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Phosphorus Limitation as a Method of Cyanobacterial Bloom Control

BY

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I, the undersigned, hereby declare that the thesis submitted herewith for the degree of Philosophiae Doctor to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other institution.

Signed:

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CHAPTER 1:

INTRODUCTION

Water bodies, both natural and man-made, are impacted by urban, industrial and agricultural activities. As a result, many aquatic ecosystems have become severely degraded and need to be restored to a level that can be permanently sustained through conservation and protection. The water quality targets should be in accordance with the quality of natural waters that are without the stress factors that cause degradation (Klapper, 2003).

Of the problems currently being experienced with natural and man-made water bodies, eutrophication is one of the most important. Eutrophication is the enhancement of the natural process of biological production in rivers, lakes and reservoirs, caused by an increase in nutrient levels, usually phosphorus and nitrogen compounds (Bartram *et al*., 1999). These increased nutrient levels usually result in an increased phytoplankton biomass, which is often dominated by toxic cyanobacterial species. The decay of the increased amount of organic matter may lead to the oxygen depletion in the water, which in turn can cause secondary problems such as fish kills from lack of oxygen. Eutrophication has a severe impact on the water quality and impairs the use of water for drinking, industry, agriculture and recreation (Carpenter *et al*., 1998).

Both the causes and effects of eutrophication need to be considered when restoring a eutrophic water body. The control of toxic cyanobacterial blooms remains a priority, but in treating the blooms in isolation, only a symptom of a greater problem is being addressed. Eutrophication management by reducing nutrient input as well as the internal source is the only feasible means of reducing the incidence of cyanobacterial blooms. It is important that the amount of nutrients entering eutrophic water bodies be drastically reduced. However, the time needed to restore eutrophic lakes and dams to their natural healthy state is longer than expected, as in many shallow lakes the phosphorus accumulated in the sediment may be many times greater than that in solution. This delays the effects of restoration measures through internal loading of phosphorus into the overlying water (Sondergaard *et al*., 2001; Lake *et al*., 2007).

Nutrient limitation through intervention is likely to be the most sustainable solution to eutrophication and its effects. Phosphorus limitation has been identified as being more achievable than nitrogen limitation, and there are chemicals available to achieve this, such as aluminium sulphate (alum), ferric salts (chlorides and sulphates), ferric

aluminium sulphate, clay particles and lime. These, however, have various disadvantages (Chorus & Mur, 1999; Lewandowski *et al*., 2003). In this study an environmentally friendly phosphorus removing product, Phoslock®, was reviewed, characterised and tested under both laboratory and field conditions.

Limiting the amount of phosphorus in a water body, and thus increasing the N:P ratio, is likely to affect the entire microbial community composition, not only that of the cyanobacteria and algae. The various methods available to investigate the structure of microbial communities were reviewed in this study. Denaturing gradient gel electrophoresis (DGGE) was the molecular tool chosen for this study to determine the effect of a reduced phosphorus concentration on the cyanobacterial and bacterial community composition in a field trial.

A bacterial species isolated from a eutrophic dam with cyanobacteriolytic capabilities was examined in the laboratory. The effect of combining this potential biological control agent with Phoslock® was investigated in order to determine whether the two agents could be used together to treat both the cause and symptoms of eutrophication simultaneously, with the Phoslock® treatment removing the phosphates released from the lysed cyanobacterial cells.

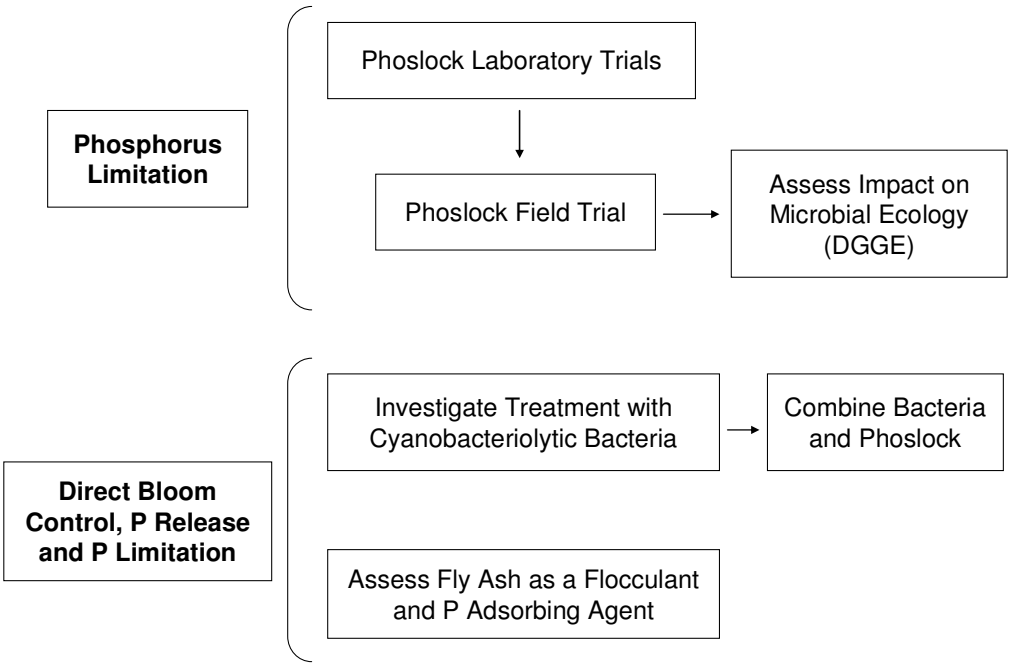
Various flocculants have been investigated for cyanobacterial removal in wastewater treatment as well as in natural water bodies. These include synthetic organic polyelectrolytes, chitosan, and various clays (Divakaran & Pillai, 2002; Sengco & Anderson, 2004; Pan, *et al*., 2006). In this study the use of fly ash, a waste product in the burning of coal for electricity generation, was investigated as a potential cyanobacterial flocculant, and its phosphate adsorbing capability was tested as an alternative to Phoslock®.

The objectives of this study were therefore:

- To characterise Phoslock® in the laboratory in terms of its kinetics, and the effect of pH and initial phosphorus concentration on the adsorption capacity.
- To determine the effectiveness of Phoslock® in water containing a high concentration of cyanobacteria with a high pH, in the laboratory as well as in a field trial.

- To assess the effect of a Phoslock® treatment and a reduction in phosphate concentration on the cyanobacterial and eubacterial species composition of a eutrophic water body using 16S PCR-DGGE.
- To identify a bacterial species isolated from Hartbeespoort Dam that appeared to have lytic activity towards *Microcystis aeruginosa*, and to determine the critical predator-prey ratio for treatment.
- To assess the possibility of combining the lytic bacteria with Phoslock® in order to produce a novel biological control product that can treat the cause of cyanobacterial blooms as well as the bloom itself, and remove the P released after bloom collapse.
- To characterise the chemical and physical properties of various fly ash samples, and test the samples for their ability to flocculate cyanobacteria and remove phophates from the water by adsorption.

The objectives can thus be illustrated as follows:



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CHAPTER 2:

LITERATURE REVIEW

1. Introduction

Eutrophication is the enhancement of the natural process of biological production in rivers, lakes and reservoirs, caused by an increase in nutrient levels, usually phosphorus and nitrogen compounds. Eutrophication can result in visible cyanobacterial or algal blooms, surface scums, floating plant mats and benthic macrophyte aggregations. The decay of this organic matter may lead to the oxygen depletion in the water, which in turn can cause secondary problems such as fish kills from lack of oxygen and liberation of toxic substances or phosphates that were previously bound to oxidized sediment. Phosphates released from sediments accelerate eutrophication, thus closing a positive feedback cycle. Some lakes are naturally eutrophic, but in many others the excess nutrient input is of anthropogenic origin, resulting from municipal wastewater discharges, industrial effluents and runoff from fertilizers and manure spread on agricultural areas (Bartram *et al*., 1999). Nutrient enrichment seriously degrades aquatic ecosystems and impairs the use of water for drinking, industry, agriculture and recreation (Carpenter *et al*., 1998).

Extensive cyanobacterial growth poses several severe implications on the general water quality as well as the maintenance of water treatment standards set for potable water. Massive cyanobacterial blooms can deplete the dissolved oxygen content resulting in fish kills and discolouration of the water by pigments released from the cells (Rae *et al.*, 1999). Because of their relatively small cell size, cyanobacteria easily penetrate and clog the fine sand filters and the primary coarse fast filters that are fundamental stages in drinking water purification (Botha-Oberholster, 2004). Biodegradation of cyanobacterial blooms contributes to the organic load of the water resulting in increased treatment costs. Non-toxic nuisance compounds such as geosmin and 2-methylisoborneol (2-MIB) that cause taste and odour problems in both dam and purified waters have been associated with cyanobacteria (Rae *et al.*, 1999).

Of greater importance is the fact that certain cyanobacteria produce toxic compounds, the consumption of which present severe health risks. Bloom-forming cyanobacterial genera with toxin producing members include *Microcystis*, *Anabaena*, *Anabaenopsis*, *Planktothrix*, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis* and *Nodularia* (Codd *et al*., 2005). The genus of most concern as a toxin producer is *Microcystis*,

predominantly *M. aeruginosa* (Botha-Oberholster, 2004). Scum formation is particularly common with *Microcystis*, *Anabaena*, *Anabaenopsis*, *Planktothrix*, and *Aphanizomenon*, and less so with the remaining genera. Mat- and biofilm-forming genera with toxigenic members include *Phormidium*, *Ocsillatoria* and *Lyngbya* (Codd *et al.*, 2005).

The dominance of cyanobacteria in water bodies is a function of many contributing factors, all of which play a role in their superior competitive ability. Cyanobacteria have a unique physiology when compared with other phytoplankton, especially in terms of their nutrient biochemistry and buoyancy. Control of cyanobacteria is a challenge, and various methods have been employed in an attempt to reduce the severity of blooms. Some of these, including biological and chemical control, aim to treat the effects of eutrophication by treating the bloom, whereas other methods focus on managing eutrophication itself by curbing the nutrient import into lakes and reducing existing high nutrient levels.

Besides the phytoplankton, other members of the microbial community of a water body will be affected, either directly or indirectly, when the water chemistry and physics are altered. Shifts in the phytoplankton composition in response to treatment measures as well as other changes in the microbial community structure can be quantified and qualified using various methods, which are reviewed here.

2. Toxins

Mechanisms of cyanobacterial toxicity are very diverse and range from hepatotoxic, neurotoxic and dermatotoxic effects to general inhibition of protein synthesis. Toxicosis associated with cyanobacterial populations and their toxins affect wild and domestic mammals, birds and fish, with human cases ranging from mild to fatal (Codd *et al.*, 2005). Cyanotoxins fall into three broad groups of chemical structure, namely cyclic peptides, alkaloids and lipopolysaccharides (endotoxins).

2.1. Hepatotoxic cyclic peptides- microcystins and nodularin

2.1.1. Microcystins

Hepatotoxins have been most often implicated in cyanobacterial toxicosis (Codd *et al*., 2005). The hepatotoxic microcystins (MC) are produced by members of several cyanobacterial genera, including *Microcystis, Anabaena, Planktothrix* (*Ocsillatoria*), *Nostoc*, *Anabaenopsis* and *Hapalosiphon* (Carmichael, 1992). Microcystins are cyclic heptapeptides consisting of seven amino acids, including several D-amino acids and two unusual amino acids, namely N-methyldehydroalanine (Mdha) and a hydrophobic b-amino acid, 3-amino-9-methoxy-2-6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Wiegand & Pflugmacher, 2005). The presence of Adda is essential for expression of biological activity (Gupata *et al*., 2003). There are over seventy different MCs, which differ mainly in the two L-amino acids at positions 2 and 4. The most common, and also the most extensively studied, are MC-LR, MC-RR and MC-YR (Jos *et al.,* 2005), with the variable amino acids being leucine (L), arginine (R) and tyrosine (Y). MC-LR has been found to be the most potent of the three toxins in mouse toxicity studies, followed by MC-YR and MC-RR (Gupta *et al*., 2003).

Microcystins are synthesized non-ribosomally by a multifunctional enzyme complex, consisting of both peptide synthetases and polyketide synthetases coded by the *myc* gene cluster (Oberholster *et al*., 2006). Microcystin synthetase genes *myc*A, *myc*B and *myc*C have been identified in *Microcystis* (Dittmann *et al*., 1996) and *Anabaena* (Meißner *et al*., 1996) as well as in microcystin producing strains of *Nostoc* and *Oscillatoria* (Neilan *et al*., 1999). Strains of other non-toxic cyanobacterial genera contain genes for similar peptide synthetase genes. Microcystin production appears to be linked to the presence of the *myc*B gene and to the occurrence of specific adenylation domains within the *myc*ABC region, although some non toxic *Microcystis* strains contain *myc*B (Tillet *et al*., 2001).

The primary effect on health is toxicity to liver cells, as a consequence of selective transport mechanisms, which concentrate the peptide toxins from the blood into the liver (Falconer, 1994). Microcystins accumulate in vertebrate liver cells due to active transport by a highly expressed unspecific organic ion transporter of the bile acid carrier

transport system. Death of vertebrate animals is mostly the consequence of severe liver damage which begins with cytoskeletal disorganization and can include cell blebbing, cellular disruption, liquid peroxidation, loss of membrane integrity, DNA damage, apoptosis, necrosis, intrahepatic bleeding and untimely death by hemorrhagic shock (Wiegand & Pflugmacher, 2005).

One toxic mechanism of MC is the specific inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) in both animals and higher plants. PP1 and PP2A are responsible for catalyzing the dephosphorylation of serine and threonine residues of phosphoproteins in eukaryotic cells, and have been shown to play an important role in the suppression of tumors in animal tissue (McElhiney, 2001). Inhibition of specific phosphatase enzymes results in hyperphosphorylation of proteins, which is exhibited by a breakdown of intermediate filaments of the cell cytoskeleton and a retraction of actin microfilaments. The cell distortion is such that the organizational structure of the liver itself is disrupted (Falconer, 1994). Chronic exposure to low concentrations of microcystins in drinking water may promote tumor growth in the human liver (Bourne *et al*., 1996). It is the introduction of Adda into the hydrophobic groove at the catalytic site of the protein phosphatase that renders it inactive, and a covalent bond forms between the Mdha residue and the protein phosphatase molecule (Sivonen & Jones, 1999). Another toxic mechanism of MC-LR involves its binding to the β-subunit of ATP synthase, which may cause mitochondrial apoptotic signaling at high MC-LR concentrations (Wiegand & Pflugmacher, 2005). External signs of poisoning include weakness, pallor, cold extremities, heavy breathing, vomiting and diarrhea (Codd, 2000). Microcystins impair photosynthesis in aquatic plants, due to a significant decrease in chlorophyll a and b as well as carotenoids. The main fish organs affected by microcystins are the liver and kidneys, with symptoms similar to those described for mammals. Furthermore, the epithelial cells of the gills undergo degeneration and necrosis, and MC inhibits ion pumps such as Na^+-K^+, Na^+, HCO_3^- and Ca^{2+}-ATPases in fish gills (Wiegand & Pfugmacher, 2005).

Besides the above-mentioned toxic mechanisms, MC-LR enhances oxidative stress in animal cells, due to the formation of reactive oxygen species (Žegura *et al*., 2004), loss of mitochondrial membrane potential and an increase in mitochondrial membrane permeability, all of which are steps to apoptosis. Similarly, in aquatic plants, exposure

to MC-LR enhances formation of hydrogen peroxide, thus increasing the oxidative stress on the plant (Wiegand & Pflugmacher, 2005).

The intracellular tripeptide glutathione acts as a co-substrate for the biotransformation enzymes glutathione S-transferases and for the antioxidative enzyme glutathione peroxidase. The hepatic glutathione content is a critical factor for preserving normal cellular redox balance and protecting hepatocytes from oxidative stress. In addition, cellular reduced glutathione is important for the regulation of cytoskeletal organization (Gupta *et al.*, 2003). Glutathione protects cells from the toxicity and oxidizing activity of MC-LR by binding to the α, β-unsaturated carbonyl group of Mdha. Binding to glutathione enhances the water solubility of the MC, aiding its excretion via the bile fluid, or, in the case of plants, deposition in the vacuole or binding to the cell walls (Wiegand & Pflugmacher, 2005). Microcystins cause depletion of hepatic GSH levels, with MC-LR causing a more significant depletion compared to MC-RR and MC-YR (Gupta *et al*., 2003). Endotoxins, especially those of toxic cyanobacterial origin, reinforce the adverse effects of microcystins by inhibiting the activity of the glutathione S-transferases, which are the key enzymes in the detoxification of microcystins (Rapala *et al*., 2002). It has been suggested that a similar detoxification mechanism occurs in plants, as the conjugation of MC-LR with glutathione has been demonstrated using glutathione transferases purified from the aquatic plant *Ceratophyllum demersum* (McElhiney *et al*., 2001).

Rapala *et al*. (1997) indicated that external growth stimuli affect the levels of microcystins produced by certain cyanobacteria. Not only does the growth of *Anabaena* and *Microcystis* increase with increasing phosphorus levels, but the levels of intracellular microcystins also increase in a similar manner. Growth-limiting phosphorus concentrations decreased the concentration of microcystins in *Oscillatoria* species. This suggests that different cyanobacterial genera respond in a similar manner to the extracellular phosphorus concentration. High and low temperatures, compared to optimal growth temperatures decrease the toxicity or concentration of microcystins. MC-LR was detected more at lower temperatures and MC-RR at higher temperatures. Microcystin production is affected by light, as demonstrated by do Carmo Bittencourt-Oliveira *et al*. (2005) who analysed the presence of MC-LR in the cyanobacterium *Microcystis panniformis* Komárek *et al*. in different times during the light:dark (L:D)

cycle. Levels of MCs per cell were at least threefold higher during the day-phase than during the night-phase, with production peaking in the middle of the day phase. The same pattern was observed under a light:light (L:L) cycle, where the cellular MC content was twice as high as the L:D cycle. Therefore, in terms of toxin production, cyanobacteria express robust circadian rhythms that are independent of the cell division cycle.

Various algae-algae interactions have been observed in eutrophic systems through changes in the abundance dynamics of phytoplankton populations, and many of these interactions are attributed to microcystins (Wiegand & Pflugmacher, 2005). Kearns & Hunter (2001) showed that the presence of MC caused paralysis in the motile green algae *Chlamydomonas reinhardtii*, causing the cells to settle faster. *Microcystis aeruginosa* increased toxin production in the presence of a non-toxic culture of *Planktothrix aghardhii* (Engelke *et al.,* 2003). Singh *et al.* (2001) also found that MC-LR produced by *Microcystis aeruginosa* inhibited the growth and photosynthetic ability of non-toxic *Nostoc muscorum* and *Anabaena*, and increased cell lysis of these species. Interestingly, Hoeger *et al*. (2004) found that the highest microcystin levels were not found to coincide with the highest cell counts of *Microcystis aeruginosa*, but rather increased at the beginning of the exponential growth phase, when *M. aeruginosa* appeared to compete with *Anabaena circinalis* for the dominant position in a fresh water lake. After suppression of its competitor, microcystin levels decreased to previous low levels. These results seem to indicate that microcystins play a role in affording producers a competitive advantage in a eutrophic system.

McElhiney *et al*. (2001) indicated that terrestrial crop plants that were exposed to microcystins through contaminated irrigation water showed a greatly reduced crop quality and yield. In addition, the plants accumulated the microcystins in their tissues. The exposure of edible crop plants is a concern for human health, as the toxins may be carried through the food chain. Aquatic macrophytes also take up and accumulate microcystins. Saqrane *et al*. (2007) investigated MC-LR accumulation, detoxication and oxidative stress induction in the free-floating aquatic vascular plant *Lemna gibba* (Duckweed) by chronically exposing the plant to the toxin. Stress oxidative processes were determined by measuring changes in peroxidase activity and phenol compound content. Following MC exposure, a significant decrease of plant growth and chlorophyll

content was observed, and it was demonstrated that *L. gibba* could accumulate and bio-transform microcystins. Changes in the peroxidase activity and qualitative and quantitative changes in phenolic compounds were observed after 24h of exposure. Aquatic ecosystems where plants co-exist with toxic cyanobacterial blooms may suffer a negative ecological impact due to toxin bioaccumulation and biotransfer through the food chain.

The role of toxins in cyanobacteria is still not understood. Microcystins, as potent inhibitors of serine/threonine protein phosphatases, have been suggested to act as protective compounds against grazing zooplankton (Jang *et al*., 2003), as intracellular chelators inactivating free cellular Fe^{2+} (Utkilen & Gjolme, 1995), or to have some specific cell regulatory function (Rapala *et al.*, 1997). It has also been suggested that the natural function of toxic cyanobacterial secondary metabolites may be cell signaling and environmental signaling (Wiegand & Pflugmacher, 2005).

2.1.2. Nodularin

Nodularin is a cyclic pentapeptide with a structure closely related to that of microcystins, but showing less structural variation. Nodularin is composed of Adda and D-erythro-β-methyl-aspartic acid (D-MAsp) as well as *N*-methaldehydrobutyrine (Mdhb), which is similar to *N*-methaldehydroalanine (Mdha) in the microcystins (Wiegand & Pflugmacher, 2005). Nodularin-producing cyanobacteria of the genus Nodularia possess a microcystin synthetase gene orthologue, and therefore a similar biosynthetic pathway for toxin production (Neilan *et al*., 1999).

The mode of action of nodularin is very similar to microcystins, in that it inhibits the catalytic subunits of serine/threonine-specific protein phosphatases PP1 and PP2. Nodularin, however, does not bind covalently to the protein phosphatases (Sivonen & Jones, 1999). Nodularin has the same effects on plants and fish as microcystins (Wiegand & Pflugmacher, 2005).

2.2. Alkaloid toxins

The alkaloid toxins are diverse, both in their chemical structures and mammalian toxicities. Alkaloids, in general, are a broad group of heterocyclic nitrogenous compounds, usually of low to moderate molecular weight (Sivonen & Jones, 1999).

2.2.1. Saxitoxins

The saxitoxins (STX) are tricyclic, neurotoxic alkaloids, which are also known as paralytic shellfish poisons (PSPs) due to their occurrence and association with seafood. The name saxitoxin is derived from the mollusc from which it was first identified, *Saxidomus giganteus*, but the toxin is primarily produced by marine dinoflagellate planktonic species (Wiegand & Pflugmacher, 2005). In addition, saxitoxins are produced by some cyanobacteria, including *Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii* (Sivonen & Jones, 1999). The toxin blocks neuronal transmission by binding to the voltage-gated Na^+ channels in nerve cells. By blocking the channel opening, the entering sodium flow is stopped which leads to muscle paralysis and death by respiratory arrest in mammals. The transport of STX through the food chain and bioaccumulation of these toxins is an important mechanism for the availability of these toxins to higher trophic levels (Wiegand & Pflugmacher, 2005).

2.2.2. Anatoxins

Three anatoxins have been described. Anatoxin-a (antx-a) and homoanatoxin-a are alkaloids described as secondary amines, whereas anatoxin-a(s) is described as a unique phosphate ester of a cyclic N-hydroxyguanidine structure. Anatoxin-a was first isolated from *Anabaena flos-aquae*, but has also been detected in other cyanobacterial species such as *Anabaena circinalis, Aphanizomenon* sp., *Cylindrospermum* sp., *Aphanizomenon* sp., *Planktothrix* sp. and *Microcystis aeruginosa* (Wiegand & Pflugmacher, 2005). Homoanatoxin-a is produced by *Oscillatoria* and anatoxin-(s) from *Anabaena flos-aquae* and *A. lemmermannii* (Sivonen & Jones, 1999).

The mode of action of anatoxin-a and homoanatoxin-a in birds and mammals is to mimic the neurotransmitter, acetylcholine, and by binding irreversibly to the nicotinic acetylcholine receptor. The irreversible activation of presynaptic acetylcholine receptors causes the sodium channel to be locked open. Muscle cells are over-stimulated by the inflowing sodium ions, and the depolarization causes a block in further electrical transmission, leading to paralysis. When respiratory muscles become affected, convulsions occur due to a lack of oxygen supply to the brain, and death by asphyxiation occurs. Anatoxin-a(s) is a potent acetylcholinesterase inhibitor, and is ten times more toxic to mice than anatoxin-a. Very few studies have shown anatoxin toxicity to aquatic organisms (Wiegand & Pflugmacher, 2005).

2.2.3. Cylindrospermopsin

Cylindrospermopsin (CYN) is a hepatotoxic cyclic guanine alkaloid (Codd, 2000), with a uracil moiety attached to a sulphated guanidine moiety. An intact pyrimidine ring is necessary for CYN toxicity. The toxin is produced by different freshwater cyanobacteria, including *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum*, *Umezakia natans, Rhaphidiopsis curvata* and *Anabaena bergii* (Wiegand & Pflugmacher, 2005).

In mouse studies, CYN causes liver, kidney, thymus and heart damage. Furthermore, CYN displays mutagenic and possibly carcinogenic activity. It induces DNA strand breakage and may disrupt the mitotic spindle, leading to chromosome loss (Wiegand & Pflugmacher, 2005). CYN does not inhibit protein phosphatases like microcystins, but is a significant and irreversible inhibitor of protein biosynthesis, probably inhibiting ribosomal translation (Hoeger *et al.*, 2004). Uptake of CYN into cells seems to be by diffusion due to its small molecular weight, although small amounts may be taken up by bile carriers (Wiegand & Pflugmacher, 2005).

2.3. Lipopolysaccharides

Endotoxic lipopolysaccharides (LPS) are generally found in the outer membrane of Gram negative bacteria, including cyanobacteria, where they form complexes with proteins and phospholipids. They can elicit irritant and allergenic reactions in human

and animal tissues that come into contact with the compounds (Sivonen & Jones, 1999). LPS and its effects are well known from enteric bacteria, such as *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae*, *Yersinia pestis* and *Pseudomonas aeruginosa* (Wiegand & Pflugmacher, 2005). Endotoxins have been associated with certain cyanobacterial genera, including *Synechococcus, Synechocystis, Microcystis, Anabaena, Phormidium, Oscillatoria* and *Schizothrix.* Of these, *Microcystis, Anabaena* and *Oscillatoria* pose the greatest threat because they often occur in great masses in nutrient rich water sources (Rapala *et al*., 2002).

LPS consist of lipid A, core polysaccharides and an outer polysaccharide chain, known as the O-antigen. Cyanobacterial LPS differ to the LPS of enteric bacteria. They have a greater variety of long chain unsaturated fatty acids and hydroxy fatty acids, including the unusual fatty acid β-hydroxypalmitic acid which is found in the lipid A moiety. Cyanobacterial LPS often lack ketodeoxyoctonate, a common LPS component of Gram negative bacterial outer membranes, and contain only small amounts of bound phosphates when compared with enteric bacteria. The cyanobacterial O-antigen, on the other hand, is reminiscent of the *Escherichia coli* O-antigen, and is responsible for cyanophage adsorption and endotoxicity in aquatic environments. Lipid A is responsible for the toxic action, whereas the O-antigen is recognized by the immune system, leading to antibody production (Hoiczyk & Hansel, 2000).

LPS cause gastroenteritis and fever in mammals, and are involved in septic shock syndrome. This may aggravate liver injury induced by other cyanobacterial toxins such as microcystins and nodularins (Rapala *et al*., 2002; Wiegand & Pflugmacher, 2005). This is achieved by the release of inflammatory mediators such as tumor necrosis factor-α and interferon-γ. Additionally, LPS from cyanobacteria decrease glutathione S-transferase (GST) induction in the liver, in the same manner as noted in enteric bacteria. Since conjugation to GST is the start of detoxification of microcystins, inhibition of this enzyme system decreases the ability of the organism to metabolise these toxins. Cyanobacterial LPS from *Microcystis aeruginosa* was shown to be ten times less toxic compared to *Salmonella* LPS. However, the cyanobacterial LPS was found to act in a more potent manner than the LPS of enteric bacteria in suppressing GST activity (Wiegand & Pflugmacher, 2005).

2.4. Toxin stability

The four most important groups of cyanotoxins; microcystins, anatoxins, PSPs and cylindrospermopsins, exhibit different chemical stabilities in water.

2.4.1. Microcystins and nodularins

Being cyclic peptides, microcystins are extremely stable and resistant to chemical hydrolysis or oxidation at near neutral pH. Microcystins and nodularins stay potent even after boiling. In natural waters and in the dark, microcystins may persist for months or even years. Jones & Orr (1994) reported that after an algicide treatment in a small embankment of Lake Centenary, Australia, the total disappearance of microcystin-LR took more than three weeks. However, at high temperatures (40°C) and at elevated or low pH, slow hydrolysis has been observed, with 90% breakdown being achieved in approximately ten weeks. Rapid chemical hydrolysis occurs under conditions that are unlikely to be found outside the laboratory, for example 6M HCl at high temperatures. Microcystins can be oxidized by ozone and other strong oxidizing agents, and are degraded by intense UV light. These processes may be relevant for water treatment processes, but are unlikely to contribute to degradation in the natural environment.

In full sunlight, microcystins undergo slow photochemical breakdown and isomerisation, with the reaction being accelerated by the presence of water-soluble cell pigments, presumably phycobiliproteins. In the presence of these pigments, the photochemical breakdown of microcystin in full sunlight can take as little as two weeks for greater than 90% breakdown, or as long as six weeks, depending on the pigment and toxin concentrations (Sivonen & Jones, 1999).

2.4.2. Anatoxins

Anatoxin-a is relatively stable in the dark, but in pure solution in the absence of pigments it undergoes rapid photochemical degradation in sunlight. Breakdown is further accelerated by alkaline conditions. The half-life for photochemical breakdown is 1-2 hours. Under normal day and night light conditions at pH 8 or pH 10, the half-life

for anatoxin-a breakdown was found to be approximately fourteen days (Sivonen & Jones, 1999).

2.4.3. Saxitoxins

No detailed studies have been carried out on saxitoxin breakdown in sunlight, either with or without pigments. In the dark at room temperature, saxitoxins have been found to undergo a series of slow chemical reactions. The half-life for breakdown reactions are in the order of 1-10 weeks, with more than three months often needed to achieve greater than 90% breakdown (Sivonen & Jones, 1999).

2.4.4. Cylindrospermopsin

Cylindrospermopsin is relatively stable in the dark, with slow breakdown occurring at an elevated temperature (50°C). Pure cylindrospermopsin is relatively stable in sunlight, but in the presence of cell pigments, breakdown occurs rapidly, being more than 90% complete in 2-3 days (Sivonen & Jones, 1999).

2.5. Toxin removal

In response to the increase in health-related problems on a global scale, the World Health Organisation (WHO) has established safe guidelines for drinking water at 1.0µg MC-LR.l^{-1} (Jos, 2005). Additionally, a health alert should be published if the concentration of 10µg MCs.l^{-1} drinking water is reached, even for a brief period. Due to the lack of reliable data, no guideline value is set yet for concentrations of nodularins, cylindrospermopsins, neurotoxins such as saxitoxins and endotoxic lipopolysaccharides (Hoeger *et al*., 2004).

Until a bloom collapses or is otherwise affected by some treatment practice, the majority of toxins will be retained within the cells, making removal of intact cells a high priority. This is achieved using processes such as direct rapid filtration, to remove suspended particulate matter, and slow sand filtration. However, under bloom conditions, it is expected that a substantial proportion of toxin will be released into the water column, making the removal of toxins an unavoidable concern (Hrudey *et al*.,

1999). The three processes usually employed for the removal of microcystins from drinking water treatment include ozonation, chlorination, and adsorption by activated carbon (Rae *et al*., 1999).

Ozonation is the most efficient method for the complete destruction of both intra- and extra-cellular microcystins, as well as nodularin and anatoxin-a. The major consideration in the application of ozone is the ozone demanded by background DOC concentrations, as only after the DOC demand is satisfied, will the ozone show an effect on the toxins (Hrudey *et al*., 1999). The effectiveness of ozone in toxin degradation is also pH dependant, with a greater reduction in toxins at low pH values (Rae *et al*., 1999). Ozone also effectively removes the non-toxic odour causing compounds geosmin and 2-MIB. Although ozone is the most efficient method for removing toxins, the cost implications of the high ozone doses required and the highly specialized mass transfer techniques that are needed for treatment mean that this option is often not feasible for water purification (Strydom, 2004).

Chlorination is very effective at destroying microcystins and nodularin, but only under the correct treatment conditions: the free chlorine residue should be 0.5mg.l^{-1} after 30 minutes contact time with a pH below 8. There is no discernable removal of anatoxin-a by chlorination (Hrudey *et al*., 1999).

Powdered activated carbon (PAC) can successfully remove microcystins and nodularins. It is recommended that this treatment method be combined with another, for example a pre-ozonation treatment, as in order to remove high amounts of toxins very high amounts of PAC are needed, and the presence of organic matter in the water interferes with toxin removal. Granular activated carbon (GAC) is more effective than PAC at removing toxins in the presence of organic compounds. Because GAC has also been shown to be more effective at adsorbing anatoxin-a than microcystin-LR, these two forms of activated carbon should be used in conjunction with one another to achieve maximum toxin removal (Hrudey *et al*., 1999). Adsorption and biodegradation mechanisms are known to be the predominant factors contributing to microcystin removal during the GAC filtration process. The presence of a biofilm within a GAC filter may increase its lifetime for the removal of problematic compounds such as the microcystins via biodegradation. Wang *et al*. (2007) demonstrated that biodegradation

was an efficient removal mechanism and that the rate of biodegradation was dependent upon temperature and initial bacterial concentration.

In terms of endotoxin treatment, the highest reductions occur during the early stages of water purification (coagulation, settling and sand filtration). Chlorination has been reported to decrease the endotoxin concentration, but activated carbon filtration was shown to increase it on some occasions. The removal of endotoxins is therefore dependant on the success of the water clarification steps (Rapala *et al*., 2002).

2.5.1. Biodegradation of toxins

Biodegradation is one of the safest means to remove cyanotoxins from water (Ishii *et al*., 2004). Jones *et al.* (1994) isolated a species of *Sphingomonas* that is capable of degrading both microcystin-LR and microcystin-RR, but not nodularin. The bacterium initiated ring opening of microcystin to produce linear microcystin as a transient intermediate. This compound was nearly 200 times less toxic than the parent toxin (Bourne *et al*., 1996). Ishii *et al.* (2004) demonstrated that *Sphingomonas* is capable of degrading microcystin-LY, -LW and-LF completely, as well as microcystin-LR. A strain of *Pseudomonas aeruginosa* isolated from a Japanese lake produced an alkaline protease that attacked the Adda side chain of microcystin-LR (Takenaka & Watanabe, 1997). A recent study by Meriluoto *et al*. (2005) demonstrated the potential use of the human probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* for microcystin removal. The fact that heat inactivated bacteria were more effective at removing microcystin-LR from solution than untreated bacteria indicated that bacterial metabolism was not involved in toxin removal, but rather that the toxin bound to the bacteria. Toxin binding to lactic acid bacteria may contribute, as one factor, to the lower oral toxicity of microcystins as compared to intraperitonial injection in mouse toxicity studies.

Little work has been undertaken on the biodegradation of anatoxins, saxitoxins or cylindrospermopsin. A *Pseudomonas* strain capable of degrading anatoxin-a at a low rate has been isolated (Sivonen & Jones, 1999).

3. Why cyanobacteria become dominant

Numerous hypotheses have been proposed to explain the success of blue green algae in eutrophic water bodies, identifying light, nitrogen or CO_2 as the limiting resource under eutrophy. The most convincing however, is that the nature of resource limitation changes during the eutrophication process, promoting cyanobacteria to a highly competitive position (Ferber *et al*., 2004).

3.1. Nutrient physiology and the importance of the N:P ratio

Because cyanobacterial blooms often develop in eutrophic lakes, it was originally assumed that cyanobacteria require high phosphorus and nitrogen concentrations, even though blooms often occur when phosphorus concentrations are at their lowest. Experimental data has shown that the affinity of many cyanobacteria for nitrogen and phosphorus is much higher than for many other photosynthetic organisms. This means that they can out-compete other phytoplankton organisms under conditions of phosphorus or nitrogen limitation. In addition to their high nutrient affinity, cyanobacteria have a substantial storage capacity for phosphorus. They can store enough phosphorus to perform two to four cell divisions, which corresponds to a 4-32 fold increase in biomass (Mur *et al*., 1999). Concentrations of phosphorus of below $0.1 mg.l^{-1}$ are sufficient to cause a cyanobacterial bloom (Bartram *et al*., 1999).

The nutrient physiology of cyanobacteria differs from that of other algae in that many species have heterocysts for nitrogen fixation in oxic well-lit waters, for example *Aphanizomenon* and *Nodularia* (Ferber *et al*., 2004). This ability allows nitrogen-fixing species to maintain high growth rates under conditions of nitrogen limitation, making them superior competitors. In addition, blue-green species that are not capable of nitrogen fixation, such as *Microcystis aeruginosa*, may be as abundant as nitrogen fixing species during times of nitrate deficiency. The low nitrogen: phosphorus (N:P) ratio hypothesis was first proposed by Pearsall (1932), and was later popularized by Smith (1983) who compiled and analysed data from 17 lakes worldwide, and observed a tendency for cyanobacterial blooms to occur when epilimnetic N:P ratios fell below about 29:1 by weight, and for blue-green algae to be rare when the N:P ratio exceeded this value. If the low N:P hypothesis is to be true, nitrogen fixing (heterocystous)

cyanobacteria overcome N shortage through fixation and would therefore always be dominant. However, this does not account for the dominance of non-heterocystous species when nitrogen is limited. Ferber *et al*. (2004) found that heterocystous cyanobacteria were dominant during periods of low nitrogen fixation (less than 2% of the required N), leading to the assumption that fixation is not the only means of heterocystous cyanobacteria outcompeting other algae. Nitrogen fixation is usually repressed at inorganic N concentrations greater than 7µM (Horne & Commins, 1987). Colonial and vacuolated non-heterocystous species such as *Microcystis*, *Oscillatoria* and *Planktothrix* are expected when the principal source of N is ammonium recycled within the water column, or a benthic ammonium source can be reached through vertical migration, and heterocystous species when neither of these sources is sufficient and fixation must be relied upon (Blomqvist *et al*., 1994; Hyenstrand *et al*., 1998). Ferber *et al*. (2004) therefore recommended that the low N:P hypothesis be modified to include the fact that both heterocystous and non-heterocystous vacuolated cyanobacteria will out-compete other species through the vertical migrations that give them superior access to nutrient sources, P as well as N. Several other factors have also been proposed to explain variation in cyanobacterial dominance below the N:P threshold, including light, temperature, pH and the effects of zooplankton (MacKay & Elser, 1998).

Xie *et al*. (2003) performed an enclosure experiment during the summer of 2001 in the shallow, subtropical Lake Donghu, China to examine the effect of TN:TP ratios and P-reduction on the occurrence of *Microcystis* blooms. The treatments were performed with an excess of N but with different amounts of P in the water column and sediment. *Microcystis* blooms occurred in the enclosures with higher concentrations of P with initial TN:TP <29 as well as TN:TP >29, indicating that the TN:TP ratio was not a deterministic factor for *Microcystis* blooms, at least in the highly eutrophic Lake Donghu. This is in agreement with Paerl *et al*. (2001), who suggested that the "N:P rule" is less applicable to highly eutrophic systems when both N and P loadings are very large and the N and P inputs exceed the assimilatory capacity of the cyanobacteria. The TP of the water in the enclosures with P-rich sediment increased dramatically after the bloom developed, with approximately 40% of the sediment P being released to the water column and assimilated by the *Microcystis*, leading to a decrease in the TN:TP ratios to about 10. The results therefore indicate that the low TN:TP ratio is not the cause of *Microcystis* blooms, but rather the result. No *Microcystis* blooms occurred in

the enclosures with low P concentrations in the water and the sediment, despite the presence of sufficient N, suggesting the effectiveness of P-reduction for the control of *Microcystis* blooms. In a comparative study of two water supply reservoirs situated in different climatic regions of Brazil, von Sperling *et al*. (2008) observed that in spite of the prevalence of high N:P values there was a clear trend in the dominance of cyanobacteria in the phytoplankton.

