosphate and nitrate) availability and water stability. Cyanobacteria are potential producers of microcystins toxins and their cell walls contain lipopolysaccharide endotoxin. Their presence in water therefore represents a threat for the health of consumers.

Cyanobacteria have been found to occur in surface waters world-wide and many cases of outbreaks have been associated to them and their toxins in recreational and drinking water, studies done in South Africa revealed the presence of cyanobacterial blooms in water sources with a dominance of *Microcystis* genus.

The present study carried out in the above area, investigated the occurrence of cyanobacteria and their toxins (microcystins and endotoxin) in water-storage containers from twenty households and in water sources.

Water samples from water-storage containers and their respective water sources were analysed at the Analytical Services of Rand Water (Vereeniging-South Africa) for the identification and enumeration of phytoplankton. The results revealed the presence of five algal classes, Bacillariophyceae, Chlorophyceae, Dinophyceae, Euglenophyceae and Cyanophyceae. Seven genera of cyanobacteria (*Microcystis spp, Oscillatoria spp, Anabaena spp, Pseudanabaena spp, Aphanocapsa spp, Radiocystis spp* and *Spirulina spp*) were identified with *Microcystis spp* being the most frequent and abundant genus.

A comparative analysis between the level of contaminants in free volume of water and in water containing dislodged biofilm showed that there were particles (later considered as biofilm) attached at the container inner surfaces. The significantly higher numbers of cyanobacteria in water stored in light containers as compared to dark containers showed that light availability influenced the accumulation of cyanobacteria in containers. Enteric bacteria (*E. coli* and total coliform) co-occurred with cyanobacteria in containers' biofilm and together

contributed to the occurrence of endotoxin in drinking water. However, there was a poor correlation between bacterial count and the concentration of endotoxin in water-storage containers.

The temporal variability of the occurrence of cyanobacteria in water sources and the use of untreated alternative sources by the population brought about the uncertainty of the original source of containers' cyanobacteria.

Of the four surface water sources (Savhani River, Luphephe River, Nwanedi River 1 and Canal) used by the population, the Nwanedi River 1 and the Canal were particularly of poor health related quality as they contain unacceptable level of microcystins and endotoxin (1 µg / ℓ and 3 µg / ℓ respectively). The Nwanedi and Luphephe impoundments with highest concentrations of cyanobacteria and their toxins contributed to the increase load of microcystins and endotoxin in water sources downstream.

The nitrate and phosphate concentration being sufficient in the entire water samples, the limiting factors for the occurrence of cyanobacteria were found to be the water stability (calm) at the water sources and light in drinking water containers.

Regarding the guidelines, consumers of water at the point of use and at the source are exposed to health problems associated to cyanobacterial toxins. A treatment process should then be implemented before water storage and the population should consider a hygienic maintenance of containers.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND

Pathogenic cyanobacteria are recognised by the World Health Organization (WHO, 2004, 1999) as pathogenic agents that should be monitored for in drinking water.

Cyanobacteria, or photo-autotrophic blue-green algae, may form blooms in water under suitable circumstances and become potentially toxics. They produce cyanotoxins that may be harmful to humans and animals (WHO, 1999; Codd et al, 1997; Sivonen, 1996; Carmichael, 1992). They occur in eutrophic waters, such as nutrients rich surface water (e.g. rivers), that is often used by a substantial number of people in rural areas, for drinking and other domestic purposes. Depending on the type of cyanotoxins (endotoxins, neurotoxins and hepatotoxins), and the dose, the impact on human health may range from gastroenteritis to dermal irritation, liver cancer and paralysis (Prescott et al, 2002; Yu, 1995; Teixera et al, 1993). There are guidelines available locally and internationally recommending a maximum acceptable level of cyanobacteria, microcystins and endotoxin present in drinking water.

Inadequate water supply services compel many people in poor and rural areas to use untreated surface water for their domestic needs. In areas where these waters are subjected to eutrophication, these people might already be exposed to pathogenic cyanobacteria and their cyanotoxins. Households fetch water from these sources in a variety of containers and store it at home, often for days, while being used (Jagals et al, 2003). This storage process of water promotes the formation of biofilm on the inner sidewalls of the containers. Considered as a reservoir of microorganisms and mineral compounds (Lechevallier, 1999), biofilms play a role in the deterioration of the microbiological quality of water stored in a container (Nala et al, 2003; Jagals et al, 2003; Momba and Kaleni, 2002).

The occurrence of cyanobacteria in water is of a great concern in South Africa as many studies have been done since the first reported cases of cyanobacterial poisoning in 1927 by a Dr Steyn from the Onderstepoort Veterinary Institute. Several cases of cyanobacterial toxicosis have been associated with animal or stock deaths in South Africa (Van Ginkel, 2004). In South Africa, assessments of cyanobacteria and their toxins in water are done by drinking water utilities (e.g. Rand Water) and researchers at Universities and Institutes (e.g. North-west University).

Studies on cyanobacteria focus mostly on the relationships between light, temperature, nutrients and their occurrence and the production of toxins in the environmental water sources (Garnett et al, 2003; Rapala et al, 1998; Sivonen, 1990). Literature searches were not

successful in finding similar research that have investigated the occurrence of cyanobacteria and their toxins in water stored in household storage containers used for drinking by a large number of mostly poor and rural people in developing countries around the world.

This study investigated the previous situation in a rural area in the northern parts of the Limpopo Province, South Africa (Figure 1)

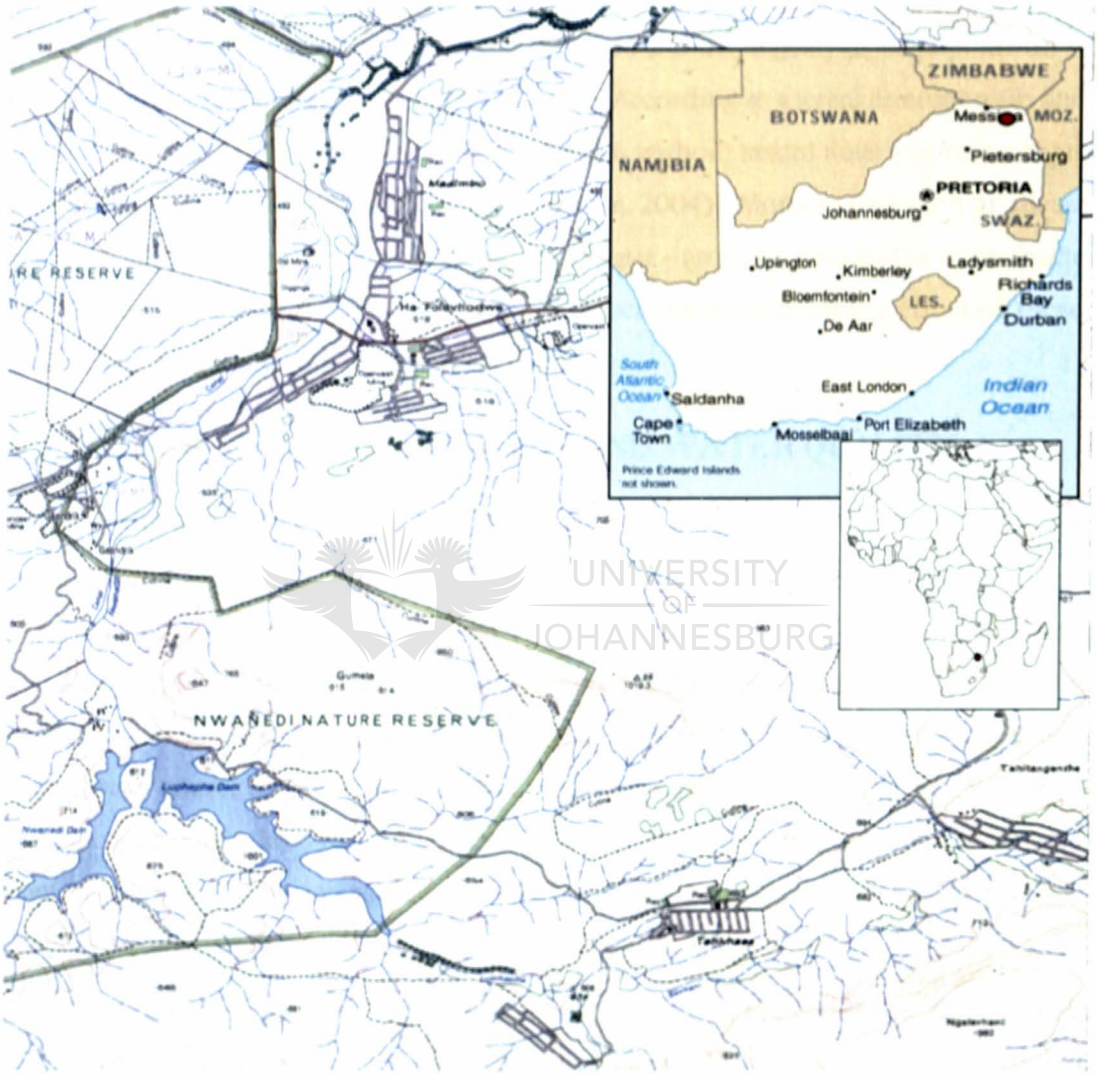


Figure 1: The study area in north-eastern Limpopo province, South Africa.

1.2 POTABLE WATER IN AFRICA

In some African countries, less than 30% of the population have access to potable water sources (Mama Africa, 2003). It is further estimated that about 264 million people in poor African communities have very limited access to potable water in terms of potable quality, quantity or distance to the source (Enterprise Works, 2000). The problem is even worse in rural areas where a considerable fraction of the population has no option than to use untreated

surface water sources, such as rivers and streams, for their potable water (Mama Africa, 2003). This contributes to negative impacts on health in Africa's continuing cycle of poverty (Enterprise Works, 2000).

In South Africa particularly there is relatively good access to potable water in urban areas where water utility companies such as Rand Water as well as local authorities as service providers ensure a permanent supply of good quality water for domestic purposes including drinking. However the rural areas are under-serviced and the majority of the population still lack access to running clean water (Nduru, 2005). According to a recent demographic and health survey, the infant mortality rate in households without treated water was twice than of infants from households with tapped water (Hemson, 2004). Mortality rates such as these may be caused by pathogens present in untreated water - amongst these are the cyanobacteria (WHO, 2004). Little is currently known about the occurrence and potential effect of these pathogens in the water used by rural households.

1.3 WATER SUPPLY, WATER USE AND WATER QUALITY

1.3.1 Rural areas

In rural areas in South Africa, people typically rely on a variety water sources, from surface to ground water with significant variation in the quality and quantity of available water (Momba and Kaleni, 2002). Many of these sources are also used for multiple purposes varying from drinking water to laundry, bathing and gardening. Often people have no choice than using a certain water source regardless of the quality (Mokgope and Butterworth, 2001). When small streams, open ponds, lakes or open reservoirs must be used as sources of water, the danger of contamination and of the consequent spread of enteric diseases such as typhoid fever and dysentery is increased (Tulchinsky et al, 2000).

In the study area (Figure 1), the villages also used a variety of sources. People from the major village (Ha-Folovhodwe), used tap water (sourced from untreated ground water), while in the other villages of Musunda, Tshitanzhe, Gumela, Tshikotoni and Tshitandani, used untreated river water (Jagals, 2005, personal communication).

Rivers in especially rural areas become polluted due to upstream activities such as washing clothes, bathing, animal activities and lack of sanitation (Nevondo and Cloete, 1999). The source-rivers in the study area were of this nature. Furthermore surface water quality is often unreliable because of flow, and is likely to be more contaminated by faecal micro organisms and cyanobacteria than ground water that has undergone a natural process of physical,

chemical and biological filtration through the soil and substrata (Conservation Technology Information Centre, 2005).

Although limited in quantity, ground waters are preferred to surface water as a source for drinking water in the areas where people rely on raw water (Taylor, 2003). However, untreated it sometimes contain unacceptable level of microorganisms and chemical compound (e.g. Dense Non-aqueous Phase Liquids), and is also associated with many drinking water disease outbreaks (Hunter, 2006). However, cyanobacteria occurrences are seldom associated with groundwater quality.

Whether people use ground or surface water, being supplied in taps or collected at the source (rivers and boreholes), these water sources are almost always some distance from the households. This forces the population to collect, transport and store water in containers for in-house use (Jagals et al, 2003; 1999; 1997).

1.3.2 Water containers

Despite the availability of cleaner water from tap, tank and borehole, problems are often experienced with accessibility to and availability of the supply water (Jagals, 2006). This leads to the inevitable practice of using containers to collect and store water from whatever source.

Developing areas may sometimes even be supplied with tap water but the distribution system does not follow the normal regulation of the taps being in-house, but rather communal taps, which often are some distances from the house (Jagals, 2006; Mokgope and Butterworth, 2001; Pollard and Walker, 2000). The distance from the closest tap to the house is often far enough to necessitate the use of containers.

Another problem is maintenance, which influences sustainability of such services. If the supply breaks down, people use alternative often polluted sources (Mokgope and Butterworth, 2001).

In both scenarios, containers are used and the source waters vary in microbiological quality, all leading to the water at the point of use being of inferior microbiological quality by the time people get to drink it (Jagals et al, 2003; Momba and Kaleni, 2002). Even when water is collected at a tap that supplies a good quality of water, the quality deteriorates during storage process.

Domestic use of water implies drinking, food preparation, washing and gardening. It is

maybe not the more important use in terms of volume required but it is the priority as it covers all the basic needs and can impact seriously on health and well being of humans (WRC, 1998). In the context of health, it is the ingestion of water that was considered above the other uses during this study.

In the study area people collect, carry and store water for drinking and food preparation in a variety of containers mostly plastic ones, of between 20-25 litres capacity each. These are filled at the source and stored in the household, where the handling (such as pouring out etc.), contribute to the deteriorating microbiological quality of water (Moabi, 2006; Nala et al, 2003). Pets, children and insects are also reported to be risk factors that can contaminate these waters in uncovered containers (Joubert et al, 2003). Jagals et al (2003; 1999; 1997) also demonstrated that storage of water in households contribute to the deterioration of the microbial water quality, with causes ranging from poor container hygiene and open containers subjected to environmental pollution, to the manner of handling of water by individuals in households.

The main factor that has been associated with the breakdown in the water quality was reported to be the formation of biofilm inside these containers. These are reported to be harbouring excessive numbers of microbes that is the cause of container water contamination and subsequent health risk to the water user (Jagals et al, 2003; Nala et al, 2003; Momba and Kaleni, 2002).

1.4 WATER QUALITY IN CONTAINERS

While container biofilm is reported to be a major cause of water quality deterioration, this was assessed by comparing what was essentially the free undisturbed volume of water in the container, to samples of the same water with biofilm from the containers somehow suspended in the free volume (Moabi, 2006; Ntsherwa, 2004; Jagals et al, 2003; Nala et al, 2003). In the context of this study it is important to review these concepts as these have a major significance for the study.

1.4.1 Free Volume of Water in Containers

This refers to the water body in a container which is directly accessible by the consumer. The microbiological quality of this water depends of the source and the hygienic conditions associated with the handling of the containers. While improved access to potable water has been shown to enhance the microbiological quality of water in containers (Jagals et al, 1999), poor container hygiene practices contributed to the contamination of the free volume (FV) of

water with micro-organisms. However it was also demonstrated that if the water in container is well protected, there will (after overnight storage) be an improvement in the microbiological quality of the FV compared to the source (Moabi, 2006, Egwari and Aboaba, 2002). In fact, the low nutrient environment as well as the absence of suitable growth temperature promote bacterial die-off in container (Wright et al, 2004). However, Jagals et al (2003) as well as Momba and Kaleni (2002) have shown that excessive numbers of micro-organisms may still occur in the free volume of water even after prolonged storage. These were demonstrated to have been released from biofilm attached to the container inner surface.

1.4.2 Biofilm in Containers

Biofilms are defined by Hall-Stoodley and Stoodley (2002) as complex communities of microorganisms that develop on surfaces in a diversity of environments – especially where water and other surfaces form interfaces. These can also be the result of the accumulation of organic and inorganic materials attaching to the strata initiated by microorganisms and vice versa (Lechevallier, 1999). During sourcing and storage, particles and microorganisms in the source water attach to the inner surfaces of water containers, eventually forming or adding to biofilm (Jagals et al, 2003). Under suitable conditions such as nutrient availability, nature of the support and stability (calmness) of water, micro-organisms in biofilm develop and colonise the surface. The interaction between cells and the attachment of cells to the surface is consolidated by the presence of a polysaccharide matrix which is generally contained in the cell-walls of Gram-negative bacteria and cyanobacteria (Sutherland, 1999). The biofilm becomes dislodged in pieces from the container sidewalls because of increased turbulence of the water in containers during handling and sourcing. These pieces of the biofilm contaminate the container water with potential pathogens, which can have a negative effect on the consumer's health when consumed (Momba and Kaleni, 2002).

1.5 CYANOBACTERIA IN DRINKING WATER

Identified in impoundments and rivers in South Africa (Van Ginkel, 2004), cyanobacteria may affect consumers' health (e.g. after consumption or skin contact), especially in rural and poor areas where people rely on raw water for their drinking needs. Cyanobacteria numbers in water vary seasonally and also depend on nutrients load in water (Du Preez and Van Baalen, 2006; Van Ginkel, 2004). They are potent producers of toxins and have been associated with water-borne disease outbreaks world-wide. For this reason, water utilities make special efforts, using many techniques to reduce their occurrence in drinking water.

1.5.1 Characteristics of Cyanobacteria

Cyanobacteria are often referred to as "blue-green algae" because they appear similar to the green algae in morphology, habitat and photosynthetic ability (Taylor, 2003). They are however classified as prokaryotes (true bacteria) because they do not have intracellular membrane-bound organelles such as a true nucleus or mitochondrion, and they possess a cell wall composed of peptidoglycan and lipopolysaccharide layers instead of the cellulose of green algae.

Morphological diversity ranges from single cells to small colonies of cells to simple branched filamentous (Whitton and Potts, 2000). The cytoplasm contains many ribosomes and appears granular. In the filamentous forms, fine plasmodesmata connect adjacent cells. There are a series of parallel membranes within the cytoplasm that are separated from the plasmalemma. The process of photosynthesis occurs on these membranes, which contain chlorophyll-a and other accessories called Phycobilisomes attached to the outside of the membranes (Weier cited in Oberholster et al, 2004).

Cell colours vary from blue-green to violet-red. Carotenoids and accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin (phycobiliproteins) usually mask the green of chlorophyll-a (Chorus and Bartram, 1999; Du Preez and van Baalen, 2006). These pigments capture light of wavelengths 550 to 650 nm and pass their light energy on to chlorophyll-a (Oberholster et al, 2004). Most of the cyanobacteria are photosynthetic and grow well in shallow eutrophic water with little turbulence, warm sunny weather, lower nitrogen to phosphorus ratios and elevated pH (DWAF, 1996). To synthesise their own organic material they require inorganic nutrients such as CO_2, phosphate and nitrogen found in eutrophic water with slow flow.

Cyanobacteria can also survive extremely high and low temperatures and can occur in various environment such as volcanic ash, desert sand and rocks (Dor and Danin, 1996; Jaag, 1945), hot springs (Chorus and Bartram, 1999; Castenholz, 1973), mountain streams (Kann, 1988), arctic and antarctic lakes (Skulberg, 1996a) and snow and ice (Laamanen, 1996; Kol, 1968). Many species are capable of living in the soil and terrestrial habitats (Whitton, 1992) but the prominent habitats of cyanobacteria are limnic and marine environments.

Cyanobacteria multiply exclusively by means of asexual reproduction; while unicellular forms multiply by binary fission; filamentous forms reproduce by trichome fragmentation, or by formation of special hormogonia (Chorus and Bartram, 1999).

Cyanobacteria can be a nuisance in water. They release compounds such as geosmin (trans-1, 10-dimethyl-trans-9-decalol); MIB (2-methylisoborneol); β-cyclocitral, IPMP (2-isopropylmethoxypyrazine) and IBMP (2-isobutylmethoxypyrazine). If released in large enough amounts, these substances cause taste and odour problems in water (Rae et al, 1999).

Most importantly, in the context of this study, they produce toxins that may harm the health of people (Chorus and Bartram, 1999). These toxins are reviewed in more detail in Section 1.8.

1.5.2 Factors affecting the Occurrence of Cyanobacteria

The occurrence of a particular genus and species of cyanobacterium in a particular part of the world is influenced by regional differences in water chemistry and climatic conditions (Whitton and Potts, 2000; Chorus and Bartram, 1999).

Cyanobacteria occur naturally in environmental water but the formations of massive blooms are generally promoted by several factors. The enrichment of water by nutrients such as phosphate and nitrate (eutrophication), play a major role in the proliferation of cyanobacteria in an aquatic system (Chorus and Bartram, 1999).

Agriculture is one of the major contributors of eutrophication, as its activities include the devastation of forests and the use of fertilizer. The clearance of forests increases the runoff of materials and mineral elements to the water bodies during rain. The fertilizer component contains elements such as nitrogen and phosphate (Chorus and Bartram, 1999).

The main driving force to the eutrophication problem is human population growth and associated economic activities with the urbanisation, high concentration of people in specific areas inevitably lead to an increase of urban waste (Chorus and Bartram, 1999). The run-off from roofs, storm and domestic sewer systems, industrial waste and detergents end in surface waters, contributing to the enrichment in nutrients. Human activities make use of numerous products and resources containing bound N and P, ultimately converting them into available N and P that are released into the aquatic environment through various pathways (Du Preez and Van Baalen, 2006; Whitton and Potts, 2000; Chorus and Bartram, 1999).

Edmonson (1991) reported that about twenty elements have been identified in nature to be essential for algal growth. The major inorganic ions such as calcium, magnesium, sodium, potassium, chloride and sulphate are present in much higher concentrations (milligram per litre) than needed for growth, while elements such as nitrogen and phosphorus are present in much smaller concentrations (microgram/l) and therefore may not always be available for algal uptake.

For this study, phosphorus and nitrogen, being the most common elements that limit growth, (Rusin et al, 2000; Ryding and Rast, 1989; Brown, 1973), have been selected for investigation. Physical factors tested for that may determine growth, were light and temperature (Chorus and Bartram, 1999).

1.5.2.1 Light

Cyanobacteria are photo-autotrophs, which means that one of their characteristic features is their ability to photosynthesise with the aid of chlorophyll and accessory pigments. Photosynthesis being their principal mode of energy metabolism (Cohen-Bazire and Bryant, 1982), they require light for this process.

In the environment, sunlight is not always directly received by cyanobacteria in the water as dissolved organic compounds absorb light energy and factors such as domestic and industrial discharge, sediment load (turbidity) and water chemistry influence the amount of light entering the water (Wetzel, 1983). But cyanobacteria have adapted to a variety of different light intensity regimes. Some require light of only a few µmolm-2s-1, and some tolerate direct sunlight (Tandeau de Marsac and Houmard, 1993). For instance, some species of cyanobacteria such as *Cylindrospermopsis raciborskii* have the ability to grow at various intensity of light (Garnett et al, 2003) while *Microcystis aeruginosa* has a narrower tolerance limits for light (64.8 to 324 µmolm-2s-1) (Abelovich and Shilo, 1972) and *Oscillatoria redekei* is adapted to use low light intensities (12 to 18 µmolm-2s-1) (Whitton and Potts, 2000).

A comparative study with *Microcystis aeruginosa* showed near zero growth at low light intensities as well as reduction at higher light intensities, having narrower tolerance limits for light (Van der Westhuizen and Eloff, 1985). The longer exposure of cyanobacteria to light conditions especially in summer, generally promotes the formation of blooms (Nicklish, 1998; Foy and Gibson, 1993).

Light also influences the production of toxins by cyanobacteria. Light intensity can have an impact on the production of microcystin by *Microcystis aeruginosa* strain (Kaebernick et al, 2000). Suitable light condition for growth does not always favour the production of the toxin by a certain species, as these secondary metabolite toxins are also maximally produced during stress conditions (Rapala et al, 1997; Utkilen and Gjolme, 1995; Kotak et al, 1995; Van der Westhuizen et al, 1986 and 1985, Watanabe and Oishi, 1985).

In the context of this study, it was assumed that the light available in the plastic water

containers will vary from the dark to light containers, enabling a crude assessment of the role of dark versus light (translucent) containers in the occurrence of cyanobacteria in the drinking water.

1.5.2.2 Temperature

Water temperature is an important environmental variable because specific organisms have definite ranges of temperature at which maximum growth and reproduction occurs (Pieterse and Janse van Vuuren, 1997). The variation of temperature has a significant impact on cyanobacteria growth, they are limited at extreme temperature but proliferate and subsequently form blooms at temperature around 25°C if the other factors are optimal (Chorus and Bartram, 1999).

Work done by Garnett et al (2003) and Van der Westhuizen and Eloff (1985) showed that toxins production can also be affected by the variation of temperature. In fact, they found that highest concentrations of toxins were produced at temperature around 20°C but decreased at temperature above 28°C.

This implies that if the temperature in sub-tropical climates may assist cyanobacteria to maintain and even increase in numbers, the potential for cyanobacteria toxin in water would also increase.

1.5.2.3 Nitrate

Nitrogen is a very important element in the ecosystem as it is used in the metabolism process by animals, plants and microorganisms including cyanobacteria (Herrero et al, 2001). Run-off carries anthropogenic products such as containing nitrogen rich sources (agricultural fertiliser and sewage) to the surface water (Global Environment Outlook, 2000). Nitrogen occurs in surface water in several forms (e.g. ammonium, nitrite, nitrate, urea and nitrogen gas). All freshwater algae are able to assimilate the first four forms, but nitrogen gas can only be utilized by certain species of cyanobacteria such as *Anabaena* spp (Walmsley, 2000).

Nitrogen plays a secondary role to phosphate in terms of sustaining cyanobacteria, but can become important at a high level of eutrophication, when N-fixing cyanobacteria such as *Oscillatoria* spp can cause nuisances much more significant than other types of algae (Korselman and Meuleman, 1996). Nitrogen concentration in water above 100 µg/ℓ will favour the growth of cyanobacteria (Rusin et al, 2000; WHO, 1999).

1.5.2.4 Phosphorus

Phosphorus has been implicated more widely than nitrogen as a limiting nutrient of

phytoplankton including cyanobacteria in freshwater systems (Hart et al, 1993). Phosphorus may be present in organic or inorganic form and in both categories; the individual phosphorus species may be present in either dissolved, precipitated or adsorbed phases. Interchange between the various categories is possible as a result of physical, chemical and biological action (Pillay and Buckley, 2001). Dissolved phosphate is the major source of phosphorus directly available to phytoplankton (Wetzel, 1983). Much phosphorus may be unavailable, because it is absorbed onto bonded particles in the water (Addiscott et al, 1991). Municipal sewage effluents contribute at least half of the total phosphorus inputs to rivers and lakes (Chorus and Bartram, 1999). Other sources of phosphorus are agricultural fertilizers run off after intensive rainfall and erosion, which release phosphate from the sediment.

The occurrence of cyanobacteria is controlled by phosphate availability in water. According to Rusin et al (2000), cyanobacteria may multiply rapidly to form blooms in eutrophic waters with levels of phosphorus greater than 10 µg/ℓ and levels of ammonia or nitrate-nitrogen greater than 100 µg/ℓ. If the other factors cited above are optimal and the phosphate concentration is below 10 µg/ℓ, the occurrence of cyanobacteria blooms will be limited (Chorus and Bartram, 1999).

Considered to be the limiting factor for the occurrence of cyanobacteria in water, it was then important for our study to measure the level of phosphate in source and container waters as this would be an indication of whether cyanobacteria could be sustained in the test waters. According to WHO (1999), the limited concentration of phosphate for the occurrence of cyanobacteria is 10 µg/ℓ.

1.5.3 Cyanotoxin

1.5.3.1 Production of cyanotoxin

Cyanotoxin is a collective name for the various toxins produced by cyanobacteria and can be influenced by environmental conditions such as pH, intensity of light, temperature, nitrate, phosphate, dissolve oxigen and CO_2 availability (Grobbelaar et al, 2004; Rapala and Sivonen, 1998). Within species of cyanobacteria, there are non-toxic strains as well as strains that can produce a particular toxin or several toxins (Grobbelaar et al, 2004; Chorus and Bartram, 1999). There are divergent opinions whether cyanotoxins are produced under stress conditions or not. According to study conducted by Sivonen (1990), high concentrations of toxins are produced under conditions which are favourable for cyanobacteria growth, while previous study by Van der Westhuizen and Eloff (1985) showed that optimum conditions for

growth did not coincide with those for toxin production by the *Microcystis aeruginosa* culture they studied. The primary metabolite cyanotoxin, lipopolysaccharide endotoxin is always produced by all cyanobacteria as it constitutes part of the cell walls (Metcalf and Codd, 2004).

1.5.3.2 Types of cyanotoxin

These can be divided according to their effect on human body. Techniques are available to identify three neurotoxins (anatoxin-a, anatoxin-a(s) and saxitoxins), one general cytotoxin which inhibits protein synthesis (cylindrospermopsin), and the much popular group of toxins termed hepatotoxins (microcystins and nodularins), which inhibit protein phosphatases. Finally there are cyanotoxins referred to as dermatotoxins – they have a particular effect on skin. In this group are included aplysiatoxin and LPS endotoxin which is poorly studied and less known; Endotoxin is also found in Gram-negative bacteria and is a constituent of the cell wall of almost all the cyanobacteria (Rapala et al., 2002; Metcalf and Codd, 2004).

1.5.3.3 Health effects of cyanotoxin

The effects of cyanotoxins on human health is classified according to region of the body affected and generally target the liver (hepatotoxins), nervous system (neurotoxins), human cells (cytotoxins) or the skin. The health effects, the toxins and their associated cyanobacteria are summarised in Table 1:

Table 1: Summary of cyanotoxin and the cyanobacteria that produce them as well as some of the recorded mammalian clinical symptoms of cyanotoxin exposure (adapted from Du Preez and Van Baalen, 2006; Falconer, 2005; NHMRC, 2004; Chorus, 2001; Chorus and Bartram, 1999; Sivonen and Jones, 1999)

TOXIN	CYANOBACTERIA GENERA	CLINICAL SYMPTOMS
Cyclic peptides		
Microcystins	Microcystis, Anabaena, Oscillatoria, Planktothrix, Nostoc	Gastro-enteritis, fever, pains in muscles and joints, nausea, vomiting, blistering around mouth, diarrhoea, swollen liver, death by liver failure
Nodularin	Nodularia	Gastro-enteritis, fever, pains in muscles and joints, nausea, vomiting, diarrhoea, swollen liver, death by liver failure
Alkaloids		
Cylindrospermopsin	Cylindrospermopsis, Aphanizomenon, Anabaena, Raphidiopsis, Umezakia,	Abdominal pains, vomiting, swollen liver, liver failure, pathological damage to the kidneys, spleen, thymus and heart
Anatoxin-a	Anabaena, Planktothrix, Oscillatoria, Aphanizomenon	Muscle weakness, respirator distress, exaggerated abdominal breathing, hyperactivity, hypersalivation, numbness around the lips, paralysis
Anatoxin-a(S)	Anabaena, Aphanizomenon	Muscle weakness, respirator distress, exaggerated abdominal breathing, hyperactivity, hypersalivation, numbness about the lips, paralysis
Saxitoxins	Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis	Numbness around the lips, complete paralysis, death from respiratory failure
Lipopolysaccharides		
Lipopolysaccharides	All	Allergic reactions, inflammatory, irritation, gastro-enteritis

1.5.3.4 Hepatotoxin (Microcystin)

Microcystin are monocyclic heptapeptide hepatotoxins (liver toxins) composed of seven amino acids with the molecular weight about 1,000 Dalton. These are the most frequently encountered group of cyanobacterial toxins (Carmichael cited in Mc Elhiney et al, 2002; Sivonen and Jones, 1999; Codd, 1995), and are produced by a number of cyanobacterial genera, the most notable of which is the widespread *Microcystis* from which the toxins take their name (New Zealand Ministry of Health [NZMH], 2000).

Microcystin consist of a seven-membered peptide ring, which is made up of five non-protein amino acids and two protein amino acids. The two protein amino acids (L-amino acids) at position 2 and 4, and methylation/demethylation on McAsp and Mdha (An and Carmichael cited in Oberholster et al, 2004; Grobbelaar et al, 2004; Mc Elhiney et al, 2002), distinguish the various microcystins from one another, while the other amino acids are more or less constant between variant microcystins. Using amino acid single-letter code nomenclature, each microcystin is designated a name depending on the variable amino acids which complete their structure. About 70 structural analogues of microcystins have been identified (Sivonen and Jones, 1999; Rinehart et al, 1994).

1.5.3.4.1 Health effects

Microcystins are potent hepato-toxins of animals and humans (Puiseux-Dao et al, 2005; Huynh-Delerme and Puiseux Dao, 1998; Ohtani et al, 1992). After ingestion of contaminated water, microcystins are absorbed in the intestines (Kuiper-Goodman et al, cited in Welker, 2004; Kotak et al, 1993; Falconer, 1991) and transported to liver via a carrier-mediated transport system. In the liver, serine/threonine protein phosphatase 1 and 2A (Romanowska-Duda et al, 2002; Honkanen et al, 1996; Eriksson et al, 1990a, b; Mackintosh et al, 1990) are inhibited. These enzymes in mammalian cells (Dondjin et al, 2003) are responsible for the dephosphorylation of amino acids serine and threonine. They control the function of hepatic cells. Microcystins are specific to hepatocytes and have difficulty to penetrate into the epithelial cells (Matsushima et al., 1990).

Studies on the mechanism of cell toxicity showed that microcystin interferes with cell structure and mitosis (Kaja, 1995; Falconer and Yeung, 1992). This action may explain the effect of microcystins as cancer promoters (Nishiwaki-Matsuchima et al., 1992; Falconer, 1991), especially liver cancer in humans exposed to long-term low doses of microcystins through drinking water (Bouaicha et al, 1998; Mez et al, 1997; Ito et al, 1997; Van Halderen et al, 1995; Carmichael, 1994).

In the same train of thought, Fitzgeorge et al (1994) demonstrated that the effect of microcystins on the liver can be cumulative. The consequence of an acute poisoning of these compounds is a rapid disorganisation of the hepatic architecture (Eriksson et al, 1990b; Falconer et al, 1981), leading to intrahepatic haemorrhage, haemodynamic shock, heart failure and death (Bhattacharya et al., 1997; Carmichael, 1992). Other organs affected are the kidneys and lungs (Hooser et al, 1990) and the intestines (Falconer and Humpage, 1996; Falconer, 1994).

To postulate the health impact of microcystin-LR on humans, studies have been done on mice. This determined a lethal dose level of microcystins. The LD50 by intraperitoneal (i.p.) route ranges from 25 to 150 µg/ kg body weight (bw) in mice. The oral LD_{50} (administered by gavage, i.e. dosing directly into the stomach through the mouth) is 5,000 µg/kg bw in one strain of mice (Yoshida et al, 1997) and higher in rats (Fawell et al, 1994). To confirm the side of action of microcystins, sub-lethal doses of variously radio-labelled microcystins were administered through intravenous (i.v.) or intra-peritoneal injection in mice, immediately after which 70 percent of the toxins were localized in liver (Lin and Chu, 1994a; Nishiwaki et al, 1992; Robinson et al, 1989, 1991; Meriluoto et al, 1990; Brooks and Codd, 1987; Falconer et al, 1986; Runnegar and Falconer, 1986).

On the basis of human life time exposure, the WHO (2004, 1999) recommended a maximum acceptable level (1 µg/ℓ) for the most toxic variant of microcystins (microcystin-LR) in drinking water.

The provisional guideline for microcystin–LR was derived using the following equation (Du Preez and Van Baalen, 2006; NZMH, 2000):

Guideline value (µg/ℓ) = (TDI x Bw x PI)/DI

Where:

TDI = An estimation of the amount of a substance in the drinking water expressed on a body mass basis (µg/kg), that can be ingested over a lifetime without significant health risks. The TDI (µg/kg/day) is calculated as (No Observable Adverse Effect Level [NOAEL] or Low Observable Adverse Effect Level [LOAEL]) / Uncertainty factors. The NOAEL is the highest dose or concentration of a substance that causes no detectable adverse health effect. The LOAEL is the lowest observed dose or concentration of a substance at which there is a detectable adverse health effect. The source of uncertainty is from interspecies variation, intraspecies variation, adequacy

of studies or databases and the nature and severity of the effect. The uncertainty values (factor of 10) thus ranges from 10 to 10000.

Bw = The average body weight of an adult (60 kg) or child (10 kg) or infant (5 kg).

PI = The portion of intake due to drinking water. This value is usually 10%. However, cyanotoxins intake is mainly via drinking water and is thus taken as 80 to 90%.

DI = The average drinking water consumption per day of an adult (2 ℓ) or child (1 ℓ) or infant (0.5 ℓ).

Therefore:

Guideline value (microcystin-LR as µg/ℓ) = [(40/1000) x 60 x 0.8)]/2

= 0.96 µg/ℓ

= 1 µg/ℓ microcystin-LR

where:

TDI = NOAEL is 40 µg/kg/day and the uncertainty factor is 1000.

Bw = The average body weight of an adult is 60 kg.

PI = The portion of intake due to drinking water is 80%.

DI = The average drinking water consumption per day of an adult is 2 ℓ.

No guideline was established by the WHO for the short-term exposure, but to address that issue, Fitzgerald et al (1999) proposed an increase of 10-fold of the previous one (Du Preez and Van Baalen, 2006).

1.5.3.5 Lipopolysaccharide Endotoxin

Lipopolysaccharides (LPS) are heat- and pH-stable endotoxins that are harmful to humans and animals. They can cause endotoxemia and gastrointestinal track disorders to human especially naïve individual (Rylander et al, 1978). The few studies carried out on cyanobacterial LPS indicate that they are less toxic than the LPS of other bacteria such as Salmonella (Razindin et al, 1983; Keleti and Sykora, 1982). Lack of axenic cyanobacterial strains has previously hindered detailed studies on structures and toxicities of cyanobacterial LPS (Chorus and Bartram, 1999).

LPS are amphiphilic macromolecules, forming the lipid matrix of the outer membrane cell

wall of all the cyanobacteria (Metcalf and Codd, 2004). LPS participate in the physiological membrane functions and are, therefore, essential for bacterial growth and viability. They contribute to the low membrane permeability and increase the resistance towards hydrophobic agents (Wiese et al, 1999).

The LPS derived from cyanobacteria and from gram-negative bacteria differ in both chemical and biological characteristics but are basically similar, (Keleti and Sykora, 1982). LPS endotoxin complexes are macromolecules composed of three regions: lipid-A, core polysaccharide, and "O" antigens (Braude, 1982). The lipid-A component is critical for all biological responses to endotoxin (Prescott et al, 2002). LPS isolated from cyanobacteria and enterobacteriaceae can be detected using the limulus amoebocyte lysate (LAL) assay (Mwaura et al, 2004, Rapala et al, 2002, Keleti and Sykora, 1982).

1.5.3.5.1 Health effects

The lipopolysaccharide (LPS) of cyanobacteria is quite similar to the one of Gram-negative bacteria, but studies carried out on cyanobacterial LPS indicate that they are less toxic than the LPS of other bacteria such as *E. coli* and *Salmonella* (Rapala et al, 2002, WHO, 1999; Razindin et al, 1983; Keleti and Sykora, 1982). LPS is released both by live and from dead bacteria where after it acts by binding to the LPS-binding protein (LBP) (Wright et al, 1990). Both LBP and BPI (bactericidal/permeability increasing protein) play an important role in the host response to endotoxin. The LBP-LPS complex binds to the CD14 receptor on the cell surface leading to activation of the cell (Grandics, 2002).

The main reactive target cells are the circulating mononuclear cells, which produce pro-inflammatory cytokines, such as IL-1 and TNF-alpha. The pro-inflammatory cytokines are involved in acute and chronic inflammation (Hailman et al, 1996). By inducing a variety of immune reactions, including the induction of inflammatory enzymes such as the inducible nitric oxide (NO) synthase (NOS II) or the inducible cyclooxygenase (Raetz et al, 1991), LPS provokes an intestinal hyper-secretion leading to diarrhoea (Closs et al, 1998).

Cyanobacteria LPS (CLPS) has been reported to be involved in many cases of gastroenteritis after ingestion of contaminated water (Rapala et al, 2002). CLPS endotoxin may reinforce the adverse effects of cyanobacterial hepatotoxins, microcystins, by inhibiting the activity of glutathione S-transferase which are the key enzymes in the detoxification of microcystins (Pflumacher et al, 1998; Pflumacher et al, 2000; both cited in Rapala et al, 2002). The only available guideline is proposed by the New Zealand Ministry of Health (NZMH, 2000).

These recommend a maximum level of 3µg/ℓ of endotoxin in drinking water.

Although the WHO (1999) has little consideration for cyanobacteria LPS in drinking water, the context of this study is different. Done in the areas where water used for drinking does not receive any treatment and the water is stored in containers, bacteria producing LPS endotoxin are allowed to accumulate in container biofilm, increasing the risk of water contamination with endotoxin. Having the ability to constitute the first step in biofilm formation (De Philippis et al, 2005), cyanobacteria could favour the attachment of other bacteria including enterobacteriaceae such as *E. coli* and total coliforms, which have been reported to contribute to the occurrence of endotoxin in water (Rapala et al, 2002). It was then important in the context of this study to link endotoxin to both cyanobacteria as well as enterobacteriaceae.

1.5.4 Some outbreaks associated with cyanobacteria

1.5.4.1 Illness attributed to cyanotoxins in recreational water

1995- Australia

The study of adverse health effects after recreational water (contaminated with cyanobacteria blooms) contact involving 852 participants in Australia in 1995 showed an elevated incidence of diarrhoea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, eye or ear irritations within seven days following exposure (Pilotto et al, 1997).

1989- England

After swimming and canoe-training in water with a heavy bloom of *Microcystis spp.*, ten of twenty soldiers became ill, and two developed severe pneumonia attributed to the inhalation of a Microcystis toxins and needed hospitalisation and intensive care (Turner et al, 1990). The degree of illness appears to be related to the swimming skills and the volume of water ingested.

1959- Canada

While swimming in a lake excessively contaminated by cyanobacteria, thirteen people become ill (headache, nausea, muscular pains, and painful diarrhoea). *Microcystis spp.* and some trichomes of *Anabaena circinalis* were identified in the excreta of one patient, a medical doctor who had accidentally ingested water (Dillenberg and Dehnel, 1960).

1.5.4.2 Illness attributed to cyanotoxins in drinking water

1996- Brazil

Caruaru dialysis incident in Brazil: In 1996 an outbreak of severe hepatitis occurred at a

Brazilian haemodialysis centre in Caruaru, Brazil. One hundred patients developed acute liver failure, of whom 52 people died after receiving routine haemodialysis treatment. The clinical symptoms included visual disturbances, nausea, vomiting, muscle weakness and painful hepatomegaly. Microcystins were found in the source water, the water in the water delivery tanker, and in the dialysis unit's holding tank as well as in the iron and carbon filters from the dialysis centre's in-house treatment system. Microcystins were also detected in the blood sera and liver tissue of both live and deceased patients (Du Preez HH and van Baalen, 2006; Chorus and Bartram, 1999).

1994- Sweden

An accidental cross connection of the drinking-water supply of a sugar factory with a river water contaminated with blooms of *Planktothrix agardhii* producing microcystins lead to intoxication of 121 of 304 inhabitants of the village (as well as some dogs and cats). Symptoms range from vomiting, diarrhoea, muscular cramps to nausea (Annadotter et al, 2001).

1993- China

A study done by Yu (1995) showed that populations using cyanobacteria-infected surface waters were more affected by liver cancer than those drinking groundwater.

1988- Brazil

One of the most serious cases of outbreak attributed to cyanobacteria toxins in drinking water occurred in the Bahia State, when the newly flooded Itaparica Dam developed an immense cyanobacterial bloom. Approximately 2,000 gastroenteritis cases, 88 of which resulted in death were reported over a 42-day periods (Teixera et al, 1993).

1985- USA

After contact with mass developments of cyanobacteria in water, Carmichael (1994) compiled case studies on nausea, vomiting, diarrhoea, fever and eye, ear and throat infections among the population.

1981- China

After exposure to surface water polluted by *Microcystis spp.*; certain peoples of the city of Armidale (in Eastern China) were found to have elevated level of liver enzyme activities (a sign of exposure to toxic agents) in their blood (Falconer et al, 1981).

1979-Australia

The use of copper sulfate to eliminate a bloom of cylindrospermopsis raciborskii in a

drinking-water reservoir on Palm Island led to release of toxins from the cells into the water, 141 people using this water became seriously ill and hospitalized (Falconer, 1993, 1994).

1968- USA

Schwimmer and Schwimmer (1968) reported numerous cases of gastrointestinal illness after exposure to mass developments of cyanobacteria in their drinking water.

1931- USA

5,000-8,000 people became ill after they drank water from the Ohio and Potomac rivers contaminated with a massive Microcystis bloom. Intensive treatment of drinking-water by precipitation, filtration and chlorination was enough to remove all the toxins (Tisdale, 1931).

1.5.5 Monitoring of cyanobacteria in water

The occurrence of cyanobacteria in water always brings a concern about water quality. In fact the formation of bloom constitutes a serious hazard to the health of consumers and could also pose a problem of clogging in the treatment plant (Du Preez and Van Baalen, 2006). The best way to avoid these problems is to practice prevention, which will mean to reduce eutrophication by restricting the excessive enrichment of water resources, thereby controlling the occurrence of potentially toxic cyanobacteria (Du Preez and Van Baalen, 2006). To manage a cyanobacterial toxin risk in water, studies have been done in order to propose a maximum value of cyanobacterial cells in water. A maximum level of 20,000 cells/mℓ in recreational water was recommended by Falconer (1994), but that value was considered as too high regarding the works done by Pilotto et al (1997), who proposed a maximum value of 5,000 cells/mℓ -a value later used by Chorus and Bartram (1999) to derive a guideline for non-cumulative health effects.

Chorus and Bartram (1999) proposed several alert levels. Alert-level 1 (Exceeding 2,000 cells/mℓ can lead to offensive odour or taste), level 2 (potentially toxic cells 2,000-15,000 cells/mℓ for 2-3 consecutive samples or confirmed toxic bloom, persistent odour/taste, and obvious bloom) and level 3 (persistent high numbers widespread, toxic, cells>15,000 cells/mℓ for toxic species, persistent bloom, and only partial success of control measures).

Water authorities in South Africa also refer to standards from the South African Bureau of Standard (SABS, 2005) to produce water of acceptable quality. However the issue of cyanobacterial toxins risk is rarely considered in these standards and the dispositions in many water utilities are not appropriate for the control of cyanobacteria and associated toxins (Du Preez and Van Baalen, 2006). Some water utilities such as Rand Water have internal

operational specifications for cyanotoxins (Du Preez and Van Baalen, 2006). When cyanobacteria occur in water at a critical level, public education and awareness should be increased by the water authorities and treatment implemented (Du Preez and Van Baalen, 2006; Chorus and Bartram, 1999).

1.6 ENTEROBACTERIACEAE IN WATER USED FOR DRINKING

Assessment of enterobacteriaceae in water was not the main focus of this study but was nevertheless included to estimate their contribution to the occurrence of LPS endotoxin in water. All enterobacteriaceae produce endotoxin. For this study *E. coli* and total coliforms were considered because these are commonly used to indicate enteric bacteria in water (Jagals, 2000). Furthermore findings of the pilot investigation showed that they occurred the most among enterobacteriaceae found in containers.

Enterobacteriaceae is a large family of bacteria, including the more familiar bacterial pathogen species such as *Salmonella* and *Escherichia coli* (*E. coli*). Bacteria of this family are generally rod-shaped, Gram-negative and are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products (Holt et al, 2000; Clesceri et al, 1999).

1.6.1 *E. coli*

E. coli generally inhabits the intestines of warm-blooded animals and is regarded as the best indicator of faecal contamination of water (Griesel and Jagals, 2002; Grabow, 1996). Steyn et al (2004) reported that the presence of *E. coli* in water also represents useful indication of risk of infection to users. The pathogenicity of *E. coli* is not only related to their enterotoxins, but also to the endotoxins contain in their cell walls, which have been reported to cause diarrhoea (Closs et al, 1998).

1.6.2 Total coliforms

These are the primary indicators of the potability and suitability for consumption of drinking water. Belonging to the family of enterobacteriaceae, they also contain endotoxin in their cell walls (Holt et al, 2000). Their presence in water sampled from containers indicates improper handling during water collection and storage or seeding from the contaminated water sources (Jagals et al, 2003).

The South African Bureau of Standard (SABS, 2005) requires the non-detection (0 cfu / 100 ml) of *E. coli* and a maximum of 10 cfu/100 ml of total coliform bacteria in drinking water.

1.7 DETECTION TECHNIQUES

1.7.1 Detection of cyanobacteria

Cyanobacteria cells can be detected under a light microscope. This is one of the frequently used techniques to detect algae. This method, while labour intensive, provide an accurate measure of the number of bacteria present, as well as provide an indication of the species (DWAF, 1996). More popular methods are the membrane filter technique and the sedimentation chamber technique (Van Baalen, 2005). The majority of cyanobacteria have a distinctive colour, which is blue-green, different to the other algae which appear green. It is important for cyanobacteria identification to sample at adequate depth considering stratification of organisms and nutrients using depth-differentiating or depth-integrating sampling techniques (Chorus and Bartram, 1999). The method to use for the identification and enumeration of cells depend mainly of the type of microscope available and the purpose of the data. The membrane filter technique employs a compound light microscope, while the sedimentation chamber technique employs an inverted light microscope (Van Baalen, 2005). Cell counts are generally done with the help of a wipple grid (counting chamber), the results are always expressed as cell/mℓ.

The postulation of potential health risk related to cyanobacteria numbers in water will be based on the WHO (1999) Guideline. Du Preez and Van Baalen (2006) also use this guideline for monitoring water for cyanobacteria contamination.

1.7.2 Detection of *E. coli* and total coliforms

A selective medium (Selective *E. coli*/Coliform Chromogenic medium, Oxide SA) is used to identify *E coli* and total coliforms. This medium contains Salmon-GAL for the detection of total coliforms by the production of β-galactosidase and X-glucuronide for the detection of *E. coli* by the production of β-glucuronidase. Gram-positive organisms and some non-enteric bacteria are inhibited by Tergitol-7 (Finney et al, 2003).

The colour of bacteria in the media varies according to the chromogen that they cleave. The coliforms in general will cleave the pink Rose-Gal chromogen, producing pink colonies while the *E. coli* will cleave the X-Glu chromogen and then produce purple or blue colonies (Oxoid, 2006).

1.7.3 Detection of toxins

1.7.3.1 Microcystins

Several techniques are available for the detection of microcystins. For this study, the Enzyme-Linked Immuno-Sorbent Assay (ELISA) technique was used. This method was preferred because of its reported sensitivity, specificity and ease of operation (Chorus and Bartram, 1999). The principle of this assay is to raise polyclonal antisera in rabbits against bovine serum albumin conjugated to microcystin. These antisera showed good cross-reactivity with microcystins-LR, -RR, -YR and nodularin, but less with -LY and –LA (Chorus and Bartram, 1999). This technique has been shown to be suitable for normal water quality testing and was used to quantify cyanobacteria hepatotoxins in domestic water supplies and biomass extracts. Its detection limit is under the guideline concentration of 1 µg/ℓ microcystin in drinking water, as established by the World Health Organisation (Chu et al, 1990). A wide range of kits are commercially available for the quantitative detection of microcystins in water.

1.7.3.2 LPS endotoxin

Weise et al (1970) was the first to isolate LPS from the cyanobacterium *Anacystis nidulans*, since then LPS endotoxins have been found in many cyanobacteria species.

The LPS endotoxin concentration can be measured by using the Limulus Amoebocyte Lysate (LAL) assay, the lysate being prepared from amoebocytes of the horseshoe crab, *Limulus polyphemus*. The chromogenic LAL assay used in this study has been modified many times (Obayashi et al, 1985; Piotrowicz et al, 1985; Bussey and Tsuji, 1984; Tsuji et al, 1984; Urbaschek et al, 1984) since its first application in 1977 (Nakamura et al, 1977), and it is now an effective technique for the detection of LPS endotoxin (Anderson et al, 2002).

It is based on the principle that the enzymes contained by LAL are activated in a series of reactions in the presence of endotoxin. The last enzyme activated in the cascade splits the chromophore, paranitro aniline (pNA), from the chromogenic substrate, producing a yellow colour in the test tube (CAPE COD, 2005). The amount of pNA released and measured photometrically at 405 nm, is proportional to the amount of the endotoxin in the system (CAPE COD, 2005). This technique has been recently used by Rapala et al (2002) and Mwaura et al (2004), to measure the concentration of endotoxins associated with cyanobacteria in drinking and environmental water samples.

1.8 THE STUDY

1.8.1 Research Problem

The occurrence of cyanobacteria and their related toxins in water used for domestic purposes by the population of the lower Nwanedi area in the Vhembe district is unknown.

1.8.2 Hypotheses

By identifying and quantifying cyanobacteria in container waters as well as in the source where the container waters are collected, will allow determining whether the water is suitable for human consumption in terms of the World Health Organisation (1999) guidelines;

Determining the levels of microcystin and endotoxin in these waters, will allow estimation of the potential for intoxication in terms of the WHO (2004) and New Zealand Ministry of Health (2000) guidelines.

1.8.3 Aim

The aim of this study was to identify and quantify the numbers of pathogenic cyanobacteria as well as quantify their related toxins (microcystin and endotoxin) in untreated water from water-storage containers and link these to surface sources from which these waters are collected.

1.8.4 Scope of the study

The scope was to form an impression of whether the potential occurrence of cyanobacteria and their toxins in container water posed a risk to the health of the consumer. This study did not conduct a full quantitative risk assessment as described by Ashbolt et al (2006) and Steyn et al (2004). Instead it compared the levels of cyanobacteria found in the various waters to alert- and other limit levels described in WHO (1999; 2004), Department of Water Affairs Forestry (1996) and NZMH (2000) guidelines and from there postulated on the probable risk posed to human health.

A pilot study prior to this study was conducted in April and July, which ware essentially in the autumn and winter seasons. Water temperatures in the containers and environmental sources were shown to be slightly below the optimum temperature for cyanobacterial activity which is 25°C. Because of resource constraints, it was decided to conduct the main study during the summer months only to ensure that waters sampled, would be at least at the optimum temperature and slightly above. The mean ambient day temperature during the very

hot summer months in the area is around 32°C. It is therefore assumed, for the purposes of this study, that the activities for cyanobacteria in the container waters as well as the environmental waters would be optimum.

1.8.5 Objectives

- Assess the occurrence of cyanobacteria in water-storage containers of households that collect their water from surface water sources;
- Assess the numbers of cyanobacteria in these surface water sources;
- Determine factors that could sustain cyanobacteria in container waters as well as in source waters;
- Determine the concentration of cyanobacteria-related toxins (microcystin and endotoxin) in container as well as surface waters;
- Assess the potential contribution of enterobacteriaceae (*E. coli* and total coliforms) to possible endotoxin levels in container water;
- Assess the probable health risk associated with the numbers of cyanobacteria, their related toxins as well as the co-occurrence of enterobacteriaceae in the various waters according to the World Health Organisation (1999; 2004), Department of Water Affairs and Forestry (1996) and New Zealand Ministry of Health (2000) guidelines.