3.2. Effect of zooplankton

Zooplankton, especially *Daphnia*, are generally rare during a cyanobacterial bloom. It is unclear whether cyanobacteria predominate in lakes with low *Daphnia* grazing pressure because they exclude large effective grazers, or because grazer biomass is decreased due to other factors allowing cyanobacteria to dominate (MacKay & Elser, 1998). There are many reasons as to why cyanobacteria may exclude *Daphnia*; they have a low nutritional value, they can be toxic (MacKay & Elser, 1998) and their shape, especially that of filamentous cyanobacteria, may mechanically interfere with the filtering mechanism of grazers (de Bernardi & Giussani, 1990). Large grazers such as *Daphnia* promote high densities of inedible colonial cyanobacteria by selectively eating competitive phytoplankton (Haney, 1987). However, there is also evidence that high *Daphnia* densities exclude cyanobacteria completely. Although cyanobacteria typically dominated the phytoplankton community in Lake Trummen, there was one year when they did not dominate, despite a low N:P ratio. Smith (1983) attributed this to a change in the food web which likely led to an increase in *Daphnia*, as this year followed a winter fish kill that eliminated planktivorous fish. Gobler *et al*., 2007 suggested there may be a threshold density of *Microcystis* which is inhibitory to *Daphnia* grazing. When *Daphnia* were able to graze in Lake Agawam, mean densities of *Microcystis* and the percentage of *Microcystis* represented within the total algal community were both significantly lower than levels and percentages present when there was no grazing detected. *Microcystis* densities were also always below 6 x 10^4 cells.ml^{-1} when grazing by *Daphnia* was detected.

Compared with other zooplankton, *Daphnia* have a higher P content in their bodies (Anderson & Hessen, 1991), and therefore, because of their low body N:P ratio, recycle nutrients at a high N:P ratio that may adversely affect cyanobacterial populations

(Sarnelle, 1992). Because *Daphnia* returns N to the environment at a much higher rate than P, recycling theory predicts that the cyanobacterial population should become P limited in the presence of these low N:P grazers (Sterner, 1990). MacKay & Elser (1998) used a field experiment to test how the differential nutrient recycling by low N:P ratio *Daphnia* affects the physiological status of cyanobacteria, including rates of nitrogen fixation, when compared to a high N:P ratio species *Epischura* and a zooplankton-free control. The ammonium concentration in the *Daphnia* treatment was twice those of the *Epischura* treatment and control, making the N:P ratio the highest in the *Daphnia* treatment. This high N:P ratio caused the cyanobacteria to become P limited. Consistent with this, the rate of nitrogen fixation was 50% lower than in the other treatments. Thus, by differentially recycling NH_4-N relative to P, *Daphnia* reduce the advantage cyanobacteria have over other phytoplankton. Combined with the observation that *Daphnia* cannot survive in severe cyanobacterial blooms, they may be more effective at preventing the occurrence of cyanobacterial blooms than in controlling existing blooms.

3.3. Buoyancy in cyanobacteria

The ability of gas-vacuolate cyanobacteria to adjust their cell density and move up and down the water column is an important factor in cyanobacterial dominance. The advantages of buoyancy regulation include a reduction in the amount of cells lost by sedimentation, an improvement in light supply as the cells are nearer the well-lit surface water layers, and the ability to balance the supply of limiting resources by altering the cell position in the water column, for example to overcome the vertical separation in light and nutrient availability that occurs in stratified water bodies (Oliver, 1994). This is especially important in shallow lakes, where there is a short distance for vacuolated cyanobacteria to migrate to the bed, allowing the cells to spend much of the day on the lake surface photosynthesizing (Ferber *et al*., 2004). Gas vesicles are exposed to intracellular turgor pressure generated by the difference in osmotic pressure between the cell cytoplasm and the surrounding medium, as well as hydrostatic pressure from the overlying water column. A high turgor pressure in the cell causes a collapse in a portion of the gas vesicles, thus reducing cell buoyancy (Oliver, 1994). Limitation of carbon, nitrogen and phosphorus have different effects on the gas vacuoles in cyanobacterial cells. When nutrients such as nitrogen and phosphorus are readily available and carbon

is not limiting, photosynthetic energy is used to synthesize cell constituents and carbohydrates do not accumulate. However, if growth is restricted by a limiting nutrient, energy capture exceeds the amount needed for growth and carbohydrates accumulate in the cell, causing an increase in turgor pressure and a decrease in buoyancy. Carbon limitation, in contrast, is expected to prevent the loss of buoyancy as the turgor pressure increase depends on carbon fixation (Klemer, 1991).

3.4. Recruitment of resting stages

Many phytoplankton species form resting vegetative stages when environmental conditions are harsh and these can survive for extended periods of time in the sediment. When environmental conditions are favourable again, they recruit to the water phase and continue growing (Ståhl-Delbanco *et al*., 2003). Many species of cyanobacteria, for example *Microcystis*, *Anabaena*, and *Aphanizomenon*, form resting stages and are, in addition, the most frequently occurring bloom-forming cyanobacteria (Willén & Mattsson, 1997). Takamura *et al*. (1994) found that the amount of *Microcystis* in the sediment can be much higher than the total amount of *Microcystis* in the water column, even during blooms. This means that a huge potential inoculum can be present in the sediment. Blooms are often formed rapidly (within days) and can often not be explained by growth of the existing pelagic population alone, suggesting that the rate of recruitment from sediment to water may be important to the formation of blooms (Ståhl-Delbanco *et al*., 2003).

Overwintering benthic cyanobacterial populations can only act as an inoculum if they remain vital and if they are able to leave the sediment. Verspagen *et al*. (2004) investigated the vitality and two possible recruitment mechanisms of benthic *Microcystis* colonies; passive re-suspension and an active increase in the buoyancy levels of the cells. They found that throughout the year benthic *Microcystis* populations were photochemically active and sufficiently vital to serve as an inoculum for the initiation of a bloom. Although *Microcystis* is able to survive under anoxic conditions by fermentation (Moezelaar & Stal, 1994), photosynthesis is sensitive to the high sulphide concentration found in the sediment. The photosynthetic vitality of *Microcystis* colonies found at the sediment surfaces of deeper parts of the lake was reduced, but colonies in the shallower parts of the lake were still in the euphotic zone and had the

highest photochemical vitality. Hence, colonies from the shallow sediments were better adapted physiologically to inoculate the water column. In terms of the mechanism of recruitment, changes in internal buoyancy seemed unlikely, as the carbohydrate content in benthic *Microcystis* is so low that a further decrease could not cause a buoyancy change, and there was no substantial increase in gas vesicle volume in spring. It was concluded that intense mixing of the water column was sufficient to re-suspend the sediment containing benthic *Microcystis*, and remove attached sediment so that buoyant colonies could enter the water column again. Bioturbation by macrofauna may also result in benthic *Microcystis* recruitment (Ståhl-Delbanco & Hansswon, 2002). Therefore, because the sediments of shallow parts of a water body are most frequently re-suspended and the vitality of colonies is the greatest in this zone, shallow areas play the most important role in cell recruitment and the development of algal blooms.

3.5. CO_2 concentration and pH

It was initially proposed by King (1970) that the onset of a cyanobacterial bloom results from low concentrations of CO_2 brought about by photosynthesis of algae early in the season, and that a low CO_2 concentration and high pH are a prerequisite for cyanobacteria to become abundant. Shapiro (1972) added to this by stating that cyanobacteria have better CO_2 uptake kinetics than green algae at low concentrations, and therefore reduce the CO_2 concentration to the degree that only they can photosynthesize and become abundant. When excess CO_2 and high nutrients (nitrogen and phosphorus) were added to an algal population containing green algae but dominated by cyanobacteria, the population shifted and was dominated by the green algae within 10d. A similar result was seen when nutrients were added and the pH lowered to 5 to make CO_2 more available. Addition of nutrients alone caused an increase in cyanobacterial growth and in the pH.

Shapiro (1997) disproved King's theory that low CO_2 and high pH are prerequisites for the formation of a cyanobacterial bloom, at least for the important species *Aphanizomenon flos-aquae* and *Anabaena flos-aquae* when he performed a whole-lake study in 1993. The south basin of Squaw Lake, Wisconsin, U.S.A. was artificially injected with CO_2 in an attempt to eliminate the massive blue-green algal bloom usually present in summer. The unmixed, un-injected north basin was the control. Despite a

great difference in the pH and CO_2 concentrations between the two basins, a cyanobacterial bloom began in both almost simultaneously and eventually reached the same size, with the predominant algal species in both basins *Aphanizomenon flos-aquae* and *Anabaena flos-aquae*. The statement that cyanobacteria do well at high pH because that is when free CO_2 concentrations are sufficient for them but not for other groups appears valid, as most of the green algae tested had a poorer ability to use low concentrations of CO_2 than the cyanobacteria.

3.6. Effect of trace metals

Trace metals are crucial for efficient carbon and nitrogen metabolism in cyanobacteria. Iron is important for photosynthesis as well as energy distribution within the cell. Addition of iron to a water body results in increased cyanobacterial photosynthesis, thus stimulating the growth rate and promoting blooms. In a study performed by Takeda *et al*. (1995), simultaneous addition of iron and nitrate stimulated algal growth more rapidly than the addition of nitrate alone. Both iron and molybdenum are involved in nitrate reduction and nitrogen fixation (Rueter & Petersen, 1987). Molybdenum enrichment of a Californian lake stimulated carbon fixation and the rate of nitrogen uptake, and had the greatest effect when nitrate was the dominant nitrogen source (Axler *et al*., 1980). Overall, three trace metal dependant processes may contribute towards dominance: efficient use of limiting light, nitrogen fixation and the production of extracellular iron binding compounds (Rueter & Petersen, 1987).

4. Methods of control

4.1. Biological control of cyanobacteria

It is important to know what controls cyanobacterial dynamics in their natural habitats. An alternative approach for the direct elimination of nuisance cyanobacteria involves the application of biological control agents. Changes in cyanobacterial populations have been attributed to a number of variables, including predation, nutrient depletion, light intensity, accumulation of metabolites, parasitism, and the pH and CO_2 content of the water. Despite the high abundance of bacteria and viruses in water bodies, the importance of lytic bacteria and viruses in regulating the population abundance of

nuisance cyanobacteria is seldom emphasized (Rashidan & Bird, 2000). Biological control has a number of advantages over chemical control. Biological control can be highly specific to the target organism, with no destruction of other organisms and no direct chemical pollution that might affect humans. Potential disadvantages include limited destruction of the target organism, limited survival of the agent or its removal by other organisms and problems with large scale production, storage and application of the biocontrol agent (Sigee *et al*., 1999).

Daft *et al*. (1985) proposed the following seven attributes that defined a good predatory bacterial agent: adaptability to variations in physical conditions; ability to search for or trap prey, capacity and ability to multiply, consumption of prey, ability to survive low prey densities (switch or adapt to other food sources), a wide host range and the ability to respond to changes in the host. In addition, Sigee *et al*. (1999) suggested that the microbial antagonists must be indigenous species of that particular lake environment, having not undergone any gene modification or enhancement.

The practice of introduction of foreign microbial agents has raised some concern with regards to environmental safety due to the so-called host specificity paradigm involving host switching (HS) and host range expansion (HRE) (Secord, 2003). The foreign microbial agents are able to reproduce naturally and may exploit the opportunities that are available in the new environment by shifting their host affinities to other host species (set of species) and/or add another target species other than the original target. The change in direction of the microbial antagonist is difficult to anticipate, and there is the possibility that the organisms may affect other economically important crops or organisms.

Viral pathogens would be ideal as biocontrol agents as they are target selective and specific for nuisance cyanobacteria. However, bacterial agents are considered more suitable than viruses as biological control agents because bacteria can survive on alternate food sources during non-bloom periods and the possibility of mutation within the host is not problematic, as bacterial predation is not reliant on unique attachment receptors (Rashidan & Bird, 2001).

4.1.1. Cyanophages

Cyanophages are extremely widespread in both freshwater and marine environments. The rapid generation time of cyanophages makes them attractive agents for controlling cyanobacterial blooms. All the known cyanophages belong to three bacteriophage families: Myoviridae, Siphoviridae, and Podoviridae (Lu *et al*., 2001; Yoshida *et al*., 2006). These phages are morphologically and genetically diverse (Zhong *et al*., 2002). Despite their abundance and significance, few cyanophages have been characterized at the genome level. Examples of those characterised include P60, P-SSP7, P-SSM2, PSSM4, and S-PM2 (Chen & Lu, 2002; Mann *et al*., 2005; Sullivan *et al*., 2005). Liu *et al*. (2007) reported the complete genome sequence of the cyanophage, Pf-WMP4, which infects the freshwater cyanobacterium *Phormidium foveolarum* Gom.

Yan-Ming *et al*. (2006) investigated the spatial distribution and morphological diversity of virioplankton in Lake Donghu, China, which contains three trophic regions: hypertrophic, eutrophic and mesotrophic. High virus diversity was observed in the lake, with cyanophages representing a significant fraction of the virus community. Numbers appeared to be directly related to the concentration of chlorophyll a, and were higher in the eutrophic region. Most morphotypes belonged to *Siphoviridae*, *Myoviridae* or *Podoviridae*. It was concluded that cyanophages play an important role in the ecology of Lake Donghu.

There is a marked difference between unicellular and filamentous cyanobacterial hosts in the dependence of the cyanophage cycle on photosynthetic activity. Unicellular cyanobacterium-cyanophage systems show an absolute dependence for phage development on their host photosynthetic machinery, for example various *Synechococcus* strains and SM-1, AS-l and AS-1M cyanophages. In filamentous organisms the cyanophage cycle can proceed independently of host photosynthesis, for example in *Plectonema* sp. and *Nostoc* sp. /*Anabaena* sp., LPP and N-l phages. This difference may be due to the altered redox state of thioredoxin *m* in filamentous cyanophage-infected cyanobacteria (Teklemariam *et al*., 1990).

An important consideration in the potential use of cyanophages as biological control agents is the rapid appearance of host mutants. These may include changes in the algae

cell envelope, preventing phage adsorption. Cyanobacterial strains that are resistant to wild type phages may, however, be susceptible to attack by mutant cyanophage strains. The high degree of host specificity, occurrence of resistant host mutants and the effect of environmental factors all contribute to the unpredictability of cyanobacteria-phage interactions in the field. Difficulties involved with producing large amounts of active inoculum also present problems in the effective use of cyanophages as biological control agents in the lake environment (Sigee *et al*., 1999).

4.1.2. Predatory bacteria

In a report by Wright & Thompson (1985), volatile products released by various *Bacillus* species, including strains of *B. licheniformis, B. pimulis and B.subtilis* were inhibitory to cyanobacterial growth, particularly that of *Anabaena*. As was found in the study by Reim *et al*. (1974), the onset of marked detectable antagonism coincided with the sporulation of the majority of the *Bacillus* cells. Wright *et al.* (1991) identified one cyanobacteriolytic volatile product produced by the *Bacillus* species as isoamyl alcohol (3-methyl-1-butanol). Isoamyl alcohol is a volatile product of peptone metabolism in some *Bacillus* species. This compound may act synergistically with other complex volatiles to cause lysis of cyanobacterial cell suspensions. Contact is not required between the bacteria and the cyanobacteria in order for lysis to occur.

The culture filtrate of an atypical strain of *Bacillus brevis* lysed seven genera of cyanobacteria, including *Plectonema boryanum, Microcystis aeruginosa* and *Anabaena flos-aquae*. These bacilli produced two main classes of filterable substances that show biological activity, namely exoenzymes and polypeptide antibiotics. The heat stability and small molecular size of the diffusible inhibitory factor present in the culture filtrate suggested that the substance was of a non-enzymatic nature, and therefore was probably an antibiotic substance. *Bacillus brevis* produced two antibiotics, gramicidin S and tyrothricin. Gramicidin S was inhibitory to the growth of *Plectonema boryanum*, while tyrothricin caused no inhibition. Gramicidin S was therefore thought to be the cyanobacteriolytic substance produced by the atypical *Bacillus brevis* strain. Most extracellular antimicrobial products synthesized by the bacilli are sporulation related, and *Bacillus brevis* is no exception, as cyanobacteriolytic activity did not appear until the early stationary phase of growth (Reim *et al*., 1974).

Nakamura *et al*. (2003a) isolated a bacterium showing high lytic activity against *Microcystis*. The bacterium was identified as *Bacillus cereus*. *B. cereus* cells first attached to the surface of the cyanobacteria to induce cyanobacterial aggregation, and extracellular products of *B. cereus* subsequently lysed the cyanobacteria. The purpose of this two-step process of *B. cereus* may have been to lyse and assimilate cyanobacteria in a more effective manner. The cyanobacteriolytic activity of *B. cereus* gradually increased following the exponential growth phase, once again indicating that the cyanobacteriolytic activity involves sporulation in *Bacillus*. The cyanobacteriolytic substance was heat stable and hydrophilic. Proteinase-K treatment had no effect on activity, indicating that the lytic substance was non-proteinaceous, and it was less than 2kDa in size. No cyanobacteriolytic activity was observed under acidic conditions, but cyanobacterial cells were immediately lysed after shifting to alkaline conditions. This indicated that the cyanobacteriolytic substance was not denatured at acidic pH values, was not affected by pH shifting and was more effective in alkaline pH conditions. This is important, as cyanobacterial blooms often alkalise the aquatic environment because some cyanobacteria, including *Microcystis*, can use HCO_3^- more effectively than CO_2. At 30°C and 25°C, almost 100% of the cyanobacterial cells were lysed, with minimal activity at 3°C. Since the cyanobacterial membrane is considered quiescent at 3°C, the minimal activity may have been due to the low activity of the cyanobacteria. Optimum conditions for cyanbobacterial growth such as high temperature and alkaline pH may accelerate the active transport of the lytic substance to the cyanobacterial cells and subsequent rapid lysis. The cyanobacteriolytic substance produced by the isolated *B. cereus* strain was not linked to the enterotoxin or emetic toxin produced by pathogenic *B. cereus* strains. It also differed from the algicide gramicidin produced by *B. brevis* (Reim *et al*., 1974), indicating the possibility of a novel algicide. Shunyu *et al*. (2006) isolated *Bacillus cereus* from Lake Dianchi of Yunnan province, China, which was capable of rapidly lysing the bloom-forming cyanobacterium *Aphanizomenon flos-aquae* through cell-to-cell contact. The bacterium also showed lytic activity towards *Microcystis viridis*, *Microcystis wesenbergi*, *Microcystis aeruginosa*, *Chlorella ellipsoidea*, *Oscillatoria tenuis*, *Nostoc punctiforme*, *Anabaena Xos-aquae*, *Spirulina maxima*, and *Selenastrum capricornutum*.

A Gram negative, rod shaped motile bacteria thought to be a new species related to *Xanthomonas* was isolated that showed lytic activity towards select cyanobacteria,

including species of *Anabaena* and *Oscillatoria*. These cyanobacteria produce the compounds geosmin and 2-methylisoborneol (MIB), which cause off-flavours in commercially produced channel catfish. Most geosmin off-flavour has been attributed to species of *Anabaena*, whereas MIB off-flavours are associated with *Oscillatoria* species. This newly isolated bacterial species therefore represents an opportunity to selectively control the nuisance cyanobacteria. The lytic characteristics of the bacteria appeared to be associated with the living cells, as no lytic activity was associated with filtered broth (Walker & Higginbotham, 2000). The mechanism of lysis therefore differed from that seen with *Bacillus* species, all of which employed an extracellular agent to achieve cyanobacterial lysis.

Numerous strains of lytic gliding bacteria, mainly members of the *Myxobacteria* and *Cytophaga* groups, were isolated, which lysed cyanobacteria by attachment and secretion of diffusible lytic substances and therefore required direct contact with the host cell. They produced a variety of different exoenzymes capable of hydrolyzing the cyanobacterial cell wall, including proteases, glucosamidase and D-alanyl-N-lysine endopeptidase, as well as antibiotics. These anti-cyanobacterial substances resulted in the lysis of cyanobacteria and release of nutrients, which then may have been taken up by lytic bacteria for their own growth (Rashidan & Bird, 2000). The cyanobacterium *Phormidium luridum* was preyed upon by *Myxococcus* species, mainly *M. xanthus* and *M. fulvus.* These bacteria displayed entrapment capabilities, causing clumping in cyanobacteria prior to lysis, and seemed to be independent of any other nutritional requirement (Burnam *et al.,* 1981; Burnam *et al.,* 1984). Rashidan & Bird (2000) isolated two *Cytophaga* strains (C1 and C2), which demonstrated host specificity. One strain showed lytic activity only towards *Anabaena flos-aquae,* the other lysed only three *Synechococcus* species and *Anacystis nidulans*. *Cytophaga* are strict aerobic bacteria, and are dependant on organic matter for growth. They need a solid substrate for gliding, which explains why they preferred to be attached to cyanobacteria, although they can grow and reproduce in the absence of their host. No special attachment organelles existed on the surface of *Cytophaga* strains C1 and C2, but because contact was required for lysis, it seemed evident that surface lytic enzymes were involved in the lytic action of these bacteria, which was consistent with their host specificity.

There are reports of *Bdellovibrio* (Burnham *et al*. 1976) and *Bdellovibrio*-like bacteria (Wilkinson 1979; Caiola & Pellegrini 1984) causing cyanobacteria lysis. Burnham *et al*. (1968) demonstrated the endoparasitic behaviour of *Bdellovibrio bacteriovorus* on *Escherichia coli*. The *Bdellovibrio* irreversibly attached to the host, with the end of the cell opposite the sheathed flagellum, commenced a grating motion which lasted for several minutes, and entered the host's cytoplasm. Once inside the prey, *Bdellovibrio* inactivated the host's metabolism and fed off its nutrients (Yair *et al*. 2003). The exhaustion of cytoplasm contents triggered the *Bdellovibrio* to undergo multiple fission replications to produce progeny called bdelloplast. The bdelloplast, now flagellated, emerged after breaking the prey cell wall leaving behind ghost prey remnants. However, when *Bdellovibrio bacteriovorus* was added to an aqueous culture of *Phormidium luridum* it caused lysis of the cyanobacteria, but the mechanism of cyanobacterial lysis was not endoparasitic as expected; an extracellular substance was released that dissolved the cyanobacteria cell wall, allowing the bacterium to gain nutrients from the cyanobacterium (Burnham *et al*. 1976). The predation mechanism of *Bdellovibrio* was therefore prey-specific.

Recently, *Streptomyces neyagawaensis* was found to have lytic activity towards four cyanobacterial species, including *Microcystis aeruginosa*, *Anabaena cylindrica*, *Anabaena flos aquae* and *Oscillatoria sancta*. Results indicated that *S. neyagawaensis* did not secrete the anti-algal substance until the bacterium met the target alga, and that the anti-algal substance was present in the periplasmic fraction of the bacterial cell (Hee-jin *et al*., 2005).

Predatory bacteria have characteristics that make them more potent and valuable as control agents when compared to cyanophages. They can survive on alternate food sources during non-bloom conditions, and mutation to non-susceptible strains in the host is far less likely because there are no unique attachment receptors. Non-obligate predatory bacteria do not require the presence of prey cells for survival, but attack and destroy prey cells when nutrients in the environment become depleted (Rashidan & Bird, 2000).

4.1.3. Fungal pathogens of cyanobacteria

There have been various reports of predation of cyanobacteria by fungi. *Oscillatoria agardhii* was parasitized by the chytridiaceous fungus *Rhizophidium planktonicum*. However, this fungus is of limited use in the control of bloom-forming cyanobacteria because of the apparent obligate nature of these parasites and difficulties in their large-scale culture (Sigee *et al*., 1999). Fungal representatives of the genera *Acremonium*, *Emiricellopsis* and *Verticillium* lysed *Anabaena flos-aquae* and, in most cases, several other filamentous and unicellular cyanobacteria. Lysis of cyanobacteria by *Acremonium* and *Emericellopsis* sp. was associated with the formation of diffusible heat-stable extracellular factors (Sigee *et al*., 1999).

4.1.4. Field application of biological control agents

Although there are non-indigenous bacterial agents that have been isolated and characterised, it appears that the studies on application of biocontrol agents are rather limited, focusing mainly on the lysis of laboratory-cultured cyanobacteria. Before application of bacterial biocontrol agents to freshwater systems, information must be available on the anti-algal activity against target algae, the effects of bacteria on other organisms in the freshwater ecosystem and the prediction of the algal dynamics after removal of target algae (Choi *et al*. 2005). Another aspect of importance is agitation. Shilo (1970) and Daft *et al*. (1971) found that cyanobacterial lysis was ineffective if there was agitation, especially where contact lysis was involved. Under natural conditions, rapid mixing may favour the proliferation of cyanobacteria and discourage attachment of predatory bacteria.

During field trials performed by Wilkinson (1979) and Caiola & Pellegrini (1984), a *Bdellovibrio*-like bacterium caused lysis of *Neofibularia irata, Jaspis stellifera* and *Microcystis* cells respectively. The bdelloplast were localised within the cell wall and cyanobacteria cytoplasm membrane. The infecting bacterium was similar in size and appearance to previously described *Bdellovibrio's*. These observations, though not replicated under controlled laboratory conditions, indicated the possibility of endoparasitism of the cyanobacteria by *Bdellovibrio*-like bacteria. The *Bdellovibrio*-like bacteria are an attractive biological control agent because in some cases they penetrate

the host cells specifically, exhaust host cell contents and replicate to form bdelloplasts, which attack further cells.

Nakamura *et al*. (2003b) immobilised *Bacillus cereus* N-14 in floating biodegradable plastic carriers, at a cell concentration of 3 x 10^7 cells/ml per 1g dry weight of starch-carrier float. This was used as an effective *in situ* control of natural floating *Microcystis* blooms, eliminating 99% of floating cyanobacteria in 4 days. The bacteria utilized the starch as a nutrient source and amino acids were derived from the lysis of *Microcystis*. The floating carrier enabled immobilized bacteria to be directed to floating cyanobacteria blooms.

4.2. Chemical control of cyanobacteria

Algicides have been used widely in some regions to control prevailing cyanobacterial blooms (Chorus & Mur, 1999). Examples of the chemicals most often used include copper sulphate ($CuSO_4$), Reglone A (diquat, 1,1-ethylene-2,2-dipyridilium dibromide) Simazine (2-chloro-4,6-bis(ethylamino)-s-trizine) alum ($(Al_2(SO_4)_3 14H_2O$) and lime ($Ca(OH)_2$). In water treatment plants, potassium permanganate ($KMnO_4$) is applied to control phytoplankton related odour problems. Of these chemicals, both alum and lime remove phosphorus from the water. The remaining chemicals remove cyanobacteria by disrupting cell functions such as new cell wall synthesis, photosynthesis or other enzymatic reactions.

Before chemical treatment, MC-LR is present in high amounts in the cyanobacterial cells, but is at a low concentration in the water. After treatment with copper sulphate, Reglone A and Simazine, there is a substantial increase in the toxin concentrations in the water due to cell lysis. Thus, improving the aesthetic value of a lake by chemically removing a toxic cyanobacterial bloom could increase the potential health risks. Treatment with alum and lime causes coagulation of the cyanobacteria, resulting in flocculation. The exocellular concentrations of MC-LR when treated with alum and lime are consistently lower than with other chemical treatments. Treatment with lime showed no increase in exocellular toxin concentrations when compared to a control, but alum shows a three-fold increase. It has been suggested that the aluminium ions in alum may cause cell lysis, but to a lesser extent than other chemicals. Lime or alum treatment

represents a more favourable treatment of toxic cyanobacterial blooms, because of their ability to remove cells with minimal toxin release (Lam *et al*., 1995).

4.3. Control using turbulent mixing

Stability of the water column is a prerequisite for bloom formation, and mixed conditions prevent bloom formation and arrest the growth of colony forming cyanobacteria such as *Microcystis* and *Aphanizomenon*. However, green algae such as *Scenedesmus* spp. tend to dominate during periods of more intensive mixing (Ibelings, *et al*., 1994). Naturally available photosynthetic photon flux densities (PPFD) for phytoplankton fluctuates as a complex function of the daily passage of the sun, weather conditions, wave action and mixing over the underwater light gradient. One of the causes of reduced growth of cyanobacteria under mixed conditions may be the reluctant acclimation of cyanobacteria to changes in the PPFD (Collins & Boylen, 1982). Ibelings *et al*. (1994) made a direct comparison between the cyanobacterium *Microcystis aeruginosa* Kützing emend. Elenkin and the eukaryotic green alga *Scenedesmus protuberance* Fritsch with respect to their acclimation to fluctuations in PPFD by simulating the conditions induced by wind mixing over the underwater light gradient in lakes. *Microcystis* exhibited a more reluctant acclimation to the fluctuating PPFD when compared to *Scenedesmus*, whose growth rate was higher in all light regimes. This implied that if *Scenedesmus* was not subject to sedimentation losses (Visser *et al*., 1996b), it would outcompete *Microcystis* in lakes. These results emphasized the importance of buoyancy regulation in cyanobacteria for increasing their daily light dose.

Artificial mixing by means of air bubbling was installed in Lake Nieuwe Meer, Amsterdam, using a system of seven perforated air tubes installed just above the lake sediment in an attempt to reduce the extensive growth of *Microcystis* in the lake. The following seasons showed a shift from cyanobacterial dominance to flagellates, green algae (mainly *Scenedesmus*) and diatoms (mainly *Cyclotella* and *Stephanodiscus*). The total phosphorus and total nitrogen concentrations were not affected by the mixing and remained high, leading to the conclusion that light limitation was responsible for the shift in the phytoplankton composition (Visser *et al*., 1996a). A distinction must be made between colony forming and filamentaous cyanobacteria in terms of their floatation velocity. Colonies have a much higher floatation velocity than filamentous or

single celled cyanobacteria, and the mixing velocity therefore needs to be high enough to keep the colonies moving in the turbulent flow (Visser *et al.*, 1997; Huisman *et al.*, 2004). Artificial mixing may temporarily reduce the amount of cyanobacterial growth, but it does not solve the problem of eutrophication.

4.4. Eutrophication management

4.4.1. Nutrient limitation

The significance of phosphorus in eutrophication has resulted in the development of many remediation plans, based on the management of the phosphorus concentration. It is accepted that phosphorus control is more achievable than that of nitrogen, because, unlike nitrogen, there is no atmospheric source of phosphorus that is bio-available. In addition, the general equation for photosynthesis (Equation 1) (Hereve, 2000) shows only one gram of phosphorus is required for every seven grams of nitrogen for the formation of the organic matter created in the process.

$$HPO_4^{2-} + 16NO_3^- + 106CO_2 + 122H_2O + 18H^+ \rightarrow (CH_2O_6)106(NH_3)16H_3PO_4 + 138O_2 \quad \ldots(1)$$

This indicates that a small degree of phosphorus reduction can achieve a much greater degree of growth reduction of cyanobacteria than a reduction of a similar magnitude in the nitrogen level. This fact, together with the availability of gaseous nitrogen to N-fixing organisms, makes phosphorus reduction strategies far more effective alternatives in eutrophication management.

In South Africa, the orthophoshate standard for effluents discharged into water bodies is $1mg.l^{-1}$ (NIWR, 1985). However, even if this standard is complied with, it may take years for phosphorus levels to decline below the threshold effective for controlling cyanobacterial biomass in dams that are already in a eutrophic state. This is due to the fact that hypertrophic aquatic ecosystems have specific positive feedback mechanisms, which stabilize the trophic state and cyanobacterial dominance. An example of this is the anoxic sediments typical of hypertrophic waters, which have a high capacity for phosphorus storage. The retention time of the dam is also an important consideration, as dams, lakes and reservoirs with high retention times will show extremely slow declines

in phosphorus concentrations, even after external inputs have been reduced to levels which should ensure a mesotrophic or oligotrophic state (Chorus & Mur, 1999). It is clear that a reduction in the phosphorus inputs into the eutrophic water body will not necessarily result in dam remediation.

4.4.2. Chemical removal of phosphorus

A solution may be to reduce the internal phosphorus concentration. Precipitation of phosphorus from the water to the sediment can be a successful measure, provided it is undertaken so that phosphorus remains permanently bound to the sediment. Experiments with precipitation of phosphorus have been undertaken with aluminium sulphate (alum), ferric salts (chlorides and sulphates), ferric aluminium sulphate, clay particles and lime as $Ca(OH)_2$ and $CaCO_3$. Ferric salts are effective in precipitating phosphorus, but are difficult to handle because of their acidity. Furthermore, the iron-phosphorus complex is stable only under oxic conditions, which means that phosphorus may be released from the anoxic sediments of eutrophic waters. In addition, iron may be a limiting micronutrient in some systems, and, in such situations, treatment with ferric salts may actually stimulate cyanobacterial growth. Hydrogen ions are liberated when alum is added to water bodies, especially lakes with a low or moderate alkalinity, leading to a sharp decrease in pH. This may consequently lead to the formation of toxic species of aluminium such as Al^{3+} and $Al(OH)^{2+}$ (Cooke *et al.*, 1993). An increase in the pH of a water body above pH 8 may result in re-release of the phosphorus from the aluminium flocs (Lewandowski *et al.*, 2003). Lime, as described previously, is used for the flocculation of intact cyanobacterial cells. Lime has also been shown to function as a longer-term algal inhibitor, as it is able to precipitate phosphorus from the water. $Ca(OH)_2$ precipitates phosphorus more efficiently than $CaCO_3$. The dose rates are quite high for sufficient phosphorus precipitation, which limits the use of this technique in large lakes and dams (Chorus & Mur, 1999). There is therefore a need for a treatment method that can bind phosphorus in a stable manner and remove it from eutrophic waters under both anoxic and oxic conditions, as well as over wide pH ranges.

4.4.3. Physical sequestering of nutrients

Asaeda *et al*. (2001) installed two vertical curtains having depths that covered the epilimnion thickness of Terauchi dam in Japan. The purpose of the curtains was to curtail the nutrient supply from nutrient rich inflows to the downstream epilimnion of the reservoir. There was a marked reduction in cyanobacterial blooms downstream from the curtain in spring and summer. The curtain prevented the direct intrusion of nutrients into the downstream zone. Epilimnion algal concentrations were higher in the upstream zones. Thus, within the upstream zone the algae consume large amounts of the inflow nutrients, reducing the nutrient supply to the downstream zone of the reservoir. Floating curtains such as these may be used to segregate *Microcystis* algal blooms, minimising turbulence. This would allow the introduction of microbial antagonists, and afford the predator ample time to attach to the prey and initiate the lytic process.

4.4.4. Phoslock® as a eutrophication management tool

Lanthanum is a rare earth element (REE) that is relatively abundant in the earth's crust compared to other REEs. Lanthanum compounds have been used in water treatment processes, as they are cheaper than those derived from other rare earth elements and the point of zero charge of lanthanum oxides is higher than that of other well-known adsorbants (Woo Shin *et al*., 2005). Examples include use of lanthanum salts for precipitative removal of Arsenic (As) ions (Tokunaga *et al*., 1997; Tokunaga *et al*., 1999), the use of lanthanum impregnated silica gel for removal of arsenic, fluoride and phosphates (Wasay *et al*., 1996a) and lanthanum oxide and lanthanum impregnated alumina for adsorptive arsenic removal (Wasay *et al*., 1996b). According to Douglas *et al*. (2000), lanthanum was highly efficient at removing phosphorus with a molar ratio of 1:1 (Equation 2), compared with sodium aluminate ($NaAlO_2$), which is relatively inefficient with a molar ratio of 7:1 needed to achieve a similar phosphorus uptake.

$$La^{3+} + PO_4^{3-} \rightarrow LaPO_4 \qquad \ldots(2)$$

Ning *et al*. (2008) developed a La(III)-modified zeolite adsorbent (LZA), and examined its phosphate adsorption capacity in sewage plant effluent, in the presence of other

anions such as sulfates, bicarbonates, and chlorides. The LZA showed good selectivity for phosphate removal, and the authors were able regenerate the LZA for re-use.

Lanthanum is toxic, depending on its concentration and application rate. It can react with cell components such as nucleoproteins, amino acids, enzymes, phospholipids and intermediary metabolites. This is because lanthanum has many physical and chemical characteristics in common with calcium. Its action is mainly mediated by the replacement or displacement of calcium in different cell functions and its high affinity for the phosphate groups of biological molecules, resulting in toxicity or impaired function. Lanthanum is considered only slightly toxic to mammals. It is, however, highly toxic to species of *Daphnia* in both acute and chronic tests (Barry & Meehan, 2000). The potential toxicity of lanthanum ions has been overcome by incorporating it into the structure of high exchange capacity minerals, such as bentonite. This lanthanum-modified bentonite, known as Phoslock®, was developed by the Australian CSIRO, and forms a highly stable mineral known as rhabdophane ($LaPO_4.nH_20$) in the presence of oxyanions such as orthophosphates (Douglas *et al*., 2000). Rare earth- anion products are stable, due to their low solubility (Firsching, 1992). The incorporation of the lanthanum ions into bentonite is obtained by taking advantage of the cation exchange capacity of clay minerals. This exchange capacity is a result of a charge imbalance on the surface of the clay platelets, which is balanced by surface adsorbed cations exchangeable in aqueous solutions. During the preparation of Phoslock®, lanthanum ions are exchanged with these surface adsorbed exchangeable cations (Douglas *et al*., 2000). As the rare earth element is locked into the clay structure, it can either react with the phosphate anion in the water body or stay within the clay structure under a wide range of physiochemical conditions. In low ionic strength water, the lanthanum remains strongly bound to the clay silicate plates, but under conditions of high ionic strength (saline water) there is a possibility of re-exchange of the bound La^{3+} for ambient Na^{+} or Ca^{2+} ions. This is not a possibility in fresh water, but may present a problem in estuaries. Any lanthanum released under these conditions is not expected to remain free, but to become strongly associated with natural humic material in the water and sediments through interaction with carboxylate groups in humic and fulvic acids (Geng *et al*., 1998; Dupre *et al*., 1999). Specific formulations of Phoslock® are used under estuarine/saline conditions to minimize lanthanum release. Phoslock is capable of

removing dissolved P under anoxic conditions, as well as over a wide pH range (pH 5-11), making it a unique water treatment product (Douglas *et al*., 1999).

As the lanthanum exchange process is carried out in solution, Phoslock® was originally prepared as slurry. However, the disadvantages of the transport of the excess water and the presence of excess residual lanthanum ions from the manufacturing process led to the formation of the granular form of Phoslock®. One of the essential features of this granular Phoslock® is that it should disperse into fine particles in water that have a similar particle size distribution to that of the parent slurry. This is necessary to ensure that the maximum number of lanthanum sites are exposed to the phosphate ions.

Two full-scale Phoslock® applications were undertaken in the summer of 2001/2002 in the impounded riverine section of two estuaries subject to cyanobacterial blooms along the coastal plain of south-western Australia. Phoslock® applied as a slurry from a small boat reduced the dissolved P in the water column to below detection limits within a few hours, and substantially reduced the amount of P released from the sediment throughout the course of the trial. The effect of the reduction in the P concentration on phytoplankton growth was clear, with the chlorophyll a concentrations of the treated areas being significantly lower than the control areas (Robb *et al*., 2003).

5. Microbial community analysis

Manipulation of the chemical and physical elements of a water body is likely to affect the microbial dynamics, both directly and indirectly. It is important to be able to describe these changes, both quantitatively and qualitatively.

According to Dejonghe *et al*. (2001), microbial diversity can be described by two components: the species richness or abundance, which is the total number of species present, and the species evenness or equitability, which is the distribution of individuals among those species. The richness component of diversity has been determined by methods such as plating, fluorescence and light microscopy and, more recently, DNA and RNA analysis (Dejonghe *et al*., 2001; Duineveld *et al*., 2001). For years, the most popular technique for investigating microbial diversity was plating (cfu). However, it is difficult to culture bacteria from environmental samples due to the selectivity of growth

media and conditions (Sekiguchi *et al*., 2002). Only 1-10% of global bacterial species are culturable (Duineveld *et al*., 2001; Von Wintzingerode *et al*., 2002). The relative proportion of bacteria growing on agar plates to those counted by fluorescence microscopy varies from 0.1% to 10%, which implies that investigations based on bacterial isolates may only include a small part of the total bacterial diversity (Amann *et al*., 1990). Although microscopic techniques can be used to obtain information about bacterial numbers and special distribution, these techniques lack the ability to assess diversity and distinguish between microbial populations (Duineveld *et al*., 2001).