CHAPTER 2

METHODOLOGY

2.1 STUDY DESIGN

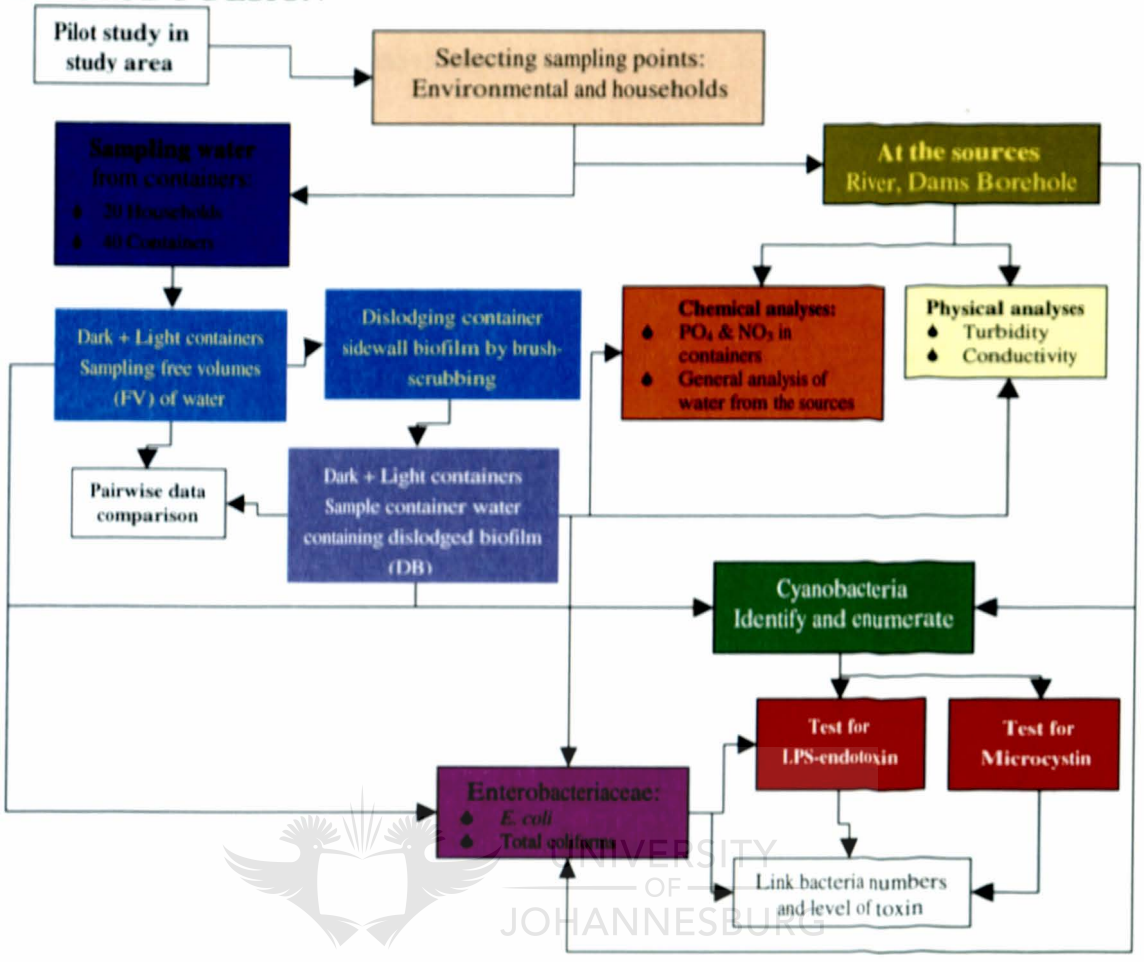


Figure 2.1 Schematic design of the project

2.2 STUDY AREA AND PILOT STUDY

This study was conducted in the lower Nwanedi area (Figure 2.2) in the Vhembe District Municipality, situated in the Limpopo Province, South Africa (Figure 1.1). The data collection took place from October 2005 until April 2006. This area is also the study site of a 5-year research programme on water and sanitation intervention studies conducted by the Water and Health Research Unit of the University of Johannesburg. The Unit samples the water of many households as well as the source waters in the area.

For the purposes of this study a short pilot study was undertaken (limited data collected April and July 2005) to assess the occurrence of cyanobacteria in water-storage containers and in water sources. This was to see whether cyanobacteria did occur in these sources.

Based on these findings, this study was focused on selected households and their water sources in six villages of the Lower Nwanedi namely Musunda, Tshitanzhe, Gumela, Tshikotoni, Tshitandahni and Folovhodwe (Figure 2.1).

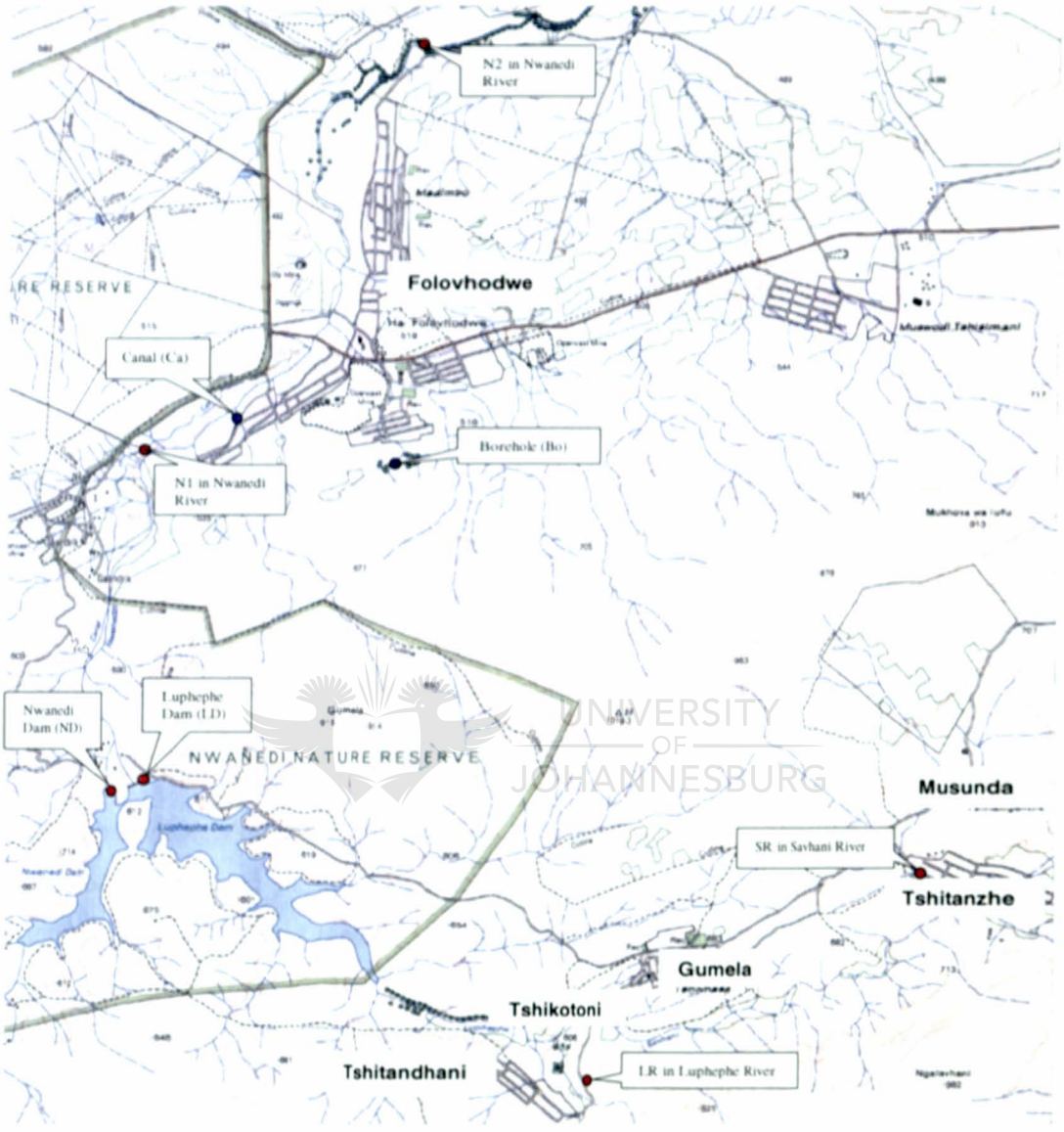


Figure 2.2 Study area with the target villages

The study communities collectively have approximately 7,000 inhabitants (Jagals, 2006 personal communication) and can be described as low-income rural communities. The villages (except for Folovhodwe) had no potable water supply and mostly used untreated waters from rivers or from hand pumps if these worked (which was not often) (Jagals, 2006; Moabi, 2006).

Households in Musunda, Tshitanzhe and Gumela sourced water from the Savhani River for their potable water needs. The Luphephe River was the only source of water for people in Tshikotoni and Tshitandahni. Folovhodwe had tap water from a distribution system supplied

by water from a groundwater source (a borehole Bo – Figure 2.2). However, the system at the time of study was prone to breakdowns during which times the people then resorted to the Nwanedi River as well as a nearby canal feeding from the Nwanedi River.

2.3 SAMPLING

Two categories of samples were taken to represent the quality of water potentially containing cyanobacteria, enteric bacteria, microcystin and endotoxin. These were ❶ from water-storage containers and ❷ at the sources where the container-waters were collected.

2.3.1 Sampling water from water-storage containers

2.3.1.1 Selection of households

During the pilot study, households were identified that had visually excessive biofilm formation in their containers. Selected households also had to be using both "light" (light penetrating) and "dark" (no light penetrating) containers. Twenty households were selected in this fashion from the villages Musunda (four households), Tshitanzhe (two households), Gumela (four households), Tshikotoni (one household), Tshitandahni (two households) and Folovhodwe (seven households); (The numbers according to the size of the village).

2.3.1.2 Selection of the type of containers

Initial observations during the pilot study revealed that biofilm (Section 1.4.2, Chapter1) in light containers were mostly green tainted (Section G2, Appendix G). Dark containers contained brown-coloured biofilm judging from particles in the relevant water samples (no photographic evidence of sidewall biofilm – these could not be photographed since we could not get a light into the dark container). Results (Section G3, Appendix G), from the preliminary samples taken during the pilot study and analysed by the laboratories of Rand Water, showed phytoplankton (including cyanobacteria), present in the green-tainted biofilm, but not in the brown biofilm.

This implied that the intensity of light reaching the insides of the two types of containers was an important factor in the occurrence of cyanobacteria in biofilms inside the containers. One light and one dark container were then selected from each of the study households for sampling the water during four research trips undertaken to the area. This was intended to establish whether cyanobacteria did occur in containers, and to what extent this occurrence differed from light to dark containers as well as whether biofilm could be associated with their occurrence if any.

2.3.1.3 Sampling biofilm-contaminated water from containers

To sample water from containers that may contain biofilm, samples were collected before and after dislodging biofilm from the inner sidewalls of containers according to the work of Jagals et al (2003):

- Water was first sampled from the container content before dislodging biofilm from the sidewalls of containers. This is referred to as the Free Volume (FV) of water;
- Biofilm was then dislodged from the same containers' inner sidewalls, using a sterile long-handled brush for each container to scrub the sidewalls and release biofilm into the container water. A second sample was then taken from the same containers directly after biofilm was dislodged. This sample was referred to as the dislodged biofilm (DB) sample.

2.3.2 Sampling at the environmental water sources

The source waters were sampled in those parts of the Savhani River, Luphephe River, and the Nwanedi River where the population wash dishes and clothes, bath, swim, fish and collect water in containers for domestic purposes including drinking. The following sampling points were selected (Figure 2.2):

- In the Savhani River at point SR; the Luphephe River (LR), Nwanedi River upstream from Folovhodwe (N1), Nwanedi River downstream from Folovhodwe (N2);
- At the canal water was collected at point Ca. Water in the canal comes from the Nwanedi River just upstream from Folovhodwe;
- At a borehole in Folovhodwe, water was collected at Point Bo directly from the pipe where the water flows from the groundwater.

Water was also sampled from dams in the Luphephe and Nwanedi Rivers just before the two rivers confluence and continues as the Nwanedi River. Water from these dams thus collectively which fed points N1, N2 and Ca downstream:

- Luphephe Dam (LD), Nwanedi Dam (ND).

2.3.3 Sampling techniques

Water was sampled 10 cm below the surface of the source waters (WHO, 1999). This was because some cyanobacteria species are more likely to occur near the surface of a water body than others depending on the intensity of light they require.

To ensure that later (in the laboratory) cyanobacteria would be identified, as they were in situ during sampling, water samples were preserved with 2% formaldehyde. For all other analyses

water samples were kept at 4-8°C during transportation to the laboratory. The physical water quality analyses were done in situ and as the enteric bacteria occurrence-analyses were done within 24 hours in a field laboratory set up close to the villages.

At each point, four types of water samples were collected:

- For cyanobacteria analyses (Section 2.4), in 100-mℓ sterile brown (to limit the penetration of light) plastic bottles containing 2 mℓ of formaldehyde (for preservation);
- For the microcystin analyses, in 100-mℓ sterile brown glass bottles. The brown glass bottles were used to limit the potential adherence of toxins to the bottle inner side as well as limit degradation by light (WHO, 1999);
- For the nitrate and phosphate assessment as well as endotoxin and enteric bacteria analyses, water was sampled in 1-ℓ sterile plastic bottles, and kept cold (4-8°C) (Appendix B);
- Samples for general chemical analyses were taken in two 1-ℓ sterile plastic bottles.

2.4 CYANOBACTERIA IDENTIFICATION AND ENUMERATION

The identification and quantification of cyanobacteria was done for three reasons; ❶ to determine their occurrence in containers – especially the container biofilm and in water sources; ❷ to determine their occurrence in the source waters and ❸ to assess their association with the levels of toxin in the water.

Cells in samples were stabilised (after sampling) with 2% formaldehyde before being transported to the laboratory of Rand Water Analytical Services. The sample was poured into a steel container, covered with a plastic lid which was then hammered with a mass (mechanically handled) several times to burst the gas vacuole inside the cyanobacterium cell as well as to homogenize the cell-distribution throughout the sample. Volumes of 3-mℓ were pipetted into sedimentation chambers and centrifuged at 3,500 rpm for 10 minutes. The centrifuged solution in each sedimentation chamber was then examined under an inverted-light microscope, with a camera linked to a computer (Du Preez and Van Baalen, 2006). Cyanobacteria were identified as blue-green colonies or filaments (Section G3, Appendix G).

2.5 CYANOTOXIN ANALYSES

2.5.1 Microcystin detection by ELISA

In this test, microcystin toxin in the sample competes with an enzyme-labelled (horseradish peroxidase) microcystin for a limited number of antibody-binding sites (Section 1.7.3.1,

Chapter 1) on the inside surfaces of test wells on 96-well micro-titre plates. Reactions were characterised by colour development. The lighter the colour, the higher the concentration of microcystin and vice versa.

To lyophilise (break the cells) algal cells (to release microcystins) in a water sample, and aliquot of sample was transferred from a glass bottle into a 50-mℓ polypropylene tubes (Merck, SA) and frozen in liquid nitrogen before thawing in a water bath (Section F1, Appendix F). Microcystin was purified by filtration (0.45 µm syringe filter) and all the reagents pipetted into the microtiter plate (Quantyplate Microcystin Kit, Envirologix Inc®).

The sample, the negative control and the calibrator were added to microcystin assay diluent in their respective well in duplicate. Prior to start the reaction, microcystin enzyme conjugate and the substrate were added to each well. Adding a stop solution to each well then stopped the reaction. The optical density (OD) was read using a microtiter plate reader (Envirologix Inc) set at 450-nm. The readings were converted from the OD to µg/ℓ using a standard curve equation (y=ax+b) (Du Preez and Van Baalen, 2006). This assay does not distinguish between microcystin variants, but detects total microcystin. For this test the minimum limit of microcystin detection was 0.18 µg/ℓ.

2.5.2 Endotoxin detection by LAL chromogenic assay

LAL contains enzymes that are activated in a series of reactions in the presence of endotoxin. The activated enzymes split the chromophore, para-nitro aniline (pNA), from the chromogenic substrate, producing a yellow colour. The concentration of endotoxin increases proportional to the intensity of the colour.

An aliquot (1 mℓ) from the water sampled in a 500-ml plastic bottle, was transferred to an Eppendorf tube to lyophilise (break the cells) by freeze-thawing technique and release endotoxin from the cell walls of all gram-negative bacteria and cyanobacteria (Section 1.5.3.5, Chapter 1) in the aliquot (Section F2, Appendix F).

The endotoxin level was then measured using Lymulus Amoebocyte Lysate chromogenic assay (LAL chromogenic QCL 1000 120T SL, from Adcock Ingram, SA). The LAL reagent, containing a chromogenic substrate, was processed in a pyrogen-free glass tube kept at 37°C in a water bath. The reaction was stopped after 16 minutes with 25% acetic acid. Each sample and standard was done in duplicate and the absorbance of each reaction tube read at 405 nm using the microplate spectro-photometer (Benchmark Plus from Bio Rad). The optical densities of the four serial dilutions of standard were used to draw the standard curve

and the endotoxin concentration was determined from the equation (y=ax+b) of the standard curve and represented by the value x [x=(y-b)/a] (Rapala et al, 2002).

2.6 ASSESSMENT OF NUTRIENT LEVELS IN WATER

Nitrates and phosphates were measured (Appendix B) using a Dr Lange Xion 500 Spectrophotometer and test tube kits for nitrate (LCK 339, CA Milsch SA) and for phosphate (LCK 349, CA Milsch SA). The general chemical analyses of samples from the water sources were performed at the Analytical Services of Rand Water.

2.7 PHYSICAL ANALYSES OF WATER

2.7.1 General analyses

The pH, conductivity and the temperature were determined in situ at each sampling point. The temperature was measured in degrees Celcius, using a digital thermometer made by Greisinger Electronic (GTH 175/pt, Germany). The pH and conductivity (in mS/m) were measured using a portable Hanna pH/EC/TDS (HI 991301) instrument made in Romania. The Dr Bruno Lange turbidity meter, Naphla (GmbH-Berlin, Germany) was used to measure the turbidity levels and values were recorded as Nephelometric Turbidity Units (NTUs).

2.7.1 Measuring biofilms in containers

These measurements were a function of turbidity in the container water. The idea of measuring this value was first to determine how "dirty" the inner sidewalls of the containers were, but more importantly to establish the density of biofilm particles release into the FV of water (Section 2.3.1.3, Chapter 2). The turbidity was then measured before and after dislodging contaminant build-up from the container's sidewalls (Ntsherwa, 2004). An increase of turbidity was then assumed an indication of the increase in biofilm particles in the water.

2.8 ENTERIC BACTERIA IDENTIFICATION AND ENUMERATION

Escherichia coli and total coliforms were tested for in the same test using the membrane filtration technique (Appendix A) with four dilutions (10^{-2}, 10^{-1}, 1 and 10 ml) and sterile 0.45 µm filters (Millipore, SA) plated on Selective *E. coli*/Coliform Chromogenic medium (Oxide, SA) and incubated at 37°C for twenty-four hours prior to counting. Blue or purple colonies were identified to be *E. coli* and pink colonies to be total coliforms. Colonies were counted per filter, the average estimated per plate and the total count expressed as colony forming unit

(cfu) per 100-mℓ of sample [(mean cell count×100/sample dilute)/applied volume] (Oxoid, 2006-Appendix A).

2.9 PROPOSED GUIDELINES TO ASSESS THE OCCURRENCE OF CYANOBACTERIA AND RELATED CONTAMINANTS

This section summarises alert levels, guidelines and standards (World Health Organisation, 1999, 2004; New Zealand Ministry of Health, 2000; South African Bureau of Standard, 2005) for the level of occurrence in drinking water of the parameters considered in this study (Sections 2.5-2.9 later on). These will be used in Chapter 3 (Results and Discussion) to postulate on the potential health risk related to the consumption of the water.

Table 2.1 Guidelines proposed for use in this study

Parameters	WHO		NZMH		SABS	
	Unit	RV	Unit	RV	Unit	RV
Cyanobacteria and cyanotoxins						
Cyanobacteria	Cell/mℓ	2,000 (WHO, 1999)				
Microcystin LR	µg/ℓ	1 (WHO, 2004)				
Endotoxin			µg/ℓ	3 (NZMH, 2000)		
Enteric bacteria						
E. coli					cfu/100 mℓ	Should not be detected i.e. <1 (SABS, 2005)
Coliforms					cfu/100 mℓ	10 (SABS, 2005)
Physico-Chemicals						
Cd					µg/ℓ	5-10 (SABS, 2005)
Cr					µg/ℓ	100-500
Al					µg/ℓ	300-500
Pb					µg/ℓ	20-50
Co					µg/ℓ	500-1,000
Ni					µg/ℓ	150-350
V					µg/ℓ	200-500
Cu					µg/ℓ	1,000-2,000
IC_F					mg/ℓ	200-600
Zn					mg/ℓ	5-10
NO3	µg/ℓ	100				
PO4	µg/ℓ	10				
pH					pH units	4-10
Conductivity					mS/cm	150-370
Turbidity					NTU	1-5

RV: Recommended Value

(WHO, 2004; 1999; NZMH, 2000; SABS, 2005)

2.10 STATISTICAL HYPOTHESES

Statistical hypotheses were formulated for each of the sections that will follow in Chapter 3: (Results and Discussion). Data were then statistically analysed to address a particular

hypothesis within sub-sections of Chapter 3, based on whether the hypotheses were accepted or rejected. The approaches that follow are summarised in tables for each sub-section.

The rationale for each specific statistical test is discussed in Appendix D.

2.10.1 Statistical comparison of parameters in drinking water containers

2.10.1.1 Bacteria and toxins concentration – light and dark containers free volume (before scrubbing) versus dislodged biofilm (after scrubbing) (Tables 2.2 - 2.5)

These analyses would reflect the occurrence of cyanobacteria, enterobacteriaceae, endotoxin and microcystins in association with container biofilm. The following approaches were followed:

- Light-permissible (light) containers before dislodging biofilm by scrubbing (minimum numbers expected) and after scrubbing (higher numbers expected);
- No light-permissible (dark) containers before scrubbing (minimum numbers expected) and after scrubbing (higher numbers expected).

The following hypotheses were developed:

2.10.1.1.1 Paired container data – all the free volume (FV) versus dislodged biofilm (DB) data sets

The Wilcoxon Signed Rank test was used because the data sets were paired.

Null hypothesis (H_0)

There would be no significant difference in the number of bacteria (cyanobacteria, and enterobacteriaceae), the concentration of toxins (endotoxin and microcystins) before and after dislodging biofilm in neither the water of the "light" containers nor that of the "Dark" containers.

Interpretation of results

- It was postulated that an increase in the levels of cyanobacteria and enterobacteriaceae in the container water (after scrubbing) would indicate that they are aggregating in the biofilm, therefore a significant increase was expected in "DB" samples.
- Cyanobacteria and enterobacteriaceae produce endotoxin, therefore a significant increase of endotoxin level was expected in "DB" samples.
- A significant increase of the concentration of microcystins was expected in dislodged biofilm samples as they are potentially produced by cyanobacteria which are supposed to accumulate in the biofilm.

Rejection of the H_0 would imply that there was a significant increase in the bacteria numbers and, the microcystins and endotoxin concentrations after scrubbing. This would indicate that the brushing activity and subsequent biofilm release brought about the significant changes. This effect was then discussed in the relevant section in Chapter 3.

2.10.1.1.2 Unpaired container data – all the light versus dark data sets

The data were not paired in the sets. The Mann Whitney Rank Sum Test was therefore used.

Null hypothesis (H_0)

There would be no significant difference in the cyanobacteria numbers in water from the light containers and water from the dark containers for both treatments (scrubbing).

Interpretation of results

- A significant increase of the numbers of cyanobacteria was expected in the dislodged biofilm samples from the light containers because of the light limiting factor assumed for the dark containers.
- The possible interaction between enterobacteriaceae and cyanobacteria in biofilm, brought us to expect a significant increase of enterobacteriaceae in dislodged biofilm samples.
- A significant increase of toxins (endotoxin and microcystins) was expected in dislodged biofilm samples as bacteria accumulate in biofilm.

Acceptance of the H_0 would simply imply no significant difference between light and dark containers. However, rejection of the H_0 would imply that there has been a significant change in the numbers of bacteria and toxins. This would indicate that light affects the subsistence of cyanobacteria in biofilm. This effect was then discussed in the relevant section in Chapter 3.

Table 2.2 ANOVA for cyanobacteria numbers of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Containers	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in cyanobacteria numbers expected-therefore reject H_0 if $LFV<LDB$
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in cyanobacteria numbers expected-therefore reject H_0 if $DFV<DDB$
Mann-Whitney Rank Sum Test	No increase of cyanobacteria numbers expected Accept H_0: $LFV=DFV$	Significant increase of cyanobacteria numbers expected Reject H_0: $LDB>DDB$	BUT expect increase $LFV<LDB \geq DFV<DDB$
	Explain differences		

Table 2.3 ANOVA for enterobacteriaceae numbers of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Containers	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in enterobacteriaceae numbers expected-therefore reject H_0 if $LFV<LDB$
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in enterobacteriaceae numbers expected-therefore reject H_0 if $DFV<DDB$
Mann-Whitney Rank Sum Test	No increase of enterobacteriaceae numbers expected Accept H_0: $LFV=DDB$	Significant increase of enterobacteriaceae numbers expected Reject H_0: $LDB>DDB$	**BUT expect** increase $LFV<LDB \geq DFV<DDB$
	Explain differences		

Table 2.4 ANOVA for endotoxin concentrations of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Containers	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in endotoxin concentrations expected-therefore reject H_0 if $LFV<LDB$
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in endotoxin concentrations expected-therefore reject H_0 if $DFV<DDB$
Mann-Whitney Rank Sum Test	No increase of endotoxin concentrations expected Accept H_0: $LFV=DFV$	Significant increase of endotoxin concentrations expected Reject H_0: $LDB>DDB$	**BUT expect** Increase $LFV<LDB \geq DFV<DDB$
	Explain differences		

Table 2.5 ANOVA for microcystins concentrations of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Containers	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in microcystins concentrations expected-therefore reject H_0 if $LFV<LDB$
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in microcystins concentrations expected-therefore reject H_0 if $DFV<DDB$
Mann-Whitney Rank Sum Test	No increase of microcystins concentrations expected Accept H_0: $LFV=DFV$	Significant increase of microcystins concentrations expected Reject H_0: $LDB>DDB$	**BUT expect** Increase $LFV<LDB \geq DFV<DDB$
	Explain differences		

2.10.1.2 Turbidity: Light and dark containers free volume versus dislodged biofilm (Table 2.2)

This section will discuss the contribution of particles release at the container inner sidewall on the increase of turbidity. The presence of biofilm was determined by analyses of water samples from:

- Light-permissible (Light) containers before scrubbing (high turbidity) and after scrubbing (higher turbidity) the container inner surface;
- No-light permissible (Dark) containers before scrubbing (high turbidity) and after scrubbing (higher turbidity) the container inner surface;

The following hypothesis was developed:

2.10.1.2.1 Paired container data – the free volume data versus dislodged biofilm data for each set of light and each set of dark containers

The Wilcoxon Signed Rank test was used because each of the respective data sets were paired (the method is defined in Appendix D).

Null hypothesis (H_0)

There would be no significant difference in the turbidity of water sampled from containers before and after scrubbing.

Expected outcomes

The H_0 would be rejected because of a significant increase in turbidity after brushing the inner sidewalls of the containers, because biofilm particles would be released into the containers' water content.

Interpreting the findings

Rejection of the H_0 would imply that there was significant increase in turbidity of the container water from the before to the after results. This indicated that the brushing activity and subsequent release of biofilm brought about the significant changes. This effect was then discussed in the relevant section in chapter 3.

2.10.1.2.2 Unpaired container data – all the light versus dark container data

The data were not paired in the sets. The Mann Whitney Rank Sum Test was therefore used (the method is defined in Appendix D).

Null hypothesis (H_0)

There would be no significant difference between the turbidity in water from the Light containers and water from the Dark containers for both scrubbing treatments.

Expected outcomes

A significant increase was expected in the dislodged biofilm results from the Light containers because more light was assumed causing higher activity in biofilm formation on the sidewall of these containers.

Interpreting the findings

Acceptance of the H_0 would imply no significant difference between the turbidity in light and dark containers. However, rejection of the H_0 would imply that there has been a significant change in the turbidity. This would indicate increased biofilms activity on the containers' inner surfaces. This effect was then discussed in the relevant section in Chapter 3.

Table 2.6 ANOVA for turbidity of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Container type	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in turbidity expected-therefore reject H_0 if LFV<LDB
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in turbidity expected-therefore reject H_0 if DFV<DDB
Mann-Whitney Rank Sum Test	No increase of turbidity expected Accept H_0: LFV=DFV	Significant increase of turbidity expected Reject H_0: LDB>DDB	**BUT expect** increase LFV<LDB ≥ DFV<DDB
	Explain differences		

2.10.2 Statistical comparison of the parameters in the water sources

2.10.2.1 Cyanobacteria numbers - River water versus impoundment (dam) water (Table 2.7)

This section will reflect the effect of water stability (calm) on the proliferation of cyanobacteria.

The following approaches were followed:

- River waters have a fast flow then not stable;
- Impoundment's waters are stable;

The following hypothesis was developed:

Null hypothesis (H_0)

There would be no significant difference between the turbidity in water from the Light containers and water from the Dark containers for both scrubbing treatments.

Expected outcomes

A significant increase was expected in the dislodged biofilm results from the Light containers because more light was assumed causing higher activity in biofilm formation on the sidewall of these containers.

Interpreting the findings

Acceptance of the H_0 would imply no significant difference between the turbidity in light and dark containers. However, rejection of the H_0 would imply that there has been a significant change in the turbidity. This would indicate increased biofilms activity on the containers' inner surfaces. This effect was then discussed in the relevant section in Chapter 3.

Table 2.6 ANOVA for turbidity of water sampled from light and dark container types before and after dislodging biofilm particles by scrubbing

Container type	Free volume (FV) of water	Dislodged biofilm (DB) into water	Paired data Wilcoxon Signed Rank Test
Light	$H_0 \rightarrow LFV=LDB$	$H_0 \rightarrow LFV=LDB$	Significant increase in turbidity expected-therefore reject H_0 if $LFV<LDB$
Dark	$H_0 \rightarrow DFV=DDB$	$H_0 \rightarrow DFV=DDB$	Significant increase in turbidity expected-therefore reject H_0 if $DFV<DDB$
Mann-Whitney Rank Sum Test	No increase of turbidity expected Accept H_0: $LFV=DFV$	Significant increase of turbidity expected Reject H_0: $LDB>DDB$	**BUT expect** increase $LFV<LDB \geq DFV<DDB$
	Explain differences		

2.10.2 Statistical comparison of the parameters in the water sources

2.10.2.1 Cyanobacteria numbers - River water versus impoundment (dam) water (Table 2.7)

This section will reflect the effect of water stability (calm) on the proliferation of cyanobacteria.

The following approaches were followed:

- River waters have a fast flow then not stable;
- Impoundment's waters are stable;

The following hypothesis was developed:

Null hypothesis (H_0)

There would be no significant difference between the numbers of cyanobacteria in water from the rivers and the impoundments.

Interpretation of results

A significant increase was expected in the water samples from the impoundment because previous studies referenced in section one showed that stagnant waters are suitable for the proliferation of cyanobacteria.

Acceptance of the H_0 would imply no significant difference between the river waters and impoundment waters. However, rejection of the H_0 would imply that there has been a significant change in the numbers of cyanobacteria. This would indicate the importance of "water stability" in the growth of cyanobacteria. This effect was then discussed in the relevant section in Chapter 3.

Table 2.7 ANOVA for cyanobacteria number for water sampled from surface waters and impoundments (Dams)

Water sources	Approaches	Null hypothesis	Unpaired data Mann-Whitney Rank Sum Test
River waters (RW)	Unstable water	$H_0 \rightarrow RW=IW$	Significant increase in cyanobacteria numbers expected therefore reject H_0 if RW<IW
Impoundments waters (IW)	Stable water		
	Explain differences		

2.10.2.2 Cyanobacteria numbers – Upstream versus downstream water sources (Table 2.8)

This section will determine if the impoundment contributes to a significant increase of cyanobacteria number in downstream water sources.

The following approaches were followed:

- The Luphephe River is located upstream, flows into the impoundment;
- The Nwanedi 1 downstream water source is fed by the impoundments;

The following hypothesis was developed:

Null hypothesis (H_0)

There would be no significant difference between the numbers of cyanobacteria in water from the Nwanedi 1 and the Luphephe River

Interpretation of results

A significant increase was expected in the water samples from the Nwanedi 1, because the impoundment is a suitable environment for the development of cyanobacteria blooms (WHO, 1999), which are expected to flow into the Nwanedi 1 downstream.

Acceptance of the H_0 would imply no significant difference between the Nwanedi 1 and the Luphephe River. However, rejection of the H_0 would imply that there has been a significant change in the numbers of cyanobacteria. This would indicate that untreated Nwanedi 1 located downstream is fed by cyanobacteria from impoundments. This effect was then discussed in the relevant section in Chapter 3.

Table 2.8 ANOVA for cyanobacteria number for water sampled from upstream (LR) and downstream (N1) water sources

Water sources	Approaches	Null hypothesis	Unpaired data ANOVA on ranks (Kruskal-wallis)
Nwanedi 1 (N1)	Downstream impoundments	$H_0 \rightarrow Imp=N1=LR$	Significant increase in cyanobacteria numbers expected therefore reject H_0 if LR<N1<Imp
Luphephe River(LR)	Upstream impoundments		
Impoundments (Imp)	In between (cyanobacteria blooms)		
	Explain differences		

CHAPTER 3

RESULTS AND DISCUSSION

The presentation of the results in this chapter is based on the Objectives (Chapter 1 Section 1.8.5), as well as the statistical parameters proposed in Section 2.10 of Chapter 2: Methodology. The potential health risk associated with the occurrence of cyanobacteria and their toxins in the sampled waters (water from storage containers as well as water from sources) is finally described by comparing the results to the guidelines proposed in Table 2.1 (Section 2.9 of Chapter 2).

This chapter starts with an overview of the occurrence of total cyanobacteria in containers as well as the sources from which the container waters were collected.

The health-related quality of water at the point of use (container water) was the main concern of the study. The results of this part of the study are therefore presented directly after the overview. For logical reporting and discussion of results, the container waters were divided into two categories of samples i.e. the free volume (FV) of water in the containers and waters from the same containers containing dislodged biofilm (DB) (Section 2.3.1.3, Chapter 2). These categories are subdivided as: FV and DB in "light" containers as well FV and DB in "dark" containers (Section 2.3.1.2, Chapter 2).

The results of the analyses of water from the sources follow after the container water results.

3.1 TOTAL CYANOBACTERIA OCCURRENCE

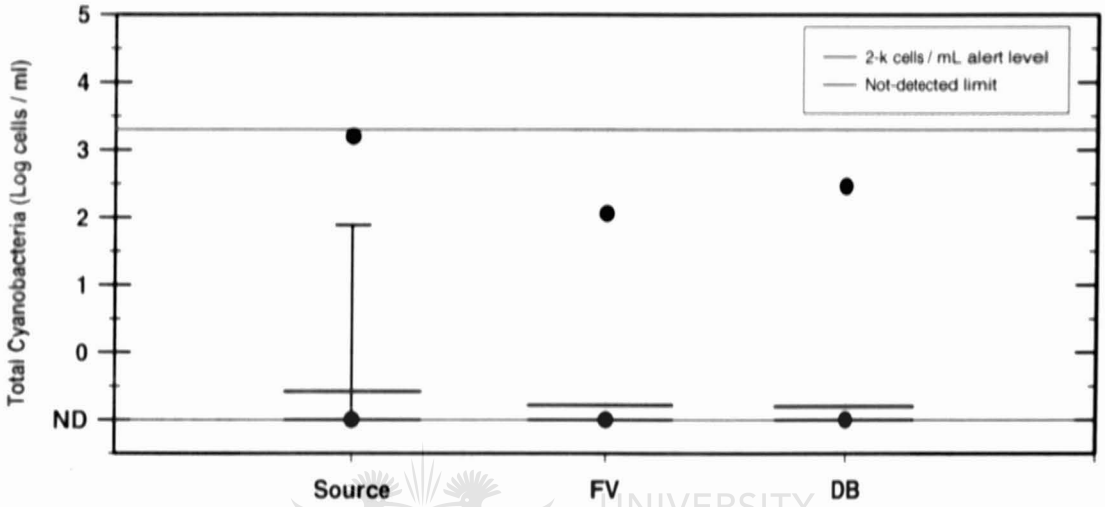
This study identified and enumerated seven cyanobacteria genera, described as potentially toxic by Jayatissa et al (2006) as well as Chorus and Bartram (1999), in the source and container waters. Microscope investigation revealed the occurrence of ❶ *Microcystis spp*, ❷ *Oscillatoria spp*, ❸ *Anabaena spp*, ❹ *Pseudanabaena spp*, ❺ *Aphanocapsa spp*, ❻ *Radiocystis spp* and ❼ *Spirulina spp* (Tables C1a and C2a-d; Appendix C). These are collectively referred to as total cyanobacteria during the reporting in this chapter.

Other non-toxic phytoplankton genera identified in the same waters were from the classes Bacillariophyceae, Chlorophyceae, Dinophyceae and Euglenophyceae.

Figure 3.1 shows an overview of the total cyanobacteria occurrence in three water categories i.e. source, free volume (FV) and dislodged biofilm (DB). For the purposes of this overview, the data from the light and dark containers were grouped for the FV and DB samples respectively. The source waters include part (Savhani River, Luphephe River, Nwanedi 1 and Canal) of the environmental water where the population (depending of the area) wash dishes and clothes, bathe, swim and collect water in containers for domestic purposes including drinking.

The water from the sources as well as storage containers (the free volume "FV" and dislodged

biofilm "DB") complied, at the 95th percentile (the upper black dot of each box in the plot - Figure D; Appendix D), with the proposed World Health Organisation (1999) alert level of ≤2,000 organisms per 1 mℓ (the red horizontal line in Figure 3.1) of water intended for consumption (Table 2.1: Section 2.9: Chapter 2). In other words, in general, the total cyanobacteria in the water sampled from all three categories did not appear to constitute a potential health problem for consumers.



2,000 cells / mℓ alert level (WHO, 1999); ND: Not-detected limit
FV: Free volume of water in container; DB: Dislodged biofilm into container's water

Figure 3.1 The occurrence of total cyanobacteria in the source and water-storage containers

Data were converted to log values to remove excessive variance. There is however, no log of results showing zero organisms. Considering that zero organisms are potentially organisms NOT DETECTED by the particular test, results showing "no organisms" are interpreted as "less than one organism" as was discussed by Jagals (2000). To show the not-detected (ND) level (blue line) on the Y-axis of the graph, 0.1 was selected as the next decimal value lower than Log-0 (which is one). The black line inside the box shows the *median* (explained in Appendix D). Coloured horizontal lines inside or approximate to the boxes (in the case of Figure 1.3 this is shown in green) show the *mean*.

Cyanobacteria numbers in the three categories of samples (Source, FV and DB) did not differ significantly (Table 3.1), which suggested that cyanobacteria occurred in containers at the same levels as in the source water (Discussed in Section 3.5.1).

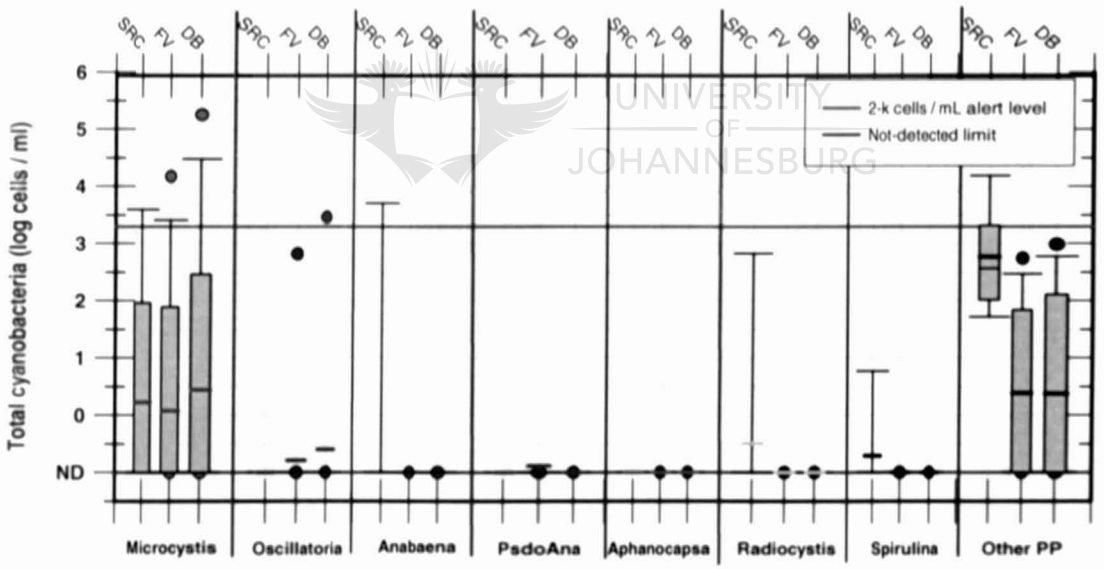
The summaries of Figure 3.1 and Table 3.1 are of <u>all</u> the occurrences. Judged by the median level of the analyses that yielded data showing organisms not detected (ND), it appears as if some or all of the genera that comprise the <u>total</u> cyanobacteria did not occur in many of the samples. This is shown in Figure 3.2 (the data can be found in Tables C1a and C2a-d; Appendix C). *Microcystis spp* occurred in substantial numbers in all sample categories but their occurrence were not statistically different (P=0.696).

Table 3.1 Comparing total cyanobacteria (log) numbers in the respective sample categories

Parameters	Source	FV	DB	ANOVA on Ranks (Kruskal-Wallis)
N	105	532	532	
Mean of the logs	-0.58	-0.78	-0.72	
Median	0	0	0	No significant difference
95th Percentile	2.83	1.75	2.35	P=0.150
Min	0	0	0	H_0 not rejected
Max	4.24	4.84	5.84	
Standard Deviation	1.25	0.93	1.14	

FV: Free volume of water in container, DB: Dislodged biofilm into container's water

The other genera of cyanobacteria were mostly absent from waters of the three categories. Of the other classes of phytoplankton, Bacillariophyceae occurred the most with the diatoms dominating, and Euglenophyceae occurring the least (Tables C2a - d; Appendix C). These organisms occurred significantly more (P=0.003) in source waters than in the containers, indicating that they can not adapt in water-storage containers.



2,000 cells / ml alert level (WHO, 1999); ND: Not-detected limit

SRC: Sourced river-water in container; FV: Free volume of water in container; DB: Dislodged biofilm into container's water

Figure 3.2 Total cyanobacteria and other phytoplankton per genus in sample categories

Figure 3.1 showed that the numbers of cyanobacteria in all sample types comply with the WHO (1999) guideline, but this was based on all occurrences. Figure 3.2 suggests that the numbers of *Microcystis spp* in source water as well as in containers alone might constitute a probability of a health risk because of their ability to produce cyanotoxins, whereas *Oscillatoria spp* and *Anabaena spp* would constitute a much lower probability of risk.

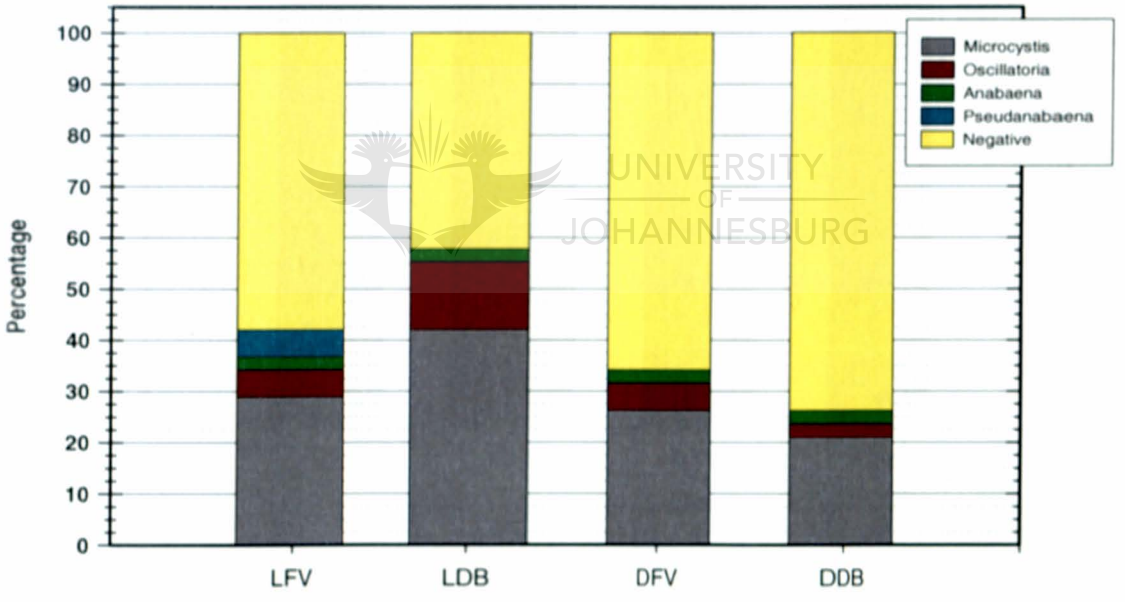
The other cyanobacteria genera posed a negligible risk in terms of cyanotoxins produced, as they occurred in low numbers. In this context the other phytoplankton were very evident but because they are not considered pathogenic, are not discussed any further.

3.2 CYANOBACTERIA IN STORAGE CONTAINERS

3.2.1 Occurrence per genus

Not all of the cyanobacteria genera identified in the waters tested during this study, occurred in the waters sampled from the storage containers (Figure 3.2). Furthermore, not all water samples collected from the containers contained cyanobacteria.

Figure 3.3 shows that cyanobacteria were not always detected in waters sampled from containers, with or without dislodged biofilm. However, four cyanobacterial genera were identified in the container samples.



LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

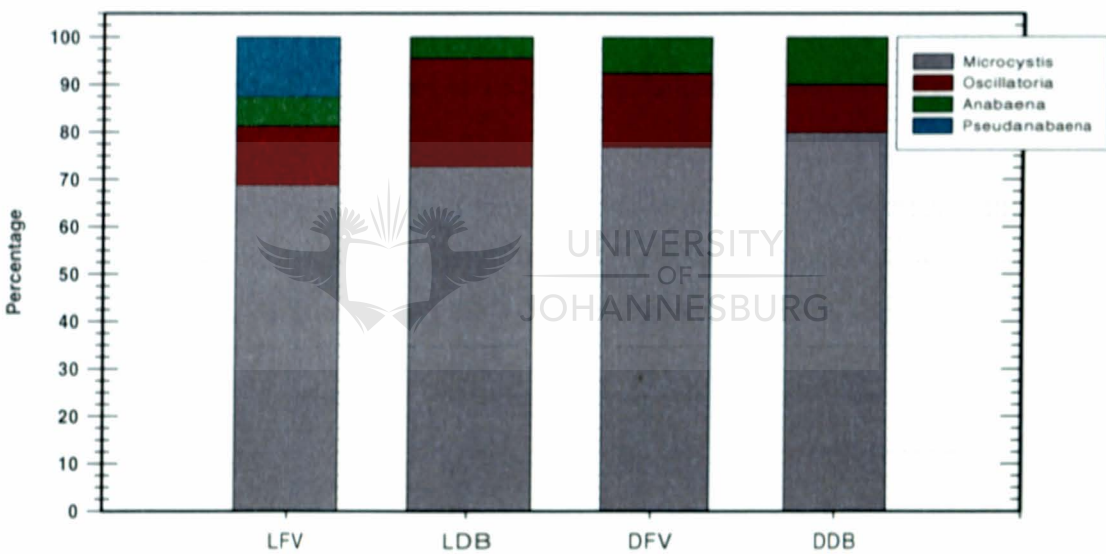
Figure 3.3 Percentage occurrences of cyanobacteria genera in all water samples from storage containers

These were *Microcystis spp*, *Oscillatoria spp*, *Anabaena spp* and *Pseudanabaena spp*. *Microcystis spp* were the most abundant in numbers and also occurred most frequently in samples, especially in the water samples from light containers containing dislodged biofilm (Table C1b, Appendix C).

Figure 3.2 also shows that of all the cyanobacteria genera detected in all three water categories, it was *Microcystis spp* occurring in numbers that exceeded the alert level of the WHO (1999)

Guideline of 2,000 cells per 1mℓ. Figure 3.3 however, shows that while cyanobacteria were not detected in the all container water samples every time (yellow parts of the box), the chances of households having containers contaminated with cyanobacteria where generally still less than fifty percent, except in water from light containers into which side-wall biofilm had been dislodged (LDB).

To postulate on the probable health risk, the percentages of the occurring cyanobacteria genera were calculated from samples where at least one of the genera occurred (a "positive" sample – i.e. this data set is presented without the "negative" or "not detected" data). Figure 3.4 compares the light FV and DB samples with each other and then the dark FV and DB samples with each other. Figure 3.4 does not reflect a comparison between the light containers to the dark containers – for this, Figure 3.3 should be consulted.



LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Figure 3.4 Percentage of cyanobacteria genera in positive samples of water from storage containers

Of the four genera of cyanobacteria detected in container water, *Microcystis spp,* in light containers, occurred in 69% of the samples in the free volume of water (FV) and in 73% in the same water samples once the biofilm was dislodged (DB). In dark containers, *Microcystis spp* occurred in 77% of FV samples and in 80% (DB) respectively. *Pseudanabaena spp* occurred in the FV of water in light containers but was not detected in the DB of light container and in the dark container (FV and DB) samples.

Since the four cyanobacteria genera identified in water-storage containers all have the potential to produce cyanotoxins, discussion from here on will refer to these as total cyanobacteria from

which probable health risk was discussed.

3.2.2 Container types and cyanobacteria occurrence

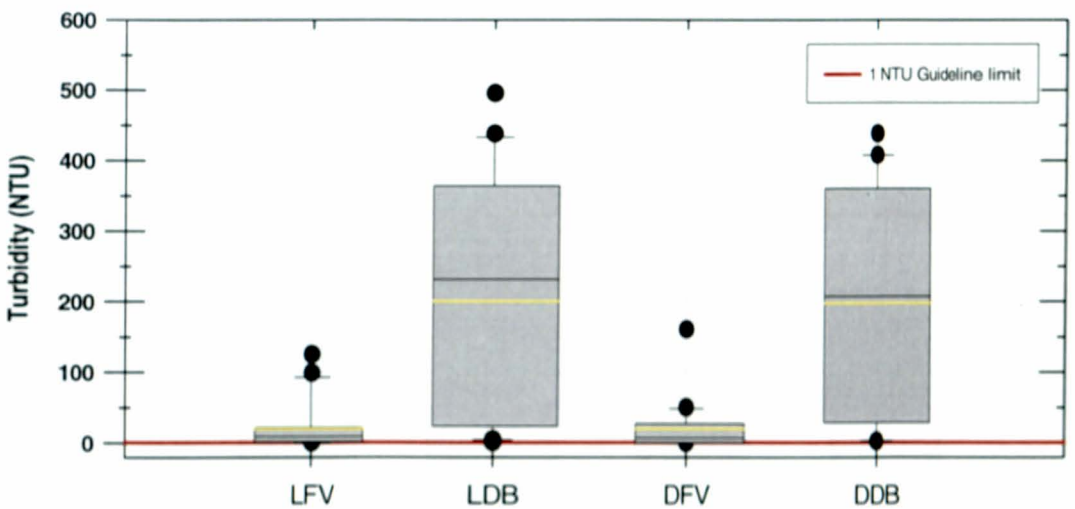
The effect of container type on the occurrence of cyanobacteria was assessed by comparing their number in the free volume of water to their numbers in the same water samples after the side-wall biofilms were dislodged and suspended into the free volume of water for each sample container (Section 2.10, Chapter 2: Methodology).

As reviewed in Section 1.4 (Chapter 1: Introduction and Literature Review) and hypothesized in Section 2.10 (Chapter 2: Methodology), changes brought about by the dislodging activity are characterised by increases of turbidity in samples after the dislodging process.

3.2.2.1 Turbidity as indicator of container-biofilm

Turbidity is the measurement of the total particles in water (SABS, 2005). For this study, this parameter also reflected biofilm particles dislodged from the inner sidewalls of the containers (Chapter 2; Section 2.8).

Figure 3.5 shows that the turbidity of water sampled from containers increased significantly ($P \leq 0.001$) after dislodging of biofilm particles from the inner side-walls for light and dark containers respectively, totally exceeding the 1-NTU standard of the SABS (2005).



1-NTU maximum limit (SABS, 2005)

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Figure 3.5 Turbidity of container water expressed in Nephelometric Turbidity Units (NTU)

Table 3.2 summarises the data as well as the results of the turbidity ANOVA between the treatments (dislodged biofilm in container water) for each container group (FV and DB).

Table 3.2 Comparing turbidity in FV and DB samples from light and dark containers

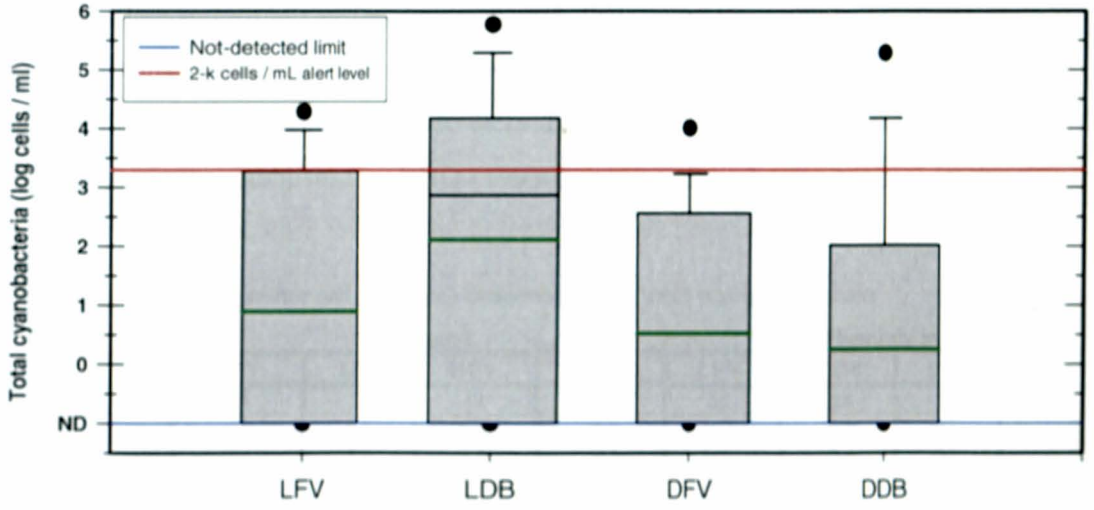
Parameters	LFV	LDB	DFV	DDB	Compared samples	Wilcoxon Signed Rank Test on paired data	Compared samples	Rank sum (Mann-Whitney) test
N	44	44	44	44	LFV vs LDB	LDB > LFV Significant increase ($P \leq 0.001$) H_0 rejected	LFV vs DFV	LFV=DFV No significant difference P=0.822 H_0 not rejected
Mean of the log	23.40	254.93	22.88	237.60				
Median	8.20	272	8.22	212.95				
Min	0.50	1.30	0.45	2.53				
Max	210.00	666.00	314.00	675.00	DFV vs DDB	DDB > DFV Significant increase ($P \leq 0.001$) H_0 rejected	LDB vs DDB	LDB=DDB No significant difference P=0.565 H_0 not rejected
Standard Deviation	44.90	180.98	49.68	191.75				
95% Confidence Interval	6.90	37.24	6.41	33.70				
95th Percentile	43.30	479.40	61.02	479.80				

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm, DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

There were however, no significant differences (P=0.822 and P=0.565) in the turbidity in waters sampled from the free volume in both light and dark containers. The same applied to the waters containing dislodged biofilm. This implies that if containers are not kept clean, biofilm will form on their inner-sidewalls regardless of the properties of the particular container type.

3.2.2.2 The role of light and the occurrence of cyanobacteria in container water

Figure 3.6 shows that for all the type of samples, the numbers of total cyanobacteria at the 95th percentile (the upper black dot of each box in the plot - Figure D; Appendix D), exceeded the WHO (1999) Guideline alert level of 2,000 (2-K) cells per 1 mℓ.



2,000 cells / mℓ alert level (WHO, 1999); Not-detected limit

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Figure 3.6 Occurrence of cyanobacteria in light and dark containers

This implies that water from the containers could constitute a potential health risk for the

consumers, should the cyanobacteria produce cyanotoxins in the waters. The risk is highest for the water in light containers containing dislodged biofilm, where cyanobacteria numbers, at the 75th percentile (upper boundary of the box is the 75th percentile - Figure D; Appendix D), exceeded the guideline alert level.

The increase of cyanobacteria number in light containers with the dislodged biofilm suspension was statistically significant (Table 3.3). In fact the number of cyanobacteria in LDB was also significantly higher (P=0.004) than in DDB. These results imply that light plays a role in the occurrence of cyanobacteria in biofilm that forms on the inner-sidewalls of light-permitting storage containers.

Table 3.3 Comparing cyanobacteria numbers (log cells/ml) in water from light and dark containers

Parameters	LFV	LDB	DFV	DDB	Compared samples	Kruskal-Wallis
n	34	34	34	34	LFV vs LDB vs DFV vs DDB	LDB Significantly higher than DDB; LFV; DFV (P=0.004) H_0 rejected
Mean of the log	0.89	2.12	0.52	0.25		
Median	ND	2.86	ND	ND		
Min	ND	ND	ND	ND		
Max	4.39	5.84	4.84	5.61		
Standard Deviation	2.14	2.51	2.01	2.10		
95% CI	0.57	1.90	0.54	0.08		
95th Percentile	4.20	5.48	3.50	4.88		

LFV: Light container's free volume water, LDB: Light container's water containing dislodged biofilm, DFV: Dark container's free volume water, DDB: Dark container's water containing dislodged biofilm

3.2.2.3 Nutrients and cyanobacteria in water sampled from storage containers

The occurrence of cyanobacteria in water is also influenced by nutrients (Section 1.5.2; Chapter 1). Phosphate and nitrate are the main nutrients that control the growth of cyanobacteria in the water. Chemical analyses of the water from the containers revealed (Table 3.4) that the concentrations of phosphate and nitrate were above the maximum limit value (10 µg/ℓ and 100 µg/ℓ respectively) established by WHO (1999), as some of the limiting factors for the occurrence of cyanobacteria.