The introduction of molecular methods to microbial community analysis provided a means to more accurately determine species richness within diversity. A first attempt to study unculturable as well as culturable species in an environment involved cloning random fragments of environmental genomic DNA and then sequencing clones that contained rRNA genes (Dejonghe *et al*., 2001; Fromin *et al*. 2002). However, this process is laborious and time consuming, and is therefore not suitable for the study of successional population changes in a microbial community. Hybridization techniques that make use of specific oligonucleotide probes are more suited to studying population dynamics, but probes rely on sequence data, and may be either too specific, targeting one population only, or too general, overlooking closely related but ecologically different populations (Muyzer, 1999). Because of the laboriousness of cloning, researchers began to use PCR to selectively amplify these rRNA genes from total microbial community DNA. This technique uses different primer sets to amplify the ribosomal genes of all types of organisms (Archaea, Bacteria or Eukarya) present in an environmental sample (Dejonghe *et al*., 2001). The DNA fragments obtained from this technique can be sequenced, or separated and visualized by various fingerprinting techniques (Dejonghe *et al*., 2001; Duineveld *et al*., 2001). Fingerprinting techniques enable the analysis of the diversity of different populations in natural ecosystems, and offer the potential to monitor community behavior over time (Muyzer, 1999).

5.1. Culture independent assessment of microbial communities

Methods for microbial community analysis that are culture-independent involve the extraction and analysis of signature biochemicals from environmental samples (Blackwood *et al*., 2003). Extracted genomic or ribosomal nucleic acids analysed using

molecular genetic techniques enables microbial community analysis to be coupled with a phylogenetic framework (Amann *et al*., 1995). The uncultured diversity of a sample reflects species that are closely related to culturable organisms as well as well as species from virtually uncultured lineages (Blackwood *et al*., 2003).

Molecular methods usually involve the separation of PCR amplicons on the basis of DNA sequence differences. These include denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), single strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP) and amplified ribosomal DNA restriction analysis (ARDRA) (Blackwood *et al*., 2003). These methods only reveal diversity if the community is relatively simple, as only a small fraction of the species indicated by DNA rehybridisation rates or clone library sequence analysis can be seen on a gel (Nakatsu *et al*., 2000). However, these methods provide a rapid means to determine the relative abundance of common species in a sample in a manner independent of culture constraints, and are valuable for testing hypotheses based on the comparison of samples (Blackwood *et al*., 2003).

Ribosomal RNA (rRNA) molecules are most often used as molecular chronometers because they are highly conserved in terms of structure and function (Kent & Triplett, 2002). Ribosomes are the organelles in which translation of RNA to proteins takes place. The relative size and density of ribosomes and their subunits is expressed in Svedberg units (S), on the basis of its sedimentation rate in a sucrose density gradient during centrifugation. Prokaryotic ribosomes consist of two subunits made up of RNA and proteins, a 30S subunit and a 50S subunit. The 30S subunit of a prokaryotic ribosome consists of a 16S molecule of rRNA, which is coded by the 16S gene of the bacterial genome, and 21 proteins (Nester *et al*., 2001). Certain domains within rRNA molecules undergo independent rates of sequence change, and are known as hypervariable regions (Yu & Morrison, 2004). Phylogenetic relationships can be determined by analysing these sequence changes over time (Kent & Triplett, 2002). Currently, 16S rDNA sequences constitute the largest gene-specific data set, and the number of entries in generally accessible databases is continually increasing, making 16S rDNA-based identification of unknown bacterial isolates more likely (von Wintzingerode *et al*., 2002). However, the taxonomic resolution of 16S rRNA genes is sometimes insufficient for the discrimination of closely related organisms. As a result,

research has also focused on the rRNA 16S to 23S internal transcribed spacer (rRNA-ITS). This region may enable high resolution analysis due to its greater degree of sequence heterogeneity when compared to 16S rDNA, as well as the considerable number of published rRNA-ITS sequences (Janse *et al*., 2003).

5.1.1. Single-strand conformation polymorphism

SSCP was developed for the detection of mutations, mainly in human genetics (Swieger & Tebbe, 1998). The method involves the separation of single strands of PCR-amplified rRNA genes with the same length but different conformational structure in a polyacrylamide gel (Lee *et al*., 1996). Under non-denaturing conditions, single stranded DNA molecules will fold into specific secondary structures according to their sequences and physicochemical environment (Swieger & Tebbe, 1998). SSCP can be used in combination with an automated sequencer to differentiate between species based on the PCR products of 16S rRNA genes (Widjojoatmodjo *et al*., 1995). A major limitation of SSCP for the analysis of community DNA is the high rate of reannealing of single stranded DNA after initial denaturation during electophoresis, especially at the high concentrations of DNA that are often required for analysis of high diversity communities. Another disadvantage of SCCP is that more than one band is detectable on a gel from a double stranded PCR product following electrophoresis. Three bands are typically visible, two single strands and one double-stranded DNA molecule. However, several conformations of one product may coexist in one gel leading to multiple bands. Also, conformations of products might be similar, resulting in the detection of fewer than three bands per organism. Finally, PCR products with similar sequences may adhere to each other, forming heteroduplex molecules (Swieger & Tebbe, 1998).

5.1.2. Terminal restriction fragment length polymorphism

T-RFLP can effectively discriminate between microbial communities in a range of environments (Blackwood *et al*., 2003). This technique uses PCR of 16S rRNA genes, in which one of the two primers used is fluorescently labeled (Dejonghe *et al*., 2001). The amplified PCR product is then cut with a restriction enzyme. Terminal restriction fragments (T-RFs) are separated by gel electrophoresis and visualized by exciting the

fluorescent label (Blackwood *et al*., 2003). With this technique, a pattern of bands is obtained, with each pattern corresponding to a different species (Dejonghe *et al*., 2001). T-RF sizes can be compared to a theoretical database derived from sequence information (Blackwood *et al*., 2003), thus providing information about changes in the community structure as well as an idea of the microbial richness of an ecosystem (Dejonghe *et al*., 2001). T-RFLP profiles have the advantage of being relatively robust to variability in PCR conditions (Blackwood *et al*., 2003).

5.1.3. Amplified ribosomal DNA restriction analysis

ARDRA is another DNA fingerprinting technique based on PCR amplification of rRNA genes in combination with restriction of the amplified fragments (Dejonghe *et al*., 2001). This technique appears to give too many bands per species to provide reliable genotypic characterisation of communities, but it is capable of monitoring specific populations within microbial communities and is useful for analysing bacterial diversity (Torsvik *et al*., 1998).

5.1.4. Reverse transcription PCR

A picture of the metabolically active members in a system can be obtained by extraction of RNA instead of DNA, followed by reverse transcription PCR (RT-PCR) (Dejonghe *et al*., 2001). The first step involves the production of complementary DNA (cDNA) from a messenger RNA (mRNA) template, employing the use of dNTPs and an RNA-dependant reverse transcriptase at 37°C. In the second step, double-stranded DNA is produced using a thermostable transcriptase and a set of upstream and downstream DNA primers. After approximately 30 cycles of PCR, the original RNA template is degraded by RNase H, leaving pure cDNA in solution. Exponential amplification using RT-PCR provides a highly sensitive technique that can detect very low copy number RNAs. The technique is widely used in genetic disease diagnosis, and the quantitative determination of RNA in a cell or tissue gives an indication of gene expression levels.

5.1.5. Denaturing gradient gel electrophoresis

Muyzer *et al*., (1993) introduced DGGE as a new approach for directly determining the diversity of complex microbial populations. DGGE relies on the sequence variation of a specific amplified region to differentiate between species, thus enabling the evaluation of genetic diversity, the monitoring of succession in microbial communities, and the determination of the dominant communities in a sample (Cocolin *et al*., 2001; Koizumi *et al*., 2002; Stamper *et al*., 2003). DGGE can also be used to determine the purity and uniqueness of isolated strains. A portion of DNA is suitable for analysis using DGGE if it can be amplified specifically from the target organism, if it has enough sequence heterogeneity for the desired resolution and if it is part of a gene for which a large amount of sequence information has been deposited in sequence databases (Janse *et al*., 2003).

The procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (Muyzer *et al*., 1993). The denaturants most commonly used are constant heat (60°C) formamide (0-40%) and urea (0-7M). The double stranded DNA fragments of 200-700 basepairs are equal in length but differ in basepair sequences (Ferris *et al*., 1996; Nakatsu *et al*., 2000, Kawai *et al*., 2002). Separation in DGGE relies on difference in the mobility of a partially melted DNA molecule during electrophoresis in polyacrylamide gels when compared with that of the completely helical form of the molecule (Muyzer *et al*., 1993). Initially, the fragments move according to their relative molecular mass. However, when a sufficiently high denaturant concentration is reached, strand separation occurs (Curtis & Craine, 1998). The “melting” of fragments proceeds in discrete “melting domains”, which are portions of the DNA fragment which require the same concentration of denaturants in order to separate. Once the melting domain with the lowest denaturing concentration requirement reaches that position in the DGGE gel, a transition from helical to partially melted molecules occurs, and migration of the molecule will practically come to a halt (Muyzer *et al*., 1993), forming a discrete band in the gel. The base pair composition and more importantly the sequence of the fragment determines the denaturant concentration at which this occurs, and analysis of a complex microbial community therefore results in a ladder of bands on the gel (Curtis

& Craine, 1998; Ferris *et al*., 1996; Wu *et al*., 1998). The technique is sensitive enough to detect single base pair differences in sequences (Myers *et al*., 1985).

The electrophoresis bands can either be probed with diagnostic oligonucleotides to identify particular sequences (Muyzer *et al*., 1993), or the bands can be excised from the gel, reamplified using PCR and then sequenced (Ferris *et al*., 1996). Alternatively, markers can be constructed using known species sequences, and the marker run alongside test samples to determine the identity of bands within the sample. This method was employed by Theunissen *et al*. (2005) for the analysis of probiotic organisms from yoghurt and lyophilized capsule and tablet preparations. Two markers consisting of the PCR-product of known lactobacilli and *Bifidobacterium* were run adjacent to test samples, and band patterns could be used for rapid species identification. Keyser *et al*. (2006) developed a marker to identify the dominant *Archaea* in upflow anaerobic sludge blanket bioreactors. They concluded that the DGGE marker holds great potential for the molecular monitoring of individual microorganisms as well as population shifts that may occur in anaerobic bioreactors.

The resolution of DGGE can be enhanced by incorporating a GC-rich sequence into one of the primers to modify the melting behavior of the fragment, allowing the detection of virtually all possible sequence variations (Curtis & Craine, 1998; Ferris *et al*., 1996; Muyzer *et al*., 1993). A GC clamp attached to the 5' end of the PCR product also prevents complete melting of the fragment during separation in the denaturing gradient (Heuer *et al*., 1997). Sheffield *et al*. (1989) attached a 40 base pair GC clamp to one end of amplified DNA fragments that encompass regions of the mouse and human β-globin genes. The clamp increased the number of mutations detectable by DGGE from 40% of all possible single base changes to close to 100%. In some cases, the attachment of a GC clamp alters the melting behavior of domain in such a way that the choice of which denaturant conditions to use is simplified. When a DGGE fragment with two or more melting domains is separated by electrophoresis on a DGGE gel, the fragment will be arrested at the position in the gel where the denaturant concentration dissociates the fragment at its lowest melting domain (Wu *et al*., 1998). Therefore, if the fragment has more than one melting domain a GC clamp may not be necessary. Wu *et al*. (1998) found that GC-clamped products with a perfect melting curve often resulted in smears or diffuse bands, whereas fragments containing a high melting domain run without a

GC clamp provided sharper bands and thus better results. They concluded that if the melting analysis of a short fragment (<200bp) predicts a high melting domain <40bp in size located at the end of the fragment and differing by not more than 5°C in melting temperature, then the fragment is suitable for DGGE analysis without a GC clamp.

5.1.5.1. Community diversity analysis using DGGE banding patterns

The variations between DGGE profiles were classically described visually on a single gel by the disappearance, appearance or the changes in the intensity of specific bands. However, an increasing number of studies propose statistical analysis of DGGE banding patterns, and employ various software packages to lead to more refined results (Fromin *et al*., 2002). Banding patterns on DGGE gels can be normalised using gel image software, using a reference pattern consisting of known type strains. By including a reference pattern consisting of six different type strains every six lanes on a gel, Temmerman *et al*. (2003) were able to normalise the gel patterns from probiotic products, enabling the comparison of different DGGE gels. For each known probiotic species, the band position of the corresponding type strain was determined and stored in a database, allowing individual bands in future gels to be rapidly identified. Normalisation software also allows images to be compared when samples are collected and analysed over a period of time, making it possible to monitor changes in community structure (van Hannen *et al*., 1999). Banding pattern similarity can be compared using dendrograms, which can identify outlier clusters and show the degree of intra-group similarity (Stamper *et al*., 2003).

Because DGGE makes it possible to screen multiple samples, it enables monitoring of fluctuations in microbial communities during seasonal and environmental changes in their habitat (Muyzer, 1999). Ward *et al*. (1998) were among the first to use DGGE of 16S rDNA fragments to study population changes in microbial communities. They examined the seasonal distribution of community members in a hot spring microbial mat community. More recently, Pierce *et al*. (2005) studied variation in the bacterioplankton community structure of three Antarctic lakes of different nutrient status subject to extremely rapid environmental change during the seasonal transition from winter to summer. Their results indicated that the changes in nutrient input and

duration of ice-cover lead to marked changes in the structure and stability of the bacterioplankton community.

DGGE fingerprint interpretation assumes that the band intensity is directly related to the species abundance, where each band represents a single species. Various software packages capable of calculating the relative band densities are employed to determine diversity indices (Fromin *et al*., 2002; Stamper *et al*., 2003). Most microbial diversity indices are based on indices developed for plant and animal studies, for example the Shannon Weaver and Simpson indices. There is some difficulty in applying these indices to microbial communities, as a clear definition of species and an unambiguous identification of each individual is necessary. An ideal bacterial index should ideally satisfy the following conditions (Watwe & Gangal, 1996): (i) the index should encompass three important dimensions of diversity, namely the species richness or number of different biotypes, their relative abundances and the differences or taxonomic distances between biotypes, (ii) it should be based on a statistically justified parameter and should not be sensitive to small changes in this parameter, (iii) possible errors or test result variability should not disproportionately affect the index, and (iv) since samples of microbial communities are small in comparison to the ecosystem, the index should not be overly sensitive to sample size. The Shannon index incorporates aspects of both species richness and species evenness, weighting individual classes by their relative abundances, and is the most common diversity index used by microbial ecologists. Nübel *et al*. (1999) quantified the diversity of oxygenic phototrophs (cyanobacteria, diatoms and green microalgae) in hypersaline microbial mats. The number of bands visible in the DGGE gels provided an estimate of richness, and the relative band intensity allowed for the calculation of the proportional abundance ("evenness") of each population and the Shannon-Weaver indices.

5.1.5.2. Limitations of DGGE

As with any molecular method, DGGE has certain limitations. More than one species may be represented by a single band on the gel, either as a result of phylogenetically related species sharing analogous sequences in the amplified area, or of similar melting profiles between phylogenetically unrelated species. The co-migration of non-related sequences to an identical point in the gel is especially a problem in complex microbial

communities (Fromin *et al*., 2002). For closely related organisms, the relationship between nucleotide sequence, phylogeny and the melting point is not well established. The retardation of a fragment in the gel matrix may therefore not properly indicate phylogenetic relatedness at a high resolution, such as the species level (Kisand & Wikner, 2003).

Jackson *et al*. (2000) used site directed mutagenesis to create *E. coli* 16S rDNA fragments differing by 1-4 base pairs. Migration on a DGGE gel was able to consistently distinguish single base changes, however, fragments with multiple base changes proved more difficult to resolve. Ferris & Ward (1997) detected artificial bands when analyzing complex banding patterns, which were most likely a result of heteroduplex molecules. Multiple bands may also be produced from a single species as a result of molecules produced by different rRNA operons of the same organism (Muyzer, 1999). It is generally accepted that only populations representing more than 0.1-1% of the target organisms in terms of relative proportion are displayed in a DGGE profile, and as a result not all populations present in a habitat appear on the gel (Fromin *et al*., 2002; Muyzer, 1999). Some of these limitations, such as the presence of heteroduplex molecules are not commonly found, whereas other limitations such as the limited sensitivity can be improved by hybridisation analysis or by the application of a group specific PCR (Muyzer, 1999). It is possible to analyse DNA fragments up to 1000 base pairs using DGGE, but larger fragments are not suitable. Large fragments migrate very slowly in polyacrylamide gels, and the degree of resolution between mutant and wild-type fragments decreases with size due to the melting of multiple domains in larger fragments (Sheffield *et al*., 1989).

It is important to note the pitfalls of molecular ecological approaches when studying microbial diversity. Each physical, chemical and biological step involved in the molecular analysis of the environment is a source of bias which may lead to a distorted view of the microbial community structure. The method of sample collection and preservation is crucial for the subsequent analysis steps (von Wintzingerode *et al*., 1997). The importance of sample handling procedures was illustrated by Rochelle *et al*. (1994). There was significant variation in 16S rRNA gene types and diversity from anaerobic deep marine sediment samples. Samples stored aerobically for 24h before freezing contained mainly beta and gamma Proteobacteria, whereas samples stored

anaerobically at 16°C contained mainly sequences representing alpha Proteobacteria. Samples taken anaerobically and frozen within 2h had the highest species diversity. von Wintzingerode *et al*. (1997) recommend releasing and stabilizing the nucleic acids immediately after sample collection. Lysis of microbial cells from environmental habitats to release the cell contents represents a crucial step in a PCR-mediated approach. Insufficient or preferential cell disruption will bias the view of microbial diversity; however, rigorous conditions may result in sheared DNA fragments which increase the formation of chimeric molecules during PCR. Contaminants must be removed from nucleic acid preparations, as certain molecules inhibit downstream reactions. Humic acids from soils strongly inhibit *Taq* polymerases.

PCR amplification has become the method of choice for obtaining rRNA sequence data from microbial communities. Although the method is routine for pure cultures, several problems arise when the method is applied to environmental samples. Co-extracted contaminants can inhibit amplification, differential amplification may occur and artefactual PCR products may form. These include chimeric molecules, which are composed of parts of two different but homologous sequences, deletion mutants due to stable secondary structures and point mutants due to misincorporation of nucleotides by DNA polymerases (von Wintzingerode *et al*., 1997). Amplified DNA can only qualitatively reflect species abundance if the efficiency of amplification is the same for all molecules. This requires several assumptions: (i) all the molecules must be equally accessible for primer hybridisation, (ii) hybridisation of the primer to the template must occur with equal efficiency, (iii) the DNA polymerase must extend with equal efficiency for all templates, and (iv) limitations imposed by exhaustion of substrate must affect the extension of all templates equally (Suzuki & Giovannoni, 1996). Lyautey *et al*. (2005) amplified the same DNA extract with three different PCR reactions. When the replicate amplicon was loaded onto the gel, dissimilarity between amplicons was only 3% of the detected bands. They confirmed that amplification is therefore not the step that introduces much variability into the analysis process. Contaminating DNA containing the specific target sequence of the PCR reaction can lead to amplification in the negative control without external DNA being added, and co-amplify in the experimental reactions. One also has to consider that 16S rRNA sequence variations due to *rrn* operon heterogeneity unavoidably lead to a biased reflection of the microbial diversity (von Wintzingerode *et al*., 1997).

Despite the limitations of DGGE, it is a well-established molecular tool in environmental microbiology and is reliable, reproducible, rapid and inexpensive. DGGE allows the simultaneous analysis of multiple samples, making it possible to monitor changes in microbial communities over time (Fromin *et al*., 2002; Muyzer, 1999).

6. Conclusion

Toxic cyanobacterial blooms have many implications for human health, as well as water quality. Eutrophication of water sources remains a problem that as yet has not been solved, mostly due to a lack of compliance with standard regulations, as well as the increasing human population. There is a need for a safe and effective treatment for eutrophic water bodies. Research into developing further understanding of the human health significance of cyanobacteria and individual cyanotoxins is a priority, and safe guideline values for toxins other than microcystin need to be established. Information concerning the efficiency of cyanotoxin removal in drinking water sources is limited. Simple, low-cost techniques for cyanobacterial cell removal, such as slow sand removal, should be investigated and developed further.

Biological control of toxic algal blooms, especially with bacteria, is an attractive solution. To date, however, the focus has been on laboratory studies when the efficiency of these agents in lysing cyanobacteria has been investigated. Although laboratory studies have an important part to play in biological control work, results obtained should be viewed with caution if they are to be interpreted in the lake context (Sigee, 1999). Laboratory data cannot simply be extrapolated to the freshwater environment. In cases where a biological control agent is shown to be effective, environmental testing as well as full-scale field trials need to be conducted.

Chemical control mechanisms have been employed often in the past to control cyanobacterial blooms, but they often lead to the release of toxins through cell lysis. Flocculants such as alum and lime result in less toxin release, but the introduction of these chemicals into aquatic ecosystems is often unfavourable. Turbulent mixing of a water body will reduce the cyanobacterial growth by giving green algae a competitive edge, but does not address the problem of eutrophication itself, only the symptoms.

Eutrophication management is the only feasible means of treating the cause of cyanobacterial blooms. It is important that the amount of nutrients entering eutrophic water bodies be drastically reduced, although highly eutrophic bodies make take many years to return to a mesotrophic state. Nutrient limitation through intervention may be the solution. Phosphorus limitation has been identified as being more achievable than nitrogen limitation, and there are various chemicals available for this purpose. The disadvantage of many of these however, is that they will release P under certain conditions of pH and anoxia, and some are toxic. Phoslock® is stable over a wide pH range, does not release P under anoxic conditions, and is non-toxic and environmentally friendly. Because P is permanently locked away and is not bioavailable, Phoslock® appears to be a viable means of eutrophication control.

Altering the chemistry of a water body by limiting certain nutrients is likely to affect the microbial community composition. Limiting P will result in an increase in the N:P ratio, and thus a shift in the algae population from cyanobacteria to green algae is expected, as well as the cyanobacterial species composition itself. Various methods for investigating microbial communities have been reviewed here. DGGE is a reliable, reproducible and well-established molecular tool in environmental microbiology that allows the simultaneous analysis of multiple samples, making it possible to monitor changes in microbial communities over time. It is the method of choice for many community studies.

Future research and goals

- More work needs to be done to determine the effects of cyanotoxins on human health, and safety guidelines for all such toxins in drinking water need to be set. The establishment of such guidelines will in turn increase the need for an effective and inexpensive toxin removal system. The use of bacteria for this purpose has shown great possibility, and more research needs to be conducted in this regard.
- The potential for the use of biological control agents in cyanobacterial bloom control needs to be investigated further. The bacterial species that are the most effective on a laboratory scale need to be applied in more large scale tests, as little data is available on the effects of up-scaling laboratory trials.

- Products and processes that focus on the removal of phosphorus rather that treatment of the cyanobacterial blooms need to be developed and improved. Treatment of the causes of eutrophication, rather than its symptoms may be the only way to remediate eutrophic water bodies.
- It is essential that the orthophoshate standard of 1mg.l^{-1} be complied with. Monitoring of industrial and sewage effluent is necessary, and there is a need for authorities to punish non-compliant offenders.

7. References

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CHAPTER 3:

CHARACTERISATION AND KINETICS OF PHOSLOCK®

Co-supervisor: Dr. F. Haghseresht

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1. Introduction

Lanthanum is a rare earth element (REE) that is relatively abundant in the earth's crust compared to other REEs. Lanthanum compounds have been used in water treatment processes, as they are cheaper than those derived from other rare earth elements and the point of zero charge of lanthanum oxides is higher than that of other well-known adsorbants (Woo Shin *et al*., 2005). Examples include use of lanthanum salts for precipitative removal of Arsenic (As) ions (Tokunaga *et al*., 1997; Tokunaga *et al*., 1999), and the use of lanthanum oxide and lanthanum impregnated alumina for adsorptive As removal (Wasay *et al*., 1996), and lanthanum impregnated silica gel for removal of As, fluoride and phosphates (Wasay *et al*., 1996). According to Douglas *et al*. (2000), lanthanum was highly efficient at removing phosphorus with a molar ratio of 1:1 (Equation 1), compared with sodium aluminate ($NaAlO_2$), which is relatively inefficient with a molar ratio of 7:1 needed to achieve a similar phosphorus uptake.

$$La^{3+} + PO_4^{3-} \rightarrow LaPO_4 \qquad (1)$$

Lanthanum is toxic, depending on its concentration and application rate. It can react with cell components such as nucleoproteins, amino acids, enzymes, phospholipids and intermediary metabolites. This is because lanthanum has many physical and chemical characteristics in common with calcium. Its action is mainly mediated by the replacement or displacement of calcium in different cell functions and its high affinity for the phosphate groups of biological molecules, resulting in toxicity or impaired function. Lanthanum is considered only slightly toxic to mammals. It is, however, highly toxic to species of *Daphnia* in both acute and chronic tests (Barry & Meehan, 2000). The potential toxicity of lanthanum ions has been overcome by incorporating it into the structure of high exchange capacity minerals, such as bentonite by taking advantage of the cation exchange capacity of clay minerals. This exchange capacity is a result of a charge imbalance on the surface of the clay platelets, which is balanced by surface adsorbed cations. These cations are exchangeable in aqueous solutions. As the rare earth element is locked into the clay structure, it can either react with the phosphate anion in the water body or stay within the clay structure under a wide range of physiochemical conditions (Douglas *et al*., 2000). Rare earth-anion products are stable, due to their low solubility (Firsching, 1992). Phoslock® forms a highly stable mineral

known as rhabdophane ($LaPO_4.nH_2O$) in the presence of oxyanions such as orthophosphates (Douglas *et al*., 2000).

In low ionic strength water, the lanthanum remains strongly bound to the clay silicate plates, but under conditions of high ionic strength (saline water) there is a possibility of re-exchange of the bound La^{3+} for ambient Na^{+} or Ca^{2+} ions. This is not a possibility in fresh water, but may present a problem in estuaries. Any lanthanum released under these conditions is not expected to remain free, but to become strongly associated with natural humic material in the water and sediments through interaction with carboxylate groups in humic and fulvic acids (Geng *et al*., 1998; Dupre *et al*., 1999). Specific formulations of Phoslock® are used under estuarine/saline conditions to minimize lanthanum release.

Metal salts, such as ferric salts and alum, can effectively precipitate phosphorus, but these have certain disadvantages. They are generally difficult to handle because of their acidity. Furthermore, the iron- or the aluminium- phosphorus complex is stable only under oxic conditions, which means that phosphorus may be released from the anoxic sediments of eutrophic waters (Chorus & Mur, 1999). Hydrogen ions are liberated when alum is added to water bodies, especially lakes with a low or moderate alkalinity, leading to a sharp decrease in pH. This may consequently lead to the formation of toxic species of aluminium such as Al^{3+} and $Al(OH)^{2+}$ (Cooke *et al*., 1993). An increase in the pH of a water body above pH 8 may result in re-release of the phosphorus from the aluminium flocs (Lewandowski *et al*., 2003).

As the lanthanum exchange process is carried out in solution, Phoslock® was originally prepared as a slurry. However, the disadvantages of the transport of the excess water and the presence of excess residual lanthanum ions from the manufacturing process led to the formation of the granular form of Phoslock®. One of the essential features of this granular Phoslock® is that it should disperse into fine particles in water that have a similar particle size distribution to that of the parent slurry. This is necessary to ensure that the maximum number of lanthanum sites are exposed to the phosphate ions.

In this study, the effects of various solution conditions on the kinetics and phosphorus adsorption capacity of Phoslock® was evaluated, as well as the effect of different

Phoslock® dosages. The effect of initial pH and phosphorus concentration was assessed in synthetic solutions, and algae-containing effluent lake water was used to analyse the performance of Phoslock® under algal bloom conditions. In this instance, the stability of the adsorbed phosphate under anoxic conditions was also examined and higher Phoslock® dosages were applied to lake water with a pH above 9 to examine the possibility of achieving a greater phosphorus removal.

2. Materials and methods

2.1. Column tests

20L perspex columns 1m long with an 8cm radius were used to evaluate Phoslock® performance under different conditions. The columns were housed in a wooden cabinet, each column surrounded by three daylight-emitting spectrum tubes and an IKA RW overhead stirrer. The columns had five taps at regular intervals along their length to facilitate sampling from different depths.

2.1.1. The effect of pH on Phoslock® performance

To evaluate the effect of different pH values on the performance of Phoslock®, synthetic solutions were prepared using reverse osmosis (RO) water. KH_2PO_4 salt (ChemSupply) was used to make a 25mg.l^{-1} phosphorus stock solution, and 800ml of this stock solution was added to 19.2L reverse osmosis water in the 20L columns to give a 1mg.l^{-1} phosphorus concentration in the columns. The conductivity of the solutions was adjusted to 0.3mS by the addition of 3.5g NaCl. The solutions were mixed overnight with the overhead stirring apparatus (IKA RW 20.n), set at the lowest speed (~200 rpm). Prior to starting the experiment the next day, the pH of the columns was adjusted to 5, 7, 8 and 9 respectively using 0.1M solutions of HCl and NaOH. An initial sample of the column test solution was taken prior to addition of Phoslock® by dispensing a quantity from a tap midway down the column into a 50ml Nalgene tube. 10ml was drawn up with a syringe and filtered through a 0.22µm filter disk (Millex-gp) into a 10ml plastic sample tube. Initial measurements of pH, conductivity (TPS Aqua-CP 1.1) and temperature were also made at this stage. pH, conductivity and temperature readings were also taken at various intervals throughout the experiment. A 230:1 ratio

of Phoslock® to phosphorus was used in all the columns. 4.5g of Phoslock® granules were measured into a 50ml Nalgene tube and RO water was added to the 15ml mark on tube, which was then vortexed for 1min to hydrate the Phoslock® granules. This slurry was then added to the columns, rinsing out remaining mixture from Nalgene tube with ≤5ml RO water from a squeeze bottle. An electronic timer was started immediately after the addition of the Phoslock® and samples were taken from the middle tap for turbidity, filterable reactive phosphorus (FRP) and particle size analysis at designated time intervals within a 6h period. Samples for turbidity were dispensed directly into the turbidimeter tube, and readings were performed on the Hach 2100A Turbidimeter, calibrated to the 100 standard range. Particle sizing was performed on the pH 5 and pH 9 solutions. 50ml was dispensed into a Nalgene tube from which 10ml was drawn up with a syringe and filtered through a 0.22µm filter disk into 10ml flat-bottomed tubes for FRP reading. Particle sizing was performed on the Malvern Mastersizer. Samples were diluted with a defined volume of tap water where necessary, and were analysed using the following particle size parameters; stirring speed 3, 3000 sweeps, low gain, and 100mm or 300mm lens depending on size of particles observed. To determine the FRP concentration of each filtered sample, 5ml was pipetted into glass test tubes. 5 drops of PO_4-1 reagent, followed by one scoop of PO_4-2 reagents from the phosphate test kit Spectroquant 0.01-5mg/l Phosphate Test Kit (Merck, catalogue #1.14848.0001), were then added to the samples, which were vortexed until the crystals were fully dissolved. Samples were then left to stand for 5 minutes before measuring absorbance with the Jasco V550 UV/Vis Spectrophotometer at 710nm. The absorbance readings were divided by the calibration coefficient 0.5061 to calculate the FRP concentration.

2.1.2. Lake water with algal bloom

Two columns were filled with environmental water samples, in this case collected from the effluent-fed lake at the University of Queensland, St Lucia Campus (Figure 1). The columns were left overnight, and a 12h day/night light schedule was applied using fluorescent bulbs under timer control (on: 6am, off: 6pm) to enhance algal growth. FRP concentration of lake water was determined prior to addition of Phoslock® (0 hour time measurement). Initial measurements at time 0 hrs were taken for pH, conductivity and temperature (TPS Aqua-CP 1.1) and turbidity (Hach Turbidimeter). A representative sample was also collected for analysis of the following parameters; Alkalinity (A),

Hardness (H), Lanthanum and Sodium (La/Na), Metals (M), and Chlorophyll a (Chl). Chlorophyll a analysis was performed using the methanol extraction method (Lorenzen, 1967; Golterman & Clymo, 1970; Holm-hansen, 1978) using the following equation:

$$\text{Chl a } (\mu g.l^{-1}) = (Abs_{665nm} - Abs_{750nm}) \times A \times Vm/V \times L \quad (2)$$

Where:
A = absorbance coefficient of Chl a in methanol (12.63)
Vm = volume of methanol used (mL)
V = volume of water filtered (L)
L = path length of cuvette (cm)
In all cases 100ml of water was filtered, 10ml of methanol was used for extraction and a cuvette with a path length of 1cm was used.

Other samples were sent away to be analysed by the Queensland Health Scientific services. Samples for alkalinity and hardness were preserved by refrigeration at 4°C, and filtered samples for lanthanum/sodium and metals were preserved with two-three drops of 1M HNO_3.

A 230:1 treatment ratio (Phoslock®: phosphorus) was added to one column. The second column was left untreated to act as a control. Samples taken for turbidity, FRP and particle size analysis at designated time intervals within a 6h period, in the same manner as for the pH column tests. The same size parameters were also applied to the particle sizing. Following the initial 6h of sampling, columns were monitored over a three-day period for changes in FRP, pH, temperature, DO and chlorophyll a. At 72h post Phoslock® addition, the column volume was increased with an additional 1L of lake water from the initial water sample. Bentonite was added to both columns at $0.5g.l^{-1}$ to flocculate the algae that remained on the surface. Fluorescent light schedule was suspended, and the columns were covered to prevent light penetration and further algal growth. Columns were monitored for a further three days following addition of bentonite, for changes in pH and FRP. At 72h post bentonite addition (6 days after initial Phoslock® treatment), Phoslock® was added to the treated column using a sediment-capping regime of $250g.m^{-2}$. Further monitoring of columns for pH, FRP, and DO continued for 5 days, and on the fifth day the columns were covered with parafilm

to accelerate the development of anoxic conditions (DO <1mg.l^{-1}). An anoxic state was achieved on the sixth day, allowing for assessment of whether the phosphorus remained bound to Phoslock® under anoxic conditions.

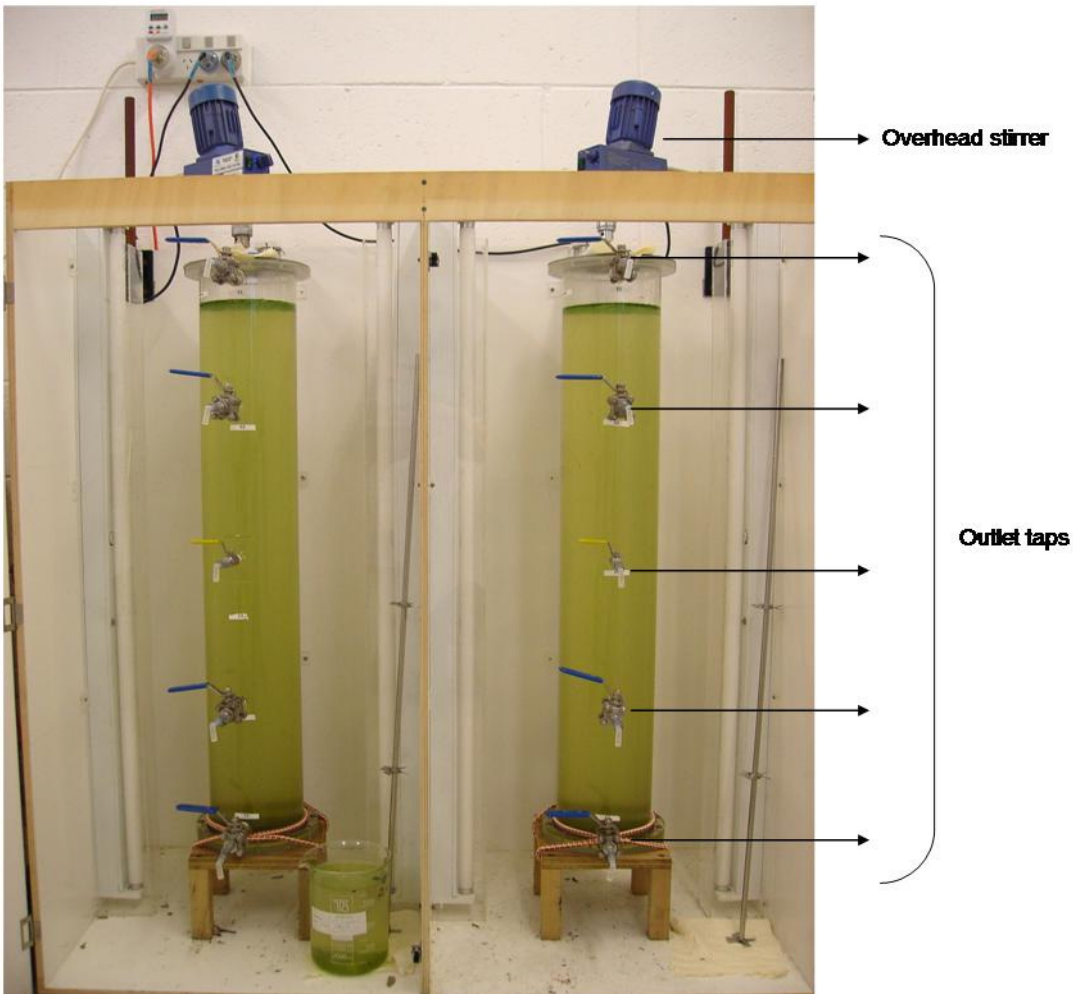

Figure 1: Columns filled with effluent lake water

2.1.3. Lake water with algal bloom treated at high dose ratios

Two further column tests were performed using the effluent lake water at pH 9, but with higher Phoslock® dosages of 340:1 and 450:1 (Phoslock® to phosphorus) respectively. The tests were performed in the same manner as the first effluent water column, except that particle sizing was not performed, and only conductivity, pH, temperature, DO, turbidity and FRP measurements were taken. Control columns were set up and monitored at the same time as the treated columns.

2.2. Beaker tests

2.2.1. Effect of initial phosphorus concentration

In order to determine the effect of different initial FRP concentrations on the adsorption capacity of Phoslock® when applied at a 230:1 dosage, solutions were prepared in 2L beakers using reverse osmosis water. KH_2PO_4 salt was added to make solutions with concentrations of 0.5mg.l^{-1}, 1mg.l^{-1}, 2mg.l^{-1} and 4mg.l^{-1} phosphorus. The pH of each solution was adjusted to 7, and the conductivity of the solutions was adjusted to 0.3mS by the addition of NaCl. The beakers were stirred continuously on a magnetic stirrer throughout the duration of the experiment to ensure maximum contact of the phosphorus with the Phoslock® particles. Phoslock® was hydrated into a slurry form in the same manner as the column experiments, and was added to the beakers. Filtered samples were taken at designated time intervals over a 3h period, and the FRP concentration determined. pH levels and conductivity were monitored throughout the test period.

2.2.2. Lake water

A beaker test was also performed on a water sample from the effluent-fed lake at the University of Queensland. A Phoslock® dosage of 230:1 was used, in order to investigate the effect of continuous stirring on the adsorption capacity of Phoslock® when compared to the non-stirring conditions of the columns. Once again, filtered samples were taken over a 3h time period to determine the FRP concentration, and measurements were taken for pH and conductivity.

3. Results

3.1. Pseudo-second order model for determining phosphorus adsorption kinetics

The sorption kinetics of Phoslock® may be described by a pseudo-second order (Ho & Chiang, 2001). The differential equation is the following:

$$dq_t/dt = k(q_e-q_t)^2 \qquad (2)$$

Where qt is the amount of phosphorus sorbed at time t ($mg.g^{-1}$), and qe is the amount of phosphorus sorbed at equilibrium ($mg.g^{-1}$).
Integrating Eq. (2) for the boundary condition t = 0 to t = t and qt = 0 to qt = qt, gives:

$$1/ (q^e-q^t) = 1/q^e + kt \qquad (3)$$

which is the integrated rate law for a pseudo second order reaction. k is the equilibrium rate constant of pseudo-second order ($g.mg^{-1}.min^{-1}$). Equation (3) can be rearranged to obtain a linear form:

$$t/q_t = 1/kq_e^2 + 1/q_e .t \qquad (4)$$

The straight-line plots of t/qt against time have been tested to obtain rate parameters. The value of k, q_e and the correlation coefficients, R^2 of phosphorus concentration under different conditions were calculated from these plots.