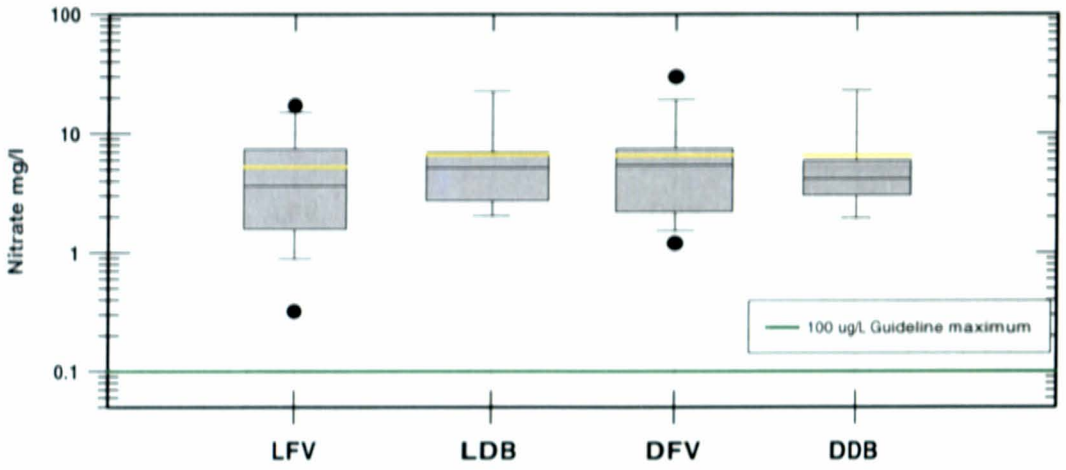
Table 3.4 Nutrients (nitrate and phosphate) concentration in water-storage containers

Parameters	Nitrate mg/l				Phosphate mg/l			
	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB
n	44	24	44	24	44	24	44	24
Mean	4.6	6.8	5.9	6.6	0.2	0.9	0.3	0.6
Median	2.4	3.9	3.2	4.1	0.1	0.6	0.1	0.3
Geomean	2.3	4.3	3.1	4.3	0.1	0.5	0.2	0.4
Min	0.2	0.8	0.3	1.1	0	0	0	0.1
Max	50.9	51.9	55.8	54.7	2.7	4.7	1.7	2.5
SD	8.4	10.1	11	10.5	0.5	1	0.4	0.6
95th Percentile	7.5	10.6	7.7	8.8	0.4	1.9	0.9	1.3

LFV: Light container's free volume water, LDB: Light container's water containing dislodged biofilm, DFV: Dark container's free volume water, DDB: Dark container's water containing dislodged biofilm

3.2.2.3.1 Nitrate

The levels of nitrate in water from the various containers (Table C6, Appendix C) were not significantly different (P=0.83). At the 95th percentile, all levels were above the WHO (1999) guideline value of 100 µg/ℓ as indicated by the green line in Figure 3.7.



100 µg/ℓ Guideline maximum limit

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

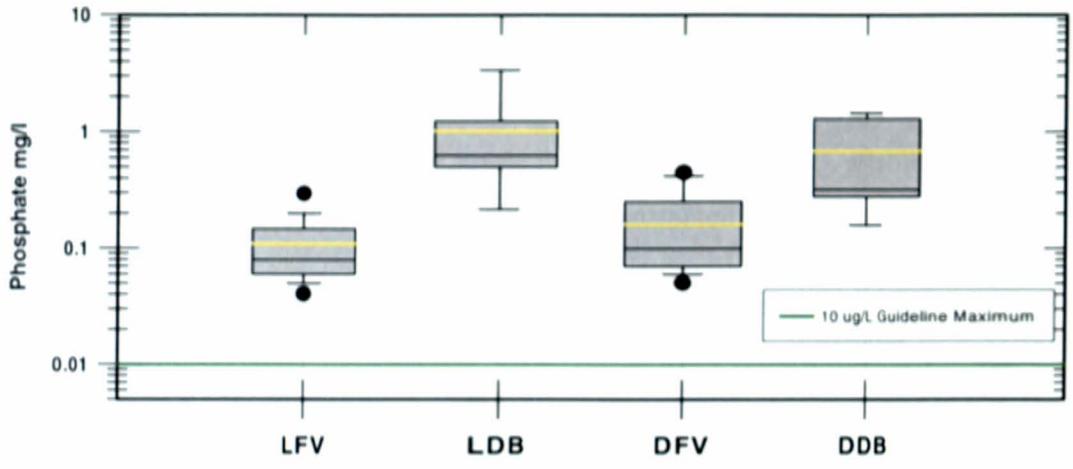
Figure 3.7 Concentration of nitrate in water from storage containers

Nitrate appeared not to have been a special factor in the higher occurrence of cyanobacteria (as shown in Figure 3.6) in the water from light containers containing dislodged container-sidewall biofilm, as the level of nitrate was quite similar in all the sample types.

3.2.2.3.2 Phosphate (as Ortho-phosphate)

Results in Figure 3.8 show that the levels of phosphate in all the samples were above the WHO (1999) Guideline limit value of 10 µg/ℓ (Table C6, Appendix C). This initially suggested that there was enough phosphate for the occurrence of cyanobacteria in water from storage containers. However, significantly higher concentrations (P=0.018 and P=0.014) of phosphate from light and dark containers respectively were measured in the water with the dislodged biofilm suspended inside.

This suggests that phosphates accumulated in the biofilm along with the other contaminants, or could also have been released from cells during dislodging. While the results showed that there were sufficient levels of phosphates to sustain cyanobacteria, the results were inconclusive as to whether these nutrients (as with nitrate) played a role in the variances of the cyanobacteria numbers in samples from the various containers.



10 µg / ℓ Guideline maximum limit

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Figure 3.8 Concentration of phosphate in water from storage containers

3.2.2.4 Physical quality of container-stored waters

The physical parameters such as temperature and pH were found to be within acceptable ranges (around 25°C and 6-9 respectively) to sustain the growth of cyanobacteria in container-stored water (WHO, 1999). In terms of health risks related to the physical factors, the SABS (2005) has established the pH range within 4-10 and turbidity at 1-10 NTU in drinking water. Turbidity was already discussed in Section 3.2.2.1. The other two parameters were also measured in containers-stored water. The results (Tables C8; Appendix C), showed that the pH of the water was within the acceptable range of the standard.

3.2.3 Toxins related to cyanobacteria in container water

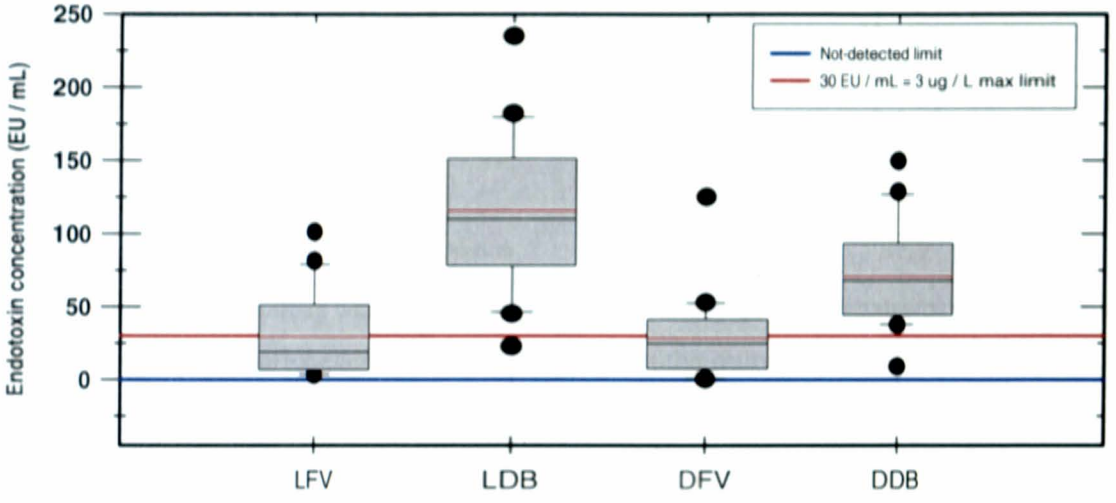
3.2.3.1 Microcystin

Only water containing dislodged biofilm sampled from light containers were analysed. The results are shown in Table C4a (Appendix C).

It was reasoned that if microcystin was to occur in any container water, this would be likely in samples where the highest numbers of cyanobacteria occurred – hence the DB samples from light containers. Results showed that microcystin could not be detected in any of these samples except for one where the toxin occurred in a concentration lower than the guideline level of 1 µg/ℓ proposed by the WHO (2004).

3.2.3.2 Endotoxin

Considerable levels of endotoxin were detected in the free volumes of sampled water as well as the same waters containing dislodged biofilm from dark and light containers. The results are shown in Figure 3.9. The maximum acceptable limit (3 µg/ℓ=30 EU /mℓ), established for acute risk by the New Zealand Ministry of Health (2000) is represented by the horizontal red line.



3 µg / ℓ maximum acceptable limit (NZMH, 2000)
LFV: Light container's free volume water, LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water, DDB: Dark container's water containing dislodged biofilm

Figure 3.9 Level of endotoxin in water-storage containers

The results show that more than fifty percent of all the samples could be considered as not suitable for drinking because the levels of endotoxin being above the maximum acceptable limit of 3 µg/ℓ. The level of endotoxin was above the maximum limit in both types of containers, in the water containing dislodged biofilm as well as in the free volume of water.

Irrespective of the type of container, there was a significant increase (P≤0.001) of the concentration of endotoxin in water containing dislodged biofilm compared to the free volume samples (Table 3.5). The water sampled from light containers with biofilm contained significantly (P≤0.001) more endotoxin than the water with DB from the dark containers. The differences were not significant (P=0.818) between the concentration of endotoxin in FV water samples.

This initially implied that biofilm in light containers were more endotoxic because of the significantly higher occurrences of cyanobacteria in water containing DB sampled from these containers (Table 3.3). However, there are other microorganisms that could contribute to the endotoxicity.

Table 3.5 Comparing endotoxin concentration (log EU / ml) in FV and DB samples of the different type of containers as well as from the same type of container

Parameters	LFV	LDB	DFV	DDB	Compared samples	Wilcoxon Signed Rank Test on paired data	Compared samples	Rank sum (Mann-Whitney) test
n	44	44	44	44	LFV vs LDB	LDB > LFV Significant increase (P≤0.001) H_0 rejected	LFV vs DFV	LFV=DFV No significant difference P=0.818 H_0 not rejected
Mean of the log	1.50	2.07	1.51	1.84				
Median	1.33	2.07	1.14	1.80				
Min	0.33	1.18	ND	0.66				
Max	2.16	2.39	2.28	2.30	DFV vs DDB	DDB > DFV Significant increase (P≤0.001) H_0 rejected	LDB vs DDB	LDB > DDB Significant increase P≤0.001 H_0 rejected
Standard Deviation	1.57	1.72	1.61	1.66				
95% Confidence Interval	1.9	2.26	1.87	2.14				

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm; DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Endotoxin found in drinking water can be from a wide variety of microorganisms (Rapala et al, 2002). Lipopolysaccharide endotoxin is a component of the outer membrane of cyanobacteria (Metcalf and Codd, 2004; Keleti and Sykora, 1982) as well as enterobacteriaceae (Wiese et al, 1999).

The pilot investigation conducted prior to this study indicated that cyanobacteria as well as enterobacteriaceae occurred in most waters sampled from containers. The abundant occurrence of enterobacteriaceae (as indicated by *E. coli* and total coliforms), in water containers and their potential health effects is also a subject currently intensively studied by the Water and Health Research Unit (UJ).

For this study, the co-occurrence of these two microorganism groups were therefore studied in conjunction with the occurrence of endotoxin levels in the container water samples. The results are presented in the next section.

3.3 ENTERIC BACTERIA AND CYANOBACTERIA CO-OCCURRING IN WATER-STORAGE CONTAINERS

Section 3.2.2.2 had shown that cyanobacteria occurred in containers water containing dislodged biofilm. This section will show *E. coli* and total coliforms also occurred more in water containing dislodged biofilm. This implied that enteric bacteria (indicated by *E. coli* and total coliforms) co-occurred with cyanobacteria in water containing dislodged biofilm.

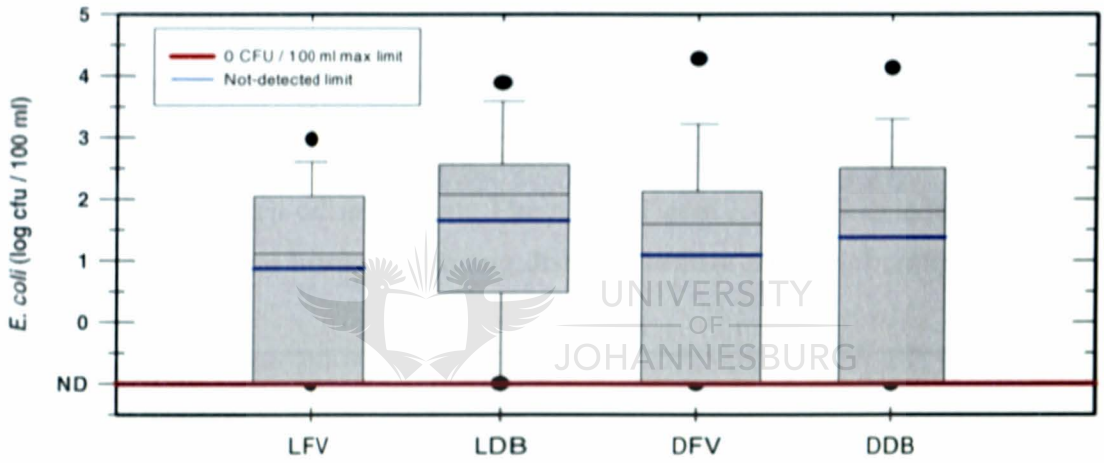
3.3.1 *Escherichia coli* in water-storage containers

Figure 3.10 shows the significantly increased numbers of *E. coli* in waters from containers that

contained dislodged biofilm. The WHO (2004) drinking water guideline as well as the South African National Standard (SABS, 2005), require that *E. coli* should not be detected in drinking water. This level is represented in Figure 3.10 by the horizontal red line at the not-detected (ND) limit.

As shown in Figure 3.10, at least seventy-five percent of all the water samples contained *E coli* (the 25th percentile of the box - Figure D; Appendix D) sitting on or above the ND level. According to the SABS (2005) as well as the WHO (2004) Guideline, the consumption of these waters could constitute a potential health risk for consumers.

The increase in *E. coli* numbers between the free volume of water and the same water containing the dislodged biofilm was significant (P=0.048) in light containers (Table 3.6).



0 CFU / 100 ml maximum limit (SABS, 2005); ND: Not-detected limit

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm
DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

Figure 3.10 *Escherichia coli* occurrence in water-storage containers

The numbers of *E. coli* in waters from dark containers containing dislodged biofilm, were not significantly different (P=0.109) from those in the free volume of water of the same container (Table 3.6). This indicated that *E. coli* were less likely to accumulate in the biofilm of dark containers than in the biofilm of light containers.

An earlier study done by De Philippis et al (2005) showed that cyanobacteria have the capability to attach and form biofilm on solid surfaces where water and solid material interface, using polysaccharide as the first step in the colonisation of the surfaces. Rapala et al (2002) also reported that cyanobacteria are able to stimulate the growth of heterotrophic bacteria. Since *E. coli* are not light dependent, this implied that more cyanobacteria occurring in biofilm could lead to more *E. coli* occurring in the same biofilm.

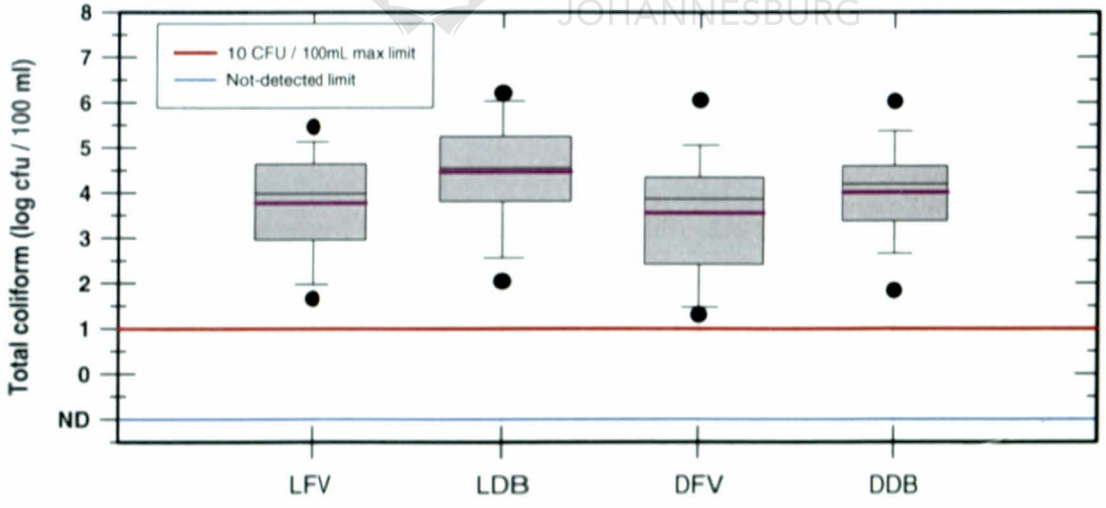
Table 3.6 *E. coli* (log CFU / 100 ml) in FV and DB samples from the same as well as alternative type of container

Parameters	LFV	LDB	DFV	DDB	Compared Samples	Wilcoxon (Signed Rank) Test on paired data	Compared samples	Rank sum (Mann-Whitney) test
N	42	42	38	42	LFV vs LDB	Significant increase ($p=0.048$) LDB>LFV H_0 rejected	LFV vs DFV	LFV=DFV No significant difference P=0.969 H_0 not rejected
Mean of the log	0.92	1.52	1.09	1.40				
Median	1.11	2.10	1.60	1.80				
Min	ND	ND	ND	ND				
Max	3.30	4.34	5.63	4.18	DFV vs DDB	No significant increase ($p=0.109$) DDB=DFV H_0 not rejected	LDB vs DDB	LDB=DDB No significant difference P=0.511 H_0 not rejected
Standard Deviation	1.49	1.60	1.78	1.58				
95th Percentile	1.81	3.00	2.14	2.73				
95% Confidence Interval	2.60	3.45	2.76	3.20				

LFV: Light container's free volume water; LDB: Light container's water containing dislodged biofilm; DFV: Dark container's free volume water; DDB: Dark container's water containing dislodged biofilm

3.3.2 Total coliforms in water-storage containers

Total coliforms, being member of enteric bacteria also have the ability to attach to the first layer of biofilm and form cell-cell interaction. The results (Figure 3.11) show an increase of total coliforms concentration in water containing dislodged biofilm especially from light containers.



10 CFU / 100ml maximum limit (SABS, 2005); ND: Not-detected limit

LFV: Light containers free volume; LDB: Light containers dislodged biofilm
DFV: Dark containers free volume; DDB: Dark containers dislodged biofilm

Figure 3.11 Total coliform occurrence in water-storage containers

The horizontal red line in Figure 3.11 representing the maximum limit established by SABS (2005), was exceeded by the numbers of total coliforms in the majority of samples, which therefore are not suitable for drinking purposes as well as indicating that the general hygienic

quality of the container waters were not good.

The numbers of total coliforms (Table 3.7) were significantly higher (P≤0.001) in water containing dislodged biofilm from both types of containers indicating the accumulation of these bacteria in the biofilms.

Table 3.7 Total coliforms (log CFU / ml) in LFV and DB samples from the same as well as alternative type of container

Parameters	LFV	LDB	DFV	DDB	Compared Samples	Wilcoxon Signed Rank Test on paired data	Compared samples	Rank Sum (Mann-Whitney) test
n	42	42	38	42	LFV vs LDB	Significant increase ($p \leq 0.001$) LDB>LFV H_0 rejected	LFV vs DFV	LFV=DFV No significant difference P=0.563 H_0 not rejected
Mean of the log	3.7	4.3	3.6	4				
Median	4	4.5	4	4.1				
Min	1.2	ND	1.2	1.6				
Max	6.3	7	6.2	6.2	DFV vs DDB	Significant increase ($p \leq 0.001$) DDB>DFV H_0 rejected	LDB vs DDB	LDB=DDB No significant difference P=0.058 H_0 not rejected
Standard Deviation	1.2	1.4	1.3	1.0				
95th Percentile	5.1	6	5	5.0				
95% Confidence Interval	7.3	8.4	7	8				

LFV: Light container's free volume water, LDB: Light container's water containing dislodged biofilm; DFV: Dark container's free volume water, DDB: Dark container's water containing dislodged biofilm

However the differences (Figure 3.11) were not significant between the LFV and DFV (P=0.563) as well as the increase of total coliforms in LDB compare to DDB (P=0.058). It is likely therefore, that dark containers do not enhance the occurrence of total coliforms.

It is also likely that the same argument about the cyanobacteria and *E. coli* (Section 3.3.1 above) is valid here.

The implication is that enteric bacteria can co-occur with cyanobacteria, at approximately the same variance in waters containing biofilm, sampled from light containers. Since enteric bacteria are not light dependent, it is plausible to reason that cyanobacteria play a role in their levels of occurrence in biofilm.

More importantly, the endotoxicity reported in Section 3.2.3.2 can be ascribed to cyanobacteria as well as enteric bacteria. The next section illustrates this.

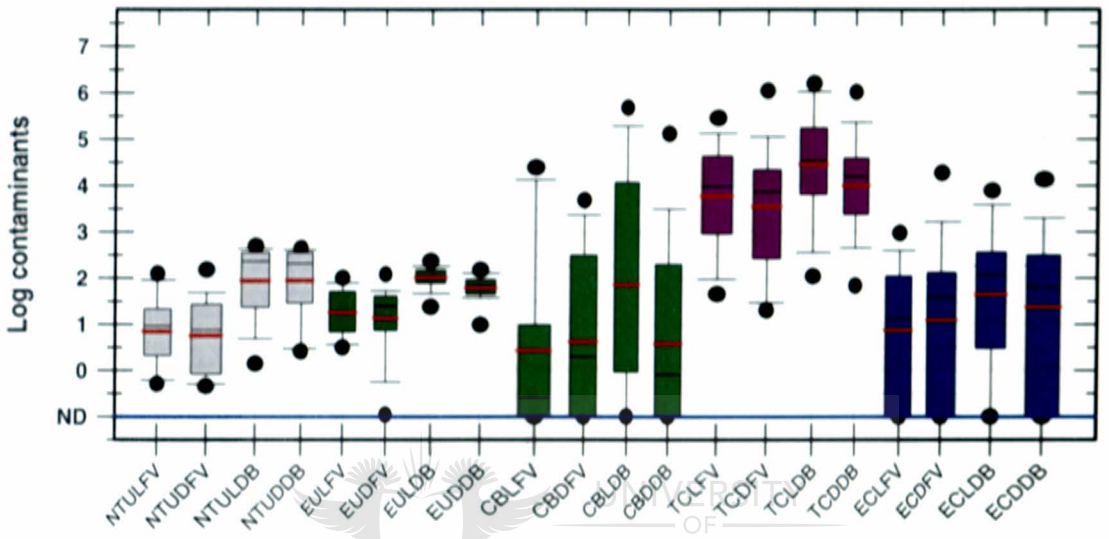
3.3.3 Linking endotoxin levels to numbers of cyanobacteria, total coliforms and *E. coli* in the same water samples

The contaminants discussed in the above sections are presented here differently. This is to illustrate relationships between the endotoxin concentration and the numbers of bacteria.

Figure 3.12 broadly shows that the levels of contaminants (toxin and bacteria) increase from the

free volume to the dislodged biofilm water samples. This implied that the contaminants mostly occurred in the biofilm growing on the inner sidewalls of the containers, especially light containers, which contained significantly higher levels of these contaminants.

Although the increase of endotoxin concentration in water containing biofilm coincided with the increase of bacteria numbers, regression analyses showed a poor correlation between the two parameters ($R<0.05$).



NTULFV: Turbidity in LFV
NTUDFV: Turbidity in DFV
NTULDB: Turbidity in LDB
NTUDDB: Turbidity in DDB
EULFV: Endotoxin unit in LFV
EUDFV: Endotoxin unit in DFV
EULDB: Endotoxin unit in LDB
EUDDB: Endotoxin unit in DDB

CBLFV: Cyanobacteria in LFV
CBDFV: Cyanobacteria in DFV
CBLDB: Cyanobacteria in LDB
CBDDB: Cyanobacteria in DDB
TCLFV: total coliform in LFV
TCDFV: total coliform in DFV
TCLDB: total coliform in LDB
TCDDB: total coliform in DDB

ECLFV: *E. coli* in LFV
ECDFV: *E. coli* in DFV
ECLDB: *E. coli* in LDB
ECDDB: *E. coli* in DDB

Figure 3.12 The levels of turbidity, endotoxin, cyanobacteria, total coliforms and *E. coli* in light and dark containers

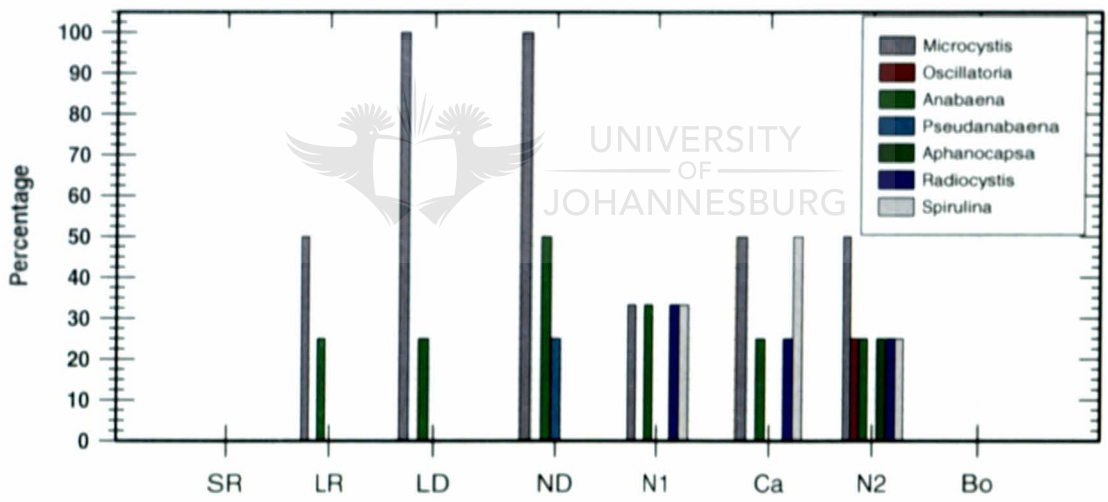
However, knowing that cyanobacteria, *E. coli* and total coliforms are producers of endotoxin, it is then plausible to reason that the increase of endotoxin levels in biofilm is a consequence of the occurrence of these bacteria in container biofilm. Furthermore, light containers appeared to enhance these higher levels of endotoxin significantly.

3.4 CYANOBACTERIA IN THE SOURCE WATERS

The members of five algal classes: Cyanophyceae, Bacillariophyceae, Chlorophyceae, Dinophyceae and Euglenophyceae were recorded at the environmental water sources during the sampling period (summer time). Genera of these classes were identified at almost all the eight sampling points: Savhani River, Luphephe River, Nwanedi and Luphephe Dams (impoundments), Nwanedi 1, Nwanedi 2, Canal and Borehole. Cyanobacteria were the most abundant group of phytoplankton in the samples taken during the study period.

3.4.1 Occurrence per genus

In general seven genera of cyanobacteria (*Microcystis spp, Oscillatoria spp, Anabaena spp, Pseudanabaena spp, Aphanocapsa spp, Radiocystis spp and Spirulina spp*) were identified in the environmental water sources, but their occurrence or frequency varied from one source to another (Figure 3.13).



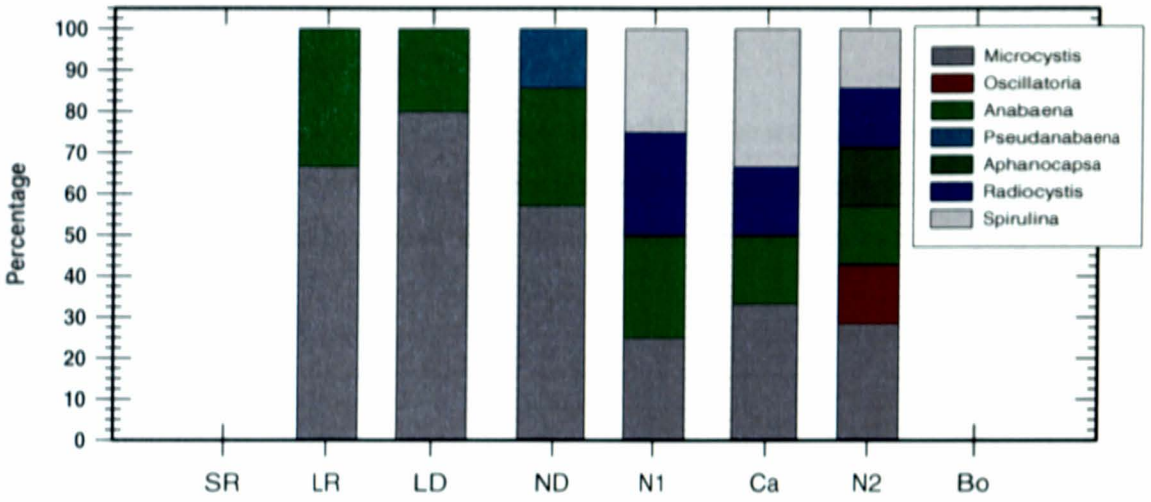
SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.13 Percentage of occurrence of cyanobacterial genera in environmental water sources

Figure 3.13 shows that cyanobacteria were not detected in any of the samples taken during the sampling period in the Savhani River. This was also the case for the Borehole water. The Cyanobacteria genera showed the widest variety in water sampled from point N2 (Nwanedi River downstream from Folovhodwe). *Microcystis spp* was the cyanobacterial genus most frequently encountered, while *Oscillatoria spp* and *Pseudanabaena spp* rarely occurred at the various sampling points (Figure 3.14). This confirms previous work done by Van Ginkel (2004) who also found that *Microcystis spp* was the predominant genus of cyanobacteria in other South

African surface waters.

Microcystis spp was particularly predominant (80%) in the Luphephe Dam, but could not be sampled from the Savhani River and the Borehole during the study.



SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.14 Predominance of cyanobacterial genera in environmental water sources

All the cyanobacteria identified at water sources have the ability to produce toxins - they will therefore be discussed in the next section as a total group capable of affecting the health of consumers.

3.4.2 Total cyanobacteria in the environmental water

3.4.2.1 Health-related water quality based on cyanobacteria numbers

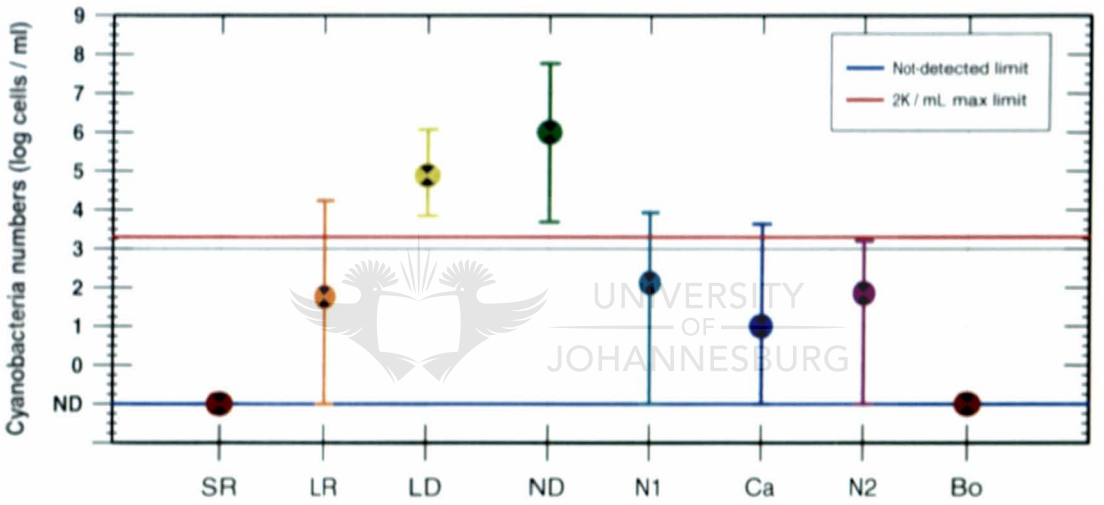
The seven genera of cyanobacteria identified in the water sources have been reported to be potentially toxic (Jayatissa et al, 2006; Chorus and Bartram, 1999). People in the study area also drink the water often directly from the sources. The postulation of potential health risk therefore will be linked to the concentration of cyanobacteria in relation to the alert level of 2,000 cells/mℓ proposed by the World Health Organization (1999), for water intended for consumption.

The numbers of cyanobacteria at the main river sources used by the population (Savhani River, Luphephe River, Nwanedi 1 and Canal), hardly exceeded the WHO (1999) alert level represented by the horizontal red line in Figure 3.15. This implied that a potential health risk related to cyanobacteria that might occur at the various sources, was not significant at the times of sampling.

The dam waters however, showed excessive levels. These dams are inside the Nwanedi game

reserve and therefore offered limited chance for people drinking its water. Nevertheless, the game animals of the park do drink from the dams. The Nwanedi Resort, situated at the foot of the two dams, also source its water from these dams before treating it for consumption by tourists staying over in the resort. More importantly, there are plans to use the dams as source water for a water treatment facility to be erected in the near future to provide potable water for the villages in the study area (Jagals, 2006).

This implies that there was an immediate risk to the health of the animals, as animals are also covered in the WHO (1999) guidelines. There is also a future risk for villages if the treatment facility cannot remove these bacteria or their toxins. Section 3.4.3 discusses the toxicity of these waters.



2,000 cells / ml maximum limit (WHO, 1999); ND: Not-detected limit

SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.15 Cyanobacteria numbers in environmental water sources

3.4.2.2 Variation in cyanobacteria numbers associated with the water sources

3.4.2.2.1 Rivers and impoundments

Cyanobacteria numbers in the impoundments were significantly higher than in the rivers (Table 3.8). This was probably due to the fact that the water in the impoundments was standing while river water was fast flowing and therefore unstable. There was then a chance for the water sources downstream in the Nwanedi River (N1, N2 and Ca) to be seeded with high numbers of cyanobacteria from the impoundments.

Table 3.8 Comparing cyanobacteria (log) numbers in rivers and impoundments

Parameters	Dam waters		River waters					Rank sum (Mann-Whitney) test
	ND	LD	SR	LR	N1	N2	Ca	
n	4	4	4	4	3	4	4	Significant difference P=0.029 H_0 rejected
Median	6.26	4.80	ND	1.90	3.43	2.60	0.70	
Mean of the log	6.00	4.80	ND	1.76	2.12	1.85	1.00	
Min	3.70	3.85	ND	ND	ND	ND	ND	
Max	7.76	6.08	ND	4.24	4.24	3.20	3.64	
SD	2.05	0.92	ND	2.15	2.71	1.98	2.37	
95th Perc	7.74	5.70	ND	3.55	3.83	3.20	3.26	

ND: Nwanedi Dam; LD: Luphephe Dam; SR: Savhani River; N1: Nwanedi 1; N2: Nwanedi 2

3.4.2.2.2 Water sources upstream and downstream of the impoundments

It was expected that the impoundments, being suitable for the proliferation of cyanobacteria (Chorus and Bartram, 1999), will contribute to an increase of cyanobacteria numbers in the Nwanedi River downstream. However statistical analyses showed that there was no significant difference (P=0.068) in the collated data of the Luphephe River upstream and the Nwanedi River downstream from the two impoundments.

Table 3.9 Comparing cyanobacteria (log) numbers in water sources upstream and downstream the impoundments

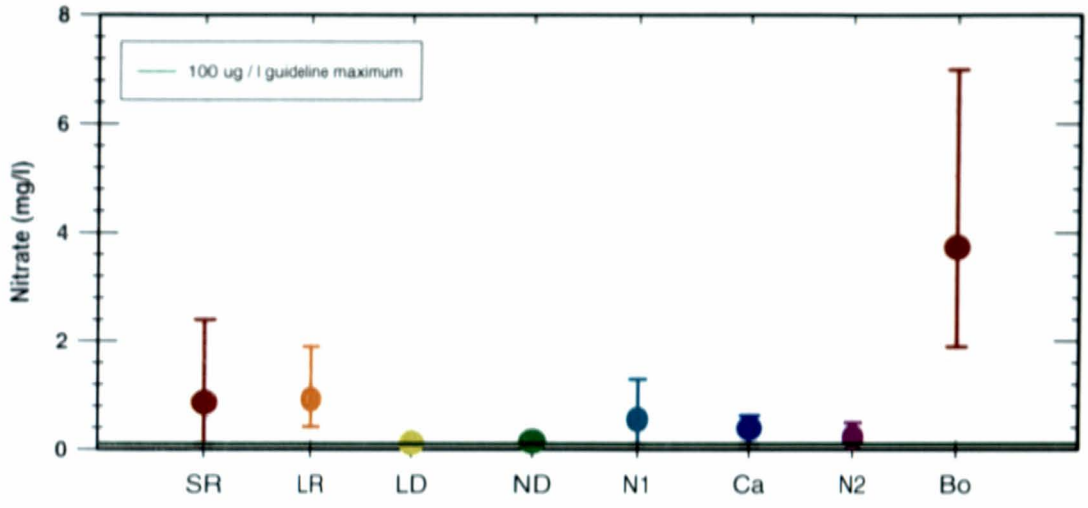
Parameters	LR	Imp	N1	Compared samples	ANOVA on ranks (Kruskal-Wallis)
n	4	4	3	LR vs Imp vs N1	No significant difference P=0.068 H_0 not rejected
Median	1.90	6.00	3.40		
Mean of the log	1.80	6.00	2.10		
Min	ND	3.80	ND		
Max	4.20	6.95	4.00		
SD	2.10	1.50	2.70		
95% CI	3.60	6.70	3.80		

LR: Luphephe River; Imp: Impoundments; N1: Nwanedi 1

3.4.2.3 Nutrients in water sources

3.4.2.3.1 Nitrate

Figure 3.16 shows the concentration of nitrate in the water sources above the horizontal green line representing the 100 µg / l, established by the WHO (1999) as a minimum requirement for the occurrence of cyanobacteria in water. Nitrate concentrations were above the guideline, showing that there was sufficient nitrate to sustain the growth of cyanobacteria in all sources.



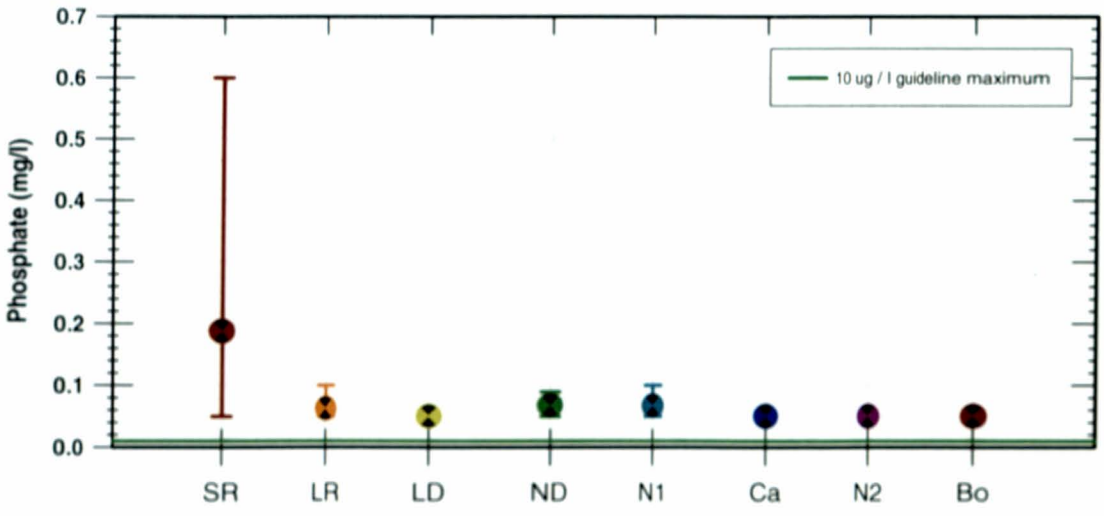
100 µg/l guideline maximum (WHO, 1999)

SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.16 Concentration of nitrate in the environmental water sources

3.4.2.3.2 Phosphate

Figure 3.17 shows that the concentration of phosphate was constant in almost all the water sources and exceeded the minimum value of 10 µg / l (horizontal green line) (WHO, 1999) for the occurrence of cyanobacteria in water. The phosphate levels were therefore sufficient to sustain the growth of cyanobacteria.



10 µg/l guideline maximum (WHO, 1999)

SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.17 Concentration of phosphate in the environmental water sources

3.4.2.4 Physico-chemical quality of water in the environmental water sources

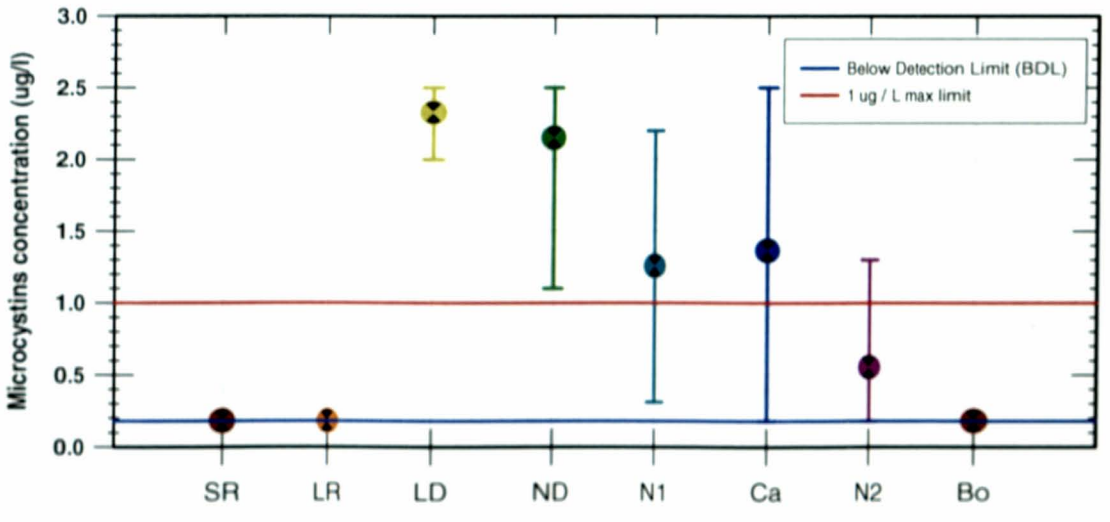
Physico-chemical analyses showed (Tables C9a - d; Appendix C) the presence of elements such as calcium, magnesium, sodium, potassium, chloride and sulphate, in sufficient concentrations required by cyanobacteria for growth (WHO, 1999). The temperature and pH were also in the acceptable range (20-35°C and 6-9 respectively) to sustain the growth of cyanobacteria.

3.4.3 Water quality related to cyanobacteria toxins

3.4.3.1 Microcystin

As can be observed in Figure 3.18, microcystin were detected in five water sources: Nwanedi and Luphephe Dams (impoundments), in the Nwanedi River at N1, N2, and in the canal (Ca). Particularly dangerous when ingested in water, microcystins are produced by many species of cyanobacteria and a maximum limit (1 µg/ℓ) for microcystin-LR in drinking water (2 ℓ/day) for life-time exposure has been established by the World Health Organization (WHO, 2004). This level is represented by the horizontal red line in Figure 3.18.

All the water sources where microcystin had been detected contained an unacceptably high level. The highest concentrations (>2.5 µg / ℓ) of microcystins were detected in the two impoundments, where the highest concentrations of cyanobacteria were also detected. The water samples from Borehole, Savhani and Luphephe Rivers were free of microcystins (the horizontal blue line in Figure 3.18 represents a level below the detection capability of the test).



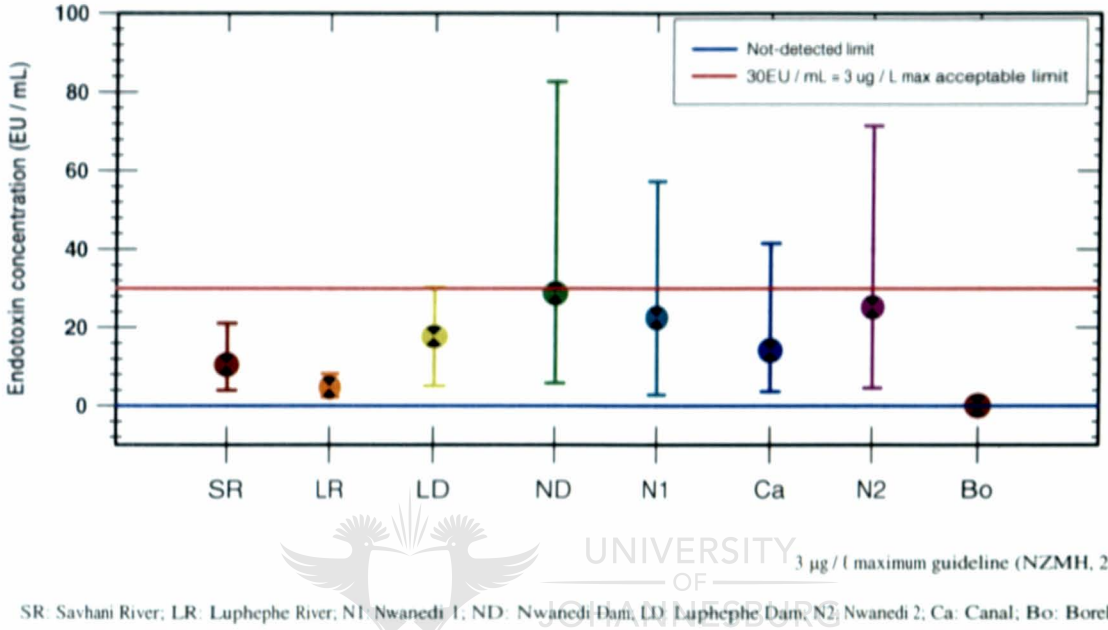
1 µg / ℓ maximum guideline (WHO, 1999); BDL: Below detection limit (0.18 µg / ℓ)

SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.18 Concentration of microcystins in the environmental water sources

3.4.3.2 Endotoxin

Endotoxin was detected in the majority of water sources (Figure 3.19) except for water from the borehole that was free of endotoxin. The highest concentrations of endotoxin were found in the Nwanedi Dam. At the 75th percentile, the levels of endotoxin in LD, ND, N1, Ca and N2 were above the maximum acceptable limit (3 µg/ℓ=30 EU/mℓ).



SR: Savhani River; LR: Luphephe River; N1: Nwanedi 1; ND: Nwanedi Dam; LD: Luphephe Dam; N2: Nwanedi 2; Ca: Canal; Bo: Borehole.

Figure 3.19 Concentration of endotoxin in the environmental water sources

3.5 DISCUSSION

3.5.1 Cyanobacteria and related toxins in containers waters

A key element of this study was whether cyanobacteria and their related toxins occurred in numbers and levels high enough to be a risk for consumers of water from domestic water-storage containers as well as environmental water sources from which the waters in the containers were collected.

Arguments about the source and accumulation of cyanobacteria in water-containers, and the poor correlation between endotoxin concentrations and the numbers of bacteria are elaborated in this section. The health-related quality of water will be also discussed by comparing the level of cyanobacteria and their toxins in water-containers to the guidelines.

3.5.1.1 Cyanobacteria in containers waters

The general overview of the occurrence of cyanobacteria in the water sources used by the

population and in the water-storage containers (Figure 3.1), certainly informs about the seeding in containers of pathogens from the sources even though some of the genera found in the containers were not found in waters sampled from the sources.

This is possibly due to the temporal variability on the occurrence of cyanobacteria at the source in response to changes in environmental factors such as season changes and with it for instance daylight lengths and temperatures. Several authors reported that the longevity and timing of a population of cyanobacteria in water can be affected by climatic and meteorological changes as well as nutrients availability (Chorus and Bartram, 1999; Whitton and Potts, 2000). Downing and Van Ginkel (2004), in a study of the major South African impoundments, also reported a temporal variability in the distribution of cyanobacteria in impoundments. It is therefore possible that cyanobacteria could be present at the study sources to seed container waters and biofilm but ceased to occur at the time of sampling.

Another explanation is that the human populations in the study area were randomly using several streams as sources - other than the ones studied. The sample points selected could not comprehensively cover all areas where people sourced water. These alternative waters could have been contaminated with the genera of cyanobacteria "missing" from the sample points but found in the containers.

There was a considerable diversity in the phytoplankton occurring in the water-storage containers with five algal classes and four genera of cyanobacteria identified. Nevertheless, as was reported from work done on environmental waters elsewhere in the world (Carmichael, 1992; Galvao et al, 2001), as well as in South Africa (van Ginkel, 2004; Du Preez and Van Baalen, 2005), *Microcystis* was the dominant genus of cyanobacteria in the source water and this was also the case of water stored in containers.

3.5.1.2 Role of light on the occurrence of cyanobacteria in container biofilm

The results showed that the number of cyanobacteria in water sampled from light-permitting containers were significantly higher to those found in the waters from the dark (not permitting light) containers. The nutrients level being similar in both types of containers, it was then likely that the occurrence of cyanobacteria in containers was controlled by the presence of light. Most cyanobacteria are autotrophic, requiring light to produce the compounds needed for their sustenance (Chorus and Bartram, 1999). It then makes sense that cyanobacteria will hardly survive in an environment where light is limited such as a dark container.

It is plausible that cyanobacteria can accumulate and even grow in light-permitting containers especially if their ability to form or contribute to biofilm forming on container inner-surfaces is

considered. Water from light containers containing biofilm dislodged from the sidewalls contained significantly higher numbers of cyanobacteria than the water in samples taken from the same containers just before dislodging the biofilm. Cyanobacteria numbers in similar waters with dislodged biofilm but from dark containers had significantly lower numbers.

3.5.1.3 Survival and / or accumulation of cyanobacteria in container biofilm

Why were the numbers of cyanobacteria so high in the container biofilm? Results show that while these could be seeded from the environmental waters (no difference in numbers between environmental and container waters except for their occurrence in waters from light containers containing dislodged biofilm), their numbers certainly did not reduce in any significant way.

Furthermore, the numbers in the water samples containing dislodged biofilm from light containers were significantly higher than in the sources – except for the dams, where nobody sourced container water. This implied that cyanobacteria could survive and accumulate in biofilm. No evidence could be found of increased seeding of container water through sourcing – therefore it could be argued that cyanobacteria not only survived in container biofilm, but could also accumulate to the high levels detected. This could happen because there were sufficient nutrients in container water and the temperatures and pH were optimal to support growth. Judging by the findings of the waters from the light containers, there was sufficient light. Why would they be more in biofilm than in the free volume of the waters sampled from the light containers? It could be argued that cyanobacteria actually play a strong role in the formation of biofilm.

De Philippis et al (2005) found that cyanobacteria could form biofilm on solid surfaces that interfaces with environmental water. De Philippis and Vincenzini (1998) as well as Sutherland (1999) also reported that exo-polysaccharides, present in the cyanobacteria cell wall, promote the attachment of the cell to solid surfaces and the formation of biofilm.

In contrast to heterotrophic bacteria that die-off during water-storage (Piriou et al, 1997; Egwari and Aboaba, 2002; Moabi, 2006), cyanobacteria, because the presence of gas vacuole in genera such as *Microcystis*, have the ability to float in water (Chorus and Bartram, 1999; Whitton and Potts, 2000). This reduces the chances to be injured or to precipitate to the bottom of containers and therefore favour their adhesion to already existing biofilm and accumulate there-in or even initiate biofilm formation. Furthermore, cyanobacteria have the ability to synthesise their own organic nutrients using only carbon dioxide and light, then do not depend on environmental carbon-derived nutrients (Chorus and Bartram, 1999).

3.5.1.4 Health-related water quality associated with the levels of cyanobacterial toxins

The occurrence of potentially toxic cyanobacteria in water stored in containers constitutes an aesthetic nuisance and a potential hazard for the health of consumers (Chorus and Bartram, 1999). The number of cyanobacteria in samples taken from both types of containers exceeded at the 95th percentile, the WHO (1999) alert level for potential health risk, implying that the water is not suitable for ingestion. But how actual was this risk? The postulation on health risk does only become more plausible if the increased occurrences of cyanobacteria could actually be associated with their toxins in the various waters.

3.5.1.4.1 Microcystin

Although cyanobacteria genera with the potential to produce microcystin were identified in many containers, microcystin was detected only in one sample, in one container in a low concentration. It is likely that microcystin did occur in other containers in very low concentrations but were below the detection level of the technique used (range of detection: 0.18 µg/ℓ - 2.5 µg/ℓ).

Another question may be whether cyanobacteria may grow in containers because of sufficient growth conditions, but that these same conditions may not be optimum for them producing microcystin? Such conditions may be different to the conditions required for survival of cyanobacteria (Van der Westhuizen et al, 1985; 1986). It is reported that environmental factors could affect the variation of the toxicity of cyanobacteria (Chorus and Bartram, 1999). Work by Sivonen (1990); Sivonen et al (1992) and Rapala and Sivonen (1998), showed the loss of toxigenicity by genera of *Microcystis* and *Anabaena* under variable light and nutrient conditions. It is therefore possible that the cyanobacteria that occurred in the containers, while having sufficient conditions to survive/accumulate, may not have found these same conditions as conducive to microcystin production.

Another factor that could have influenced the results, were the low number of container-water samples tested for microcystin. These numbers were too low (Clescerl et al, 1999) to be representative of the entire range of container samples collected during this study.

3.5.1.4.2 Endotoxin

In contrast to microcystin, which is not always produced by cyanobacteria, lipopolysaccharide endotoxin is part of the cyanobacterium. This implied that high levels of cyanobacteria mean high levels of endotoxin. An Australian study (Stewart et al, 2006) reported that cyanobacterial endotoxin on its own should not be conclusively considered as harmful to humans until proven so. Therefore, in the context of this study, health risk associated to cyanobacterial endotoxin,

simply constitutes an equivocal postulation based on the guidelines.

Endotoxin was detected in almost all the water stored in containers at concentrations exceeding the available guideline (3 µg/l) of the New Zealand Ministry of Health (2000). This implied that consumers coming in contact with or ingesting water from containers in the area could be exposed to skin irritation or diarrhoea (Closs et al, 1998; WHO, 1999, Rapala et al, 2002).

While the work of the Water and Health Research Unit focuses on the incidences of diarrhoea associated with poor microbial water quality in the area, this study did not go into the diarrhoea incidences or skin irritation in the households investigated during this study.

It was therefore not possible to postulate on the effect that the endotoxin would have had on the consumers – in particular whether such diarrhoea incidences would be associated with the occurrence of cyanobacteria.

3.5.1.5 Relationship between endotoxin concentrations and the numbers of bacteria

The co-occurrence of cyanobacteria and enteric bacteria (*E. coli* and total coliforms) contributed to high concentration of endotoxin in water stored in containers, particularly in biofilm where the potential interaction between the two groups of bacteria promote their accumulation at the container inner surfaces (Sutherland, 1999). During previous studies (Momba and Kaleni, 2002; Jagals et al, 2003) pathogenic as well as heterotrophic bacteria were also found to co-occur in biofilm on the sidewalls of water-storage container.

Although the results showed an increase of endotoxin concentration coinciding with an increase of bacteria numbers in biofilm, regression analyses showed the correlation between the concentrations of endotoxin and bacteria numbers was poor. The tendency was nevertheless there – especially in DB water from light containers. At best this implied that the water could cause an effect in consumers but the source of the toxin or whether such levels would actually affect a consumer remain inconclusive.

Another possible explanation can be that some other waterborne Gram-negative pathogenic bacteria such as *Salmonella* spp. which have strongly active endotoxin (Rapala et al, 2002), could have been present in the samples waters and contributed to the occurrence of endotoxin. This is a plausible explanation when considering the fact that *E. coli*, which are considered indicators of other enteric pathogens (WHO, 2004b), also occurred in the waters.

Furthermore, LAL Chromogenic assay also detects endotoxin from non-viable and / or dead bacteria (Jorgensen et al, 1979). This means that the test could have been picking up remnants of dead bacteria or signals from viable but not culturable pathogenic bacteria.

3.5.2 Cyanobacteria and related toxins in water sources

This section discusses the variability and the distribution of cyanobacteria in water sources as well as the quality of water related to cyanobacteria and their toxins.