3.2. Column tests

3.2.1. The effect of pH on Phoslock® performance

The effect of pH on the phosphate uptake of Phoslock® is shown in Figure 2. Linear plots of t/q_t against t in Figure 3 shows the applicability of the pseudo-second order equation for the system with initial pH ranging from 5 to 9. Values of k and q_e calculated from equation (4) and the correlation correlation coefficient (R^2) calculated from Figure 3 are listed in Table 1. It is clear that the kinetics of phosphorus adsorption onto Phoslock® followed the pseudo-second order model with correlation coefficients higher than 0.999 for all the systems. The equilibrium adsorption capacity of Phoslock® (q_e) decreased from 4.38$mg.g^{-1}$ to 3.19$mg.g^{-1}$ as the initial pH of the solution increased from 5 to 9. However, the adsorption capacity of Phoslock® remained similar within the range of pH 5 to 7 (Figure 3). The conductivity of the solution was not affected by the addition of Phoslock® and remained at 0.3$mS.cm^{-1}$ throughout the 6h test period.

The turbidity of the solutions decreased after the addition of Phoslock®, with all four solutions having a final turbidity of 5NTU or lower after 6h (Figure 4). However, the turbidity showed a more rapid decrease at the higher initial pH values of 8 and 9 than at pH 5 and 7.

Figures 5 and 6 present the particle size distribution (D) of the pH 5 and pH 9 solutions expressed as a volume diameter (µm). The values D[v, 0.1], D[v, 0.5] and D[v, 0.9] refer to particle diameters below which 10%, 50% and 90% of the particle volume is contained, respectively. In the pH 5 column, the values obtained for D[v, 0.1] decreased from 2.37µm to 2.11µm, the D[v, 0.5] value decreased from 8.02µm to 6.3µm and the diameter for D[v, 0.9] decreased from 23.44µm to 17.68µm over the 6h study period. In the pH 9 column, the D[v, 0.1] value decreased from 2.6µm to 1.81µm, the D[v, 0.5] value decreased from 12.16µm to 6.25µm and the diameter for D[v, 0.9] decreased from 46.15µm to 33.04µm. There was a similar decrease in the D[v, 0.1] value in both columns, but the values for D[v, 0.5] and D[v, 0.9] were higher in the pH 9 column, and decreased by greater amounts.

Table 1: Kinetic parameters for phosphorus adsorption onto Phoslock® at different initial pH values

pH	k ($g.mg^{-1}min^{-1}$)	q_e ($mg.g^{-1}$)	R^2
5	0.046	4.37	0.9999
7	0.031	4.36	1
8	0.036	3.38	1
9	0.038	3.19	1

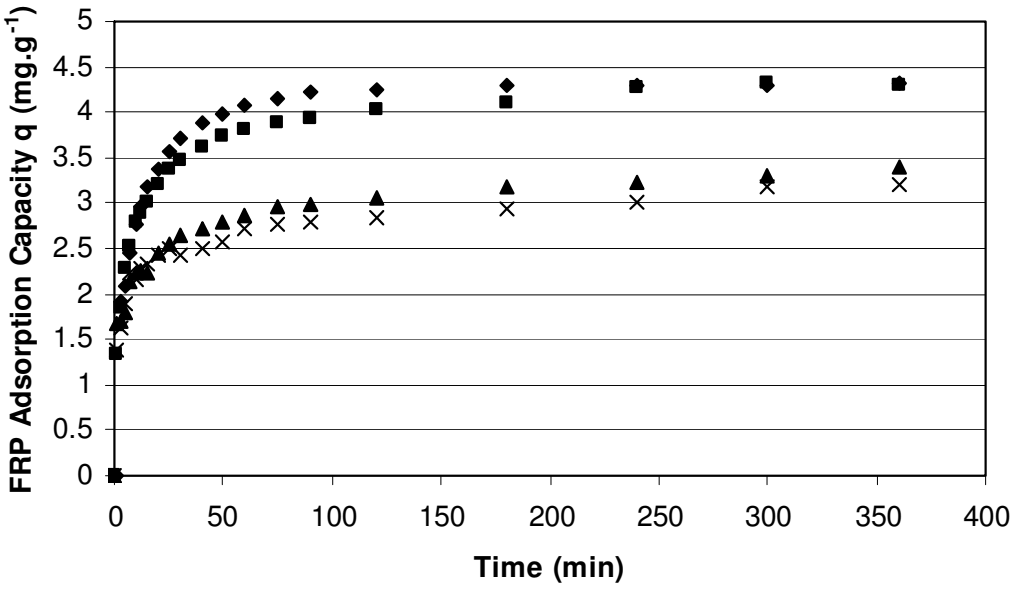

Figure 2: FRP adsorption capacity of Phoslock® versus time at various initial pH values (♦) pH 5 (■) pH 7 (▲) pH 8 (x) pH 9

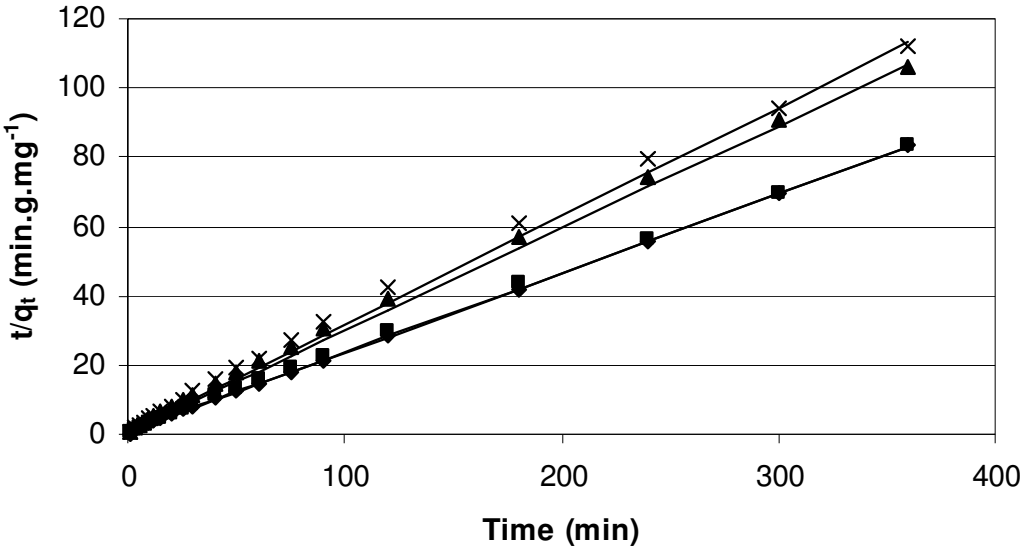

Figure 3: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® at various initial pH values. (♦) pH 5 (■) pH 7 (▲) pH 8 (x) pH 9. Conditions: Initial FRP = 1mg.l^{-1}, Initial conductivity = 0.3mS

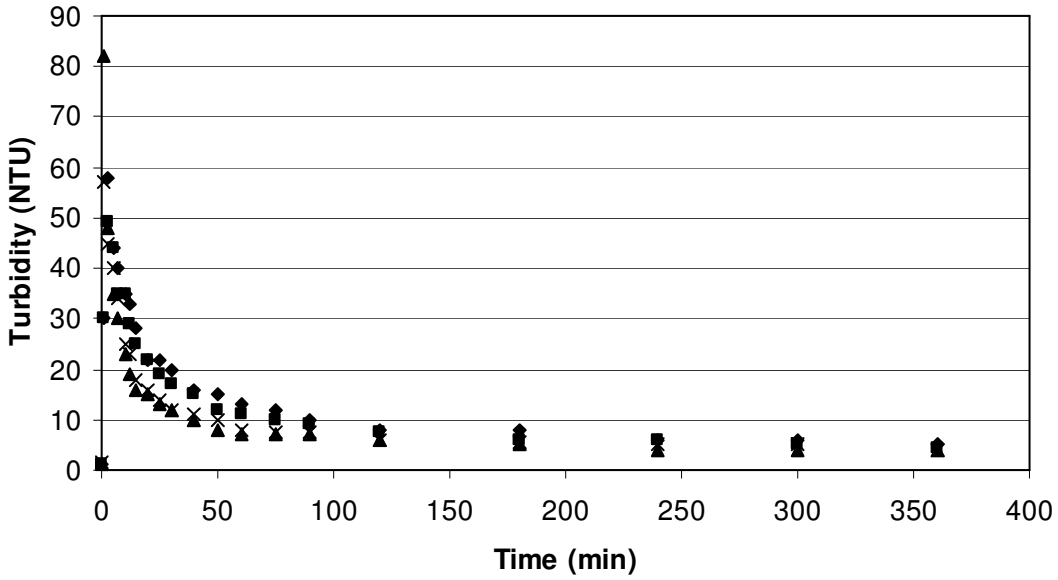

Figure 4: Turbidity of column solutions over time at various initial pH values. (♦) pH 5 (■) pH 7 (▲) pH 8 (x) pH 9

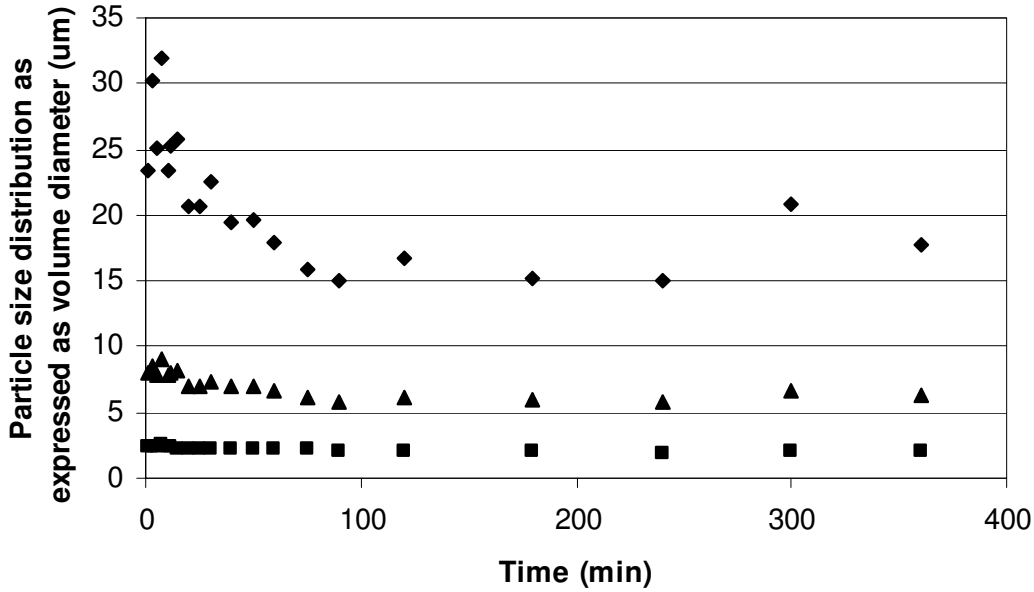

Figure 5: Particle size distribution of the Phoslock® grains in the pH 5 column expressed as volume diameter at each time interval over the 6h period of study. (■) D[v, 0.1], (▲) D[v, 0.5] and (♦) D[v, 0.9]

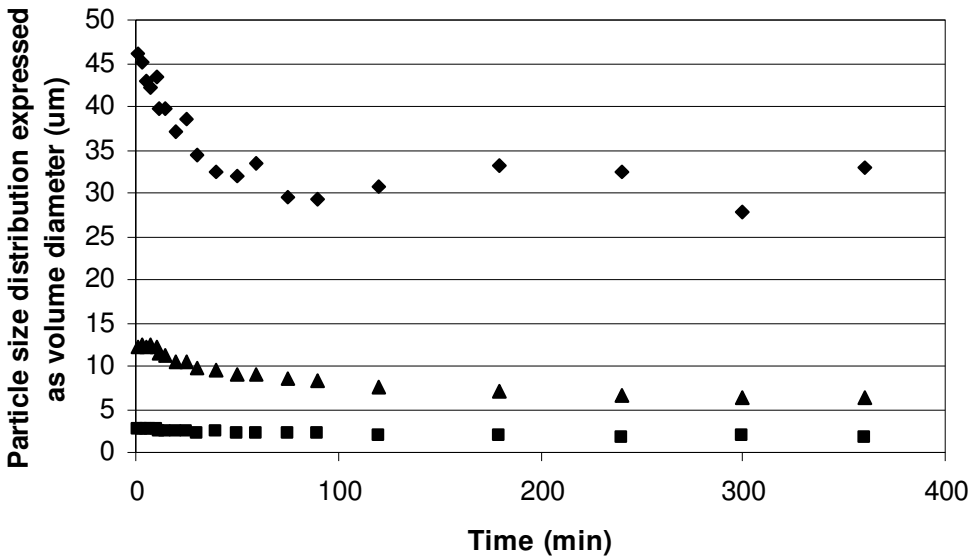

Figure 6: Particle size distribution of the Phoslock® grains in the pH 5 column expressed as volume diameter at each time interval over the 6h period of study. (■) D[v, 0.1], (▲) D[v, 0.5] and (♦) D[v, 0.9]

3.2.2. Lake water with algal bloom

The initial FRP concentration of the lake water at the start of the experiment was 0.82mg.l^{-1}, the initial pH 8.45, DO 12.5mg.l^{-1} and the conductivity 0.4mS/cm. The initial chlorophyll a concentration was 11.7µg.l^{-1}. Following the addition of a 230:1 dosage of Phoslock®, the FRP concentration decreased to 0.4mg.l^{-1} after 6h (Figure 7). The FRP concentration in the control column fluctuated over the 6h period but remained above 0.75mg.l^{-1}. The conductivity remained unchanged at 0.4mS/cm.

Linear plots of t/q_t against t in Figure 8 show the applicability of the pseudo-second order equation for the system. Values of k and q_e calculated from equation (4) and the correlation correlation coefficient (R^2) were calculated and are listed in Table 2. The equilibrium adsorption capacity of Phoslock® (q_e) in the effluent lake water was 2.38mg.g^{-1}, which was less than that observed in the synthetic water columns at either pH 8 or pH 9.

The chlorophyll a concentrations in the treated and control columns at various time intervals is shown in Table 3. Although the initial values differed in the two columns before the addition of Phoslock®, the chlorophyll a concentration in the control column

increased more than the treated column in the first 6h. This may be attributed to the higher turbidity in the treated column, which may have prevented algal growth by blocking the light. After 24 and 72h, the chlorophyll a concentration decreased by similar amounts in both columns, so it is unlikely that Phoslock® was responsible for this decrease.

The initial lanthanum concentration of the lake water was less than 0.003mg.l^{-1}, and increased to 0.023mg.l^{-1} 15min after the addition of Phoslock® (Table 4). After 24h, the lanthanum concentration had stabilized at 0.025mg.l^{-1}. The sodium concentration remained constant after treatment (Table 4), and the conductivity remained at 0.4mS. The alkalinity and hardness of the water was measured before treatment, and the concentration of various metals was measured before treatment and 24h after treatment (Table 5). The metal concentrations were not affected by the addition of Phoslock®.

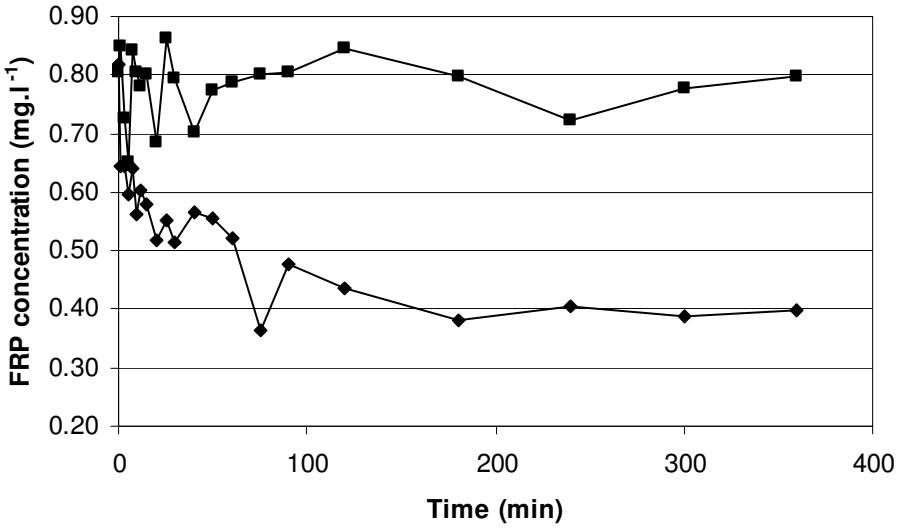

Figure 7: Comparison of the FRP concentration in the Phoslock® treated and control columns for the first 6h following the 230:1 dosage (♦) Treated (■) Control

Table 2: Kinetic parameters for phosphorus adsorption onto Phoslock® in effluent lake water following a 230:1 treatment and a subsequent sediment capping treatment of 250g.m^{-2}

Dosage	k (g.mg^{-1}min^{-1})	q_e (mg.g^{-1})	R^2
230:1	0.029	2.38	1
250g.m^{-2}	0.446	0.47	1

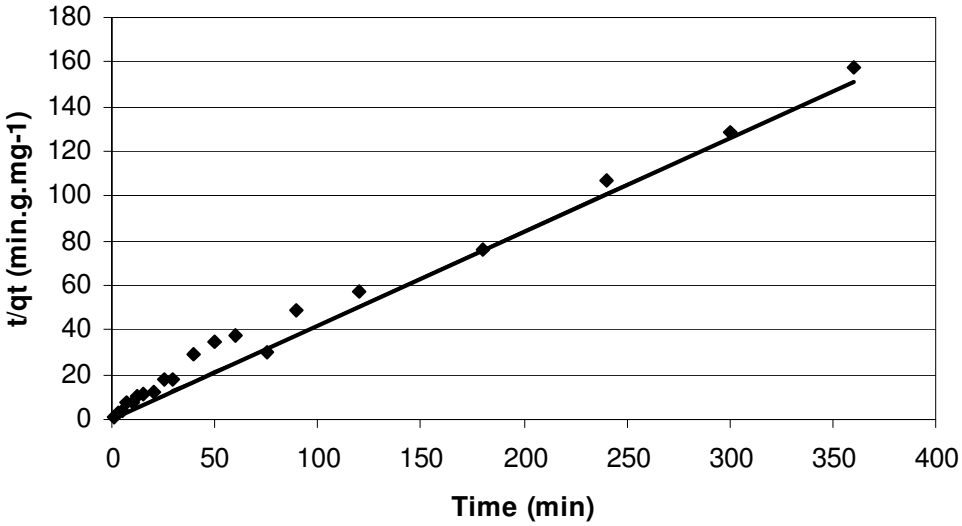

Figure 8: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® in effluent lake water. Conditions: Phoslock® dosage 230:1, initial FRP concentration = 0.82mg.l^{-1}, initial pH = 8.45, water temperature = 25.5°C, conductivity = 0.4mS

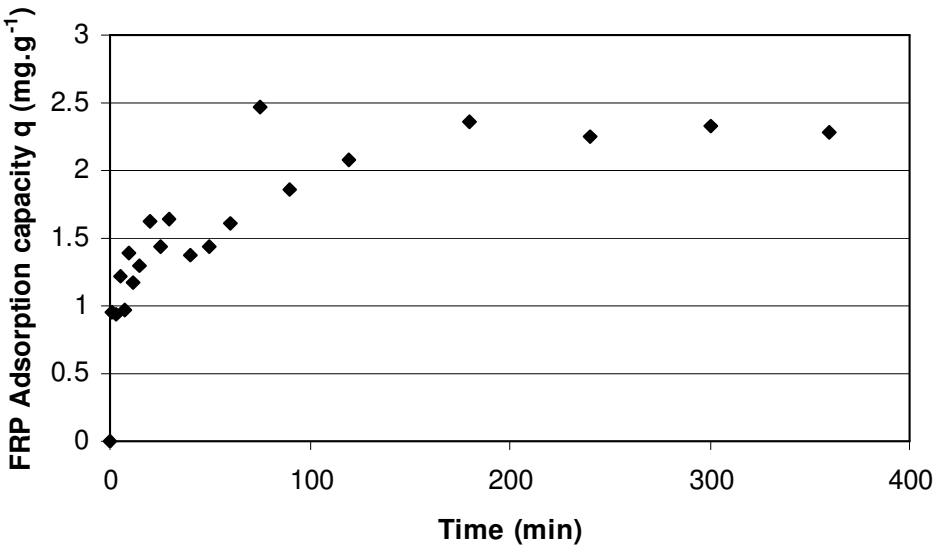

Figure 9: FRP adsorption capacity of Phoslock® versus time in effluent lake water following a 230:1 dosage

Table 3: Chlorophyll a concentrations (µg.l^{-1}) in the 230:1 treated and control columns at various time intervals after treatment

Time (h)	Treated	Control
0	11.7	17.1
6	10.21	22.56
24	13.3	18.7
72	7.53	11.35

Table 4: Lanthanum and sodium concentrations in the effluent lake water prior to Phoslock® treatment and at various times after treatment

Time (h)	La (mg.l^{-1})	Na (mg.l^{-1})
0	<0.003	61
0.25	0.023	62
1	0.024	62
2	0.022	62
24	0.025	62

72h after the 230:1 Phoslock® treatment, the lights were turned off and bentonite added to flocculate some of the algae on the surface. By this stage the FRP concentration of the treated column had decreased to 0.07mg.l^{-1}, and the control column had decreased to 0.24mg.l^{-1}, most likely as a result of algal uptake during growth. The pH of the treated column had increased to 10.07, and the control column to 10.17. A further 72h after adding the bentonite, the FRP concentration had increased to 0.13mg.l^{-1} in the middle of the treated column and 0.3mg.l^{-1} at the bottom of the column, and the pH had decreased to 8.55 at the top and 8.74 at the bottom. The FRP concentration of the control column also increased to 0.16mg.l^{-1} in the middle and 0.6mg.l^{-1} at the bottom, and the pH decreased to 9.09 at the top and 9.04 at the bottom. The increase in FRP was most likely due to the breakdown of dead algal cells and subsequent release of phosphorus into solution. The DO of the treated column was 9.1mg.l^{-1} at the top and 13.3mg.l^{-1} at the bottom, and that of the control column was 11.7mg.l^{-1} at the top and 11.4mg.l^{-1} at the bottom. At this point a sediment capping treatment of Phoslock® was applied.

Table 5: Concentrations of various metals ($mg.l^{-1}$) in the effluent lake water both prior to Phoslock® treatment and 24h after treatment, as well as the alkalinity and hardness of the water prior to treatment

	0h	24h
Alkalinity	112	
Hardness	98	
Calcium	21.8	
Magnesium	10.6	
Aluminium	<0.04	<0.04
Arsenic	<0.04	<0.04
Boron	0.43	0.4
Barium	0.024	0.02
Beryllium	<0.0002	<0.0002
Cadmium	<0.004	<0.004
Cobalt	<0.005	<0.005
Chromium	<0.004	<0.004
Copper	<0.03	<0.03
Iron	0.025	0.017
Manganese	0.002	0.002
Molybdenum	0.012	0.012
Mercury	<0.010	<0.010
Nickel	<0.005	<0.005
Lead	<0.01	<0.01
Selenium	<0.04	<0.04
Sodium	62	62
Vanadium	<0.003	<0.003
Zinc	<0.004	<0.004

Values of k and q_e calculated from equation (4) and the correlation correlation coefficient (R^2) calculated from Figure 10 for the sediment capping treatments are listed in Table 2. The equilibrium adsorption capacity of Phoslock® (q_e) in the effluent lake water was $0.47mg.g^{-1}$ (Figure 11).

The FRP concentration in the control column remained constant for the 3h period after the sediment capping treatment, whereas the FRP concentration in the treated column decreased by 86% to $0.02mg.l^{-1}$ (Figure 12), indicating that Phoslock® was responsible for the decrease in FRP concentration.

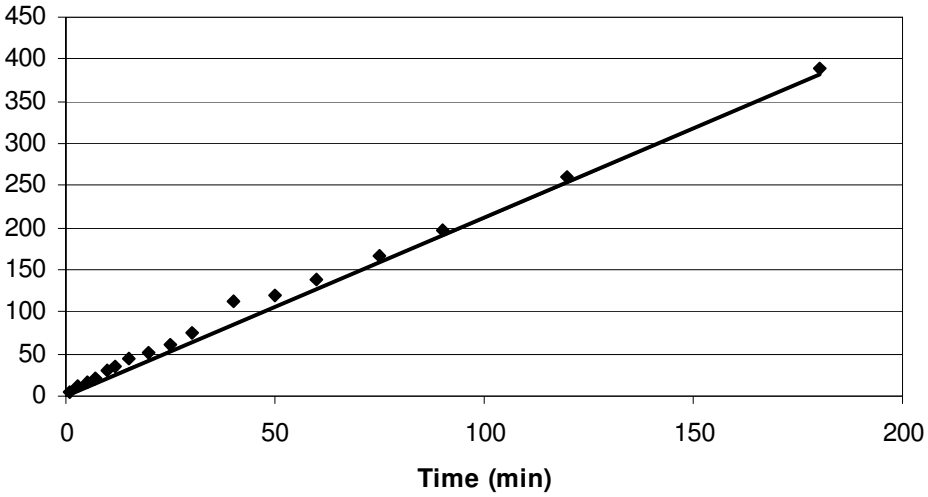

Figure 10: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® in effluent lake water. Conditions: Phoslock® dosage = 250g.m^{-2}, initial FRP concentration = 0.14mg.l^{-1}, initial pH at top of column = 8.55, initial pH at bottom of column = 8.74, initial DO top = 9.1mg.l^{-1}, initial DO bottom = 13.3mg.l^{-1}

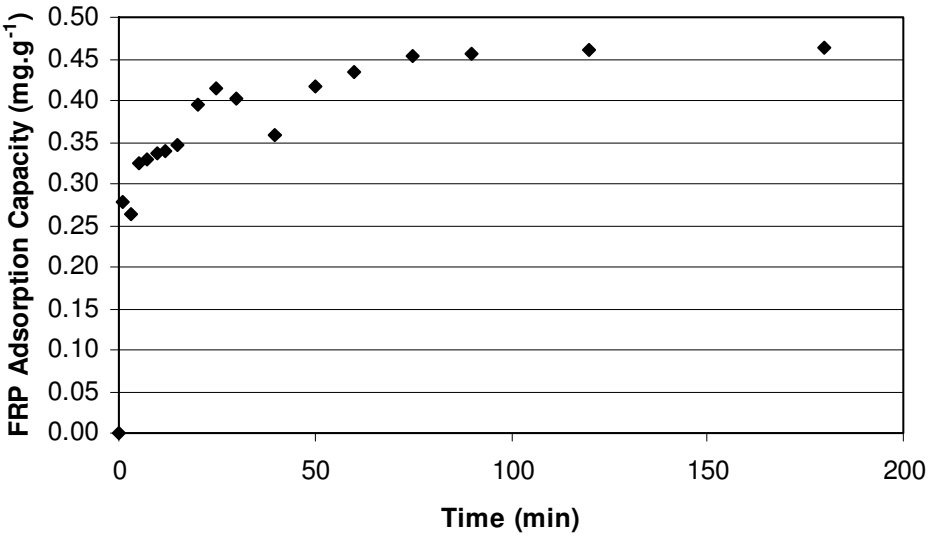

Figure 11: FRP adsorption capacity of Phoslock® versus time in effluent lake water following a sediment capping dosage of 250g.m^{-2} (6d after 230:1 dosage)

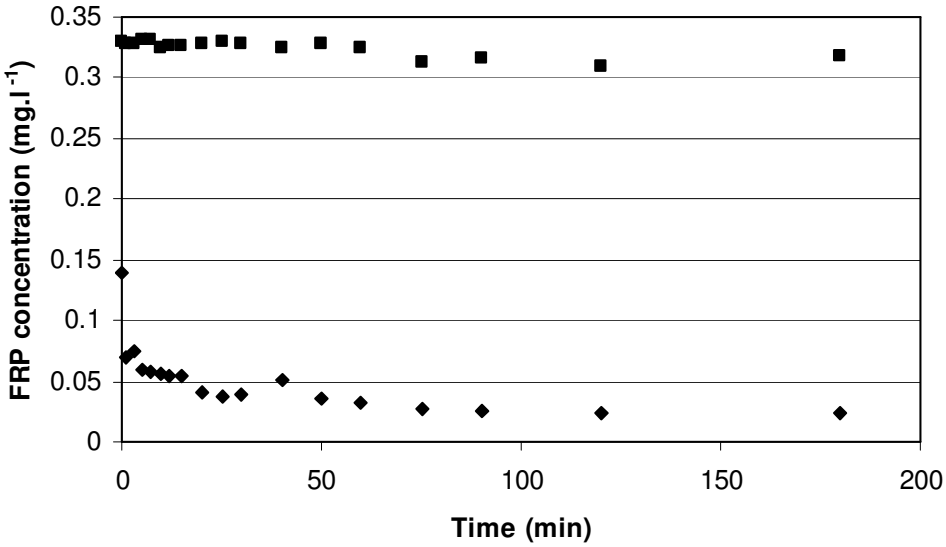

Figure 12: Comparison of the FRP concentration in the Phoslock® treated and control columns for the first 3h following the sediment capping treatment. (♦) Treated (■) Control

The pH of both columns continued to decrease after the sediment capping treatment, especially after the columns were covered with parafilm 5d after treatment. The pH of the water at the bottom of the treated column decreased from 9.02 to 7.12, and that of the control column decreased from 9.12 to 7.61 (Figure 13). Similarly, there was a decrease in the DO concentration of both columns (Figure 14). The control column reached an anoxic state (DO <1mg.l^{-1}) after 4 days, and the treated column only after covering with parafilm. After 6 days the DO concentrations at the bottom of the treated and control columns were 0.45mg.l^{-1} and 0.3mg.l^{-1} respectively. The FRP concentration of the control column increased over the 6d period, from 0.39mg.l^{-1} to 0.731mg.l^{-1}. However, the FRP concentration of the treated column remained below 0.1mg.l^{-1} (Figure 15).

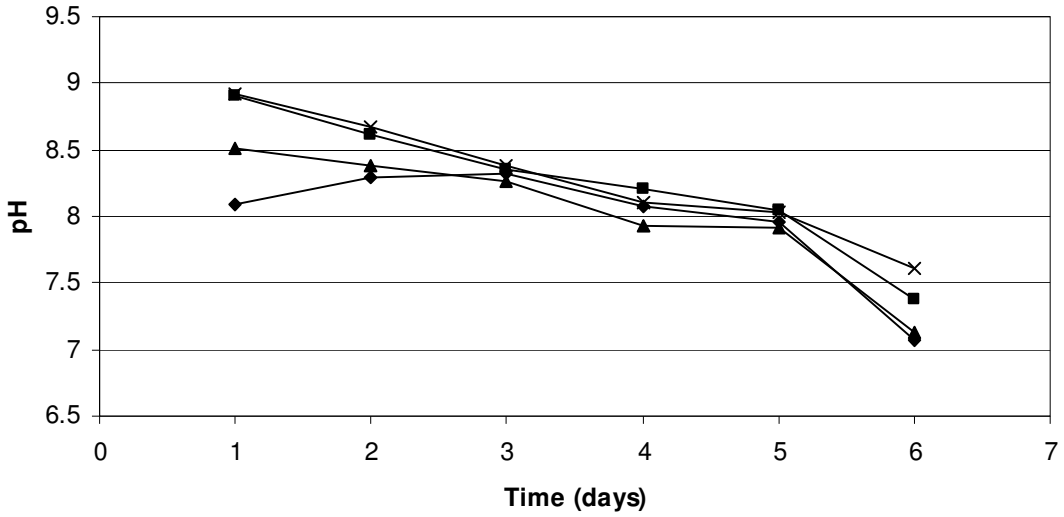

Figure 13: Change in pH at the top and bottom of the treated and control columns for 6 days following the sediment capping treatment. (♦) Treated top (■) Control top (▲) Treated bottom (x) Control bottom

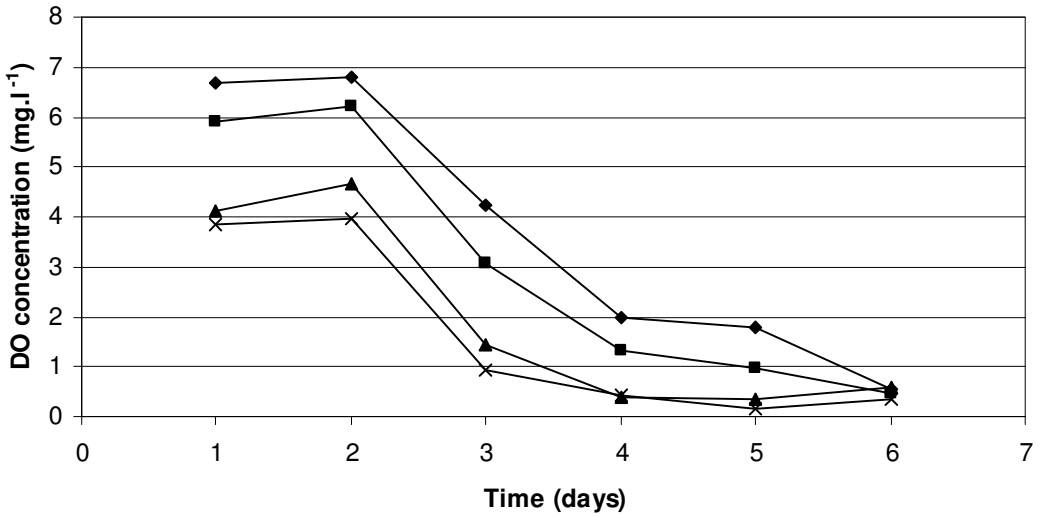

Figure 14: Dissolved oxygen concentration at the top and bottom of the control and treated columns for 6 days following the sediment capping treatment. (♦) Treated top (■) Treated bottom (▲) Control top (x) Control bottom

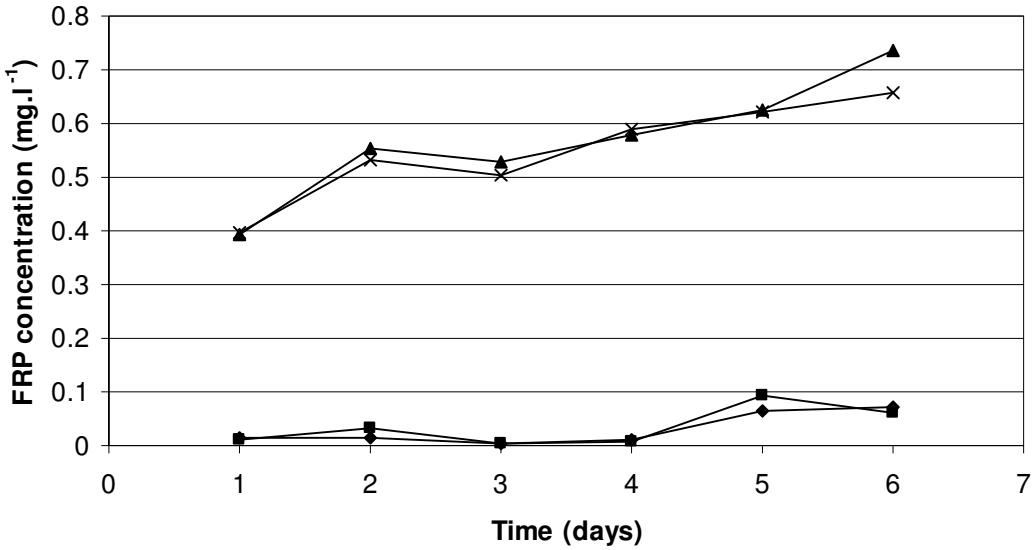

Figure 15: FRP concentrations in the middle and bottom of the treated and control columns for 6 days following the sediment capping treatment. (♦) Treated middle (■) Treated bottom (▲) Control middle (x) Control bottom

3.2.3. Lake water with algal bloom treated at high dose ratios

The initial FRP concentration of the effluent lake water for both the 340:1 treatment and the 450:1 treatment was 0.5mg.l^{-1}; the pH was 9.22 in the 340:1 treatment and 9.04 in the 450:1 treatment. Both had similar initial conductivity (0.4mS/cm) and DO (12.9mg.l^{-1}) concentrations. Linear plots of t/q_t against t in Figure 16 show the applicability of the pseudo-second order equation for the system. Values of k and q_e calculated from equation (4) and the correlation coefficient (R^2) were calculated and are listed in Table 6. The equilibrium adsorption capacity of Phoslock® (q_e) in the effluent lake water treated at a 340:1 ratio of Phoslock® to phosphorus was 1.43mg.g^{-1}, and that of the 450:1 treatment was lower, at 1.34mg.g^{-1} (Figure 17), which is close to the adsorption capacity of the 340:1 treatment. In the 340:1 treated column there was a decrease in FRP concentration from 0.57mg.l^{-1} to 0.32mg.l^{-1} and the FRP concentration of the control column decreased from 0.56mg.l^{-1} to 0.5mg.l^{-1} (Figure 18). In the 450:1 treatment, the FRP concentration decreased from 0.52mg.l^{-1} to 0.2mg.l^{-1}, and decreased in the control from 0.52mg.l^{-1} to 0.4mg.l^{-1} (Figure 19).

Table 6: Kinetic parameters for phosphorus adsorption onto Phoslock® in effluent lake water following treatment dosages of 340:1 and 450:1

Dosage	k ($g.mg^{-1}min^{-1}$)	q_e ($mg.g^{-1}$)	R^2
340:1	0.032	1.43	1
450:1	0.06	1.34	1

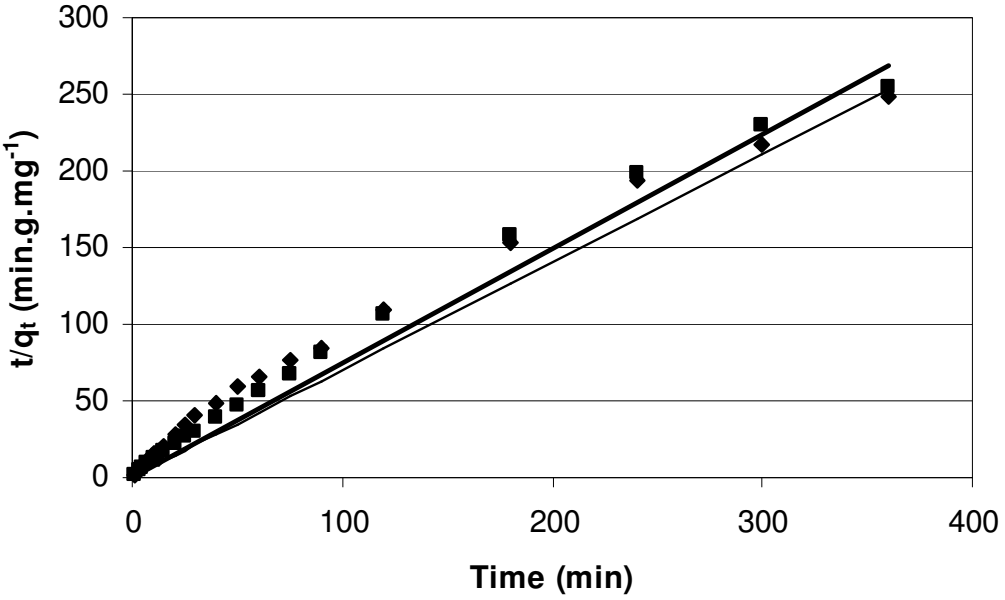

Figure 16: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® in effluent lake water above pH 9.

(♦) 340:1 Phoslock® dosage, (—) Linear trendline for 340:1 dosage. Conditions: initial FRP concentration = 0.5mg.l^{-1}, initial pH = 9.22, Conductivity = 0.4mS, DO = 12.2.mg.l^{-1}, Temperature = 24°C.

(■) 450:1 Phoslock® dosage, (▬) Linear trendline for 450:1 dosage. Conditions: initial FRP conc. = 0.5mg.l^{-1}, initial pH = 9.04, Conductivity = 0.4mS, DO = 12.9mg.l^{-1}, Temperature = 24°C

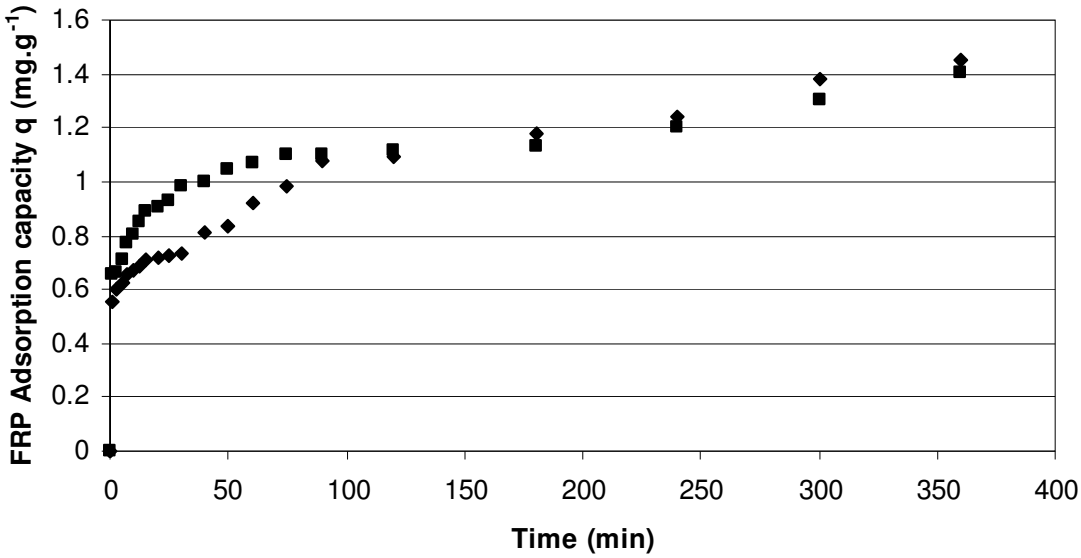

Figure 17: FRP adsorption capacity of Phoslock® versus time in effluent lake water following a (♦) 340:1 Phoslock® dosage, and (■) 450:1 Phoslock® dosage

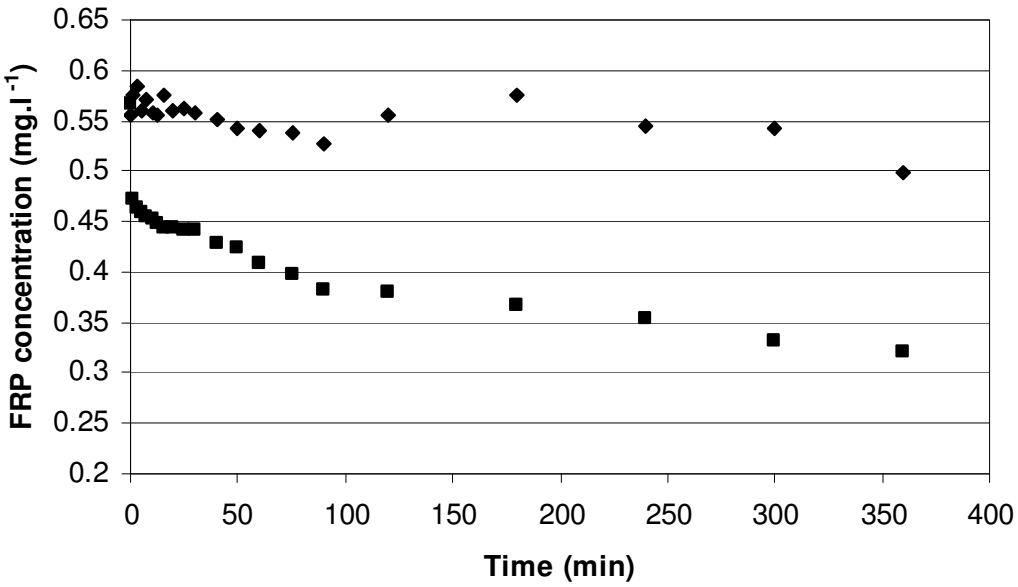

Figure 18: Comparison of the FRP concentration in the Phoslock® treated and control columns for the 6h following the 340:1 treatment (♦) Control (■) Treated

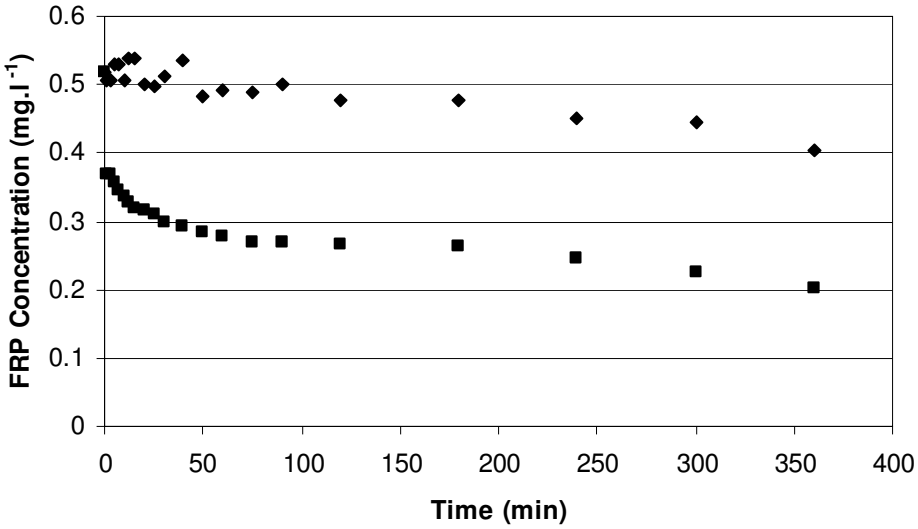

Figure 19: Comparison of the FRP concentration in the Phoslock® treated and control columns for the 6h following the 450:1 treatment

3.3. Beaker tests

3.3.1. Effect of initial phosphorus concentration

Values of k and q_e calculated from equation (4) and the correlation correlation coefficient (R^2) calculated from Figure 20 are listed in Table 4. With increasing FRP concentration, the rate constant (k) decreased and the adsorption capacity of Phoslock® increased (Figure 21). When the beaker experiment at 1mg.l^{-1} was compared with the results from the synthetic solution column experiment at pH 7 and an FRP concentration of 1mg.l^{-1} (Table 1), the adsorption capacity of 4.37mg.g^{-1} was slightly higher in the column than in the beaker (4.26mg.g^{-1}), but the rate constant was higher in the beaker.

Table 4: Kinetic parameters for phosphorus adsorption onto Phoslock® at different initial FRP concentrations

FRP concentration (mg.l^{-1})	k (g.mg^{-1}min^{-1})	q_e (mg.g^{-1})	R^2
0.5	0.72	2.23	0.9982
1	0.11	4.26	0.9979
2	0.01	8.01	0.9991
4	0.02	8.01	0.9972

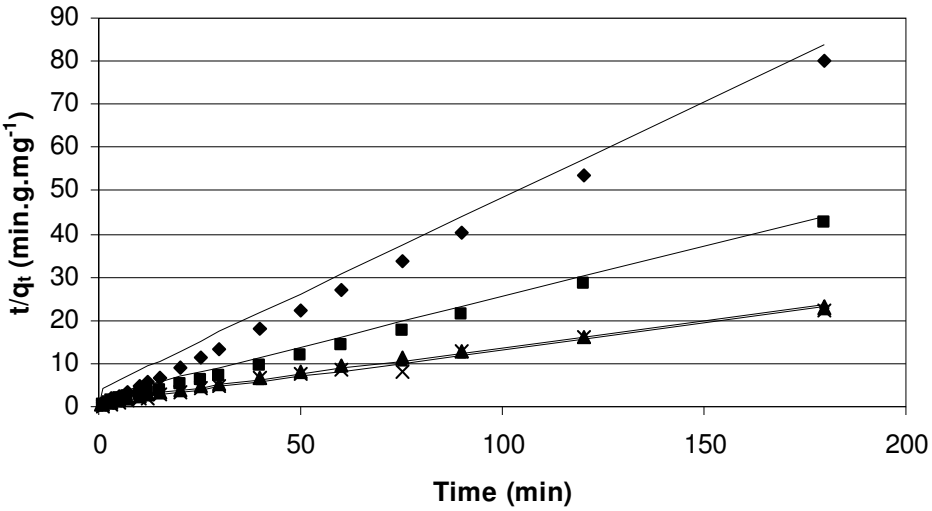

Figure 20: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® at different initial phosphorus concentrations (♦) 0.5mg.l^{-1} (■) 1mg.l^{-1} (▲) 2mg.l^{-1} (x) 4mg.l^{-1} Conditions: continuous stirring, pH = 7, conductivity = 0.3mS

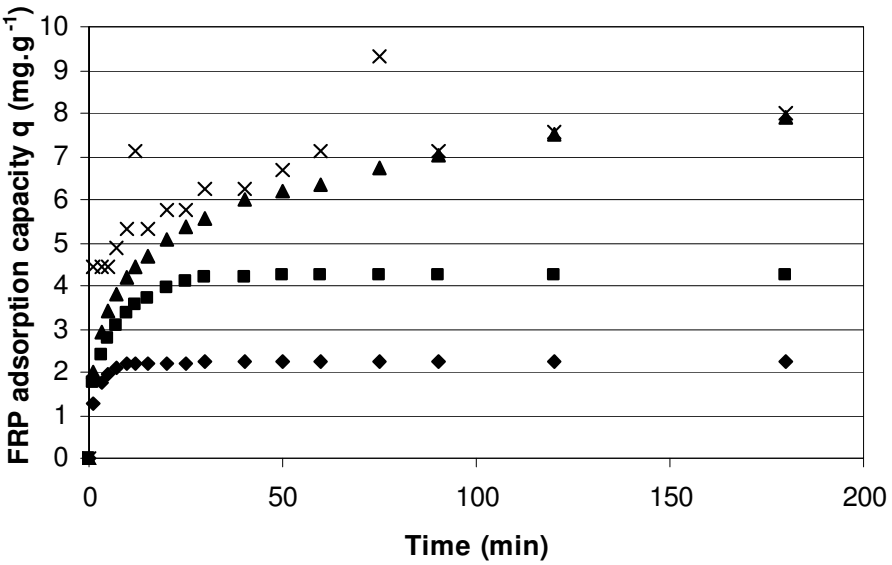

Figure 21: FRP adsorption capacity of Phoslock® versus time at different initial phosphorus concentrations (♦) 0.5mg.l^{-1} (■) 1 mg.l^{-1} (▲) 2 mg.l^{-1} (x) 4 mg.l^{-1}

3.3.2. Lake water

The pH of the effluent lake water was 7.02, and the initial FRP concentration was 0.9mg.l^{-1}. The kinetic parameters for phosphorus adsorption onto Phoslock® in effluent lake water under conditions of continuous stirring following a Phoslock® dose of 230:1 (Figure 22) are shown in Table 5. The effect of humic acids in the effluent lake water is

obvious when the adsorption capacity of 3.84mg.g^{-1} is compared to the synthetic solution beaker experiment at 1mg.l^{-1} FRP and pH 7, which had an adsorption capacity of 4.31mg.g^{-1} (Figure 23). The rate constant in the effluent lake beaker test was higher than that of the synthetic water. The FRP concentration decreased in the treated beaker by 94% from 0.9mg.l^{-1} to 0.05mg.l^{-1}, over the 3h test period, and that of the control beaker stayed constant (Figure 24). The reduction in phosphorus can therefore be attributed to Phoslock® and not algal uptake.

Table 5: Kinetic parameters for phosphorus adsorption onto Phoslock® in effluent lake water under conditions of continuous stirring following a Phoslock® dose of 230:1

Dosage	k (g.mg^{-1}min^{-1})	q_e (mg.g^{-1})	R^2
230:1	0.037	3.84	0.9999

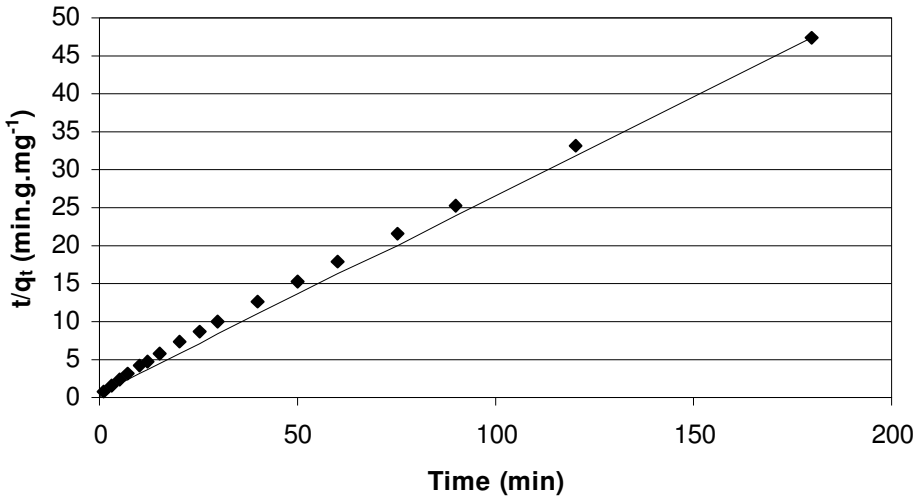

Figure 22: Pseudo-second order kinetics of phosphorus adsorption onto Phoslock® in effluent lake water. Conditions: Continuous stirring, initial FRP concentration = 0.9mg.l^{-1}, Phoslock® dosage = 230:1, pH = 7.02, conductivity = 0.2mS

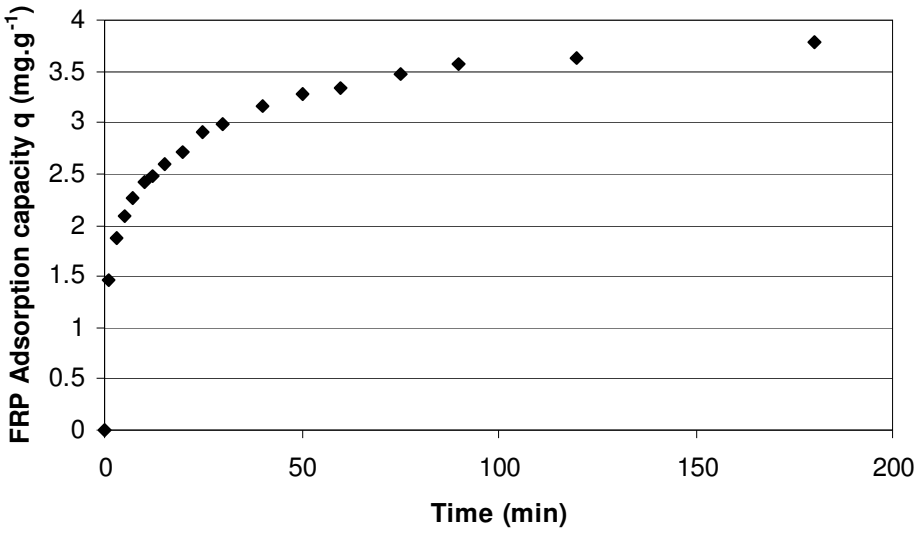

Figure 23: FRP adsorption capacity of Phoslock® versus time in effluent lake water under continuous stirring conditions

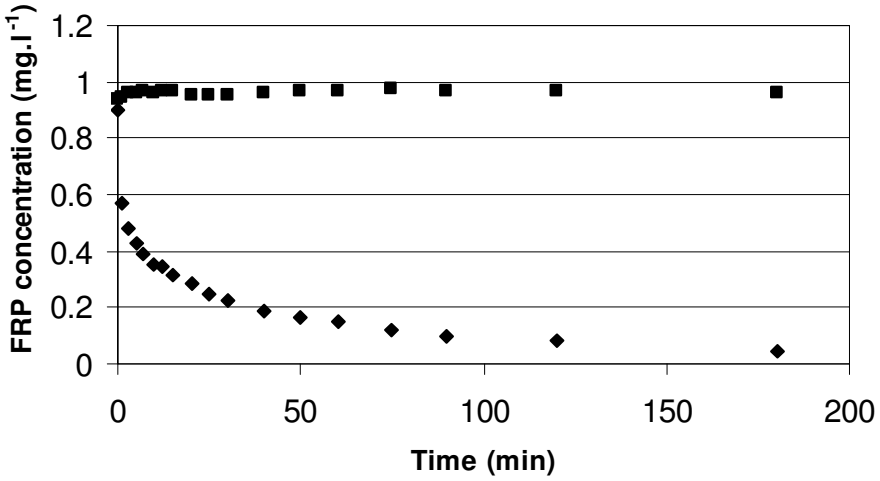

Figure 24: Comparison of the FRP concentration in the Phoslock® treated (♦) and control (■) beakers

4. Discussion

4.1. Column tests

4.1.1. The effect of pH on Phoslock® performance

The extent of phosphorus removal decreased rapidly as the pH was increased from 7 to 9. This can be attributed to the formation of the hydroxyl species of the lanthanum ions, decreasing the number of phosphorus binding sites on the Phoslock® surface. Lanthanum hydroxides begin to precipitate at pH 8.35 (Dibtseva *et al.,* 2001), so a rapid decrease in adsorption capacity is therefore expected above this pH.

The solution turbidity following the Phoslock® application decreased at a faster rate when the initial solution pH was 9, when compared to the pH 5 solution. This is supported by the particle size data. There was a similar decrease in the D[v, 0.1] value in both columns, but the values for D[v, 0.5] and D[v, 0.9] were higher in the pH 9 column, and decreased by greater amounts. The particles were therefore bigger in the pH 9 column, and settled out at a faster rate, as a result of the aggregation of the smaller particles at this pH. The faster settling time at high pH values may contribute to the reduced performance of Phoslock® due to a shorter contact time with the solution. Niriella & Carnahan (2006) reported that bentonite particles displayed a negative zeta potential (the overall charge that a particle acquires in a particular medium) at all pH values between pH 4 and pH 10, with no reverse in charge at any point. However, bentonite particles in distilled water showed an increase in zeta potential value (a larger negative) above pH 8, which could be due to charge development at the edges by direct transfer of H^+ from clay to water. If particles in a solution have a high negative or positive zeta potential then they will tend to repel each other and resist the formation of aggregates. However, if the particles have a low zeta potential (close to zero) there is nothing to prevent the particles from approaching one another and aggregating. Because one would expect the zeta potential of Phoslock® to become more negative at high pH values in the same manner as bentonite, especially because of the increase in the hydroxyl ion species of lanthanum, the increased aggregation of Phoslock® particles observed at high pH is unexpected. It may be explained by the fact that the negatively charged edges are attracted to the positively charged lanthanum ions. This would also contribute to the decrease in phosphorus adsorption capacity of Phoslock® at high pH

values. The presence of counterions in the suspension as a result of the added salt may have caused a reduction in surface charge, thereby contributing to the formation of larger aggregates and the possibility for more rapid settling. Apart from the loss of lanthanum sites to hydroxylation, another reason for the observed decrease in the adsorption capacity, q_e, could also be due to the unavailability of the lanthanum sites, caused by aggregation of the small particles. Aggregation of the smaller particles reduced the available surface area; hence less lanthanum ions per unit surface become available for reaction with the phosphate anions.

4.1.2. Lake water with algal bloom

The FRP concentration in the treated column decreased by approximately 50% from 0.82mg.l^{-1} to 0.4mg.l^{-1} after 6h, but the FRP concentration in the control column remained above 0.7mg.l^{-1}. As a result, the reduction in FRP in the treated column was attributed to Phoslock® and not to algal uptake during growth.

The equilibrium adsorption capacity of Phoslock® (q_e) in the effluent lake water was less than that observed in the synthetic water columns at either pH 8 or pH 9. This may be due to the presence of humic acids in the water, which lowered the phosphorus adsorption capacity of Phoslock®, especially at higher pH values (Douglas *et al*., 2000).

The chlorophyll a concentrations in the treated and control columns at various time intervals is shown in Table 3. Although the initial chlorophyll a values differed in the two columns before the addition of Phoslock®, that of the control column increased more than the treated column in the first 6h. This may be attributed to the higher turbidity in the treated column, which may have prevented algal growth by blocking the light. After 24 and 72h, the chlorophyll a concentration decreased by similar amounts in both columns, so it is unlikely that Phoslock® was responsible for this decrease.

In examining the stability of the adsorbed phosphorus under anoxic conditions, the FRP concentration in the control column remained constant for the 3h period, whereas the FRP concentration in the treated column decreased by 86% following the addition of a sediment capping dosage, indicating that Phoslock® was responsible for the decrease.

After the columns became anoxic, the FRP concentration of the control column increased from 0.39mg.l^{-1} to 0.731mg.l^{-1} over a 6d period, whereas the FRP concentration of the treated column remained below 0.1mg.l^{-1}, even though the system was anoxic, as indicated by the large decrease in the dissolved oxygen (DO) concentration. This demonstrated that Phoslock® was unaffected by the anoxic conditions in the column and the adsorbed phosphorus was not re-released. This is important, as the sediments of water bodies, especially those in a eutrophic state, are usually anoxic (Sweerts *et al*., 1991; Cermelj & Faganeli, 2003).

4.1.3. Lake water with algal bloom treated at high dose ratios

The rate constant (k) was higher for the 340:1 treatment than the 450:1 treatment because the ratio of available FRP to Phoslock® was higher. The equilibrium adsorption capacity of Phoslock® (q_e) in the effluent lake water treated at a 340:1 ratio of Phoslock® to phosphorus was 1.43mg.g^{-1}, and that of the 450:1 treatment was lower, at 1.34mg.g^{-1}, which is close to the adsorption capacity of the 340:1 treatment. In the 340:1 treated column there was a 44% decrease in FRP concentration from 0.57mg.l^{-1} to 0.32mg.l^{-1}. The control showed a 10.1% decrease in FRP from 0.56mg.l^{-1} to 0.5mg.l^{-1}. Therefore only about 34% of the reduction can be attributed to Phoslock® and the rest to algal uptake during growth. In the 450:1 treatment, there was a 61% decrease in FRP concentration from 0.52mg.l^{-1} to 0.2mg.l^{-1}, but there was a 23% decrease in the control from 0.52mg.l^{-1} to 0.4mg.l^{-1}. Therefore, only 38% of the decrease in the FRP concentration can be attributed to Phoslock®, which is similar to the 34% noted in the 340:1 column. The large increase in Phoslock® dosage to 450:1 therefore did not improve the phosphorus removal at this high pH (above pH 9). The dosage may need to be even higher to have an effect.

4.2. Beaker tests

4.2.1. Effect of initial phosphorus concentration

The adsorption capacity of Phoslock® increased with an increase in the FRP concentration, although the equilibrium adsorption capacity of Phoslock® at an FRP

concentration of 1mg.l^{-1} was similar to that at 2mg.l^{-1}. The removal of FRP increased rapidly at the beginning and then more slowly until equilibrium, though more steeply at higher FRP concentrations. When the beaker experiment at 1mg.l^{-1} was compared with the results from the synthetic solution column experiment at pH 7 and an FRP concentration of 1mg.l^{-1}, the adsorption capacity was slightly higher in the column than in the beaker, but the rate constant was higher in the beaker. This may be due to the effect of continuous stirring in the beaker, which allowed for maximum contact between the Phoslock® and the solution.

4.2.2. Lake water

The adsorption capacity of 3.84mg.g^{-1} in the effluent lake water was lower than that of the synthetic solution beaker experiment at 1mg.l^{-1} FRP and pH 7. This is most likely due to the presence of humic acids in the water, which reduce the adsorption capacity of Phoslock®. The FRP concentration decreased by 94% in the treated beaker over the 3h test period, but that of the control beaker stayed constant. The reduction in phosphorus can therefore be attributed to Phoslock® and not algal uptake.

5. Conclusions

- Phoslock® was the most effective at removing phosphorus from the water at pH values between 5 and 7, and the adsorption capacity decreased greatly above pH 9.
- Phoslock® did not affect the conductivity of the water.
- The settling rate of Phoslock® increased with an increase in pH.
- The adsorption capacity of Phoslock® was lower in lake water than in a synthetic water solution at the same pH, most likely due to the effect of humic acids.
- Other than lanthanum, Phoslock® does not have an effect on the concentration of metals in the solution.
- Phosphorus remains bound to Phoslock® under anoxic conditions.
- Above pH 9, the negative effects of pH cannot be overcome by increasing the Phoslock® dosage.

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CHAPTER 4:

PHOSLOCK® FIELD TRIAL AT K'SHANI LAKE LODGE, HARTBEESPOORT DAM

JANUARY - DECEMBER 2006

1. Introduction

Hartbeespoort Dam is located 37km west of Pretoria on the Crocodile River (NIWR, 1985). It is classified as hypertrophic due to the runoff from fertilized fields and the inflow of sewage plant effluents from the Northern suburbs of Johannesburg that contain high amounts of salts, phosphates and nitrates. On reaching the dam, these effluents stimulate cyanobacterial growth, which further accelerates eutrophication. For most of the year, Hartbeespoort Dam is dominated by dense populations of the cyanobacterium *Microcystis aeruginosa* (Robarts & Zohary, 1986), usually representing more than 90% of the total algal biomass (NIWR, 1985). During calm weather the buoyant *M. aeruginosa* accumulate to form thick, crusted, floating mats called hyperscums, which usually form in winter in the shelter of the dam wall (Robarts & Zohary, 1985).

Extensive cyanobacterial growth poses several severe implications on the general water quality as well as the maintenance of water treatment standards set for potable water. Massive blooms such as those found in the Hartbeespoort Dam can deplete the dissolved oxygen content resulting in fish kills and discolouration of the water by pigments released from the cells (Rae *et al.*, 1999). Cyanobacteria easily penetrate and clog the fine sand filters and the primary coarse fast filters that are fundamental stages in drinking water purification because of their relatively small cell size (Botha-Oberholster, 2004). Biodegradation of cyanobacterial blooms contributes to the organic load of the water resulting in increased treatment costs. Non-toxic nuisance compounds such as geosmin and 2-methylisoborneol (2-MIB) that cause taste and odour problems in both dam and purified waters have been associated with cyanobacteria (Rae *et al.,* 1999). Of greater importance is the fact that certain cyanobacteria produce toxic compounds, the consumption of which present severe health risks (Botha-Oberholster, 2004).

High phosphorous levels remain the greatest factor influencing the development of algal blooms. It is accepted that phosphorus control is more achievable than that of nitrogen, because, unlike nitrogen, there is no atmospheric source of phosphorus that is bio-available. This indicates that a small reduction in the phosphorus concentration can

achieve a much greater degree of cyanobacterial growth control than a reduction of a similar magnitude in the nitrogen level, making phosphorus reduction strategies a far more effective alternative in eutrophication management.

Phoslock®, lanthanum-modified bentonite clay, is able to bind phosphorous from the water body and form a cap on the sediment to prevent phosphate re-release. Phoslock® is capable of removing dissolved P under anoxic conditions, as well as over a wide pH range (Douglas *et al*., 1999).

Two full-scale Phoslock® applications were undertaken in the summer of 2001/2002 in the impounded riverine section of two estuaries subject to cyanobacterial blooms along the coastal plain of south-western Australia. Phoslock® was applied as a slurry from a small boat, the first application in October and subsequent applications in December and January. The dissolved P in the water column was reduced to below detection limits within a few hours, and the amount of P released from the sediment was substantially reduced throughout the course of the trial. The effect of the reduction in the P concentration on phytoplankton growth was clear, with the chlorophyll a concentrations of the treated areas being significantly lower than the control areas (Robb *et al*., 2003). Previous Phoslock® applications have involved treating eutrophic waterways prior to the development of a cyanobacterial bloom, which is recommended, because the maximum amount of phosphorus will be available for binding in solution. For this field trial on Hartbeespoort Dam, the Phoslock® was applied in January, during a cyanobacterial bloom.

Prior to commencement of the trial, results from water samples taken from the trial site indicated that the water had a high pH. Laboratory-scale tests (see Chapter 5) were performed on columns containing lake water in a state of a cyanobacterial bloom, with a high pH (>9) and high soluble phosphorus concentration. The recommended dosage of Phoslock® (230:1 ratio of Phoslock® to phosphorus) was only capable of reducing the filterable reactive phosphorus (FRP) by 50%. This is due to the fact that the phosphorus adsorption capacity of Phoslock decreases at a high pH, especially above pH 9. A higher Phoslock® dosage (450:1) improved the amount removed. The pH of the sediment of a eutrophic dam is lower than that of the overlying water body, enabling the Phoslock® to reach equilibrium in the sediment, so a 400:1 dosage was tested.

The aims of the field trial on Hartbeespoort Dam were therefore to evaluate the ability of Phoslock® to reduce the phosphorus concentration of the water under high pH conditions and during a cyanobacterial bloom, and to determine the long-term effect of treatment on the FRP concentration and the severity of the cyanobacterial bloom.

2. Materials and methods

2.1. The site

The site used for the field trial was a man-made bay at K'shani Lake Lodge, a housing development on Hartbeespoort Dam. Figures 1 and 2 present the local layout of the site and the sample sites respectively. The maps were drawn using GIS from measured GPS points (indicated on the trial site in Figure 1), courtesy of Africa Geo-Environmental Services (AGES). The site was approximately 2.5 hectares in size, had an average depth of 3m, and had an opening into the main dam about 8m wide. This was blocked off with floating logs, to which a tarpaulin curtain was attached to form a moveable boom. The bottom of the tarpaulin was weighed down with chains. A further area was blocked off in a similar manner within the test site to serve as an untreated control area. The site was in a state of cyanobacterial bloom at the time of treatment in January 2006.

2.2 Calculation of Phoslock® quantity needed for treatment

The site was monitored throughout December 2005. The FRP (filterable reactive phosphorus) levels of the water body were between 0.2 and 0.8mg.l^{-1}, and sediment FRP values ranged from 0.6 to 3.84 mg.l^{-1}. The water pH was, on average, 9.2, whereas that of the sediment was 7.5.

Samples were taken three days prior to treatment (Table 1). The phosphorus levels had decreased from those seen in December, to 0.09mg.l^{-1}. The pH had increased to 9.8. However, because it was presumed that the phosphorus concentration in the sediment was still high, an FRP value of 0.2mg.l^{-1} was used to calculate the amount of Phoslock® necessary to treat the site. At neutral pH, a ratio of 230:1 Phoslock® to phosphorus is recommended, but the high pH of the Hartbeespoort Dam water required a higher dosage.

As the water body was approximately 2.5ha in size (25 000m^2 surface area), 6000kg of Phoslock® was used. At a 0.2$mg.l^{-1}$ phosphorus concentration, this resulted in a Phoslock® to phosphorus ratio of 400:1. This dose would overcome some of the negative effects of the high pH, and the Phoslock® would ultimately reach equilibrium in the sediment, meaning that its adsorption capacity would increase with time, enabling it to continue binding FRP after application.

2.3. Product application

The product was first mixed into a slurry prior to application; 125kg of Phoslock® was added to 1000L of water. This was mixed well in a large tank upon a barge, and the slurry was sprayed onto the water surface using a pump and hose.

2.4. Sampling strategy

Samples were taken three days prior to application, on the day of application immediately prior to treatment, and daily for six days following application. Further samples were taken weekly for five weeks, and then bi-weekly, for a period of one year.

In terms of the samples taken daily prior to application, as well as for six days following application, ten samples were taken each day, from the control and treated areas. The sample sites were chosen so as to best represent the conditions of the site as a whole. Results for the parameters tested (Table 1) are averages of these ten samples. Only 5 samples were taken from the treated and control areas in the following weeks of monitoring (See Figure 2).

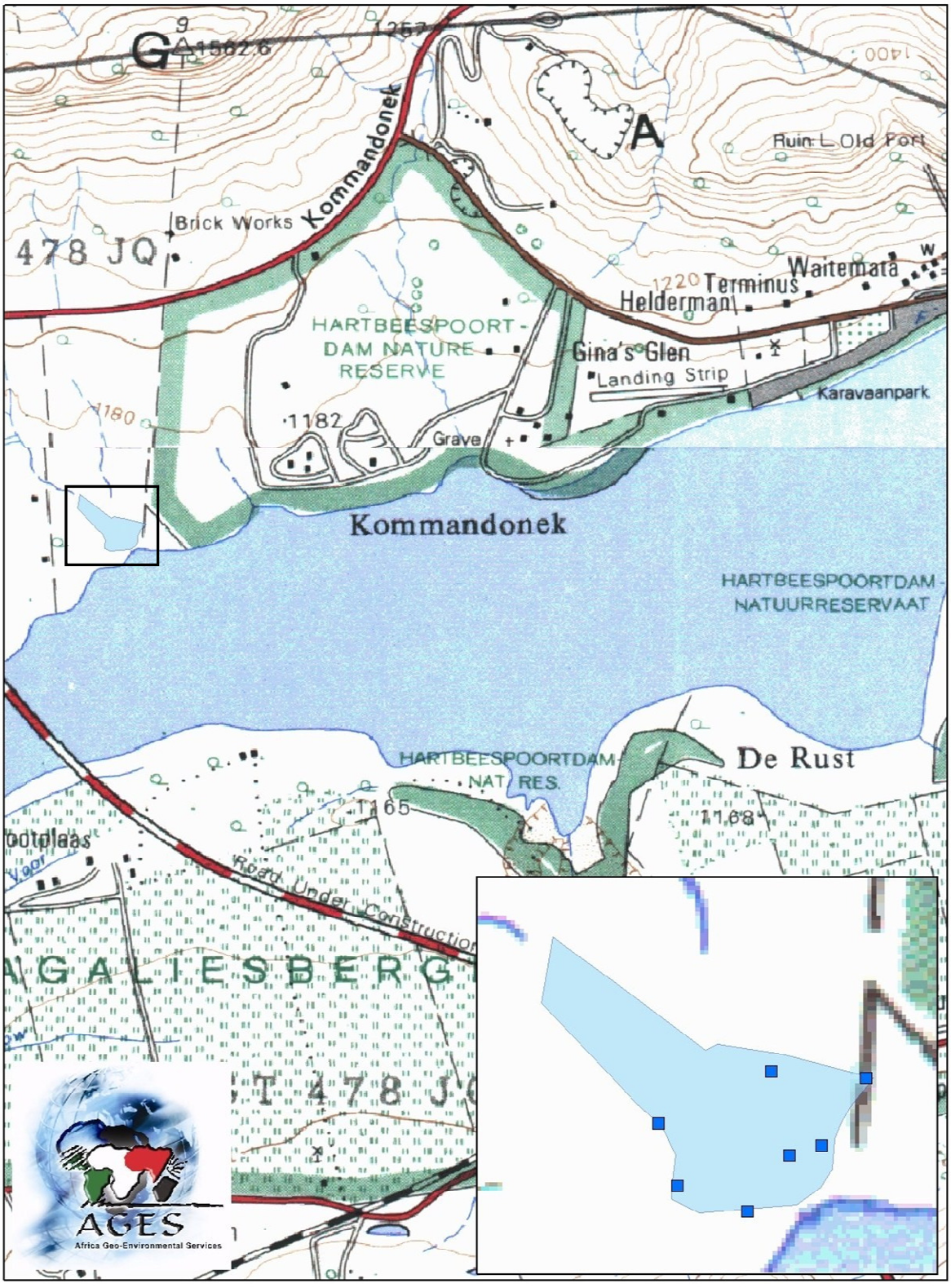

Figure 1: Phoslock trial site showing the regional and local layout. Blue squares on the trial site map indicate points used for GPS determination.

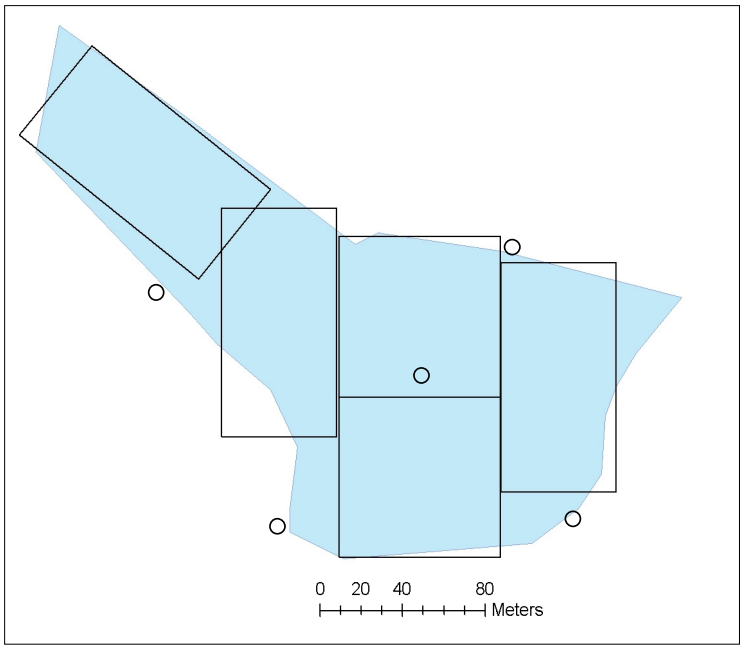

Figure 2: Sizing distribution of the Phoslock® trial site- Each block represents 0.5ha, and the circles represent the sample sites of the treated area

3. Results

The concentration of FRP decreased by more than 50% in the first 24h after treatment from 0.09mg.l^{-1} to 0.043mg.l^{-1}. The FRP of the control area remained constant. After 48h, the FRP concentration in the treated area had decreased to 0.017mg.l^{-1}, and then stabilised at approximately 0.02mg.l^{-1} for the reminder of the first week of testing. The FRP concentration of the control area remained high (Table 1; Figure 3). A decrease in the amount of surface algae was observed following application, and after 6 days the algae had not yet returned to its former state. The difference in the FRP (PO_4-P) concentration between the control and treated areas on day 6 was significant; when a two tailed *t*-test was performed at a significance level of 0.01, the *p*-value was 4.59 x 10^{-9}, which rejects the null hypothesis.

Unusually high rainfall during the second week of the trial resulted in partial flooding of the test site. The subsequent rise in the level of the dam also resulted in water and algae being washed into the test site from the main dam over the top of the floating logs. This inflow of water and algae was most likely responsible for the increase in FRP concentration in both the treated and control areas to 0.29mg.l^{-1} and 0.22mg.l^{-1} respectively (Figure 4). By the third week, the FRP concentration of the control area

had once again increased, whereas that of the treated area had decreased to approximately 0.1mg.l^{-1}. During the weeks that followed, the FRP concentration of the control area fluctuated, but remained above 0.2mg.l^{-1}. The treated area continued to show an improvement and decreased to 0.015mg.l^{-1} by the seventh week after treatment. The FRP concentration of the treated area remained below 0.02mg.l^{-1} throughout the winter months, despite the fact that the algae started to die off when the water temperature dropped below 15ºC, in mid May. There was a decrease in the FRP concentration in the control area from 0.72mg.l^{-1} in week 3 (16 February) to 0.04 mg.l^{-1} in week 27 (2 August). However, after week 26, the FRP concentration increased steadily in the control area, but remained low at below 0.02mg.l^{-1} in the treated area until December (week 46), by which time the control area had increased to 0.22mg.l^{-1}. The difference in the FRP (PO_4-P) concentration between the control and treated areas on day 324 was significant; when a two tailed *t*-test was performed at a significance level of 0.01, the *p*-value was 5.71 x 10^{-15}, which rejects the null hypothesis.