3.5.2.1 Cyanobacteria distribution in water sources

Findings of this study indicated the variable distribution of cyanobacteria in some surface waters of the studied area as also found elsewhere around South Africa (Van Ginkel, 2004). The results from different points on the Nwanedi River system showed a distinct variation in the nature of phytoplankton, the genera of cyanobacteria and the numbers in which they occurred. A higher degree of phytoplankton biodiversity was observed particularly in waters sampled at Nwanedi 2 and in the canal. Cyanobacteria (75% occurrence) and Bacillariophyceae (100%) appeared to be the dominant classes of phytoplankton in all the water sampled at the various points.

As with work done elsewhere by Jayatissa et al (2006), this study found that diatoms were the predominant genera of Bacillariophyceae with a dominance of the group Pennate diatom. Already considered in previous studies by Du Preez and Van Baalen (2006), as well as Van Ginkel et al (2004) as the dominant cyanobacterial genus locally, *Microcystis spp* occurred in almost all the water bodies studied and often at the highest concentration. The high frequency of occurrence of *Anabaena spp* second to *Microcystis spp* in water sources also supports the work of Downing and Van Ginkel (2003) who found it to occur commonly in South Africa.

The Nwanedi River sampling points N1 and N2 were free of Dinophyceae and Euglenophyceae, six potentially toxic genera of cyanobacteria: *Microcystis spp*, *Oscillatoria spp*, *Anabaena spp*, *Aphanocapsa spp*, *Radiocystis spp* and *Spirulina spp* were identified in these points. The occurrence in water sources of cyanobacteria genera such as *Aphanocapsa spp*, *Radiocystis spp* and *Spirulina spp* not often identified in South African surface waters (Van Ginkel and Conradie, 2001) highlights the diversity of cyanobacteria populations in the water sources of the studied area.

Cyanobacteria were identified in the Luphephe and Nwanedi Rivers; in fact water from the Nwanedi and Luphephe impoundments (which contained cyanobacteria blooms) merge down stream to continue as the Nwanedi River flowing past the sampling points (N1, N2) as well as feeding the canal (Ca). The significantly higher numbers of cyanobacteria in the impoundments as compared to the rivers can be ascribed to the calm conditions with turbulence lower than what it appeared to be in the rivers. This stability of surface water could promote the development of cyanobacterial blooms when the other factors (Chapter 1, section 1.5.2) are optimal (Whitton and Potts, 2000).

It was expected that the impoundments would contribute to an increase of cyanobacteria numbers in water sources downstream compared to those upstream, but the statistical analysis showed that there was no difference between the numbers of cyanobacteria upstream and downstream the impoundments. This implied that there was not significant release of cyanobacteria from the impoundments flowing downstream.

In contrast to the impoundments, the fast-flowing water in Savhani River was not suitable for the development of cyanobacteria.

The absence of microorganisms in water sampled from the borehole was expected. Groundwater is a result of surface waters that naturally infiltrates the soil, leaving behind in the topsoil many of the constituents it collects during the process of precipitation and infiltration including cyanobacteria (Taylor, 2003). In addition there is no light underground, which would have been part of the reasons for the zero occurrences of cyanobacteria in these borehole samples (Mur et al, 1999).

3.5.2.2 Health-related quality of source water associated with the levels of cyanobacteria and their toxins

3.5.2.2.1 Microcystin

The occurrence of microcystin in water sources almost reflected the presence of cyanobacteria, as no microcystins occurred in Savhani River and borehole not containing cyanobacteria. Maybe the concentration of microcystin produced was very small and not detectable by our technique or the strains of cyanobacteria in the Luphephe River were not toxic since not all cyanobacteria strains are toxic (Sivonen et al, 1992; Sivonen and Jones, 1999; Utkilen and Gjolme, 1995; Rapala and Sivonen, 1998).

Regarding the WHO (2004) guideline (1 µg/ℓ) for lifetime exposure to microcystin in drinking water, the levels of microcystin in Ca, N1, N2, Nwanedi Dam and Luphephe Dam was unacceptable and thus liable to affect the health of consumers. This finding is supported by the report of Van Ginkel (2004) on the potential nuisance posed by microcystins in other South African water sources.

3.5.2.2.2 Endotoxin

Endotoxin was detected in all the water sources except the borehole, which was free of cyanobacteria and enteric bacteria reported to produce endotoxin in environmental water (Rapala et al, 2002). The concentration of endotoxin was above the available guideline (3 µg / ℓ) in Nwanedi 1 and canal (main sources of domestic water) exposing the consumers to health

problems such as skin irritation and diarrhoea (Closs et al, 1998; Rapala et al, 2002). This implies that the quality of water at those sources was not suitable for domestic use (drinking, bathing and washing).

3.5.3 Comparison between the health-related water quality in water-storage containers and in water sources

In general there was not a significant difference (P=0.150) between the numbers of cyanobacteria in the water-storage containers and in the water sources. However the occurrence of microcystin in water-storage containers did not reflect the number of cyanobacteria present, as it was the case in water sources.

The assessment of the compliance of the water used for domestic purposes in the study areas to the available guidelines of cyanobacteria related toxins revealed a different risk exposure at the source compared to the point of use. The findings elaborated on above allow postulating the health risks related to the levels of cyanobacteria and their toxins in water. These showed that consumers were most exposed to risks if ingesting the water at source where the concentrations of microcystin as well as endotoxin were unacceptable in terms of the respective guidelines (1 µg/ℓ and 3 µg/ℓ respectively) by the WHO (1999, 2004) and the New Zealand Ministry of Health (NZMH, 2000).

On the other hand, a health risk at the point of use (water-storage container) also existed, not of microcystin but because of unacceptable levels of endotoxin in free volumes of water as well as water containing dislodged biofilm. Furthermore the accumulation of pathogenic bacteria in biofilm constituted a microbial infection risk as these can be released into the free volume of container water during handling of the containers (Momba and Kaleni, 2002; Ntsherwa, 2004).

Beside the risk related to cyanobacteria and their toxins, it was also found that in the water-storage containers as well as in the environmental water sources, the levels of *E. coli* and total coliforms were often above the South African Bureau of Standard (2005), exposing the consumers to risk of infections, especially of those leading to enteric disease.

Despite the fact that this study focused essentially on microcystins and endotoxin as sources of water-borne intoxication associated with cyanobacteria, it was not excluded that other cyanotoxins (not tested for) such as neurotoxins produced by Anabaena could have been present in the water exposing consumers to nerves inhibition (paralysis).

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

4.1 CONCLUSION

The aim of this study was to identify and quantify the numbers of pathogenic cyanobacteria as well as quantify their related toxins (microcystins and endotoxin) in untreated water from water-storage containers and their surface sources.

This was within the scope of determining whether the potential occurrence of cyanobacteria and their toxins in container water posed a risk to the health of the consumer.

The results of this study showed that seven potentially toxic genera of cyanobacteria (*Microcystis spp, Oscillatoria spp, Anabaena spp, Pseudanabaena spp, Aphanocapsa spp, Radiocystis spp* and *Spirulina spp*) occurred in waters used by the population for various needs including drinking. *Microcystis spp* occurred the most frequently and could indirectly constitute a health problem as its numbers exceeded alert levels proposed by the WHO (1999) guidelines.

Contaminated water from environmental sources, transported in water-storage containers for later use, did not constantly reflect the quality of stored water in term of cyanobacteria occurrence or genus variability. The incoherence was considered in Section 3.5.1 (Chapter 3) to be possibly due to the temporal variation in cyanobacteria occurrence at the water sources as well as the random use of alternative untreated sources by the population.

The numbers of cyanobacteria in water-storage containers varied from light to dark containers as genera such as *Microcystis spp* and *Oscillatoria spp* that accumulated in biofilm at the inner surface of light containers, did not appear to survive in dark containers. Light was thus the limiting factor for the occurrence of cyanobacteria in water-storage containers.

Judging the health-related quality of the water, based on the occurrence and concentrations of cyanobacterial toxins in the water, the potential risk for consumers to be affected was higher at the source compared to the point of use.

Although occurring at unacceptably high (≥1 µg/l) concentrations in the Nwanedi River and the canal (main sources of domestic water), microcystin was detected in only one container but in a very low concentration. Since the production of microcystins by cyanobacteria is being controlled by environmental factors (WHO, 1999; Garnett et al, 2003), it is possible that the containers' environment were not suitable for its production by cyanobacteria.

Lipopolysaccharide-endotoxin occurred in unacceptably high levels both at the sources as well as in water-storage containers. In addition to cyanobacteria, enteric bacteria (indicated by *E. coli* and total coliforms) were also likely to contribute to the occurrence of endotoxin in containers. Considering the numbers of cyanobacteria and the concentration of endotoxin, the quality of

water at the point of use (water-storage containers) did not comply with relevant health-related water quality guidelines at the 95th percentile. This implied a health risk, either from water ingestion or direct contact (intoxication and irritation). The extent to which cyanobacteria on their own could affect consumers' health remained uncertain in the context of this study as microcystin did not occur meaningfully in water-storage containers while high endotoxin concentrations coincided, although not significantly, with high numbers of enteric bacteria in waters sampled from containers.

4.2 RECOMMENDATIONS

4.2.1 Further research

Further studies for improved understanding of health effects link to the consumption of water contaminated with cyanobacteria need to be done and should include:

- The detection of more cyanotoxins such as neurotoxins using High Performance Liquid Chromatography (HPLC);
- The toxic effect of cyanotoxins using cytotoxicity methods based on human cell cultures;
- The structure of biofilm, the steps of its formation and the interaction of microorganisms during this process;
- The impact of container's material (plastic polyvinyl chloride etc) on biofilm formation.

4.2.2 Water service authorities

- Programmes for regular monitoring of the health-related quality of water are strongly recommended;
- The installation of a reliable water supply system that can deliver potable water to the population in a general or specific way;
- Until such time that this happens, a continuous education and assistance programme for the population in hygienic handling and storage of water should be implemented. These should include:
    - Improving storage conditions: by relying on river water contaminated with abnormal concentrations of cyanobacteria, microcystins and endotoxin for their drinking needs, villagers are exposed to intoxication. To improve the quality of their water using affordable methods they should:
    - Filter the water from the river to reduce the load of cyanobacteria;
    - Sanitise the inner side of their containers (a sodium hypochlorite solution typically found in household bleach or a detergent) regularly to prevent biofilm formation.

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INDICATOR ANALYSES

ENTEROBACTERIACEAE CULTURE MEDIUM

The medium used for the culture of enterobacteriaceae is the Selective *E. coli*/coliform chromogenic medium CM1046 from Oxoid SA. It is a selective, chromogenic medium for the detection and enumeration of *Escherichia coli* and other coliforms from water samples.

A1 Composition of the medium

Formula	gm/litre
Peptone	**8.0**
Di-sodium hydrogen phosphate	2.2
Sodium chloride	5.0
Potassium di-hydrogen phosphate	1.8
Sodium lauryl sulphate	0.1
Chromogenic mix	0.35
Agar	10.6

PH 6.7 ± 0.2

A2 Preparation of the medium

Suspend 28.1 g of Selective *E. coli*/Coliform Chromogenic Medium in 1 litre of distilled water. Bring the medium gently to the boil, to dissolve completely. Either pour the medium into sterile Petri dishes or keep at 45°C for pour plate technique.

A3 Principle

The recovery and enumeration of *Escherichia coli* and coliforms are important indicators of environmental hygiene. Detection of β-glucuronidase activity is widely used to differentiate *Escherichia coli* bacteria, as the enzyme, which is encoded by the uidA gene, is present in *Escherichia coli*, but not other members of the coliform group. As coliforms are lactose positive, β-galactosidase activity, encoded by the lacz gene, is then used to differentiate this group from the other organisms able to grow on the selective medium.

Selective E. coli/Coliform Chromogenic Medium contains two chromogenic agents:-Rose-Gal-which detects β-galactosidase activity; X-Glu-which detects β-glucuronidase activity. The medium also contains sodium lauryl sulphate which acts as a selective agent, inhibiting

the growth of Gram-positive organisms. Most organisms in the coliform group are able to ferment lactose, so will cleave the pink Rose-Gal chromogen, producing pink colonies. *Escherichia coli* strains can be differentiated from the other coliforms as they also possess the enzyme β-glucuronidase (which has been shown to be highly specific to *Escherichia coli*). The X-Glu chromogen is targeted by this enzyme. The ability of *Escherichia coli* species to cleave both chromogens means that typical colonies will be purple.

A4 Technique

Heavily contaminated water samples should first be diluted so that the number of colonies to be counted is of a readable number e.g. 20-100 colonies.

APPENDIX B

NUTRIENTS ASSESSMENT

B1 NITRATE (NITRATE-NITROGEN)

B1.1 Principle

Nitrate ions in solutions containing sulfuric acids react with 2,6-dimethylphenol to form 4-nitro-2,6-dimethylphenol.

B1.2 Reaction

1 Pipette 1 ml of sample in the Nitrate kit (LCK 339, from CA Milseh, SA);

2 Add 0.2 ml solution LCK 339 A to the mixture;

3 Close cuvette and invert a few times until no more streaks can be seen;

4 After 15 min thoroughly clean the outside of the cuvette and evaluate with the Dr Lange Xion 500 spectrophotometer (GmbH & co.kg. Berlin, Germany).

B2 PHOSPHATE (TOTAL PHOSPHATE)

B2.1 Principle

Phosphate ions react with molybdate and antimony ions in an acidic solution to form an antimonyl phosphomolybdate complex, which is reduced by ascorbic acid to phosphomolybdenum blue.

B2.2 Reaction

1 Carefully remove the foil from the screwed-on DosiCap Zip

2 Unscrew the DosiCap Zip

3 Pipette 2 ml sample into the cuvette

4 Screw the DosiCap Zip back; fluting at the top

5 Shake firmly

6 Heat at high temperature (>150°C) for 15 min or at 100°C for 60 min

7 Cooled the cuvette (18-20°C) and add 0.2 ml reagent B (LCK 348/349/350 B)

8 Screw a grey DosiCap C (LCK 348/349/350 C) onto the cuvette

9 Invert a few times. After 10 min invert a few times more, thoroughly clean the outside of the cuvette and evaluate with the Dr Lange Xion 500 spectrophotometer (GmbH & co.kg. Berlin, Germany).

1) – 8) Total phosphate; 1), 7) – 8) Phosphate

APPENDIX C

DATA

Tables for C1 Occurrence of cyanobacteria and other phytoplankton in water from storage container

Table C1a Composition of cyanobacteria and other phytoplankton in water from storage containers

Parameters	Microcystis				Oscillatoria				Anabaena				Pseudanabaena			
	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB
n	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38
Geomean	0.12	1.07	0.05	-0.18	-0.77	-0.37	-0.80	-0.83	-0.89	-0.91	-0.89	-0.88	-0.76	ND	ND	ND
Median	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Min	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Max	4.39	5.84	4.84	5.18	3.32	5.11	2.97	5.61	3.12	2.32	3.07	3.63	3.79	ND	ND	ND
StDev	1.87	2.58	1.82	1.71	0.97	1.67	0.88	1.07	0.67	0.54	0.66	0.75	1.04	0	0	0
95th Perc	4.18	5.40	3.43	2.93	-0.36	3.46	-0.43	ND	ND	ND	ND	ND	-0.34	ND	ND	ND

Parameters	Aphanocapsa				Radiocystis				Spirulina			
	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB
n	38	38	38	38	38	38	38	38	38	38	38	38
Geomean	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Median	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Min	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Max	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
StDev	0	0	0	0	0	0	0	0	0	0	0	0
95th Perc	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Parameters	Other phytoplankton			
	LFV	LDB	DFV	DDB
n	38	38	38	38
Geomean	0.42	0.76	0.36	0.00
Median	ND	1.531479	ND	ND
Min	ND	ND	ND	ND
Max	2.82	3.81	2.77	3.30
StDev	1.54	1.75	1.56	1.53
95th Perc	2.54	2.99	2.74	2.74

LFV: Light free volume; LDB: Light dislodged biofilm; DFV: Dark free volume; DDB: Dark dislodged biofilm; ND: Not-detected

Table C1b Percentage occurrence of cyanobacteria in water-storage containers

Sample types	Cyanobacteria genera	Percentage of general occurrence	Percentage in sample types containing cyanobacteria
LFV	Microcystis	29	69
	Oscillatoria	2	13
	Anabaena	3	6
	Pseudoana	5	13
	Aphanocapsa	0	0
	Radiocystis	0	0
	Spirulina	0	0
LDB	Microcystis	42	73
	Oscillatoria	13	23
	Anabaena	3	5
	Pseudoana	0	0
	Aphanocapsa	0	0
	Radiocystis	0	0
	Spirulina	0	0
DFV	Microcystis	26	77
	Oscillatoria	5	15
	Anabaena	3	8
	Pseudoana	0	0
	Aphanocapsa	0	0
	Radiocystis	0	0
	Spirulina	0	0
DDB	Microcystis	21	80
	Oscillatoria	3	10
	Anabaena	3	10
	Pseudoana	0	0
	Aphanocapsa	0	0
	Radiocystis	0	0
	Spirulina	0	0

LFV: Light free volume; LDB: Light dislodged biofilm; DFV: Dark free volume; DDB: Dark dislodged biofilm

Tables for C2 Total cyanobacteria and other phytoplankton (cell/ml) in the water sources

Table C2a October (2005)

Sample ID	Cyanophyceae		Other Phyto-groups							
			Bacillanophyceae		Chlorophyceae		Dinophyceae		Euglenophyceae	
	Species	Number	Species	Number	Species	Number	Species	Number	Species	Number
ND	Microcystis sp	44,006,312	ND		ND		ND		ND	
	Pseudanabaena	3,721,462								
LD	Microcystis sp	64,505	Pennate diatoms	2,481	ND		ND		ND	
SR	ND		Centric diatoms	34	ND		ND		ND	
			Pennate diatoms	138						
LR	Anabaena sp	17,367	Pennate diatoms	29,772	ND		ND		ND	
N1	ND		Centric diatoms	1,240	Scenedesmus	7,443	ND		ND	
			Pennate diatoms	1,240						
N2	Oscillatoria sp	340	Centric diatoms	34	Scenedesmus	345	ND		ND	
			Pennate diatoms	207						
	Radocystis sp	103								
	Spirulina sp	103								
Ca	Microcystis sp	241	Centric diatoms	896	Chlamidomonas	276	ND		Trachelomonas	103
			Pennate diatoms	34	Scenedesmus	551				
Bo	ND		Pennate diatoms	34	ND		ND		ND	

ND: Nwanedi Dam, LD: Luphephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal, Bo: Borehole

Table C2b December (2005)

Sample ID	Cyanophyceae		Other Phyto-groups								Phyto problem			
			Bacillanophyceae		Chlorophyceae		Dinophyceae		Euglenophyceae		Filter clogg	Organic pollution	Taste odour	Toxic
	Species	Number	Species	Number	Species	Number	Species	Number	Species	Number				
LD	Microcystis sp	1,212,583	ND		ND		ND		ND				1,212,583	1,212,583
ND	Microcystis sp	57,856,632	Centric diatoms	18,607	Scenedesmus	12,405	ND		ND		80,632	24,810	57,918,656	57,918,656
	Anabaena sp	62,025	Pennate diatoms	6,202	Tetraedron	6,202								
Ca	ND		Centric diatoms	103	Scenedesmus	138	ND		ND		103	207		
			Pennate diatoms	69										
N2	Aphanocapsa sp	245	Pennate diatoms	276	Scenedesmus	69	ND		ND			345	1,551	1,551
	Microcystis sp	1206												
SR	ND		Pennate diatoms	69	ND		ND		ND			69		
LR	ND		Centric diatoms	276	Cosmarium	103	ND		ND		276	2,998	241	
			Pennate diatoms	2,826	Chlamidomonas	241								
					Monoraphidium	69								
					Scenedesmus	69								

ND: Nwanedi Dam, LD: Luphephe Dam, SR: Savhani River, N2: Nwanedi River 2, Ca: Canal.

Table C2c February (2006)

Sample ID	Cyanophyceae		Other Phyto-groups								Phyto problem			
			Bacillariophyceae		Chlorophyceae		Dinophyceae		Euglenophyceae		Filter clogg	Organic pollution	Taste odour	Toxic
	Species	Number	Species	Number	Species	Number	Species	Number	Species	Number				
LD	Anabaena sp	1,723	Centric diatoms	402	Pandorina morum	459	Dinophyte	57	ND		2,125	517	63,460	62,944
	Microcystis sp	61,221	Pennate diatoms	57										
ND	Microcystis sp	69,158	Pennate diatoms	413	Scenedesmus	413	Dinophyte	310	ND			827	69,468	69,158
Ca	ND		Centric diatoms	34	Scenedesmus	69	ND		ND		34	69		
N2	ND		Centric diatoms	34	Scenedesmus	138	ND		ND		34	689		
			Pennate diatoms	551	Monoraphidium	207								
SR	ND		Pennate diatoms	517	Monoraphidium	103	Dinophyte	103	ND			1,861	103	
					Cosmarium	1,344								
LR	Microcystis sp	92	Pennate diatoms	1,516	Actinastrum hantzchii	368	ND		ND			1,562	92	92
					Cosmarium	46								
					Monoraphidium	46								
Bo	ND		ND		ND		ND		ND					
N1	Spirulina	2,688	Pennate diatoms	69	Chlamydomonas	34	ND		ND			69	34	

ND: Nwanedi Dam, LD: Lephephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal; Bo: Borehole.

Table C2d April (2006)

Sample ID	Cyanophyceae		Other Phyto-groups								Phyto problem			
			Bacillariophyceae		Chlorophyceae		Dinophyceae		Euglenophyceae		Filter clogg	Organic pollution	Taste odour	Toxic
	Species	Number	Species	Number	Species	Number	Species	Number	Species	Number				
ND	Microcystis sp	4,689	Centric diatoms	207	Monoraphidium	69	ND		ND		413		4,893	4,893
	Anabaena sp	207	Nitzschia sp	207										
LD	Microcystis sp	7,112	Centric diatoms	83	ND		ND		ND		83		7,112	7,112
SR	ND		Pennate diatoms	34	ND		ND		ND			34		
			Nitzschia sp	34										
LR	Microcystis sp	69	Centric diatoms	34	ND		ND		ND		34		69	69
N1	Microcystis sp	5,709	Pennate diatoms	248	Coccomonas sp	83	ND		ND		2,977	496	7,939	7,939
	Anabaena sp	2,233	Centric diatoms	744	Staurastrum tetracerum	83								
	Raphocystis sp	662			Scenedesmus sp	165								
N2	Microcystis sp	512	Centric diatoms	138	ND		ND		ND		620		1,585	1,585
	Anabaena sp	482												
Ca	Microcystis sp	3,067	Pennate diatoms	34	Scenedesmus sp	207	ND		ND		1,240	276	3,687	3,653
	Anabaena sp	584	Centric diatoms	655	Euastrum sp	34								
	Raphocystis sp	689	Navicula sp	34	Coccomonas sp	34								
					Tetraedron sp	34								
					Chlamydomonas sp	34								
Bo	ND		ND		ND		ND		ND					

ND: Nwanedi Dam, LD: Lephephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal, Bo: Borehole.

Table C3 Comparison of *Microcystis spp* numbers (log) in sample categories

Group	n	Mean of the logs	5th percentile	95th percentile	Anova on ranks (Kruskal-Wallis)
Sources	19	0.23	ND	3.7	No significant difference P=0.696 H_o not rejected
FV	83	0.09	ND	4.0	
DB	83	0.45	ND	5.2	

Mcs *Microcystis spp*, FV: Free volume, DB: Dislodged biofilm

Tables for C4 Microcystin in water

Table C4a Microcystin in water-storage containers

	Sample names (LDB)								
	MH 03	MH 17	MH 85	GHO 41	GHO 42	TOH 09	DAH 12	DAH 13	FH 76
Microcystins µg/l	ND	ND	ND	ND	0.36	ND	ND	ND	ND

LDB: Water from light containers with dislodged biofilm

Table C4b Microcystin in environmental water sources

Parameters	Microcystins µg/l							
	ND	LD	SR	LR	N1	N2	Ca	Bo
n	4	4	2	2	2	3	4	2
Mean	2.15	2.33	0.18	0.18	1.26	0.55	1.36	0.18
Median	2.50	2.40	0.18	0.18	1.26	0.18	1.39	0.18
Geomean	2.04	2.32	0.18	0.18	0.83	0.35	0.94	0.18
Min	1.10	2.00	0.18	0.18	0.31	0.18	0.18	0.18
Max	2.50	2.50	0.18	0.18	2.20	1.30	2.50	0.18
SD	0.70	0.24	0.00	0.00	1.34	0.65	1.01	0.00
95th Perc	2.50	2.50	0.18	0.18	2.01	1.08	2.29	0.18

ND: Nwanedi Dam, LD: Luphephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal, Bo: Borehole.

Tables for C5 Endotoxin in water

Table C5a Endotoxin in container water

Parameters	LFV	LDB	DFV	DDB
n	44	44	44	44
Mean of the log	1.50	2.07	1.51	1.84
Median	1.33	2.07	1.14	1.80
Min	0.33	1.18	ND	0.66
Max	2.16	2.39	2.28	2.30
Standard Deviation	1.57	1.72	1.61	1.66
95% Confidence Interval	1.9	2.26	1.87	2.14

Table C5b Endotoxin in water sources

Parameters	Endotoxin EU/ml							
	ND	LD	SR	LR	N1	N2	Ca	Bo
n	4	4	4	4	3	4	4	3
Mean	28.67	17.64	10.44	4.66	22.43	25.20	14.11	ND
Median	13.09	17.53	8.30	4.05	7.20	12.35	5.62	0.00
Geomean	16.93	14.66	8.73	4.15	10.49	14.40	8.16	ND
Min	5.80	5.10	4.00	2.23	2.80	4.60	3.60	ND
Max	82.70	30.40	21.15	8.30	57.30	71.50	41.60	0.00
SD	36.18	10.64	7.43	2.63	30.28	31.31	18.39	0.06
95th Perc	61.93	27.48	17.38	7.22	47.28	55.15	31.23	0.00

ND Nwanedi Dam, LD Luphephe Dam, SR Savhanu River, N1 Nwanedi River 1, N2 Nwanedi River 2, Ca Canal, Bo Borehole.