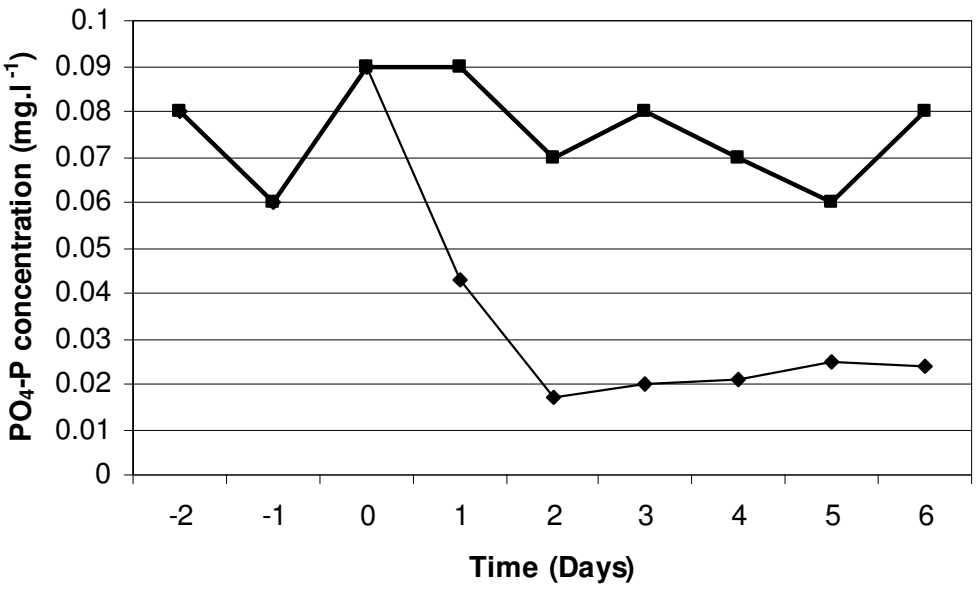

Figure 3: FRP values of the treated and control areas two days prior to treatment and 6 days after treatment, Day 0 represents the day of treatment (— ♦ —) Treated area (—■—) Control area

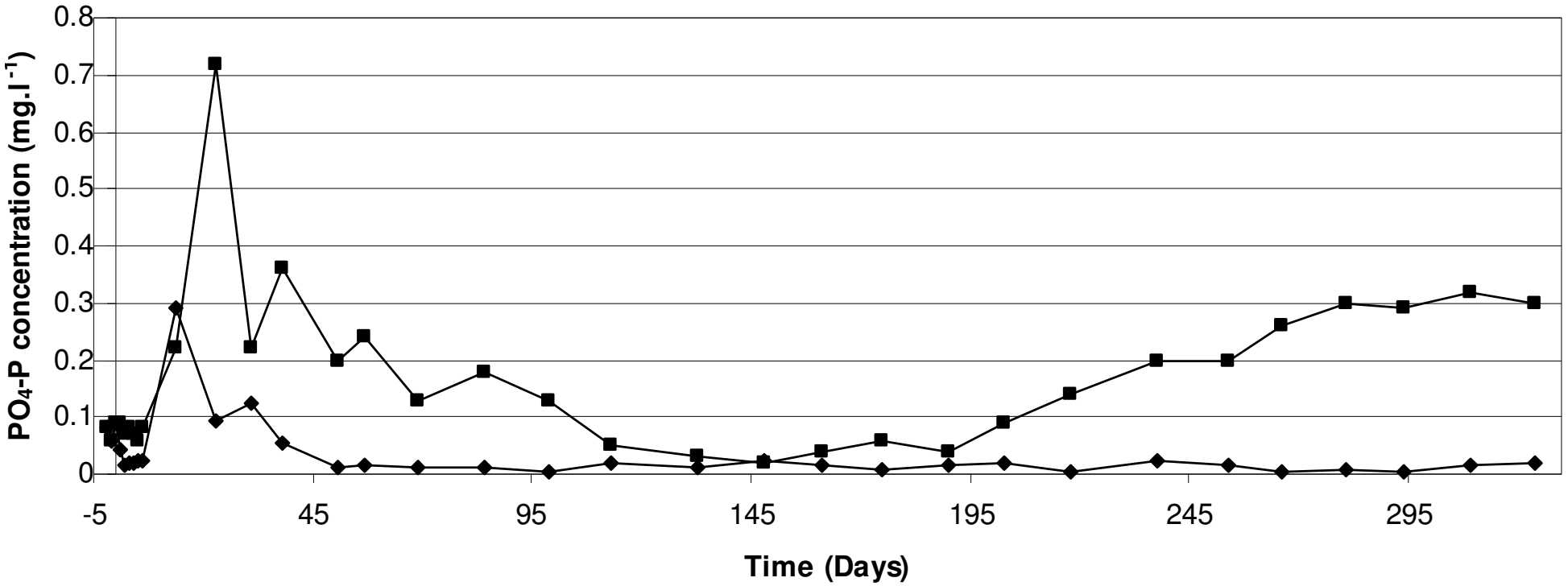

Figure 4: FRP values of the treated and control areas for the duration of the trial (♦) Treated area (■) Control area

The amount of algae present in the test site varied throughout the initial 7 weeks of testing. Immediately after application there was a visible decrease in the quantity of surface algae, as the Phoslock® has a flocculating effect. There was a large amount of algae present after the heavy rains in week 2, due to algae being washed in from the main dam. Despite the boom, small amounts of algae were still able to flow into the test area from the main dam. The algae remained present in the treated area throughout the period of testing, although the amount decreased once the water temperature decreased to below 15°C. There appeared to be a smaller amount of algae in the treated area when compared with the control area. Both the treated and control areas were free of algae through the winter months, but the cyanobacterial growth began in September (week 34) when the FRP concentration reached 0.2mg.l^{-1} and the water temperature 14.9ºC. At this stage the FRP concentration in the treated area was 0.02mg.l^{-1} and there was no cyanobacterial growth. The water level in the main dam as well as the trial site decreased throughout winter, and by September the level had dropped by approximately 1m. The summer rains began in October, and there was a rapid rise in the water level. By early November the water had risen back to its original level, with water containing phosphates and algae flowing in from the main dam. Up to that point no cyanobacterial growth was visible in the treated site.

The pH values of the control and treated areas remained very similar throughout the trial. Following Phoslock® application, the treated area showed a decrease in pH when compared to the control (Figure 5). However, by the second week after treatment, both the control and treated areas were once again of similar pH, and followed a similar trend thereafter (Figure 6). The pH of both sites decreased with time to below 9 by the end of August. However, as the water temperature increased (from 12ºC to 26ºC from August to December, Figure 7) and cyanobacterial growth occurred, the pH of both areas increased, following a similar trend until December.

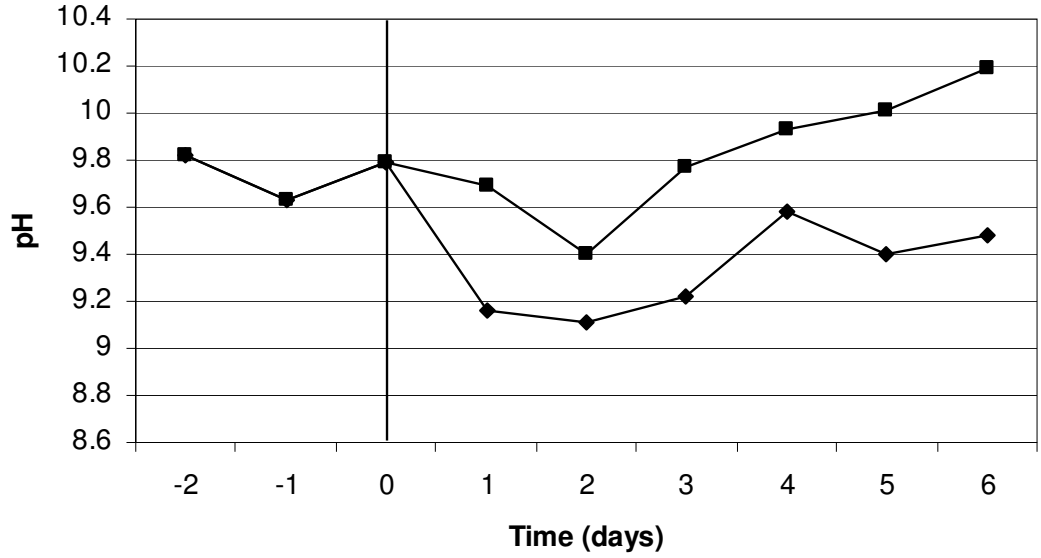

Figure 5: pH values of the treated and control areas two days prior to treatment and 6 days after treatment (Day 0 represents the day of treatment) (♦) Treated area (■) Control area

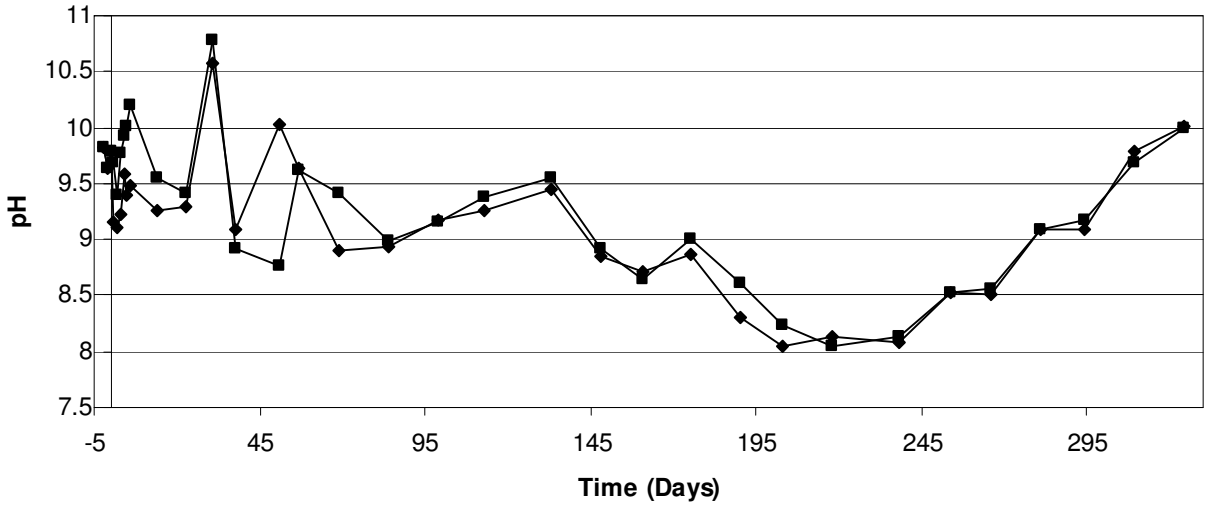

Figure 6: pH values of the treated and control areas for the duration of the trial (♦) Treated area (■) Control area

The nitrate concentration varied in both the treated and control areas throughout the duration of the trial, although both areas showed a similar trend (Figure 8). There was an increase in nitrates in both areas in the second week, which can once again be contributed to the heavy rain and inflow of water into the test site from the main dam, and runoff from the land into the test site. In the following weeks, the nitrate concentration decreased and stabilised, ranging from 1 to 4mg.l^{-1} in both the control and the treated areas. From week 10 (April), both sites

showed an increasing trend, but the nitrate concentration was greater in the control area (23.3 mg.l-1) than the treated area (9.6mg.l^{-1}) by week 27 (2 August). From 15 August to 17 October (week 29 to week 38) the nitrate concentration was lower in the treated area than the control area, after which time it increased to once again follow the same trend as the control. By week 46 (14 December) the nitrate concentration had increased to 9.15mg.l^{-1} in the treated area and 10.1mg.l^{-1} in the control.

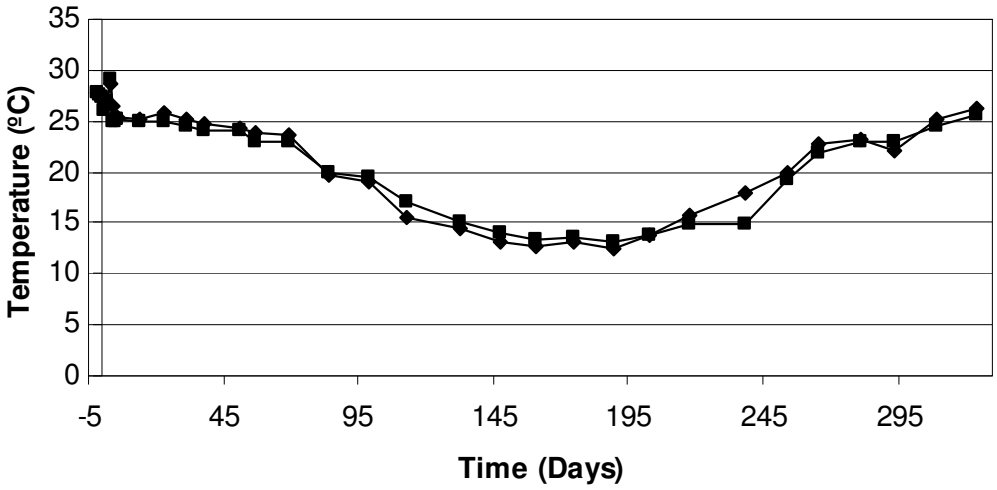

Figure 7: Water temperature of the treated and control areas for the duration of the trial (♦) Treated area (■) Control area

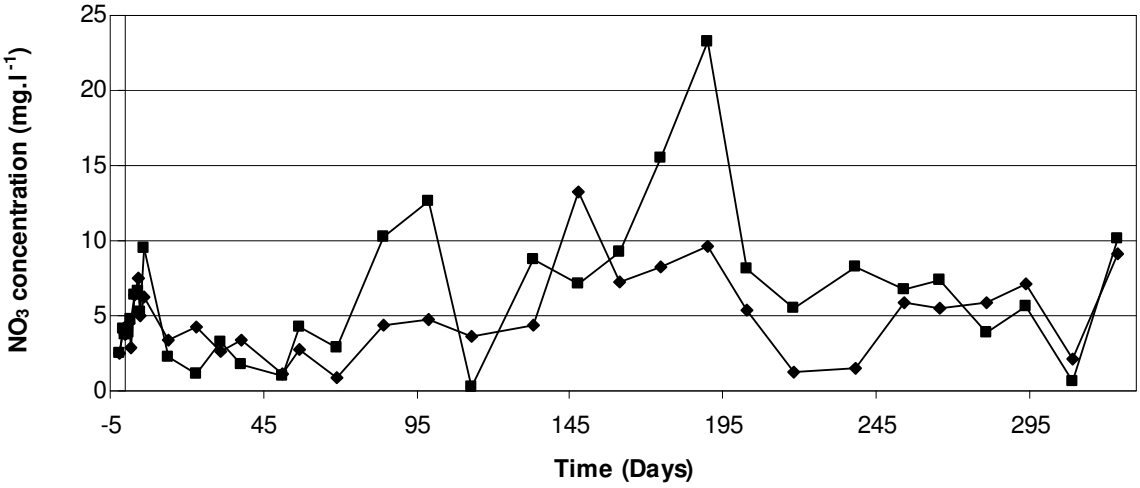

Figure 8: Nitrate concentration of the treated and control areas for the duration of the trial (♦) Treated area (■) Control area

Table 1: Results of tested parameters before and after treatment with Phoslock®

Treated

Parameter	Day -2[a] 22 Jan	Day -1 23 Jan	Day 0 24 Jan	Day 1[b] 25 Jan	Day 2 26 Jan	Day 3 27 Jan	Day 4 28 Jan	Day 5 29 Jan	Day 6 30 Jan	Day 14 Week 2 7 Feb	Day 23 Week 3 16 Feb	Day 31 Week 4 24 Feb
FRP ($mg.l^{-1}$)	0.08	0.06	0.09	0.043	0.017	0.02	0.021	0.025	0.024	0.2925	0.0925	0.1225
Nitrate ($mg.l^{-1}$)	2.5	4.15	3.75	4.53	2.9	6.4	7.5	5	6.2	3.4	4.2	2.575
pH	9.824	9.63	9.791	9.16	9.11	9.22	9.58	9.4	9.48	9.255	9.2875	10.5725
Turbidity (NTU)	36.8	32.7	24.2	13.3	9.6	12.2	15.2	19	18.4	38.75	32.75	63.75
Dissolved oxygen ($mg.l^{-1}$)	9.04	7.45	6.83	3.75	6.03	3.65	5.78	4.5	4.83	4.875	4.95	5.65
Water Temperature (ºC)	27.7	27.6	27.3	26.8	26.6	28.6	26.5	25.2	25.4	25.25	25.75	25.25

Control

	Day -2 22 Jan	Day -1 23 Jan	Day 0 24 Jan	Day 1 25 Jan	Day 2 26 Jan	Day 3 27 Jan	Day 4 28 Jan	Day 5 29 Jan	Day 6 30 Jan	Day 14 Week 2 7 Feb	Day 23 Week 3 16 Feb	Day 31 Week 4 24 Feb
FRP ($mg.l^{-1}$)	0.08	0.06	0.09	0.09	0.07	0.08	0.07	0.06	0.08	0.22	0.72	0.22
Nitrate ($mg.l^{-1}$)	2.5	4.15	3.75	3.9	4.7	6.4	6.6	5.2	9.5	2.2	1.1	3.3
pH	9.824	9.63	9.791	9.69	9.4	9.77	9.93	10.01	10.19	9.55	9.41	10.77
Turbidity (NTU)	36.8	32.7	24.2	39	26	17	25	45	23	23	18	75
Dissolved oxygen ($mg.l^{-1}$)	9.04	7.45	6.83	4.4	6.5	4.4	5.8	5.2	4.2	4.6	4.9	5.1
Water Temperature (ºC)	27.7	27.6	27.3	26	27	29	25	25	25.2	25	25	24.6

[a] Days –2 to 0 represent days before treatment (Day 0 samples taken immediately prior to treatment).

[b] Day 1 onward represents post application sampling

Table 1 continued

Treated

Parameter	Day 38 Week 5 3 Mar	Day 51 Week 7 16 Mar	Day 57 Week 8 22 Mar	Day 69 Week 10 3 Apr	Day 84 Week 12 18 Apr	Day 99 Week 14 3 May	Day 113 Week 16 17 May	Day 133 Week 19 6 Jun	Day 148 Week 21 21 Jun	Day 161 Week 23 4 Jul	Day 175 Week 25 18 Jul	Day 190 Week 27 2 Aug
FRP ($mg.l^{-1}$)	0.055	0.01	0.015	0.01	0.0125	0.005	0.0175	0.0125	0.0225	0.015	0.0075	0.015
Nitrate ($mg.l^{-1}$)	3.425	1.125	2.725	0.85	4.4	4.75	3.625	4.425	13.275	7.225	8.275	9.575
PH	9.0825	10.0225	9.635	8.9025	8.935	9.1775	9.2525	9.44	8.8475	8.7075	8.8575	8.305
Turbidity (NTU)	21.75	45.5	78	92.5	55.75	69.25	54.5	28.25	15.75	11	20.5	12.5
Dissolved oxygen ($mg.l^{-1}$)	6	7.05	7.85	8.82	7.9	7.3	7.975	8.05	8.2	9.225	6.775	7.35
Water Temperature (ºC)	24.65	24.3	23.9	23.7	19.75	19.125	15.5	14.5	13.225	12.6	13.075	12.5

Control

	Day 38 Week 5 3 Mar	Day 51 Week 7 16 Mar	Day 57 Week 8 22 Mar	Day 69 Week 10 3 Apr	Day 84 Week 12 18 Apr	Day 99 Week 14 3 May	Day 113 Week 16 17 May	Day 133 Week 19 6 Jun	Day 148 Week 21 21 Jun	Day 161 Week 23 4 Jul	Day 175 Week 25 18 Jul	Day 190 Week 27 2 Aug
FRP ($mg.l^{-1}$)	0.36	0.2	0.24	0.13	0.18	0.13	0.05	0.03	0.02	0.04	0.06	0.04
Nitrate ($mg.l^{-1}$)	1.7	1	4.2	2.9	10.3	12.6	0.2	8.7	7.1	9.2	15.5	23.3
PH	8.92	8.77	9.62	9.41	8.99	9.15	9.38	9.55	8.91	8.65	9.01	8.61
Turbidity (NTU)	18	15	68	115	68	27	94	21	9	23	32	10
Dissolved oxygen ($mg.l^{-1}$)	5.6	6.2	8.1	9.6	8.8	7.2	9	10.8	9.5	8.3	8.3	8.5
Water Temperature (ºC)	24.1	24	23	23	20	19.5	17	15	14	13.4	13.6	13.1

Table 1 continued

Treated

Parameter	Day 203 Week 29 15 Aug	Day 218 Week 31 30 Aug	Day 238 Week 34 19 Sep	Day 254 Week 36 5 Oct	Day 266 Week 38 17 Oct	Day 281 Week 40 1 Nov	Day 294 Week 42 14 Nov	Day 309 Week 44 29 Nov	Day 324 Week 46 14 Dec
FRP ($mg.l^{-1}$)	0.0175	0.005	0.0225	0.015	0.005	0.0075	0.005	0.015	0.02
Nitrate ($mg.l^{-1}$)	5.35	1.275	1.475	5.925	5.45	5.85	7.15	2.125	9.15
pH	8.04	8.1325	8.0875	8.5275	8.5	9.0825	9.0875	9.9525	10.005
Turbidity (NTU)	8.5	16.25	5.5	14.25	17.75	16.5	33	22.25	38.75
Dissolved oxygen ($mg.l^{-1}$)	7.175	8.425	6.65	9.3	8.975	9.45	7.825	9.7925	10.925
Water Temperature (ºC)	13.7	15.65	17.875	19.8	22.675	23.275	22.025	25.125	26.225

Control

	Day 203 Week 29 15 Aug	Day 218 Week 31 30 Aug	Day 238 Week 34 19 Sep	Day 254 Week 36 5 Oct	Day 266 Week 38 17 Oct	Day 281 Week 40 1 Nov	Day 294 Week 42 14 Nov	Day 309 Week 44 29 Nov	Day 324 Week 46 14 Dec
FRP ($mg.l^{-1}$)	0.09	0.14	0.2	0.2	0.26	0.3	0.29	0.32	0.22
Nitrate ($mg.l^{-1}$)	8.1	5.5	8.3	6.7	7.4	3.9	5.6	0.6	10.1
pH	8.24	8.04	8.13	8.53	8.56	9.08	9.18	9.68	9.99
Turbidity (NTU)	3	9	3	5	15	9	57	28	32
Dissolved oxygen ($mg.l^{-1}$)	6.4	6.9	7	9.1	9.2	7.9	10.6	11.8	10.5
Water Temperature (ºC)	13.7	14.9	14.9	19.3	21.8	23	22.9	24.5	25.5

4. Discussion

The FRP was reduced by more than 50% in the 24h following Phoslock® application. There was no change in the control area over this period, so it can be concluded that Phoslock® was responsible for removing the phosphorus from the water, despite the high pH of the surface waters. After 48h, the FRP concentration in the treated area decreased to 0.017mg.l^{-1}, and then stabilised at around 0.02mg.l^{-1} for the reminder of the first week of testing, whereas the FRP concentration of the control area remained high at 0.08mg.l^{-1}. The difference in the average FRP concentration for the control and treated area after day 6 was statistically significant when tested with a two-tailed *t*-test. Phoslock® therefore successfully removed 73% of the FRP from the treated site. Because the amount of algae visible on the surface was reduced immediately after treatment, and this did not reappear in the following 6 days after treatment, it can be concluded that Phoslock® also acted as an efficient flocculant of some of the algal cells.

Heavy rain in the second week of the trial caused the water level of the dam to rise substantially, and resulted in inflow of water and a large amount of algae into the test site. This caused an increase in FRP and nitrate concentrations in both the treated and control areas. However, the FRP concentration in the treated area did not increase to the same degree as the control area, and decreased at a much faster rate, and by week 7 the FRP concentration was once again below 0.02mg.l^{-1}. This was most likely due to the remaining phosphorus binding potential of the Phoslock®, as it was in the sediment where the pH was lower, allowing for improved adsorption capacity. It is also possible that the FRP concentration decreased following the introduction of new algal cells to the site as a result of phosphorus uptake of the cells during growth. However, this does not explain why the phosphorus concentration of the control area did not show a similar decrease. The level of the boom was subsequently raised, to prevent the re-occurrence of such an event. The phosphorus concentration remained low in the treated area throughout the trial period, despite the fact that the algal cells died off as the water temperature decreased. The FRP concentration of the control area decreased from 0.72mg.l^{-1} in February, to 0.04mg.l^{-1} in August. This was unexpected, as the decomposing algae release phosphorus back into the water. It is likely that the dying algae sank to the sediment, and the phosphorus concentration was subsequently increased at the sediment level, but not in the rest of the water body because of a lack of

water circulation typical of the winter months due to the development of a thermocline. After week 27 (August), the FRP concentration increased steadily in the control area, but remained low (Below 0.02mg.l^{-1}) in the treated area until December (week 46), by which time the control area had increased to 0.22mg.l^{-1}. The temperature of the water increased from 12ºC to 26ºC from August to December. It can therefore be concluded that the event of increased water circulation as the water warmed caused the nutrients at the sediment layer of the control area to be brought to the surface. The FRP concentration of the treated area remained low even after the water temperature increased and rainfall caused inflow of nutrient and algae laden water into the site. This means that Phoslock® effectively and irreversibly bound the FRP, and that there were still some available binding sites to cope with the inflow of phosphates after the rain.

The pH values of the control and treated areas remained very similar throughout the trial period. After Phoslock® application, the treated area showed a decrease in pH when compared to the control, but by the second week of the trial the two areas were once again of similar pH. Therefore the apparent pH lowering effect of Phoslock® is short lived, and is not a dominant feature of this product. It is possible that the lower pH value was as a result of increased water circulation in the treated site during treatment, and not a result of Phoslock® itself. The decrease in pH in both the control and treated areas throughout the winter months was most likely a result of the decrease in the amount of algae in the water. The pH increased in both areas from August to November. This is due to the increased water circulation and algal growth. It was not expected that the treated area would increase to the same degree as the control area because there was less growth in the treated area. This may be a result of mixing between the two areas, as well as the inflow of new water from the main dam.

Phoslock® had no effect on the concentration of nitrates in the treated area, which remained similar to that of the control area. The nitrate concentration varied in both the control and treated sites, but there was a general increase in the nitrate concentration in both areas from April to August and a decrease in the nitrate concentration from August to November. This decrease may be a result of uptake of nutrients by the algae, as well as due to the large amount of rain in October that may have had a diluting effect. The concentration increased in both areas in December, possibly as a result of run-off from rain, or inflow of nutrient laden water from the main dam.

The amount of cyanobacterial growth in the treated site did not appear to increase after the inflow from the dam, nor did it seem to decrease in the first 7 weeks after treatment, despite the fact that the FRP concentration was almost below detectable limits. Cyanobacteria have a substantial storage capacity for phosphorus. They can store enough phosphorus to perform two to four cell divisions, which corresponds to a 4-32 fold increase in biomass (Mur *et al*., 1999). This means that the algae present before treatment would still have had the ability to grow, despite the removal of large amounts of phosphorus. The fact that the FRP concentration remained low through winter despite the decomposition of algal cells indicates that active sites remained on the Phoslock® that were able to bind the phosphorus released from the algae. However, the decrease in water circulation as the water temperature dropped resulted in a similar decrease in the FRP concentration in the control area. As the water temperature increased to above 15ºC from August, the high phosphorus concentration at the sediment level of the control area caused an increase in the FRP concentration in the whole water body, up to 0.32mg.l^{-1} by November. This subsequently led to the development of a cyanobacterial bloom in the control area. The FRP concentration remained low (0.015mg.l^{-1}) in the treated area even after the water temperature increased to above 20ºC in October. Through winter the water level gradually dropped to approximately 1m below the original level. During October the water level increased again by approximately 1m due to heavy rain, causing water from the main dam containing a high FRP concentration and algae to flow into the trial area. The FRP concentration in the treated area was unaffected by this inflow, indicating that active sites remained on the Phoslock® in the sediment.

Takamura *et al*. (1994) found that the amount of *Microcystis* in the sediment can be much higher than the total amount of *Microcystis* in the water column, even during blooms. Overwintering benthic cyanobacterial populations can only act as an inoculum if they remain vital and if they are able to leave the sediment. Verspagen *et al*. (2004) investigated the vitality and two possible recruitment mechanisms of benthic *Microcystis* colonies; passive resuspension and an active increase in the buoyancy levels of the cells. They found that throughout the year benthic *Microcystis* populations were photochemically active and sufficiently vital to serve as an inoculum for the initiation of a bloom. Colonies in the shallower parts of the lake were still in the euphotic zone and had the highest photochemical vitality, and in addition these areas were most frequently

re-suspended. It was concluded that intense mixing of the water column may be sufficient to resuspend the sediment containing benthic *Microcystis* and remove attached sediment, and that shallow water bodies, such as the Phoslock® trial site, will be more prone to high inoculation. The fact that the cyanobacterial bloom occurred in the control area first, followed by a lag before the treated area showed cyanobacterial growth (and then only after inoculation from the main dam) indicates that recruitment in the treated area did not occur, at least not to the same degree as in the control area. It is possible that the reduced phosphorus concentration in the treated sediment may have affected the vitality of the overwintering cyanobacteria.

5. Conclusion

It can be concluded that the product successfully reduced the phosphorus levels of the test site. Phoslock® was also able to flocculate a noticeable amount of algae from the surface on application, and had no effect on the pH or nitrate concentration of the treated area. Following the increase in water temperature after winter, the phosphorus concentration remained low in the treated site when compared with the control, even after a large amount of nutrient containing water entered the site. The cyanobacterial growth was more severe in the control area than the treated area, and was visible from much earlier. The low phosphorus concentration in the water body and the reduced concentration in the sediment therefore prevented the onset of an algal bloom, and may have affected the algal species composition.

6. References

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CHAPTER 5:

ANALYSIS OF THE MICROBIAL COMMUNITY DIVERSITY IN PHOSLOCK® TREATED AND CONTROL AREAS OF HARTBEESPOORT DAM USING PCR-DGGE

1. Introduction

Many fresh water lakes and dams worldwide have been affected by eutrophication, largely as a result of high external nutrient loading with nitrogen and phosphorus compounds (Van der Gught *et al*., 2005). Eutrophication can result in visible cyanobacterial blooms which are often toxic and present severe health risks (Codd *et al*., 2005). The significance of phosphorus in eutrophication has resulted in the development of many remediation plans based on the management of the phosphorus concentration. It is accepted that phosphorus control is more achievable than that of nitrogen, because, unlike nitrogen, there is no atmospheric source of phosphorus that is bio-available. In addition, the general equation for photosynthesis shows that only one gram of phosphorus is required for every seven grams of nitrogen for the formation of the organic matter created in the process (Hereve, 2000). This indicates that a small degree of phosphorus reduction can achieve a much greater degree of growth reduction of cyanobacteria than a reduction of a similar magnitude in the nitrogen level.

Traditional classification systems for cyanobacteria- the bacteriological approach (Rippka *et al*., 1979) and the botanical approach (Anagnostidis & Komárek, 1985)- rely mainly on the morphology of cells and colonies and do not always lead to the identification of phylogenetically coherent taxa (Castenholz, 1992; Wilmotte & Golubic, 1991). At all taxonomic levels, especially above species level, the sequence analysis of genes encoding small-subunit ribosomal RNA (16S RNA) is currently the most promising approach for the phylogenetic classification of cyanobacteria (Nübel *et al*., 1997).

16S rDNA PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) is one of the most frequently used techniques to assess the genetic diversity of microbial communities (Muyzer, 1999). Sequences of 16S rRNA genes are independent from cultivation or growth conditions and can be retrieved by PCR of small amounts of DNA extracted from natural environments. Currently, 16S rDNA sequences constitute the largest gene-specific data set, and the number of entries in generally accessible databases is continually increasing, making 16S rDNA-based identification of unknown bacterial isolates more likely (von Wintzingerode *et al*., 2002).

Several approaches to 16S rRNA analysis in cyanobacteria have been suggested, all of which focused on extending the analysis of the cyanobacterial 16S rRNA beyond axenic cultures. Wilmotte *et al*. (1992) used antibiotics inhibiting peptidoglycan synthesis to suppress the growth of contaminating heterotrophic bacteria in non-axenic cultures of cyanobacteria in order to extract workable amounts of RNA. Garcia-Pichel *et al*. (1996) used micromanipulation to isolate representative samples of field populations of the cyanobacterium *Microcoleus chthonoplastes* from their environment, and thus obtained seven corresponding cultured strains. Mat samples of *M. chthonoplastes* were cleaned by being dragged through agarose gel which removed other cyanobacteria, diatoms and heterotrophic bacteria, and DNA was extracted directly from the cleansed bundles and amplified by PCR to obtain the 16S rRNA gene. Weller *et al*. (1991) used random priming of the 16S rRNA to allow cDNA synthesis anywhere along the molecule. Fragments were cloned and screened for plasmid inserts of interest by sequencing.

However, it was Nübel *et al*. (1997) who developed a set of oligonucleotide primers for the specific amplification of 16S rRNA gene segments from cyanobacteria and plastids by PCR, namely CYA359F (forward), CYA781R(a) and CYA781R(b) (reverse). CYA781R(a) and CYA781R(b) differ by two polymorphic bases situated at positions 7 and 23 (5'to 3'), and were designed to be used in combination as an equimolar mixture. These primers produced a PCR product corresponding to variable regions V3 and V4, which contain significant information for phylogenetic assignments (Yu & Morrison, 2004). PCR products were obtained from all cultures of cyanobacteria and diatoms that were tested, but not from other bacteria and archaea. Gene segments retrieved from cyanobacteria in unialgal but non-axenic cultures could be directly sequenced. The use of this specific PCR in combination with DGGE to probe cyanobacterial diversity in complex microbial communities was also demonstrated (Nübel *et al*., 1997).

The primers designed by Nübel *et al*. (1997) have been used in numerous studies investigating cyanobacterial diversity in environmental samples. Geiß *et al*. (2004) used CYA359F and CYA781R, an equimolar mixture of CYA781R(a) and CYA781R(b), to amplify cyanobacterial 16S rDNA fragments in order to investigate the cyanobacterial diversity of a shallow estuary at the Southern Baltic Sea. The cyanobacterial component of the microbial assemblages of Lake Cisó and Lake Vilar in Spain were analysed by performing PCR-DGGE and sequence analysis of 16S rRNA gene fragments using

CYA359F and CYA781R, with the addition of a GC clamp to the 5' end of primer CYA359F for DGGE purposes (Casamayor *et al*., 2000). Zwart *et al*. (2005) specifically amplified cyanobacterial rDNA for DGGE, but adapted the protocol of Nübel *et al*. (1997) to enable direct comparison of cyanobacterial community profiles with overall bacterial profiles in Lake Loosdrecht in the Netherlands. A single step amplification procedure was used for the bacteria, and a nested PCR for the cyanobacteria. The first round of the nested procedure was performed with cyanobacterial specific primers, and the general bacterial primers were used in the second round. Cyanobacterial bands that were not detectable in the general bacterial pattern were identified in the cyanobacterial specific DGGE. Boutte *et al*. (2006) investigated the variation in banding profiles caused by the position of the GC clamp on the forward or reverse primer, and the combination of the primers designed by Nübel *et al*. (1997) which allowed an optimum investigation of the cyanobacterial community diversity. They found that, irrespective of the position of the GC clamp, the diversity of the bands obtained was lower when both reverse primers were used together than the sum of the bands obtained separately with the primers (a) and (b). This indicates that, when used together, the reverse primers compete for template hybridization, making the genetic fingerprint less complete. In addition, sequence results showed that when the (a) reverse primer was used, filamentous cyanobacterial species were preferentially amplified, whereas the (b) reverse primer targeted unicellular cyanobacteria. This is because the polymorphism at position 23 is situated in the region critical for the specificity of annealing during PCR; the reverse primer (a) amplifies preferentially the filamentous cyanobacteria, whereas the reverse primer (b) targets mainly the unicellular cyanobacteria. It was recommended that the reverse primers CYA781R(a) and CYA781R(b) be used separately with CYA359F in order to give a more complete view of the cyanobacterial community composition, rather than in an equimolar mixture as was originally described by Nübel *et al*. (1997).

This study aims to compare the changes in the cyanobacterial and general bacterial community diversities of two areas of Hartbeespoort Dam over time using DGGE, one area that received a Phoslock® treatment and one that remained untreated as a control. The treated area had a phosphorus concentration significantly lower than that of the control area (Chapter 4). Samples were taken from mid-winter until the end of summer

in order to observe the effect of phosphorus limitation on both the cyanobacterial community and directly or indirectly, on the heterotrophic bacterial community.

2. Materials and Methods

2.1. Sampling and DNA extraction

Water samples were taken from both the Phoslock® treated area and the untreated control area monthly from July 2006 to February 2007. 100ml of water from each sample was ultrasonicated at 50Hz for 30s to break apart cyanobacterial colonies and reduce buoyancy, after which the samples were centrifuged at 10 000g for 15min to obtain a cell pellet. The pellets were resuspended in 567µl of 10mM Tris-1mM EDTA, pH 8, and treated with 30µl of 10% sodium dodecyl sulphate and 3µl Proteinase K (Sigma-Aldrich) (20mg.ml^{-1}) for 60min at 37°. 100µl of 5M NaCl and 80µl of 10% CTAB in 0.7M NaCl was added to each tube and the solutions incubated at 65°C for 10min. Following addition of an equal volume of chloroform-isoamyl alcohol (24:1) was the tubes were centrifuged for 5min at 10 000g. The supernatants were transferred to new tubes and mixed with an equal volume of phenol-chloroform isoamyl alcohol (25:24:1) and centrifuged for 5min at 10 000g. The DNA in the supernatant was precipitated with 0.6vol isopropanol and collected by centrifugation for 15min (10 000g). Finally, DNA was cleaned by washing with 500µl of 70% ethanol and the pellets recovered by centrifugation for 5min. The supernatant was removed and the pellets dried under vacuum at room temperature. DNA was resuspended in 20µl of DNase/RNase free water and maintained at -20°C.

2.2. Polymerase chain reactions

2.2.1. General bacterial PCR

A portion of the 16S eubacterial gene was amplified by means of PCR from the total extracted DNA using the primers PRUN518r (K) and pA8f-GC (M) (Table 1). pA8f-GC was designed specifically for DGGE and thus a GC clamp is included at the 5' end. A reaction with no template DNA was included as a negative control. 0.5µl of DNA

(~25ng.µl^{-1}) was added to 19.5µl of amplification mixture containing 12.8µl sterile MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10x) (Fermentas), 2µl dNTPs (2.5µM), 1µl PRUN518r (10pM), 1µl pA8f-GC (10pM), 0.2µl Taq DNA polymerase (Super Therm) (5U.µl^{-1}) to give a final volume of 20µl.

DNA amplification was performed in a PCR thermal cycler (Biorad) using the following program: 10min at 95°C, 35 cycles of 30s at 94°C, 30s at 58°C and 1min at 72°C, followed by 10min at 72°C then held at 4°C. The PCR product was analysed on a 1% TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3)) agarose gel.

Table 1 Oligonucleotide primers used in this study

Primer	Sequence (5'- 3')	Reference
PRUN518r (K)	5'ATTACCGCGGCTGCTGG3'	(Muyzer *et al*., 1993)
pA8f-GC (M):	5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGAGTTTGATCCTGGCTCAG3'	(Fjellbirkeland *et al.* 2001)
CYA359F	5'CGCCCGCCGCGCCCCGCGCCGGTCCCGCCGCCCCCGCCCGGGGGAATYTTCCGCAATGGG3'[a]	(Nübel *et al*., 1997)
CYA781R(a)	5'GACTACTGGGGTATCTAATCCCATT3'	(Nübel *et al*., 1997)
CYA781R(b)	5'GAC TAC AGG GGT ATC TAA TCC CTT T3'	(Nübel *et al*., 1997)

[a] Y, a C/T nucleotide degeneracy (Liébecq, 1992).

2.2.2. Cyanobacterial specific PCR

A portion of the conserved region of the cyanobacterial 16S gene was specifically amplified using the primers CYA359F, CYA781R(a) and CYA781R(b) as recommended by Boutte *et al*. (2006) (Table 1). CYA359F, the forward primer, has a 40-nucleotide GC-clamp attached at the 5' end for better resolution during DGGE. A reaction with no template DNA was included as a negative control. 1µl of DNA (~25ng.µl^{-1}) was added to 19µl of amplification mixture containing 12.3µl sterile distilled MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10x) (Fermentas), 2µl dNTPs (2.5µM), 1µl CYA359F (10pM), 1µl CYA781R(a) (10pM) or 1µl CYA781R(b) (10pM), 0.2µl Taq DNA polymerase (Super Therm) (5U.µl^{-1}) to give a final volume of 20µl.

DNA amplification was performed in a PCR thermal cycler (Biorad) using the following program, modified from Nübel *et al*. (1997): 5min at 94°C, 35 cycles of 1min at 94°C, 1min at 60°C and 1min at 72°C, followed by 5min at 72°C then held at 4°C. The PCR product was analysed on a 1% TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3)) agarose gel.

2.3. DGGE

16S PCR products from the general bacterial and cyanobacterial specific (using (a) and (b) reverse primers separately) reactions were analysed by DGGE according to the method described by Muyzer *et al*. (1993). 10µl each of the general bacterial and cyanobacterial specific PCR products containing approximately 250ng of 16S rDNA were loaded per lane on three separate denaturing gradient gels. A standard DNA was not added as each DGGE gel was treated as a separate data set. The gel for general bacteria contained a 35-55% formamide/urea denaturing gradient, whereas the gels for the cyanobacterial specific (a) and (b) PCRs had a 40-50% denaturing gradient (Table 2). Gels were run at 70V for 17h at a constant temperature of 60°C. From the gels graphic cluster representations of the banding patterns were drawn using Gel2K (Norland, 2004). The program estimates band peak intensity along the lane. Peaks can be manipulated to ensure that, should more than one peak be registered per band, they can be grouped together. Dominant species per lane are indicated as dark prominent bands across the lane. CLUST (Norland, 2004) was used to compile a dendrogram of each banding pattern drawn in order to analyse species diversity. CLUST is based on Shannon index algorithms and groups the species profiles in each sample according to how similar in community composition the samples are. Dominant bands were picked from the gels under blue light, placed into 30µl sterile MilliQ and allowed to stand over night at 4°C to dissolve, before being used for sequencing.

Table 2: Denaturing gradient table showing volumes in millilitres of denaturing stock solution A (DSSA): 8% acrylamide in 0.5x TAE (40nM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3) buffer) and denaturing stock solution B (DSSB): 8% acrylamide, 7M urea, 40% formamide in 0.5x TAE buffer, mixed to form a gradient within the gel.

Denaturing percentage	DSSA	DSSB	Total volume
15	12.3	2.2	14.5
20	11.6	2.9	14.5
25	10.9	3.6	14.5
30	10.2	4.4	14.5
35	9.4	5.1	14.5
40	8.7	5.8	14.5
45	8.0	6.5	14.5
50	7.3	7.3	14.5
55	6.5	8.0	14.5
60	5.8	8.7	14.5
65	5.1	9.4	14.5
70	4.4	10.2	14.5
75	3.6	10.9	14.5

2.4. Sequencing and phylogenetic analysis

DNA from each dominant DGGE band was first amplified in an up-PCR (as described above) using the K and M primers for DNA picked from the general bacterial gel, and CYA359F, CYA781R(a) and CYA781R(b) for the DNA from the cyanobacterial gels. Up-PCR product was cleaned by transferring the entire volume to a 0.5ml Eppendorf tube, adding 2µl of 3M sodium acetate (pH 4.6) and 50µl 95% ethanol, and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000rpm for 30min. The ethanol solution was removed, the pellet rinsed in 150µl 70% ethanol and centrifuged at 10 000rpm for 5min. The ethanol was aspirated and the pellet dried under vacuum for approximately 10min. The pellet was then re-suspended in 20µl sterile water. Each amplified PCR was then sequenced in an Eppendorf tube containing 1µl clean PCR product, 2µl Big Dye sequencing mix (Roche), 0.32µl primer and 1.68µl deionised filter-sterilised water. For the bands from the general bacterial DGGE gel, partial sequences of the 16S bacterial gene were obtained using the K primer above, and nucleotide sequence order was confirmed by comparing it to the sequence obtained

using the M primer. Similarly, the CYA359F and CYA781R(a) primers were used for the DNA from the cyanobacterial gel that targeted filamentous cyanobacteria, and CYA359F and CYA781R(b) for the gel targeting unicellular cyanobacteria. Sequence PCR products were cleaned in the same manner as the amplification PCR, except that 15µl of sterile water was added to the PCR before transferring it to a 0.5ml tube, and the dried pellet obtained at the end was not re-suspended in water. Tubes were transferred on ice to the sequencer, and DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase Applied Biosystems, UK). Sequences were deposited in GenBank, and the accession numbers are presented in Tables 3 and 4.

Each sequence was subjected to a BLAST analysis on the GenBank database, and by determining the sequences with the highest percentage match and coverage, tentative species identification was possible. A phylogenetic analysis was performed on the cyanobacterial sequences from the DGGE gels. Separate trees were drawn, one for each cyanobacterial DGGE gel and one that combined the sequences from both gels. Closely related sequences for each cyanobacterial sequence were selected from GenBank for alignment and inclusion in the trees. Sequence orientation was checked using Vector NTI (Invitrogen), and where necessary the orientation was changed. Sequences were then aligned with Clustal X (Thompson et al., 1994) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 Phylogenetic Analysis Using Parsimony (Swofford 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping, MULPAR-effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was assessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention indices (RI) were determined for all data sets. The phylogenetic tree of sequences from the DGGE gel targeting filamentous cyanobacteria was rooted with *Calothrix*, and that of the unicellular cyanobacteria was rooted with *Thermatoga maritima.* The tree combining sequences from both gels was rooted with *T. maritima* and a *Pseudomonas* species. Bootstrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

3. Results

3.1. DGGE targeting filamentous cyanobacteria

The DGGE gel that targeted the filamentous cyanobacteria in the monthly samples is illustrated in Figure 1. The species diversity between the months is compared in the dendrogram in Figure 3. The months of July, August, October and November fall in the same dominant clade (III) for both the treated and control areas, whereas September, December, January and February group together (II). This means that the species diversity was similar in these months in the control and treated areas. October and November therefore showed a species diversity in both the treated and control areas that was similar to the winter months, but September had a diversity comparable to the summer months. The diversity of the control and treated areas for most months appears to be similar, as they are grouped together at the lowest level in most cases. One exception is the control area in January, which had a very low diversity. In contrast, the diversity of the treated area in January was much higher.

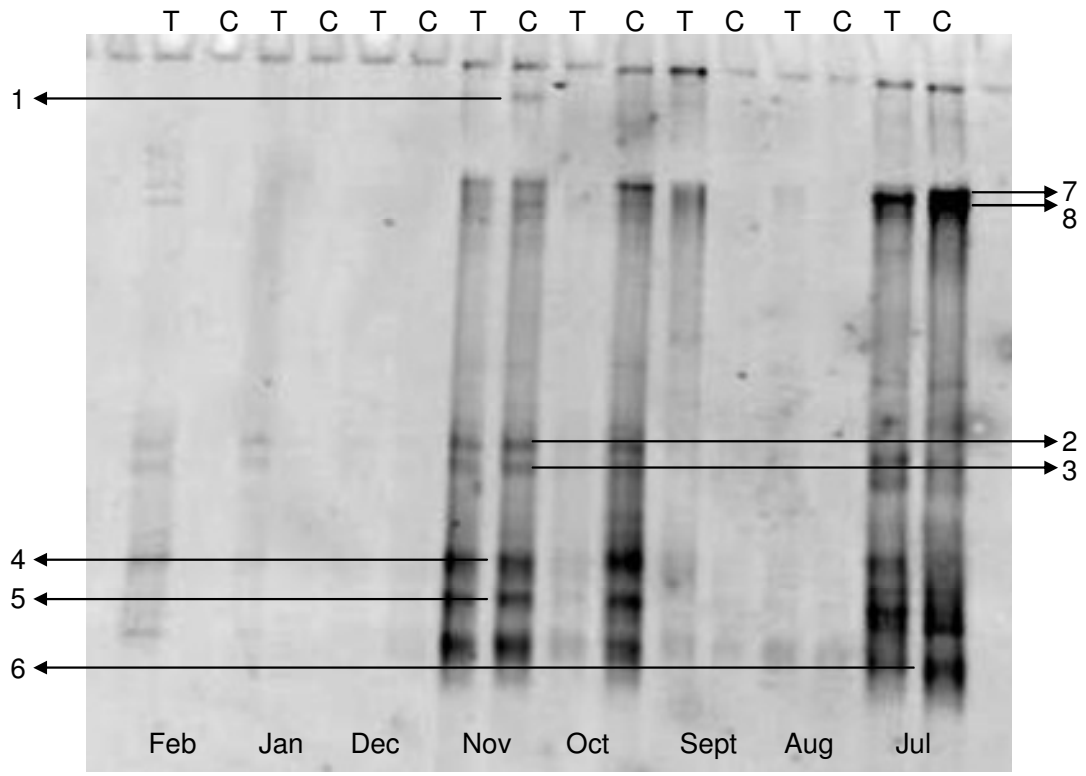


Figure 1: DGGE gel of filamentous cyanobacteria ('a' reverse primer) showing the banding patterns for each month. C= control area T= area treated with Phoslock®

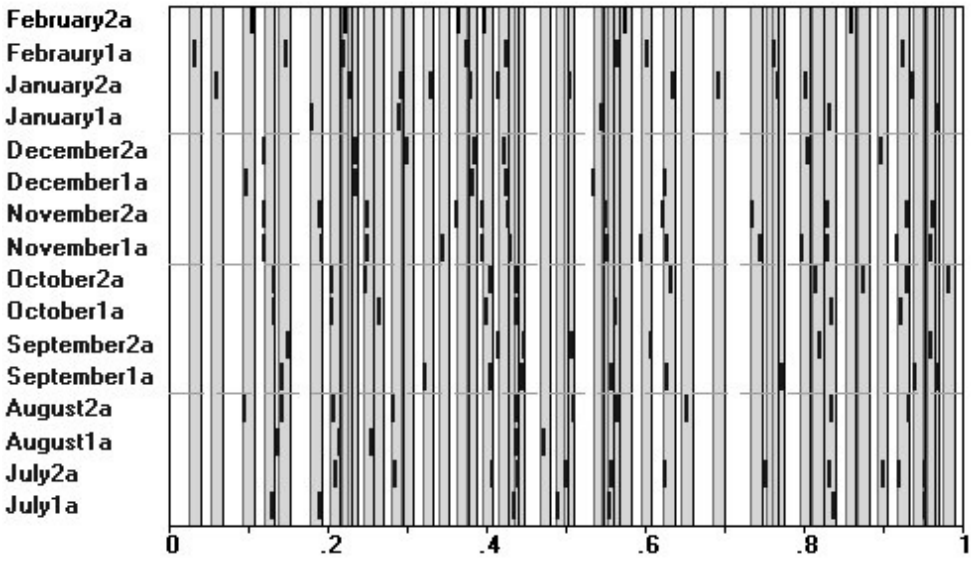


Figure 2: Schematic representation of the banding pattern of the DGGE gel targeting filamentous cyanobacteria (a). Black bars represent dominant species in each sample 1a= control area, 2a= treated area

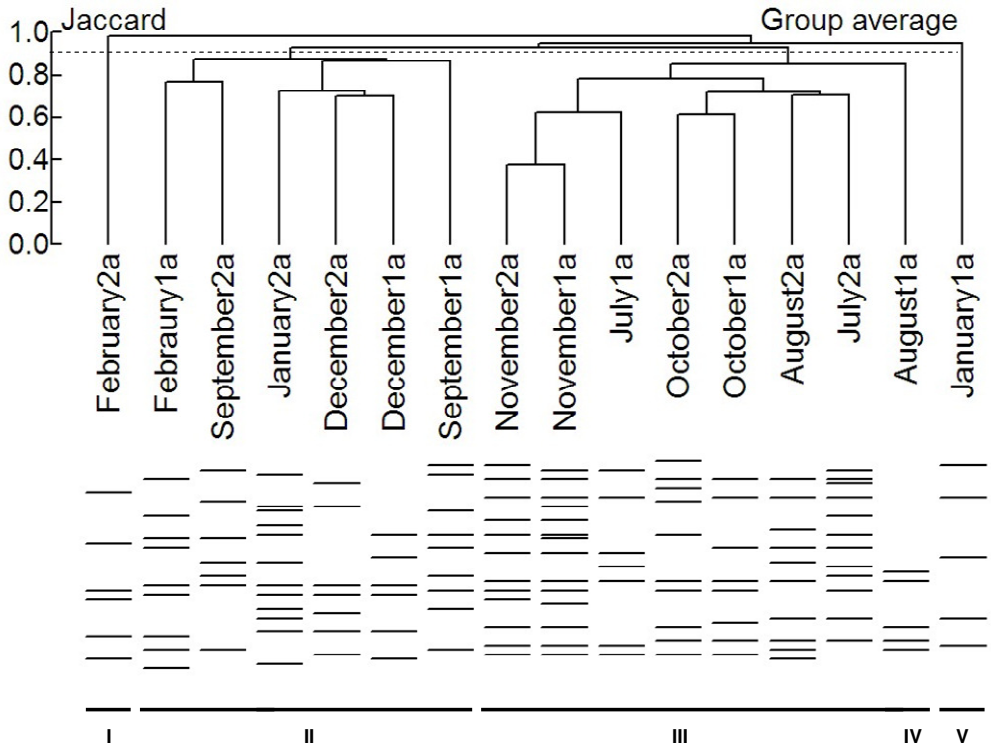
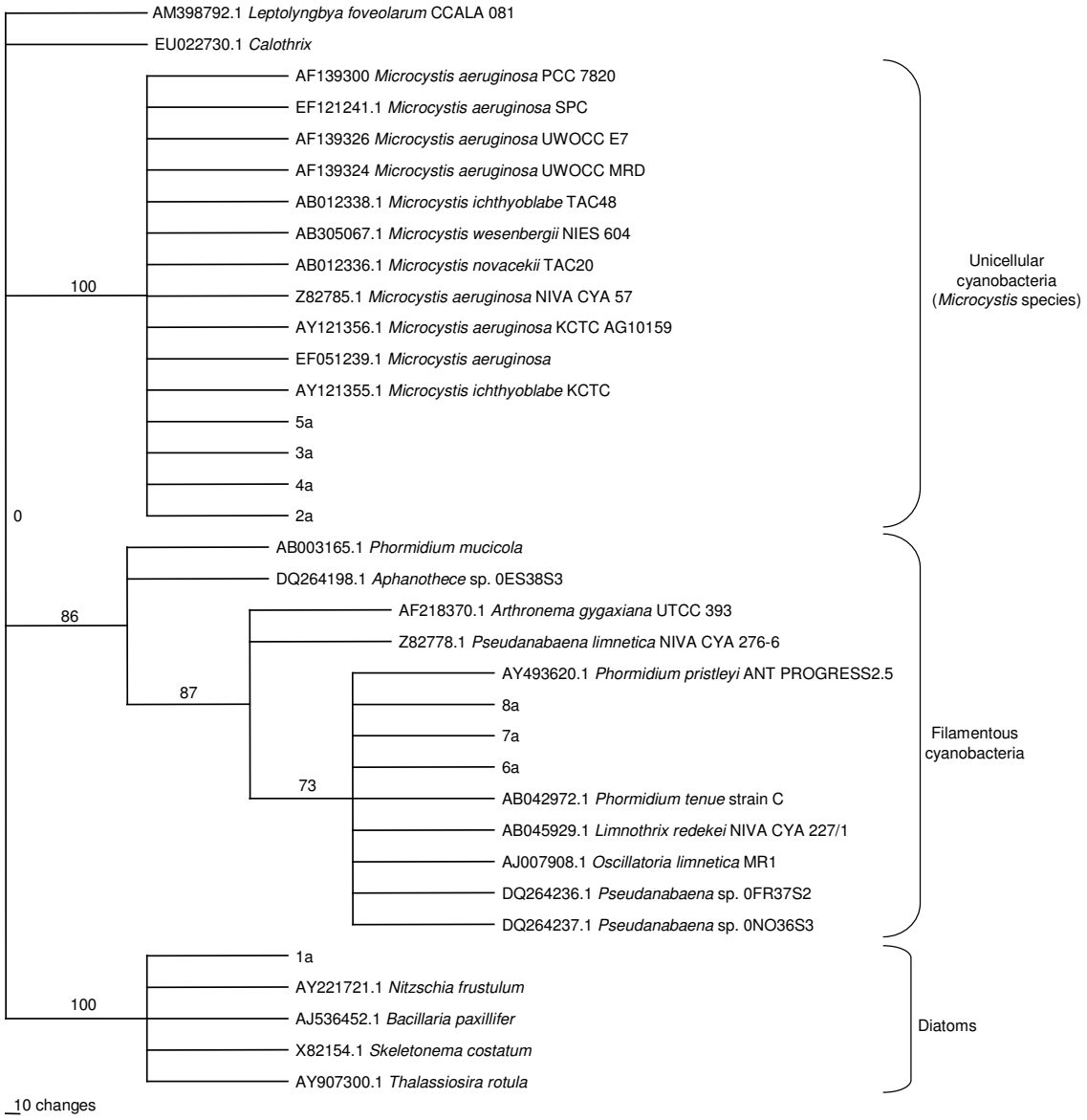


Figure 3: Dendrogram to show the differences in the species diversity of monthly samples with targeted filamentous cyanobacteria (a) using a group average, Jaccard setting. 1a= control area, 2a= treated area

In order to determine the species composition, dominant bands were picked from the gel (1-8 on Figure 1) and sequenced (Appendix A). The closest matching species are presented in Table 3. The sequence in band 1 matched closely to the chloroplast 16s rRNA gene of *Nitzschia frustulum*, a diatom. Sequences in bands 2-5 were close matches to various *Microcystis* species (unicellular cyanobacteria), and bands 6-8 matched with sequences of filamentous cyanobacteria such as *Pseudanabaena* sp., *Limnothrix redekei* and *Oscillatoria limnetica.* These are non-heterocystis species (not capable of nitrogen fixation). The primer combination of CYA359F and CYA781R(a) therefore picked up both unicellular and filamentous species of cyanobacteria, and not just the filamentous species as was expected. Band 1 was only visible until November, indicating that the diatom *Nitzschia frustulum* was not present during the summer months of December through to February. Interestingly, the *Microcystis* species (bands 2-5) were prominent during July, but disappeared in August and September. During October, *Microcystis* was dominant in the control area, whereas in the treated area bands 2-5 were very faint. During January and February, *Microcystis* species, with the

exception of band 5, were present in the treated area but not the control, which was unexpected. In terms of the filamentous cyanobacterial species, band 6, most likely a *Pseudanabaena* sp., was prominent in both the treated and control areas from July until November. However, in January and February it was present only in the treated area. Bands 7 and 8 were present in both the treated and control areas in July, October and November, but only in the treated area in September. The bands disappeared until January, when they were only present in the treated area. According to the gel, the control area in January and February had no cyanobacterial species, as no bands are visible. However, the Gel2K software picked up 5 bands in January and 6 in February (Figure 2). The species diversity in the treated area was higher during January and February than in the control area.

The phylogenetic tree of the sequences obtained from the gel in Figure 1 (1-8) and their closely related sequences obtained from BLAST is presented in Figure 4. The sequences from the gel grouped with the expected sequences: sequence 1 grouped with the diatoms, sequences 2-5 grouped with the unicellular *Microcystis* species, and sequences 6-8 grouped with the filamentous cynobacteria. Therefore the tentative identifications presented in Table 3 appear to be correct, at least up to species level. The high retention index of 0.8483 indicated that the data set was significant.



Tree length = 103.28666
Consistency index (CI) = 0.5338
Retention index (RI) = 0.8483

Figure 4: Phylogeny of cyanobacterial 16s rRNA gene amplicons recovered from the DGGE gel in Fig. 1 and closely related sequences obtained from Genbank (Distance values are indicated above branches)

3.2. DGGE targeting unicellular cyanobacteria

The DGGE gel which targeted the unicellular cyanobacteria in the monthly samples is illustrated in Figure 7. The diversity between the months is compared in the dendrogram in Figure 6. The treated and control areas in July group together (clade I), as they have a similar low diversity, as is expected for the winter months. In clade II, November, January and February of the treated area group closely with August and September of the control area. The treated area in December had a similar diversity to the control area in October (clade III). This indicates that the diversity of the control area in spring is comparable with that of the treated area in summer. An exception to this was the treated area in September, which grouped with the control area in November (clade IV). In terms of the diversity, therefore, the reduced phosphorus in the water due to the Phoslock® treatment appeared to have a greater effect on the unicellular cyanobacteria than was obvious in the gel that targeted filamentous cyanobacteria.

In order to determine the species composition, dominant bands were picked from the gel (9-16 on Figure 5) and sequenced (Appendix A). The closest matching sequences obtained from BLAST are presented in Table 3. Band 9 closely matched the chloroplast 16S rDNA of the diatoms *Aulacoseira ambigua* and *Haslea wawrikae*. Bands 10-16 were all close matches to species of *Microcystis*, predominantly *M. aeruginosa*, *M. viridis*, *M. botrys* and *M. wesenbergii*. For each sequence that was run on BLAST, the closest matching sequences had the same percentage match as well as coverage, so it was not possible to identify the sequences up to species level. The combination of CYA359F and CYA781R(b) primers only amplified unicellular cyanobacteria, no filamentous cyanobacterial sequences were detected. Band 9 (diatom chloroplast 16S rDNA) was present from July until December, but appeared to be more dominant in the treated area from September. Bands 13, 14 and 15 (near the top of the gel) were present in both the treated and control areas for all the months sampled. Bands 10, 11, 12 and 16 (near the bottom of the gel) were only predominant until November. It is possible that the *Microcystis* species in bands 13-15 were able to out-compete those present in bands 10-12 and 16 when bloom conditions were experienced.

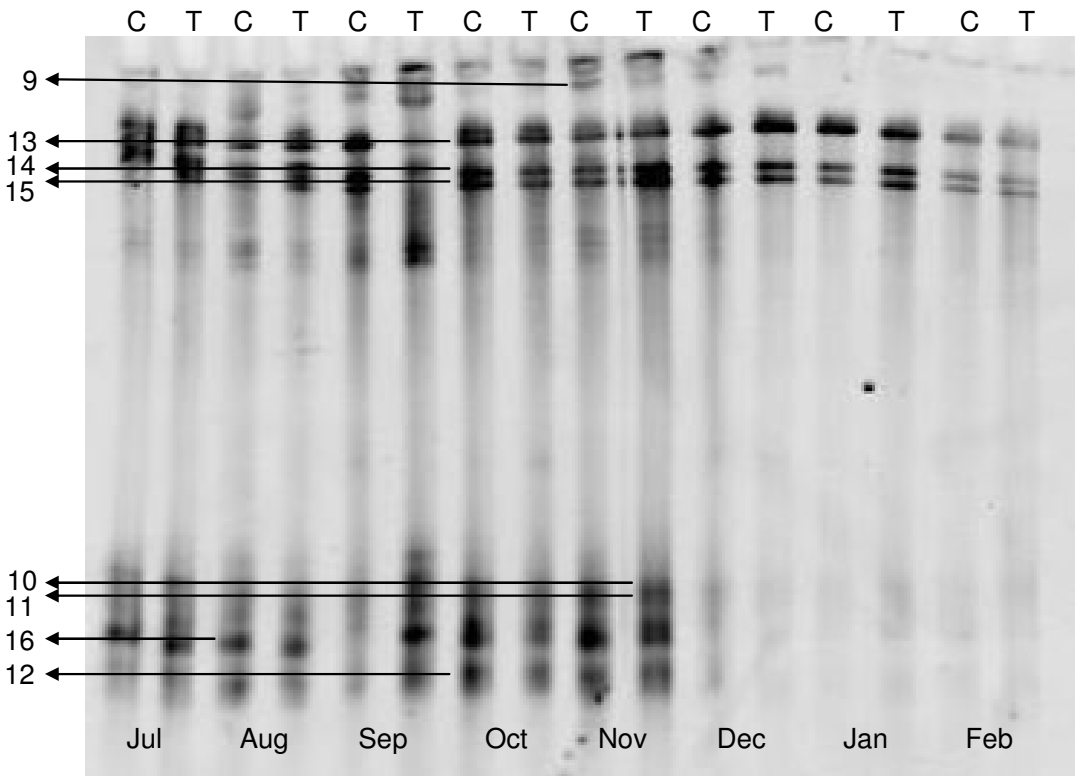


Figure 5: DGGE gel of unicellular cyanobacteria ('b' reverse primer) showing the banding patterns for each month. C= control area T= Area treated with Phoslock®

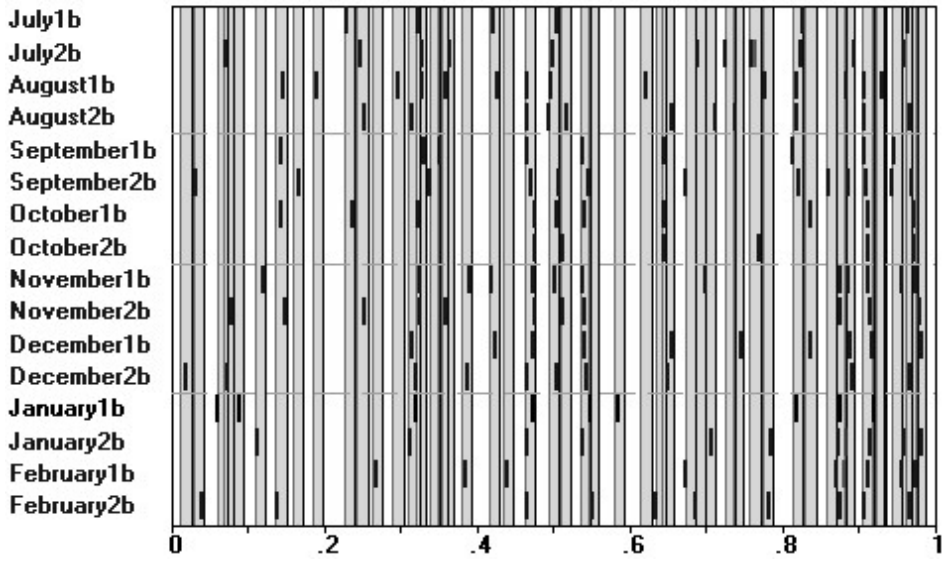


Figure 6: Schematic representation of the banding pattern of the DGGE gel which targeted unicellular cyanobacteria (b). 1b= control area, 2b= treated area

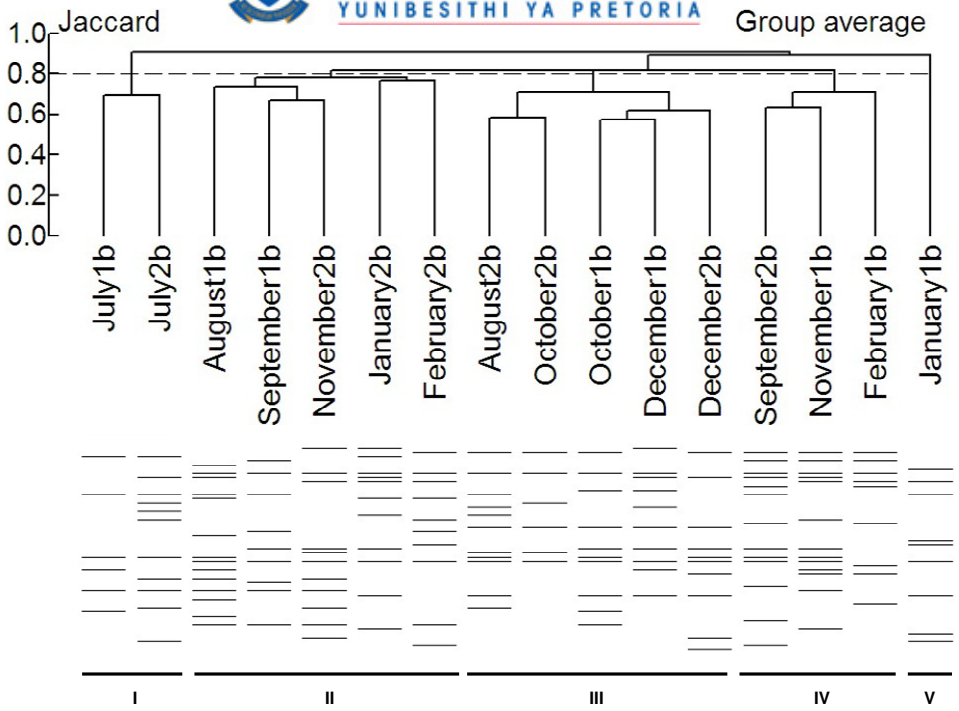
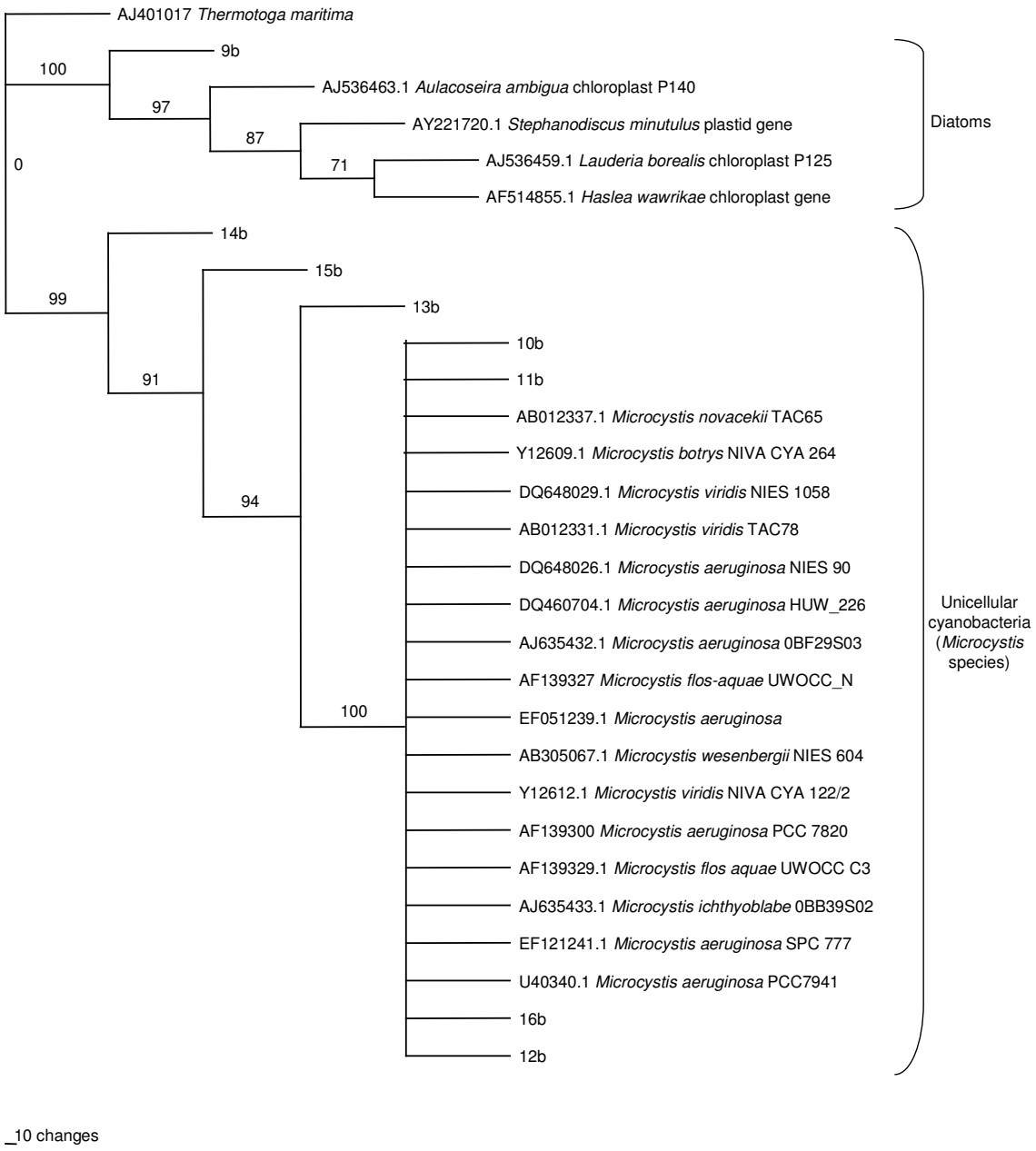


Figure 7: Dendrogram to show the differences in the species diversity of monthly samples with targeted unicellular cyanobacteria (b) using a group average, Jaccard setting 1b= control area, 2b= treated area

The phylogenetic tree of the sequences obtained from the gel in Figure 4 (9-16) and their closely related sequences obtained from BLAST is presented in Figure 8. The high retention index of 0.9352 indicated that signal within the data set was significant. The sequences from the gel grouped with the expected sequences. Sequence 9 grouped with the diatoms, and sequences 10-16 grouped with the unicellular *Microcystis* species, although 13, 14 and 15 were basal to the main clade containing the related sequences and 10, 11, 12 and 16. This difference in grouping corresponds to the banding pattern described above, with the sequences from the bands near the top of the gel grouping with the related *Microcystis* species, and the sequences from the bands near the bottom of the gel falling basal.

Figure 9 presents a phylogenetic tree combining the sequences from the gels in figures 1 and 5. Sequences 2-5 group with the unicellular *Microcystis* species along with 10, 11, 12 and 16, but once again 13, 14 and 15 were basal to this clade, although they did not group with the filamentous cyanobacteria or the diatoms. The filamentous cyanobacterial sequences appeared to be more closely related to the diatom chloroplast 16S rDNA that the unicellular cyanobacteria.



Tree length = 111.21071
Consistency index (CI) = 0.8360
Retention index (RI) = 0.9352

Figure 8: Phylogeny of cyanobacterial 16s rRNA gene amplicons recovered from the DGGE gel in Figure 5 and closely related sequences obtained from Genbank (Distance values are indicated above branches)

Tree length = 148.33752
Consistency index (CI) = 0.6739
Retention index (RI) = 0.9037

Figure 9: Phylogeny of cyanobacterial 16s rRNA gene amplicons recovered from the DGGE gels in Figure 1 and Figure 5 and closely related sequences obtained from Genbank (Distance values are indicated above branches)

3.3. DGGE targeting all bacteria, including cyanobacteria

The DGGE gel that was run with the DNA amplified with K and M primers capable of amplifying all bacterial (including cyanobacterial) 16S rDNA is presented in Figure 10, as well as the bands (1-18) that were picked for sequencing. The sequences from BLAST that were the closest match to the sequences obtained from the gel (Appendix A) are presented in Table 4. Bands 7, 8, 10-13 and 18 were close matches to cyanobacteria. Band 7 (most likely *Microcystis aeruginosa*) was not present in the control area until October, and only appeared in November in the treated area. This band was also less bright in the treated area for January than the control area. Bands 10 and 11, which were also close matches to *Microcystis* species, were present in all the months sampled, but band 10 was only present in the control area in January and February. Band 13, most likely *Anabaena flos-aquae*, a filamentous cyanobacterium, was present in the treated and control areas until October, after which it was more dominant in the treated areas until February. This heterocystous species was not present in the DGGE gel which targeted filamentous cyanobacteria, but followed the same pattern. The sequences from bands 1-6, 9, and 14-17 correspond to bacterial 16S rDNA sequences, mainly uncultured α-, β- and δ-proteobacteria, as well as uncultured actinobacteria. Bands 3, 5, 6, 9 and 14-17 were more dominant through the winter months. Bands 3 and 5 disappeared after September, and bands 9 and 14-17 were only present until October. Band 6 was only present until October, but was dominant in the treated area through winter. Bands 1 and 4 (β- and δ-proteobacteria) were not present during the winter months, and only appeared in October in both the treated and control areas. Band 2 was present throughout the sampled months, in both the treated and control areas.

The diversity between the months is compared in the dendrogram in Figure 12. The winter and spring months of July, August, and September grouped together (clades III and IV), and the summer months (October to February) grouped together in clades I and II. On the whole, the Phoslock® treatment did not appear to affect the species diversity, as the treated and control areas for each month were grouped according to season.