Table C6 Nutrients (nitrate and phosphate) concentration in water-storage containers

Parameters	Nitrate mg/l				Phosphate mg/l			
	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB
n	44	24	44	24	44	24	44	24
Mean	4.67	6.80	5.89	6.56	0.26	0.90	0.28	0.62
Median	2.44	3.85	3.22	4.14	0.08	0.56	0.10	0.34
Geomean	2.32	4.34	3.11	4.29	0.11	0.52	0.15	0.41
Min	0.28	0.83	0.30	1.14	0.02	0.03	0.03	0.11
Max	50.90	51.90	55.80	54.70	2.71	4.74	1.76	2.52
SD	8.39	10.13	11.03	10.52	0.52	1.04	0.41	0.61
95th Perc	7.46	10.57	7.67	8.80	0.44	1.91	0.88	1.31

LFV Light free volume, LDB Light dislodged biofilm, DFV Dark free volume, DDB Dark dislodged biofilm

Table C7 Nutrients (nitrate and phosphate) concentration in the water sources

Parameters	Nitrate mg/l								Phosphate mg/l							
	ND	LD	SR	LR	N1	N2	Ca	Bo	ND	LD	SR	LR	N1	N2	Ca	Bo
n	4	4	2	2	2	3	4	2	4	4	2	2	2	3	4	2
Mean	0.14	0.11	0.10	0.44	0.18	0.13	0.39	2.10	0.07	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Median	0.13	0.10	0.10	0.44	0.18	0.14	0.42	2.10	0.07	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Geomean	0.13	0.11	0.10	0.43	0.16	0.13	0.32	2.09	0.07	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Min	0.10	0.10	0.10	0.42	0.10	0.10	0.10	1.90	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Max	0.20	0.15	0.10	0.45	0.26	0.16	0.63	2.30	0.09	0.05	0.05	0.05	0.05	0.05	0.05	0.05
SD	0.05	0.03	0.00	0.02	0.11	0.03	0.22	0.28	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
95th Perc	0.19	0.14	0.10	0.45	0.24	0.16	0.58	2.26	0.09	0.05	0.05	0.05	0.05	0.05	0.05	0.05

ND Nwanedi Dam, LD Luphephe Dam, SR Savhanu River, N1 Nwanedi River 1, N2 Nwanedi River 2, Ca Canal, Bo Borehole

Table C8 Physical quality of water in water-storage containers

Parameters	Temperature				pH			
	LFV	LDB	DFV	DDB	LFV	LDB	DFV	DDB
n	44	44	44	44	44	44	44	44
Mean	26.04	25.97	26.10	26.10	7.07	6.95	7.02	6.90
Median	25.20	25.20	25.20	25.20	7.06	6.90	7.10	6.98
Geomean	25.79	25.74	25.85	25.85	7.04	6.91	6.99	6.87
Min	18.60	18.60	18.60	18.60	4.99	4.90	4.84	4.99
Max	35.20	35.20	35.20	35.20	8.60	8.41	8.79	8.12
SD	3.69	3.57	3.74	3.74	0.68	0.70	0.70	0.63
95th Perc	32.34	31.50	31.85	31.85	7.77	7.76	7.72	7.62

LFV Light free volume, LDB Light dislodged biofilm, DFV Dark free volume, DDB Dark dislodged biofilm

Tables for C9 Physico-chemical quality of the source water

Table C9a October (2005)

Constituents	LD	Reservoir	ND	Ca
Conductivity at 25°C (mS/m)	12.00	46.00	10.00	11.00
pH at 25°C	7.50	7.70	7.50	7.20
Turbidity in nephlelometric units	7.50	0.30	26.00	2.10
M Alkalinity as $CaCO_3$ in mg/ℓ	30.00	105.00	24.00	28.00
Fluoride as F in mg/ℓ	0.05	0.95	0.05	0.05
Sulphate as SO_4 in mg/ℓ	5.00	25.00	5.00	5.00
Chloride as Cl in mg/ℓ	16.00	58.00	15.00	15.00
Cobalt as Co in mg/ℓ	0.02	0.05	0.02	0.02
Molybdenum as Mo in mg/ℓ	0.01	0.01	0.01	0.01
Calcium as Ca in mg/ℓ	6.30	14.00	5.00	6.60
Magnesium as Mg in mg/ℓ	4.00	11.00	3.50	3.90
Sodium as Na in mg/ℓ	8.70	60.00	7.80	7.80
Potassium as K in mg/ℓ	0.58	2.40	0.41	0.48
Cadmium as Cd in mg/ℓ	0.00	0.00	0.00	0.00
Chromium as Cr in mg/ℓ	0.01	0.01	0.01	0.01
Copper as Cu in mg/ℓ	0.01	0.01	0.01	0.01
Iron as Fe in mg/ℓ	0.03	0.01	0.06	0.42
Manganese as Mn in mg/ℓ	0.00	0.16	0.00	0.00
Lead as Pb in mg/ℓ	0.01	0.01	0.01	0.01
Zinc as Zn in mg/ℓ	0.01	0.04	0.01	0.03
Aluminium as Al in mg/ℓ	0.01	0.01	0.02	0.01
Sulphur as S in mg/ℓ	0.82	8.10	0.76	0.71
Boron as B in mg/ℓ	0.02	0.07	0.02	0.02
Ammonia as N in mg/ℓ	0.05	0.05	0.05	0.05
Nickel as Ni in mg/ℓ	0.02	0.02	0.02	0.02
Phosphar as P in mg/ℓ	0.04	0.04	0.04	0.67
Total silica as Si in mg/ℓ	1.40	44.00	4.20	3.80
Ortho Phosphate as P in mg/ℓ	0.05	0.05	0.08	0.05
Active Silica as Si in mg/ℓ	0.65	21.00	2.00	1.80
Strontium as Sr in ug/ℓ	8.70	11.00	6.00	9.00
Nitrate as N in mg/ℓ	0.10	1.60	0.16	0.38
Nitrite as N in mg/ℓ	0.07	0.10	0.31	0.12

ND: Nwanedi Dam, LD: Luphephe Dam, Ca: Canal; Reservoir

Table C9b December (2005)

Constituent	LD mg/l	ND mg/l	Ca mg/l	N2 mg/l
PO4	0.05	0.09	0.05	0.05
NO3	0.10	0.20	0.63	0.14
IC_SO4	5.0	5.0	0.05	15.00
IC_F	0.59	0.28	5.0	0.78
IC_Cl	19.00	18.00	13.00	65.00
Cd	0.003	0.003	0.003	0.003
Cr	0.010	0.010	0.010	0.010
Mo	0.010	0.010	0.010	0.010
Pb	0.010	0.010	0.010	0.010
Co	0.015	0.015	0.015	0.015
Ni	0.015	0.015	0.02	0.015
V	0.030	0.030	0.030	0.030
Cu	0.02	0.02	0.03	0.02
Mn	0.04	0.01	0.01	0.003
P	0.07	0.0410	0.05	0.07
B	0.09	0.03	0.03	0.07
Fe	0.10	0.03	0.85	0.10
Al	0.11	0.010	0.85	0.07
Zn	0.14	0.02	0.02	0.01
S	0.96	0.94	0.90	5.90
K	1.10	0.76	0.77	1.20
Si	1.50	2.10	3.50	3.40
Na	11.00	7.80	9.20	38.00
Totalsilica	3.20	4.60	7.50	7.30
Mg	4.30	3.50	4.50	15.00
Ca	7.20	5.10	7.60	16.00
Sr	38.00	23.00	36.00	120.00
Conductivity	12.00	9.30	12.00	40.00
M Alk	37.00	26.00	35.00	83.00
pH	6.55	6.74	6.96	7.96
NO2	0.03	0.09	0.13	0.10
Turb	495.00	17.00	40.00	24.00
Temp	29.80	27.60	24.60	25.50

ND: Nwanedi Dam, LD: Luphephe Dam, N2: Nwanedi River 2, Ca: Canal.

Table C9c February (2006)

Constituent	LD mg/l	ND mg/l	Ca mg/l	N2 mg/l	LR mg/l	Bo mg/l	SR mg/l	N1 mg/l
NH4	0.05	0.05	0.27	0.05	0.05	0.05	0.05	0.31
NO3	0.15	0.10	0.46	0.16	0.45	2.30	0.10	0.26
PO4	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
IC_SO4	5.00	5.00	5.00	9.00	5.00	22.00	5.00	5.00
IC_F	0.06	0.05	0.05	32.00	0.05	0.68	0.05	0.05
IC_Cl	15.00	11.00	14.00	32.00	11.00	54.00	10.00	14.00
Cd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cr	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Mo	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Pb	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Co	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Ni	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
V	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Cu	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Mn	0.01	0.00	0.01	0.01	0.01	0.20	0.01	0.01
P	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
B	0.02	0.02	0.02	0.05	0.01	0.07	0.01	0.02
Fe	0.11	0.11	2.50	0.62	0.41	0.01	0.19	2.30
Al	0.07	0.03	0.05	0.07	0.07	0.02	0.05	0.08
Zn	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S	0.90	0.76	0.56	3.10	0.53	7.10	0.40	0.57
K	0.31	0.31	0.31	0.57	0.31	2.00	0.31	0.31
Si	1.90	2.50	3.00	3.60	3.70	19.00	2.90	3.00
Na	7.20	4.90	6.50	20.00	4.90	57.00	4.20	6.70
Totalsilica	4.00	5.40	6.30	7.70	8.00	42.00	6.10	6.50
Mg	3.30	2.20	3.40	9.60	2.00	13.00	1.30	3.30
Ca	5.10	3.10	5.10	11.00	1.50	15.00	1.50	5.10
Sr								
Conductivity	8.80	5.90	8.90	24.00	4.80	45.00	4.20	8.90
M Alk	31.00	21.00	33.00	64.00	14.00	115.00	16.00	32.00
pH	7.19	6.88	6.95	7.88	6.30	7.43	6.68	7.19
NO2	0.03	0.03	0.03	0.03	0.03	0.05	0.03	0.04
Turb	4.80	6.10	5.50	8.00	1.70	0.22	3.60	17.00
SiO2	1.50	2.00	2.20	2.50	2.90	14.00	2.40	2.20
Temp	29.30	30.20	24.70	30.50	24.80	34.80	24.70	29.40

ND: Nwanedi Dam, LD: Luphephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal, Bo: Borehole.

Table C9d April (2006)

Constituent	ND mg/ℓ	LD mg/ℓ	SR mg/ℓ	LR mg/ℓ	N1 mg/ℓ	N2 mg/ℓ	Ca mg/ℓ	Bo mg/ℓ
NH4	0.05	0.27	0.05	0.05	0.05	0.05	0.05	0.05
IC_SO4	5.00	5.00	5.00	5.00	5.00	5.80	5.00	26.00
IC_F	0.05	0.05	0.05	0.05	0.05	0.07	0.05	0.98
IC_Cl	9.92	13.00	9.30	9.70	12.00	26.00	12.00	42.00
Cd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cr	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01
Mo	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Pb	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Co	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Ni	0.02	0.02	0.02	0.02	0.02	0.07	0.02	0.02
V	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Cu	0.05	0.06	0.03	0.03	0.03	0.05	0.01	0.04
Mn	0.01	0.01	0.01	0.00	0.04	0.02	0.01	0.20
P	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
B	0.01	0.01	0.01	0.01	0.02	0.03	0.02	0.06
Fe	0.15	0.06	0.29	0.26	0.34	0.45	0.28	0.02
Hardness	18.00	24.00	6.70	9.20	22.00		21.00	85.00
Al	0.02	0.02	0.07	0.03	0.05	0.16	0.01	0.01
Zn	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01
S	0.64	0.74	0.56	0.66	0.91	2.50	0.89	8.00
K	0.49	0.57	0.31	0.31	0.31	0.31	0.31	1.80
Si	2.30	2.00	3.00	3.70	2.70	3.80	2.60	20.00
Na	6.00	7.70	4.30	4.60	6.30	16.00	6.10	55.00
Totalsilica	5.00	4.20	6.50	8.00	5.80	8.00	5.50	43.00
Mg	2.40	3.00	1.10	1.60	2.80	7.30	2.60	12.00
Ca	3.20	4.50	0.84	1.00	4.10	8.10	3.90	14.00
Sr	15.00	22.00	6.20	8.10	19.00	62.00	20.00	98.00
Conductivity	5.20	7.20	3.10	3.80	6.70	17.00	6.60	45.00
M Alk	19.00	25.00	9.00	9.30	24.00	47.00	24.00	120.00
pH	6.74	6.94	6.31	6.05	6.80	7.22	6.71	7.22
NO2	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Turb	3.70	2.30	4.10	1.40	6.00	9.30	3.00	0.38
SiO2	1.70	1.40	2.00	2.40	1.60	2.20	1.70	14.00
P alk	5.00	5.00				5.00		
Tl	5.00	5.00						
Ti	2.90	3.70						
Te	2.00	2.00						
Ba	12.00	13.00						
Be	2.00	2.00						
PO4	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
NO3	0.10	0.10	0.10	0.42	0.10	0.10	0.10	1.90

ND: Nwanedi Dam, LD: Luphephe Dam, SR: Savhani River, N1: Nwanedi River 1, N2: Nwanedi River 2, Ca: Canal, Bo: Borehole

STATISTICAL ANALYSES

D1 DATA MANAGEMENT

In the field, the physical data were recorded on sample sheets then later transferred to Excel spreadsheets. To remove any excessive variance data were converted to logarithmic values. These logs were then transferred to a plotter programme (SigmaPlot V9®) and expressed in mostly vertical box plots, with a number of scatter and line plots used as well. For Descriptive Statistical Analyses, the following parameters were used:

D1.1 Central values

- For the average of the logs the mean was used;
- To visualise normality the median was used;
- For variance, the 95th confidence interval was used based on the standard deviation;
- Compliance was measured at the 95th percentile (SANS, 2005).

D2 HYPOTHESES

Setting up and testing hypotheses is an essential part of statistical inference.

Statistical tests were used to determine whether the hypothesis should be accepted or rejected. Hypotheses were formulated (Section 2.10; Chapter 2: Methodology) and later used to discuss the results in chapter 3.

D2.1 Zero hypothesis (H_0)

The zero hypothesis is given special consideration in statistical tests, this is due to the fact that this hypothesis relates to the statement being tested (Helsel and Hirsch, 2002).

In this study the zero hypothesis (H_0) represents a theory that has been put forward, either because it is believed to be true or because it is to be used as a basis for argument, but has not been proved.

The final conclusion once the test has been carried out is always given in terms of the zero hypothesis. This means the H_0 was either rejected or accepted.

D2.2 Significance of P-values

The P-value represents the statistical significance of the difference between the average of the analyzed parameters in the free volume and dislodged biofilm samples. Depending of the interval to which the P value belongs, the difference was considered as follow:

- P<0.05: There was a significant difference between two types of samples for the parameter analyzed;
- P>0.05: There was no significant difference between two types of samples for the parameter analyzed.

D3 ANALYSES OF VARIANCE: FREE VOLUME AND DISLODGED BIOFILM

To indicate the role of containers in the occurrence of cyanobacteria in storage containers, this study investigated, indirectly, attachment of biofilm to the inner-sides of containers (Section 2.10, Chapter 2: Methodology). To demonstrate whether the turbidity caused by dislodged biofilm (DB) suspended into the initial free volume (FV) of container water, the FV and DB values were compared using the Signed Rank (Wilcoxon) test. The same statistical method was used to compare the numbers cyanobacteria, *Escherichia coli*, total coliforms, and toxins.

The Rank Sum (Mann-Whitney) test was used to compare the numbers of cyanobacteria in samples from the FV and DB waters from the light as well as the dark containers.

The ANOVA on Ranks (Kruskal-Wallis) was used to compare group of data sets.

These two tests are non-parametric, meaning that they are used to analyze data that do not fit a normal distribution and are based on the rank order of measurements rather than their values (Helsel and Hirsch, 2002). The used of these tests in this study doesn't imply that the data were non-parametric but because they strengthen the analyses as non-parametric tests are also capable to analyze parametric data sets.

The principles of the two statistical methods used are as follows:

D3.1 Sign Rank test

The Signed-Rank test was used to determine the significant difference between paired data sets (i.e free volume of water and dislodged biofilm) at the 95th percentile level, but the data were not assumed parametric (t-test discarded).

D3.2 Rank sum test:

The Rank-sum test is a non-parametric test, used to test whether one group tends to produce larger observations than the second group.

This test can also be used to determine whether the two groups come from the same population (same median and other percentiles), or whether they differ only in location (central value or median) (Helsel and Hirsch, 2002).

In the context of this study, the Rank-sum text was used to determine the significant difference between the data sets from light and dark containers or from the different environmental sources.

D3.3 Kruskal-Wallis ANOVA on Ranks

The Kruskal-Wallis statistic is the non-parametric equivalent of the one-way ANOVA. It is used when there are 3 or more groups of non-parametric data (i.e. various water sources used by the population). It is a generalization of the rank sum test to three or more groups (Helsel and Hirsch, 2002).

D4 GRAPH INTERPRETATION

The data recorded in excel spreadsheet were expressed graphically using the vertical box plot. Box plot can be used to inspect a single data as well as to compare multiple data sets.

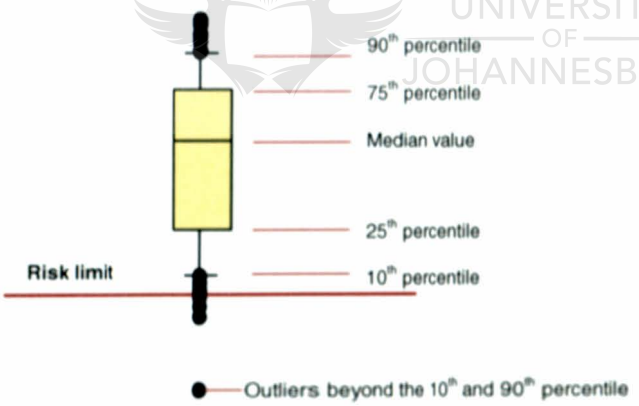


Figure D: Example of box plot.

In this study, microcystins, endotoxin, microorganisms and physico-chemical data sets from water sources and in water-storage containers were plotted on Sigmaplot V9® and parameters such as skewness, median and the interquartile range observed, giving clues for interpretation.

- **Skewness**: They represent the extreme values, meaning that data sets are not symmetries around the mean or median

- **Median**: The median, or 50th percentile, is the central value of the distribution when the data are ranked in order of magnitude. For an odd number of observations, the median is the data point which has an equal number of observations both above and below it. For an even number of observations, it is the average of the two central observations.

Interquartile range (IQR): It is the most commonly used resistant measure of spread; it is defined as the 75th percentile minus the 25th percentile. The 75th percentile, also called the upper quartile, is a value which exceeds no more than 75 percent of the data and is exceeded by no more than 25 percent of the data. The 25th percentile or lower quartile is a value which exceeds no more than 25 percent of the data and is exceeded by no more than 75 percent. The 95th percentile not shown on the figure above was used in this study to determine the compliance of water quality and represent the value, which exceeds no more than 95 percent of the data and is exceeded by no more than 5 percent of the data.

APPENDIX E

CLASSES OF PHYTOPLANKTON INCLUDING CYANOBACTERIA

E1 SOME GROUPS OF CYANOBACTERIA

Order (Family)	Representative Genera
Chroococcales	**Gloeothece, Synechoccus,** (Anacystis, Agmenellum) Gloeocapsa, Chroococcus, Synechocystis, Microcystis, Merismopedia
Chamaesiphonales	**Chamaesiphon, Dermocarpa**
Dermocarpella, Chroococcidiopsis	
Pleurocapsales	Xenococcus, Myxosarcina, Pleurocapsa, Hyella
Nostocales	Oscillatoria, Microcoleus, Spirulina, Pseudanabaena, Plectonema, Lyngbyna, Phormidium, Schizothrix
Nostocaceae	Anabaena, Aphanizomenon, Nostoc, Nodularia, Anabaenopsis Cylindrospermum
Rivulariaceae	Calothrix, Dichothrix, Gloeotrichia, Rivularia
Scytonemataceae	Scytonema, Tolypothrix
Stigonematales	Mastigocoleus, Nostochopsis, Mastigocladus, Westiella, Fischerella, Hapalosiphon, Stigonema, Chlorogloeopsis

E2 SOME TOXINS-PRODUCING STRAINS (CYANOBACTERIA)

Microcystis aeruginosa Kutz.
Microcystis viridis (a. Br.) Lemm
Microcystis wesenbergii Kom
Nodularia spumigena Mertens
Nostoc rivulare Kutz
Oscillatoria actissima Kuff
Oscillatoria agardhii/rubescens group
Oscillatoria nigro-viridis Thwaites
Anabaena circinalis Rabenh
Anabaena flos-aquae (Lyngb.) Breb.
Anabaena spiroides var. contracta Kleb
Anabaena variabilis Kutz.

Aphanizomenon flos-aquae (L.) Ralfs
Oscillatoria agardhii Gom.
Cylindrospermopsis raciborskii (Wolos)
Lyngbya wollei

E3 CYNOBACTERIA AND OTHER PHYTOPLANKTON IDENTIFIED

Classes	Species
Bacillariophyceae	Pennate diatom
	Centric diatom
Chlorophyceae	Scenedesmus
	Chlamidomonas
	Coccomonas
	Monoraphidium
	Cosmarium
	Tetraedron
	Tetrastrum
	Pandorina morum
	Actinastrum hantzchii
Dinophyceae	Dinophyte
Euglenophyceae	Trachelomonas
Cyanophyceae	Microcystis
	Pseudanabaena
	Anabaena
	Oscillatoria
	Aphanocapsa
	Spirulina
	Radiocystis

APPENDIX F

CYANOBACTERIAL TOXINS

F1 NON-SPECIFIC MICROCYSTIN ANALYSES

(Du Preez and Van Baalen, 2005)

1 For microcystin assessment water should be sampled in dark glass bottle and then transferred in a freezable container.

2 Lyophylize the algal cells, by freezing the sample in liquid nitrogen and thawing in water bath or other hot container.

3 Filter the sample by using a 0.45 µm syringe filter for each sample

4 Allow reagents to reach room temperature (18°C to 24°C) before pouring in 96-wells microtiter plate.

5 Calibrate the microtiter plate reader (Envirologix Inc) before commencing with reaction.

6 Set-up automated washer and incubator

7 Mark strips with sample name to prevent confusion

8 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 strip and the actual samples the second strip

9 Mix all reagents with the vortex shaker before use in analysis

10 Pipette 125 µl of microcystin assay diluent to each well that will be used

11 Add 20 µl of negative control, 20 µl of each calibrator and 20 µl of each sample to their respective well in duplicate

12 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

13 Start the timer and add 100 µl of microcystin enzyme conjugate to each well. Repeat step 12

14 After incubation, reset timer, remove the plate covering and then wash plate with the automated microtiter plate washer with wash solution

15 Start timer and add 100 µl of substrate to each well then repeat step 12

16 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

17 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)

Calculation of microcystin concentration

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

2- The percentage coefficient of variance of each pair of calibrators or samples should not exceed 20 %

3- If the microcystin toxin concentration exceeds the concentration of the highest calibrator the sample may be either diluted with reagent water to fall in the range of the calibrators or the concentration may be reported as >2.5 µg/l

F2 LIPOPOLYSACCHARIDE ENDOTOXIN

F.2.1 Composition of LPS endotoxin

LPS consists of three components or regions, **Lipid A, an R polysaccharide and an O polysaccharide:**

- **Region 1. Lipid A** is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of lipid A is highly conserved among Gram-negative bacteria. Among Enterobacteriaceae Lipid A is virtually constant, but vary in cyanobacteria;
- **Region 2. Core antigen or R polysaccharide** is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO-Hep-Hep-Glu-Gal-Glu-Glu-Glu-Nac. Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin). With minor variations, the core polysaccharide is common to all member of a Gram-negative bacterial genus (e.g. Salmonella), but it is structurally distinct in other genera

of Gram-negative bacteria. Salmonella, Shigella and Escherichia have similar but not identical cores.

- **Region 3. Somatic (O) antigen or O polysaccharide** is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3-5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide

F2.2 Detection of LPS endotoxin

In this study the Limulus Amebocyte Lysate (LAL) chromogenic assay (LAL chromogenic QCL 1000 120T SL from Adcock Ingram, SA) was used to detect LPS endotoxin in water sample

F2.2.1 Test procedure

1 Carefully dispense 50 µl of sample or standard into the appropriate endotoxin-free glass tube in a 37°C block or waterbath. Each series of determinations must include a blank plus the four endotoxin standards run in duplicate. The blank tubes contain 50 µl of LAL Reagent Water instead of sample. All reagent additions and incubation times are identical. As the sample is warming reconstitute the reagents as indicated in the prescription provided by the manufacturer.

2 At time T=0, add 50 µl of LAL to the reaction vessel. Begin timing as LAL is added to the first reaction vessel. It is important to be consistent in the order of reagent addition from vessel to vessel and in the rate of pipetting. Thorough mixing of the two solutions is essential, but do not vortex.

3 At T=10 minutes, add 100 µl of substrate solution (prewarmed to 37°C). Pipette the substrate in the same order as in step 2. Maintain a consistent pipetting rate. Assure thorough mixing of solutions.

4 At T=16 minutes, add 100 µl of stop solution. Maintain the same pipetting order and rate as in steps 2 and 3. Mix well.

5 Read the absorbance of each reaction tube at 405-410 nm.

The standard curve is drawn from the OD of the standard solutions knowing the concentration of endotoxin. The equation of the standard curve is expressed as y=ax+b; Where: y is the OD and x is the endotoxin concentration, the equation x=(y-b) allow to determine unknown x.

APPENDIX G

G1 EXAMPLES OF WATER-STORAGE CONTAINERS

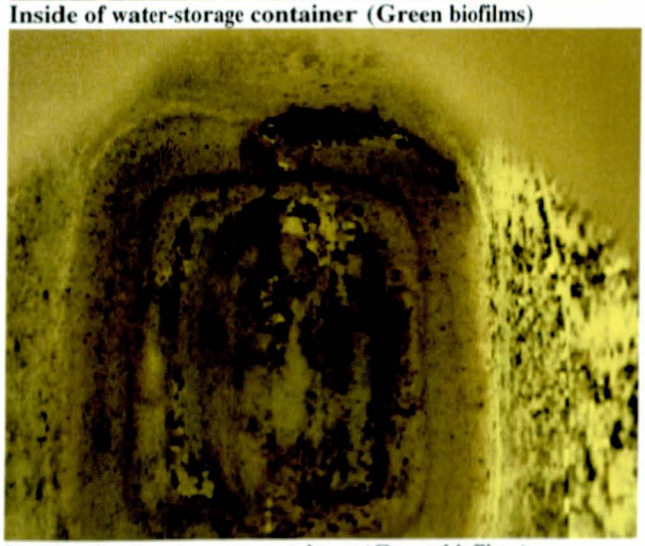
Example of a dark container

Example of light and dark containers

G2 BIOFILM IN LIGHT WATER-STORAGE CONTAINERS

Inside of water-storage container (Green biofilm)

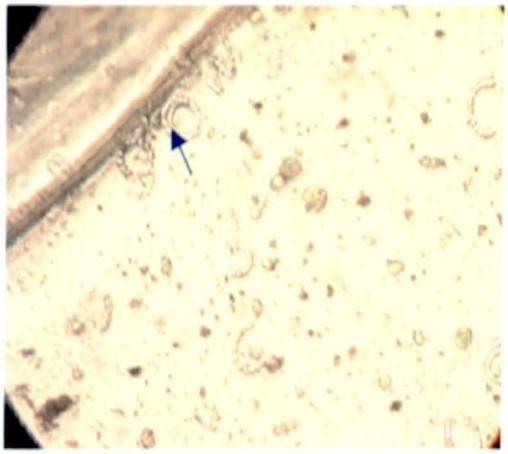
Inside of water-storage container (Green biofilms)



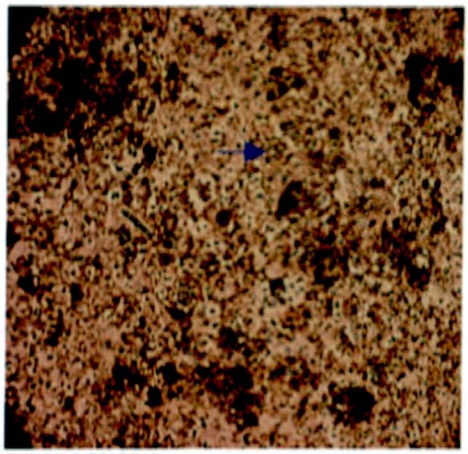
Inside of water-storage container (Green biofilms)

G3 EXAMPLES OF SPECIES IDENTIFIED IN SAMPLES

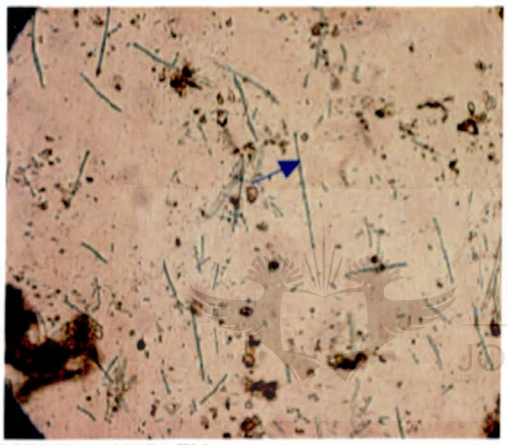
CYANOBACTERIA



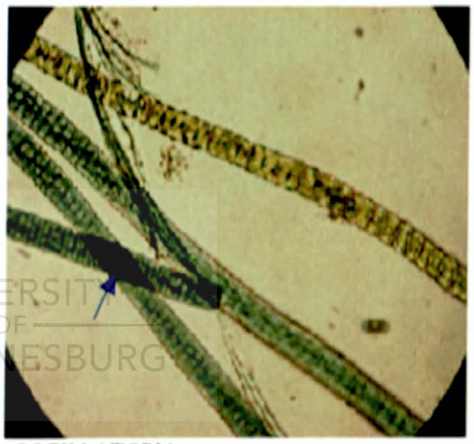
ANABAENA



MICROCYSTIS



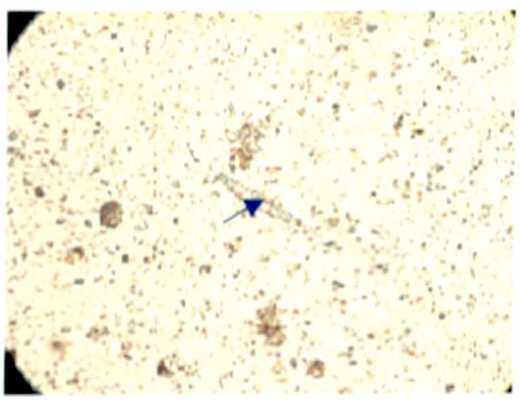
PSEUDOANABAENA



OSCILLATORIA

OTHER PHYTOPLANKTONS

PENNATE DIATOMS



PANDORINA