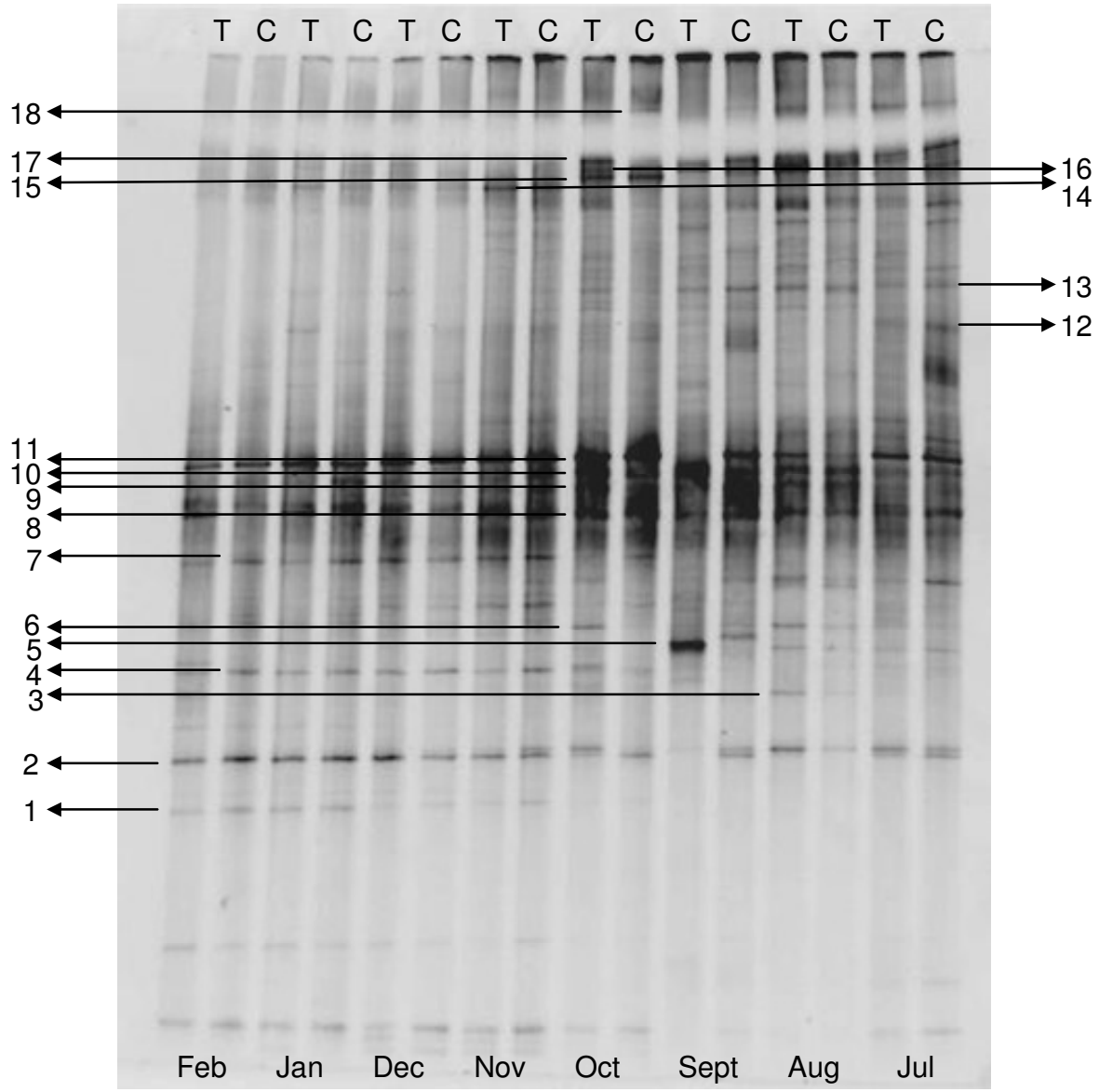


Figure 10: General bacterial DGGE gel showing the banding patterns for each month. C= control area T= Area treated with Phoslock®

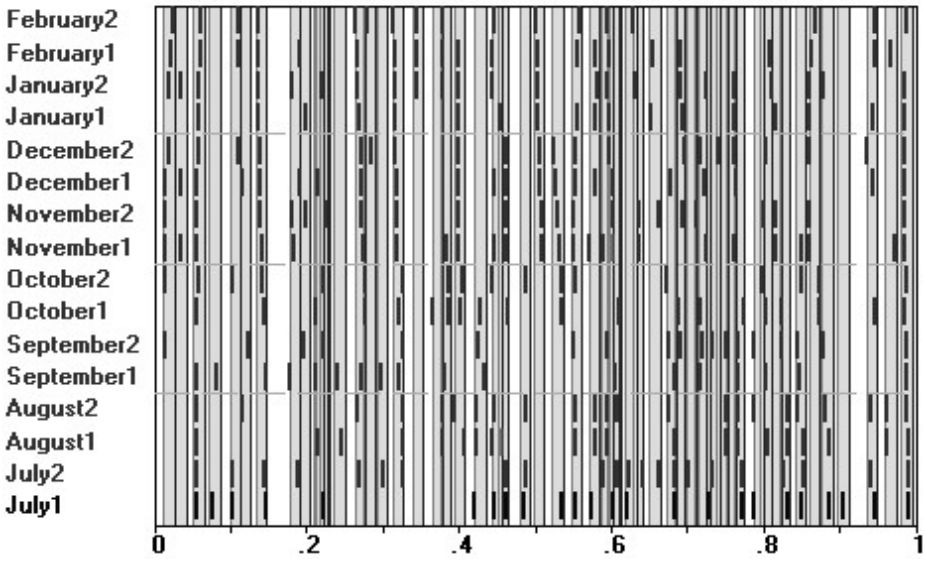


Figure 11: Schematic representation of the banding pattern of the bacterial DGGE gel. 1= control area, 2= treated area

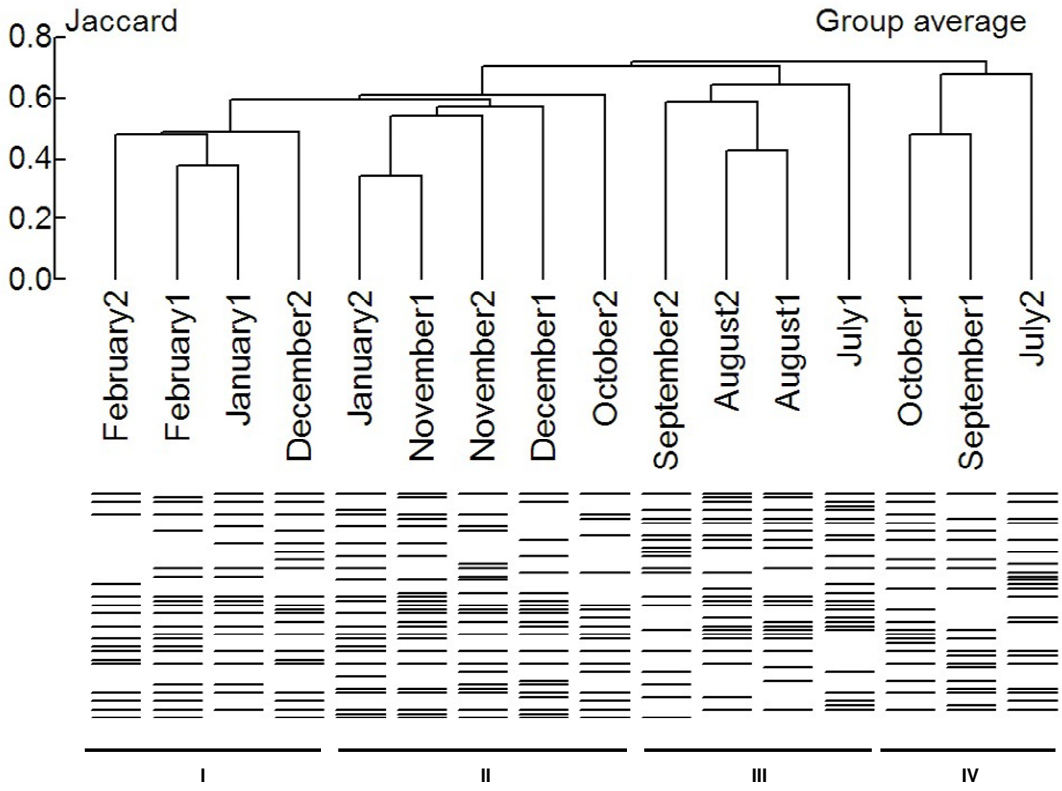


Figure 12: Dendrogram to show the differences in the species diversity of bacteria in the monthly samples using a group average, Jaccard setting 1= control area, 2= treated area

Table 3: Cyanobacterial 16s rDNA sequences from bands picked from DGGE gels in Figures 1 and 2. Species that had the highest percentage match with the highest coverage and their descriptions are presented

Band number (Accession number)	Matching GenBank accession numbers	Closest species identification	Percentage match (Query coverage)	Description
1 (EU94509)	AY221721.1	*Nitzschia frustulum*	91% (82%)	16S ribosomal RNA gene, partial sequence; plastid gene for plastid product
	AJ536452.1	*Bacillaria paxillifer*	89% (88%)	Chloroplast 16S rRNA gene
2 (EU94510)	EF051239.1	*Microcystis aeruginosa*	92% (86%)	16S ribosomal RNA gene, partial sequence
	EF121241.1	*Microcystis aeruginosa*	93% (84%)	Strain SPC 777, 16S ribosomal RNA gene, partial sequence
3 (EU94511)	Z82785.1	*Microcystis aeruginosa*	92% (88%)	Strain NIVA-CYA 57, 16S rRNA gene
	AB012337.1	*Microcystis novacekii*	92% (88%)	Isolate TAC65, gene for 16S rRNA, partial sequence
	AB305067.1	*Microcystis wesenbergii*	92% (88%)	Strain NIES-604, gene for 16S ribosomal RNA, partial sequence
4 (EU94512)	EF051239.1	*Microcystis aeruginosa*	88% (93%)	16S ribosomal RNA gene, partial sequence
	AY121356.1	*Microcystis aeruginosa*	88% (89%)	Strain KCTC AG10159, 16S ribosomal RNA gene partial sequence
	AY121355.1	*Microcystis ichthyoblabe*	88% (89%)	Strain KCTC AG10160, 16S ribosomal RNA gene partial sequence
	AY074802.1	*Microcystis novacekii*	88% (89%)	Strain NIER-10029, 16S ribosomal RNA gene partial sequence
5 (EU94513)	EF051239.1	*Microcystis aeruginosa*	96% (92%)	16S ribosomal RNA gene, partial sequence
	DQ786006.1	*Microcystis aeruginosa*	96% (89%)	Strain PCC 7820, 16S ribosomal RNA gene, partial sequence
	DQ264219.1	*Microcystis ichthyoblabe*	96% (89%)	Strain 9EH38S1, 16S ribosomal RNA gene, partial sequence
6 (EU94514)	DQ264237.1	*Pseudanabaena* sp.	91% (86%)	0NO36S3, 16S ribosomal RNA gene, partial sequence
	AB045929.1	*Limnothrix redekei*	90% (86%)	Strain NIVA-CYA 227/1, 16S ribosomal RNA gene, partial sequence
	AF218370.1	*Arthronema gygaxiana*	90% (86%)	Strain UTCC 393, 16S ribosomal RNA gene, partial sequence
7 (EU94515)	DQ264236.1	*Pseudanabaena* sp.	94% (90%)	0NO36S3, 16S ribosomal RNA gene, partial sequence
	AB045929.1	*Limnothrix redekei*	93% (90%)	Strain NIVA-CYA 227/1, 16S ribosomal RNA gene, partial sequence
8 (EU94516)	AJ007908.1	*Oscillatoria limnetica*	89% (88%)	strain MR1, 16S rRNA gene, partial

	AB045929.1 AB003165.1	*Limnothrix redekei* *Phormidium mucicola*	88% (88%) 83% (84%)	Strain NIVA-CYA 227/1, 16S ribosomal RNA gene, partial sequence gene for 16S ribosomal RNA
9 (EU94517)	AJ536463.1 AF514855.1 AJ536459.1	*Aulacoseira ambigua* *Haslea wawrikae* *Lauderia borealis*	96% (90%) 96% (90%) 95% (90%)	Strain P140, chloroplast 16S rRNA gene, 16S ribosomal RNA gene partial sequence; chloroplast gene for chloroplast product Strain P125, chloroplast 16S rRNA gene
10 (EU94518)	EF121241.1 AF139329.1 Y12612.1	*Microcystis aeruginosa* *Microcystis flos-aquae* *Microcystis viridis*	98% (100%) 98% (100%) 98% (100%)	Strain SPC 777, 16S ribosomal RNA gene, partial sequence Strain UWOCC C3, 16S ribosomal RNA gene, partial sequence Strain NIVA-CYA 122/2, partial16S ribosomal RNA gene sequence
11 (EU94519)	DQ460704.1 DQ648029.1 Y12609.1	*Microcystis aeruginosa* *Microcystis viridis* *Microcystis botrys*	98% (97%) 98% (97%) 98% (97%)	Strain HUW 226 16S ribosomal RNA gene, partial sequence Strain NIES-1058 16S ribosomal RNA gene, partial sequence Strain NIVA-CYA 264 16S ribosomal RNA gene, partial sequence
12 (EU94520)	DQ648028.1 AJ635432.1 AB012336.1	*Microcystis wesenbergii* *Microcystis aeruginosa* *Microcystis novacekii*	95% (100%) 95% (100%) 95% (100%)	Strain NIES-107 16S ribosomal RNA gene, partial sequence Strain 0BF29S03 partial 16S rRNA gene Isolate TAC20, 16S rRNA gene, partial sequence
13 (EU94521)	AB012331.1 AF139327 DQ460704.1	*Microcystis viridis* *Microcystis flos-aquae* *Microcystis aeruginosa*	92% (99%) 92% (99%) 92% (99%)	Isolate TAC78, 16S rRNA gene, partial sequence Strain UWOCC N 16S ribosomal RNA gene, partial sequence Strain HUW 226 16S ribosomal RNA gene, partial sequence
14 (EU94522)	DQ460704.1 Y12609.1	*Microcystis aeruginosa* *Microcystis botrys*	91% (94%) 91% (94%)	Strain HUW 226 16S ribosomal RNA gene, partial sequence Strain NIVA-CYA 264 16S ribosomal RNA gene, partial sequence
15 (EU94523)	DQ460704.1 DQ648028.1 DQ648029.1	*Microcystis aeruginosa* *Microcystis wesenbergii* *Microcystis viridis*	90% (99%) 90% (99%) 90% (99%)	Strain HUW 226 16S ribosomal RNA gene, partial sequence Strain NIES-107 16S ribosomal RNA gene, partial sequence Strain NIES-1058 16S ribosomal RNA gene, partial sequence
16 (EU94524)	EF051239.1 AJ133174.1	*Microcystis aeruginosa* *Microcystis wesenbergii*	99% (99%) 99% (97%)	16S ribosomal RNA gene, partial sequence Strain NIES 104 partial 16S rRNA gene

Table 4: Bacterial 16s rDNA sequences from bands picked from DGGE gel in figure 7. Species that had the highest percentage match with the highest coverage and their descriptions are presented

DGGE gel band sequence number	Matching GenBank accession numbers	Closest species identification	Percentage match (Query coverage)	Description
1 (EU94525)	EF665917.1	Uncultured δ-proteobacterium	91% (47%)	Clone GASP-MB3W2 C12, 16S ribosomal RNA gene, partial sequence
2 (EU94526)	AY509417.1	Uncultured α-proteobacterium	97% (100%)	Clone LiUU-3-194, 16S ribosomal RNA gene, partial sequence
	AF538712.1	*Roseomonas mucosa*	94% (100%)	Strain MDA5527, 16S ribosomal RNA gene, partial sequence
3 (EU94527)	DQ628961.1	Uncultured Microbacteriaceae bacterium	93% (96%)	Clone SOC1 6H, 16S ribosomal RNA gene, partial sequence
	DQ316367.1	Uncultured Actinobacterium	93% (96%)	Clone ST11-6, 16S ribosomal RNA gene, partial sequence
4 (EU94528)	AY824332.1	Uncultured β-proteobacterium	79% (83%)	Clone cloRDC+39, 16S ribosomal RNA gene, partial sequence
	AB211233.1	*Ideonella* sp.	79% (83%)	Strain 0-0013, gene for 16S rRNA, partial sequence
	AF244133.1	*Burkholderia cepacia*	68% (98%)	16S ribosomal RNA gene, partial sequence
5 (EU94529)	EF428988.1	*Aeromonas veronii*	98% (100%)	16S ribosomal RNA gene, partial sequence
6 (EU94530)	EF520353.1	Uncultured Actinobacterium	76% (64%)	Clone ADK-GRe02-60, 16S ribosomal RNA gene, partial sequence
	AY337957.1	Uncultured Microbacteriaceae bacterium	76% (64%)	Clone M13-99, 16S ribosomal RNA gene, partial sequence
7 (EU94531)	DQ887510.1	*Microcystis aeruginosa*	80% (100%)	FC-070, 16S ribosomal RNA gene, partial sequence

	AF139328.1 DQ648028.1	*Microcystis flos-aquae* *Microcystis wesenbergii*	80% (100%) 79% (100%)	Strain UWOCC C2, 16S ribosomal RNA gene, partial sequence Strain NIES-107, 16S ribosomal RNA gene, partial sequence
8 (EU94532)	AJ133171.1 DQ648029.1	*Microcystis aeruginosa* *Microcystis viridis*	85% (50%) 84% (50%)	Strain PCC 7941, partial 16S rRNA gene Strain NIES-1058, 16S ribosomal RNA gene, partial sequence
9 (EU94533)	AJ518316.1 AY371926.1	Unidentified bacterium Bacteroidetes bacterium	96% (24%) 91% (27%)	Clone Neu2P1-29, partial 16S rRNA gene JS5 16S ribosomal RNA gene, partial sequence
10 (EU94534)	AB193613.1 DQ648029.1 AJ635429.1	Uncultured Clostridiales bacterium *Microcystis viridis* *Microcystis aeruginosa*	89% (54%) 86% (54%) 86% (54%)	Clone RsC01-042, gene for 16S rRNA, partial sequence Strain NIES-1058, 16S ribosomal RNA gene, partial sequence Strain 1BB38S07, partial 16S rRNA gene
11 (EU94535)	DQ887510.1	*Microcystis aeruginosa*	84% (70%)	Strain FC-070, 16S ribosomal RNA gene, partial sequence
12 (EU94536)	AF139295.1 AB012331.1	*Microcystis aeruginosa* *Microcystis viridis*	95% (77%) 94% (77%)	Strain UWOCC 019, 16S ribosomal RNA gene, partial sequence Isolate TAC78, 16S rRNA gene, partial sequence
13 (EU94537)	AY887021.1	*Anabaena flos-aquae*	80% (26%)	Strain CCAP, 1403/13F 16S ribosomal RNA gene, partial sequence
14 (EU94538)	AJ853587.1	Uncultured bacterium	72% (86%)	Clone GZKB93, partial 16S rRNA gene,
15 (EU94539)	AF107335.1 DQ316386.1	Uncultured freshwater bacterium Uncultured Actinobacterium	71% (75%) 71% (75%)	LCK-79, 16S ribosomal RNA gene, partial sequence Clone STH5-5, 16S ribosomal RNA gene, partial sequence
16 (EU94540)	EF516194.1	Uncultured bacterium	94% (21%)	Clone FCPT473, 16S ribosomal RNA gene, complete sequence
17 (EU94541)	AM690823.1	Uncultured α-proteobacterium	88% (96%)	Clone TH1-19, partial 16S rRNA gene
18 (EU94542)	DQ887510.1 AB035553.1	*Microcystis aeruginosa* *Microcystis wesenbergii*	90% (100%) 89% (100%)	Strain FC-070, 16S ribosomal RNA gene, partial sequence Gene for 16S rRNA, partial sequence

4. Discussion

Both cyanobacterial primer combinations amplified diatom chloroplast 16S rDNA. Plastids are believed to be of origin early in the cyanobacterial evolutionary line (Nelissen *et al*., 1995). After what was probably a single primary endosymbiotic event, a nearly simultaneous radiation of the ancestors of recent cyanelles, rhodoplasts and chloroplasts occurred, and other plastids evolved from secondary endosymbioses (Bhattacharya & Medlin, 1995). The primers have one or more mismatches to a large amount of chloroplast sequences. For most mismatching sequences however, the mismatches are few and only rarely at the 3' end (Zwart *et al*., 2005). Therefore it can be expected that most chloroplast 16S rDNA sequences will be amplified. In the gel targeting filamentous cyanobacteria, the band corresponding to the chloroplast 16S rDNA of the diatom *Nitzschia frustulum* was only present until November, and in the unicellular specific gel, the diatom band (corresponding to the diatoms *Aulacoseira ambigua* and *Haslea wawrikae*) was only present until December, but was more dominant in the treated area from September. These results indicate that the diatoms were out-competed by the cyanobacteria in both the control and treated areas after December, despite the fact that the N:P ratio of the treated area was higher than that of the control area. However, because the diatoms were more prevalent in the treated area than the control area between September and December, it would appear that the lower phosphorus level did favour diatom growth.

The DGGE gel which targeted filamentous cyanobacteria also contained bands that closely matched *Microcystis* species, which was confirmed by their grouping in the phylogenetic tree. This was unexpected, as Boutte *et al*. (2006) tested 381 sequences from unicellular strains, and found that 92.6% matched with the primer CYA781R(b), but only 5.0% matched with primer CYA781R(a), none being *Microcystis* species. During October, the bands corresponding to *Microcystis* were dominant in the control area, whereas in the treated area they were very faint. It seems that the *Microcystis* bloom occurred earlier in the control area, which may have been a result of the low phosphorus concentration in the treated area. During January and February, bands corresponding to the filamentous cyanobacterial species were only present in the treated area. The low relative phosphorus concentration may have allowed for greater

cyanobacterial species diversity in the treated area by preventing dominance by one species, although it was not able to prevent the occurrence of the bloom.

The DGGE gel targeting unicellular cyanobacteria showed that the diversity of the control area in spring was comparable with that of the treated area in summer. This indicated that the reduced phosphorus in the water due to the Phoslock® treatment had an effect on the diversity of the treated area. The combination of CYA359F and CYA781R(b) primers only amplified unicellular cyanobacteria, which was expected. When the sequences were run on BLAST they were close matches to species of *Microcystis*, however it was not possible to identify the sequences up to species level. The *Microcystis* species represented by the bands near the top of the gel (13-15) were present in the treated and control areas for all the months sampled, but the four species represented by bands at the bottom of the gel (10-12 and 16) were only present until November. The sequences in these bands also grouped differently in the phylogenetic tree, indicating that they were not the same species. It is possible that, once bloom conditions were experienced, certain *Microcystis* species were able to out-compete others for dominance within the bloom.

Better species resolution (at or below species level) is possible through cyanobacterial specific amplification of other regions of the DNA apart from the 16S rDNA region. The *rpoC1* gene, which encodes the γ subunit of cyanobacterial RNA polymerase that is absent in other bacteria, has been used to analyse cyanobacterial phylogeny (Bergsland & Haselkorn, 1991) and community structure (Palenik, 1994) However, sequence data for this gene is limited (Nübel *et al*., 1997). DGGE of *hetR*, a gene involved in heterocyst differentiation, has been used to study isolated strains of the cyanobacterial genera *Trichodesmium* and *Nostoc* (Rasmussen & Svenning, 2001; Orcutt *et al*., 2002). *nifH*, a gene encoding nitrogenase reductase in many organisms including cyanobacteria was used by Lovell *et al*. (2001) in the DGGE analysis of nitrogen fixing cyanobacterial species. Phylogeny based on *nifH* is generally in agreement with the phylogeny inferred by 16S rRNA gene sequences (Ueda *et al*., 1995) and is currently one of the largest non-ribosomal datasets (Zehr *et al*., 2003). More recently, Roeselers, *et al*., (2007) used *nifD*, a gene encoding the dinitrogenase enzyme, as a phylogenetic marker, and found it to give more resolution than *nifH* among closely related diazotrophic cyanobacteria, although compared to *nifH* there are relatively few *nifD* sequences available for

phylogenetic analysis. An important drawback of these protein-encoding genes is that they are present in only a limited number of cyanobacterial genera. Janse *et al*. (2003) focused their research on the rRNA 16S to 23S internal transcribed spacer (rRNA-ITS), which allowed high-resolution discrimination of a variety of cyanobacteria, including *Microcystis* spp. The difference in resolution with 16S and ITS DGGE in *Microcystis* can be explained by the fact that the average sequence diversity of rRNA 16S is less than 1%, (Boyer *et al*., 2001), whereas that of rRNA-ITS is up to 7% (Otsuka *et al*., 1999). It may therefore be possible to gain more information on the specific *Microcystis* species in each sample by performing an rRNA-ITS DGGE.

Some of the apparently different *Microcystis* species in the two cyanobacterial DGGE gels may in fact be multiple bands of one species. Nikolausz *et al*. (2005) observed that dominant amplicons could be distributed at different positions in the same pattern. If several domains have similar melting properties, stochastic effects may cause one to denature before the other in a fraction of the amplicon population and could also explain the presence of different bands with the same sequence in one lane (Boutte *et al*., 2006). Thus, cyanobacterial sequences from bands 10, 11, 12 and 16 in the unicellular specific gel may in fact be one species of *Microcystis*, as they all group together in the phylogenetic tree.

The general bacterial 16S rDNA DGGE gel provided further information on the cyanobacterial species in the treated and control areas. Generally, the *Microcystis* species became dominant earlier in summer in the control area than in the treated area, and in some cases were absent from the treated area during January and February. Band 13 was a close match to the heterocystous filamentous cyanobacterium *Anabaena flos-aquae*, and was the only filamentous cyanobacterial species to be detected on the gel. Interestingly, it was not present on the filamentous specific cyanobacterial gel. This band was present in the treated and control areas until October, after which it was more dominant in the treated areas until February. It thus followed the same pattern as the filamentous cyanobacteria in the filamentous specific gel. The general bacterial DGGE gel therefore provided a confirmation of the information already gained from the cyanobacterial specific gels, but did not provide as much detail. The use of cyanobacterial specific primers prevents the amplification of the abundant DNA of non-cyanobacterial microbes in field samples. The resulting DGGE profiles are less complex

than those generated with general bacterial primers, making detection of cyanobacteria that are less abundant or have lower amplification efficiencies more feasible (Janse *et al*., 2003). This is clear when the DGGE gels in this study are compared, as the profile generated from general bacterial primers contained less bands corresponding to cyanobacteria than in the cyanobacterial specific gels for each sample, especially the filamentous species.

The bacterial species composition represented in the general DGGE gel appeared to be affected by the presence of the cyanobacteria in the water, or at least by the seasonal changes experienced in the water body which coincided with the increase in cyanobacterial growth. As the cyanobacteria became more dominant in the treated and control areas from October, there appeared to be a shift in the bacterioplankton population. Species of Actinobacteria and Bacteroidetes were present in both the treated and control areas only until October, with one species of Actinobacteria only being present in the treated area (represented by band 6). From November, the bacterioplankton population was dominated by β- and δ-proteobacteria. An α-proteobacteria, represented by band 2, was present in both areas throughout the months sampled. Van der Gught *et al*. (2005) investigated the bacterial community composition of four lakes with different nutrient loads (eutrophic and hypertrophic) and turbidity (turbid and clearwater). They found that in shollow eutrophic and hypertrophic lakes, the bacterioplankton was dominated by α- and β-proteobacteria, Bacteroidetes and Actinobacteria, with a low frequency of δ-proteobacteria. In the hypertrophic turbid Lake Blankaart, Actinobacteria were dominant, whereas in the eutrophic turbid Lake Visvijver, β-proteobacteria were dominant. In both clearwater lakes (one hypertrophic and one eutrophic) β-proteobacteria were dominant. The clearwater lakes had a higher percentage of Bacteroidetes, and the turbid lakes a higher percentage of cyanobacteria. This is in agreement with Zwart *et al*. (2002) who found a similar species composition in eutrophic water bodies. The results from this study agree in part with these findings. The treated and control areas revealed an almost identical species composition to those investigated by Van der Gught *et al*. (2005). The treated and control areas were both turbid from November, but were dominated by β- and δ-proteobacteria rather than Actinobacteria. In fact, the Actinobacteria species were not present after October in either the treated or control area, but the Bacteroidetes disappeared as expected as the water shifted from a clearwater to a turbid state. The β- and δ-proteobacteria present

appear to be tolerant to the turbid conditions resulting from a cyanobacterial bloom, and in fact may be species that associate with the bloom. The species composition of the bacterioplankton population therefore appeared to be affected more by the turbidity caused from the presence of cyanobacteria than from the nutrient composition of the water, as there was very little difference between the control and treated areas.

Although the Phoslock® treatment did appear to affect the cyanobacterial species composition in the treated area when compared to the control area, in both the treated and control areas the greatest effect on the cyanobacterial and bacterial populations seemed to be related to seasonal changes. The Phoslock® treatment did not prevent the development of an algal bloom, but this is likely due to the fact that a large amount of nutrient rich water flowed into the treated site at the start of the rainy season in October (Chapter 4).

5. Conclusion

It can be seen from the results that using cyanobacterial specific primers to analyse the cyanobacterial community composition by DGGE was necessary, as general bacterial primers did not give a detailed picture of the cyanobacterial species present in a sample. Using the 16S rRNA gene as a target was practical, as the database of these sequences is the largest. However, the resolution of certain species, the most notable of these being *Microcystis* spp., is low when this region is used. If resolution is required below species level for *Microcystis*, DGGE of the rRNA-ITS region should be considered.

The lower phosphorus concentration in the treated area encouraged the presence of diatoms, which are indicators of healthy species diversity. In terms of the cyanobacteria, the difference in trophic status between the treated and control areas had a greater effect on the filamentous cyanobacterial population, which were more prevalent in the treated area during the summer months than in the control area. The unicellular cyanobacteria were present in both areas, but there appeared to be a lag in the appearance of these species in the treated area. The Phoslock® treatment therefore appeared to affect the cyanobacterial species composition, resulting in an increase in diversity and a slower bloom time.

The bacterioplankton species in both the treated and control areas were similar to those found in other eutrophic and hypertrophic dams. The presence of cyanobacteria in the water appeared to cause a population shift in the bacterial population, which was most likely due to an increase in the turbidity of the water as the cyanobacterial bloom developed. The Phoslock® treatment did not appear to affect the bacterial population, as the treated and control areas displayed similar patterns.

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CHAPTER 6:

THE CONTROL OF TOXIC CYANOBACTERIAL BLOOMS USING BIOLOGICAL CONTROL IN THE FORM OF PREDATORY BACTERIA, ALONE AND IN COMBINATION WITH PHOSLOCK®

1. Introduction

There are currently various methods in use to treat cyanobacterial blooms and remove the toxins and undesirable compounds from the water. Chemicals that have been tested for use in the control of phytoplankton related problems in surface waters include copper sulphate, Reglone A, Simazine, alum and lime (Lam *et al.,* 1995) as well as algicides such as phenolic compounds, amide derivatives and quaternary ammonium compounds (Botha-Oberholster, 2004). These chemicals cause cell lysis, removing the algal bloom, but increasing the potential health risks by releasing toxins into the water (Lam *et al.,* 1995). Toxins persist for a minimum of 21 days, but may still be present for 2-3 months following treatment (Lam *et al.,* 1995; Botha-Oberholster, 2004). Alum and lime are exceptions, displaying cell coagulation and causing cells to flocculate, thus clearing the water without releasing toxins (Lam *et al.,* 1995). Other methods of water treatment to remove toxins include flocculation with aluminium sulphate, addition of powdered activated carbon, sedimentation, sand filtration and chlorination. Although these methods effectively remove cyanobacterial cells, they do not necessarily acceptably eliminate the toxins they produce (Hoeger *et al.,* 2004). Ozonation has been found to be very effective at removing microcystin (Rae *et al.,* 1999), as well as the odour causing compounds geosmin and 2-MIB. However, the cost implications of the high ozone doses that are required and the highly specialised mass transfer techniques that are needed (Strydom, 2004), make this a non-viable option.

There is therefore a need for an alternative method of cyanobacterial bloom control. Numerous studies have demonstrated that certain aquatic bacteria are capable of lysing cyanobacterial cells. *Bacillus cereus* produced a novel, non-proteinaceous substance which has high lytic activity against *Microcystis* (Nakamura *et al.,* 2003). Other previous studies have implicated both volatile and non-volatile compounds produced by *Bacillus* species in cyanobacterial growth inhibition and lysis, particularly *Anabaena* species (Reim *et al.,* 1974; Wright & Thompson, 1985). One such volatile substance, isoamyl alcohol, was thought to be a toxic metabolite of peptone degradation in some *Bacillus* species (Wright *et al.,* 1991). A Gram negative, rod shaped bacteria thought to be a new species related to *Xanthomonas* was isolated that lysed select cyanobacteria, including species of *Anabaena* and *Oscillatoria* (Walker & Higginbotham, 2000). Lytic gliding bacterial strains such as members of the *Myxobacteria* and *Cytophaga* strains

C1 and C2 showed host specific lysis that required direct contact with the host cell (Rashidan & Bird, 2001). The cyanobacterium *Phormidum luridum* was preyed upon by *Myxococcus* species, mainly *M. xanthus* and *M. fulvus.* These bacteria displayed entrapment capabilities causing clumping in cyanobacteria prior to lysis, and seemed to be independent of any other nutritional requirement (Burnam *et al.,* 1981; Burnam *et al.,* 1984). Bacteria displaying cyanobacteriolytic activity could potentially be used for biological control, as an alternative method to costly and environmentally damaging chemical treatments.

Phoslock® is a lanthanum modified bentonite clay that can reduce the dissolved phosphorus concentrations available to phytoplankton and limit their growth. It is effective over a wide range of pH and dissolved oxygen conditions, and is able to bind phosphorus under the same anoxic conditions experienced by many eutrophic waterways (Robb *et al*., 2003). It is applied as a slurry to the surface of the water body, and binds P as it settles. A reactive layer forms on bottom sediments to block phosphorus release from the sediment into the overlying water. Phoslock® acts fast enough to bind dissolved phosphorus as it settles through the water column. In a large-scale trial of Phoslock® in the Canning River in Australia, 95% of the filterable reactive phosphorus (FRP) was removed from the water. Phosphorus with the potential to become available over time, including the phosphorus bound to particles, contained in organic matter, and that already present in phytoplankton cells was also significantly reduced. Phoslock® is non-toxic and has no known negative environmental impacts, with no effect on macro-invertebrates, fresh-water shrimps or periphyton. Even with Phoslock® application there is still sufficient phosphorus for microbial communities to function in both water and sediment (Greenop & Robb, 2001). There is widespread support for the idea that phosphorus uptake in P-limited aquatic systems is dominated by bacteria because their uptake systems have higher affinities than those of algae (Coveny & Wetzel, 1992). This is of great importance, as Phoslock® can be used in conjunction with predatory bacteria to control the cyanobacterial blooms, and can be used as a vehicle to transport bacterial cells through the water column.

A species of bacteria was isolated from Hartbeespoort Dam which displayed cyanobacteriolytic activity towards *Microcystis aeruginosa.* This study aims to assess the predatory activity of this strain by determining the critical predator-prey ratio of

bacteria and *M. aeruginosa* as well as to evaluate whether the bacteria can use the algae as their sole nutrient source. The potential use of Phoslock® as a biological control aid was examined by determining its effect on bacterial growth, and whether combining bacteria and Phoslock® together had a synergistic effect on the cyanobacteria.

2. Materials and Methods

2.1. Culture of bacterial strains

Bacterial cultures used in this study were isolated previously from water samples taken from the Hartbeespoort Dam and have cyanobacteriolytic activity. All cultures were grown on nutrient agar plates or in nutrient broth at 28°C for 18-24h unless otherwise mentioned.

2.2. Host cyanobacteria and cultivation

An axenic culture of *Microcystis aeruginosa* PCC7806 was obtained from the Department of Biochemistry and Microbiology, University of Port Elizabeth to use as a representative of the Hartbeespoort Dam cyanobacterial population. The cyanobacterium was grown in modified Allen's BG-11 medium (Table 1), (Krüger & Eloff, 1977). The cultures were grown in 250ml cotton plugged sterile Erlenmeyer flasks at ambient temperatures (24-26°C) with shaking to allow for aeration. Continuous lighting of 2000lux (Extech instruments Datalogging lightmeter model 401036) was provided by 18W cool white fluorescent lamps (Lohuis FT 18W/T8 1200LM) suspended above the flasks.

Table 1: Mineral composition of modified BG-11 medium

Component	Concentration
$NaNO_3$	1.500g.l^{-1}
K_2HPO_4	0.040g.l^{-1}
$MgSO_4.7H_2O$	0.075g.l^{-1}
$CaCl_2.2H_2O$	0.036g.l^{-1}
Na_2CO_3	0.020g.L^{-1}
$FeSO_4$	0.006g.L^{-1}
$EDTA.Na_2H_2O$	0.001g.L^{-1}
Citric acid	0.0112g.L^{-1}
Trace metal solution (Table 1.1)	1ml.l^{-1}

Table 1.1: Trace metal solution for modified BG-11 media

Trace metal solution component	Concentration (g.l^{-1})
H_3BO_3	2.8600
$MnCl_2.4H_2O$	1.8100
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.5H_2O$	0.300
$CO(NO_3)_2.H_2O$	0.0494
$CuSO_4.5H_2O$	0.0790

2.3. Bacterial characterisation and identification

Gram stains were performed on the unknown bacterial culture after overnight cultivation on nutrient agar. This was followed by microscopic observation using a Nikon optiphot microscope with standard bright field 40X and 100X objectives to determine Gram staining ability as well as cell morphology. Pictures were taken with a Nikon digital camera DMX1200 using an oil immersion.

Hugh-Liefson's oxidation/fermentation test as well as catalase and oxidase tests were performed on the culture to gain information on its biochemical metabolism. Glucose was added to melted Hugh-Liefson's media as the carbon source, and the media was allowed to solidify. Two tubes were inoculated with each culture respectively, and a thin layer of sterile paraffin added to one tube of each culture to create an anaerobic environment. The tubes were incubated at 37°C for two days. The catalase test was performed by placing a drop of H_2O_2 on a microscope slide. A single bacterial colony was placed into the H_2O_2. The oxidase test was performed in a similar manner, but with tetramethyl-*p*-phenylenediamine (Wuster's blue).

In order to identify the unknown bacteria, sequencing of the 16S ribosomal rRNA gene was performed. A portion of the 16S rDNA operon was amplified by means of colony PCR using the following primers:

PRUN518r: 5'ATT ACC GCG GCT GCT GG3' (Muyzer *et al*., 1993)

pA8f-GC: 5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC CTG GCT CAG3' (Fjellbirkeland *et al.* 2001)

pA8f-GC was designed specifically for DGGE and thus a GC clamp is included in the 5' end. A reaction with no template DNA was included as a negative control. 0.5µl 10^{-1} bacterial suspension was added to 24.5µl of amplification mixture containing 18.7µl sterile distilled MilliQ water, 2.5µl PCR buffer with $MgCl_2$ (10x), 2µl dNTPs (2.5µM), 0.5µl PRUN518r (50µM), 0.5µl pA8f-GC (50µM), 0.3µl Taq DNA polymerase (Promega) (5U.μl^{-1}) to give a final volume of 25µl.

DNA amplification was performed in a PCR thermal cycler (Biorad) using the following program: 10min at 95°C, 35 cycles of 30s at 94°C, 30s at 51°C and 1min at 72°C, followed by 10min at 72°C then held at 4°C. The PCR product was analysed on a 1% TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3)) agarose gel.

The PCR product was cleaned by transferring the entire volume to a 0.5ml Eppendorf tube, adding 2µl of 3M sodium acetate (pH 4.6) and 50µl 95% ethanol, and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000rpm for 30min. The

ethanol solution was removed, the pellet rinsed in 150µl 70% ethanol and centrifuged at 10 000rpm for 5min. The ethanol was aspirated and the pellet dried under vacuum for approximately 10min. The pellet was then re-suspended in 20µl sterile water. Each amplified PCR was then sequenced in an Eppendorf tube containing 1µl clean PCR product, 2µl Big Dye sequencing mix (Roche), 0.32µl primer and 1.68µl deionised filter-sterilised water. Partial sequences of the 16S eubacterial gene of the rDNA were obtained using the K primer above, and nucleotide sequence order was confirmed by comparing it to the sequence obtained when using the M primer. Sequence PCR products were cleaned in the same manner as the amplification PCR, except that 15µl of sterile water was added to the PCR before transferring it to a 0.5ml tube, and the dried pellet obtained at the end was not re-suspended in water. Tubes were transferred on ice to the sequencer, and DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase Applied Biosystems, UK).

The sequence was subjected to a BLAST analysis on the GenBank database, and by determining the sequences with the highest percentage match and coverage, tentative species identification was possible.

2.4. Critical predator-prey ratio

1200ml of BG-11 in a 2L Erlenmeyer flask was inoculated with 10ml of an established *M. aeruginosa* PCC7806 culture and grown for 14d with shaking to prevent adherence to the flask and formation of colonies. After 14d, 200ml volumes were transferred to 6 x 500ml Erlenmeyer flasks, resulting in uniform algal growth in all flasks. Cyanobacterial cell count after 14d growth was determined microscopically using the 10x objective and a Petroff-Hausser counting chamber according to an established method (Burnam *et al.*, 1973). 10µl of the cyanobacterial culture was placed directly in the 0.02mm deep counting chamber with improved Neubauer ruling. Counts were performed in duplicate. Original *M. aeruginosa* cell count was found to be 2.09 x 10^7 cells.ml^{-1}.

For each group squares in the chamber, the total number of cells present is given by Equation 1:

$$xy/v \text{ cells.ml}^{-1} \quad \ldots\ldots. (1)$$

where

x is the number of cells counted per 16 small squares

y is the dilution used (1 in this case as dilution was unnecessary)

1/v is the reciprocal of the chamber volume, 1.25×10^6

(http://whitewolf.newcastle.edu.au/techinfo/proc_bacto_counts.html)

The bacterial culture was grown for 12h on nutrient agar, and the bacterial colonies washed off the plate with sterile Ringers into a sterile test tube. This suspension was made up to a 10ml volume, and a serial dilution performed with Ringers to determine the cell count in colony forming units (cfu) in the original tube by plating 100µl of each dilution onto nutrient agar plates. A count of 5.1×10^9 cfu.ml^{-1} was observed. Serial dilutions from 10^{-1} to 10^{-4} were then made with the bacterial culture and 10ml of each dilution added to the cyanobacterial culture flasks, leaving one untreated as a control. This resulted in a 1:1, 1:10, 1:100, 1:1000 and 1:10 000 predator-prey ratios, as adding 10ml of the bacteria to 200ml algal culture diluted the bacteria a further 200x resulting in 2.5×10^7 cells.ml^{-1} in the 1:1 flask, which closely matched the algal count. Cyanobacterial cell counts were performed as described after 24, 48 and 72h followed by counts every three days up to 15d. Flasks were shaken before counting, and all counts were performed in duplicate. Because no bacterial nutrient source was provided, this test also helped determine whether the bacteria can use the algae as their sole nutrient source. 100µl samples were taken from the flasks at 3, 6 and 12d to determine the bacterial viability by performing ten-fold serial dilutions in 900µl Ringers, plating on nutrient agar and counting cfu.ml^{-1} after overnight incubation at 28°C. All bacterial plate counts were performed in duplicate.

2.5. Collection, treatment and processing of environmental samples

For use in the Phoslock® trials, water samples were taken directly from the Hartbeespoort Dam. Sterile 1 and 2L Schott bottles were used, and samples were taken at an approximate depth of 15cm to ensure a high algal sample density. Samples were taken at three points, namely near the dam wall, off-shore of the Kosmos boat launching

site and a sample at the shore line. Samples were immediately put on ice to slow any bacterial growth that may result in changes in pH and nutrient composition. Bottle screw caps were tightened to minimize aerobic bacterial growth. Samples were stored over-night at 4°C before processing.

Phosphorus concentration of the water samples were measured with Spectroquant Phosphortest (PMB) 1.14848.001 (Merck), according to the manufacturer's instructions, using the Photometer SQ118. pH levels of the water samples were measured with a Beckman Φ34 pH meter.

2.6. Effects of Phoslock® on bacterial growth

5ml of nutrient broth was inoculated with the bacterial culture and grown overnight at 37°C with shaking. 150ml of nutrient broth was added to 2 x 250ml Erlenmeyer flasks and 1ml of the overnight culture added to each flask. 1.5g of Phoslock® was added to one flask, and the second left untreated as a control. These flasks were shake-incubated at 37°C. 1ml samples were taken from the flasks after 6 and 12h to determine the cell counts by serial dilution. Plating out was performed in duplicate.

2.7. Combined Phoslock® and bacteria treatment

Using the same method as that used to determine the critical predator-prey ratio, *Microcystis aeruginosa* PCC7806 was cultured in 800ml BG-11 media, and 200ml transferred to each of four 500ml Erlenmeyer flasks, resulting in uniform algal growth in all the flasks. Cyanobacterial cell count after 14d growth was determined microscopically using the 10X objective and a counting chamber according to an established method (Burnam *et al.*, 1973). A different counting chamber was used for this experiment to that of the predator-prey experiment, with a depth of 0.1mm, and Neubauer improved ruling (Marienfield). The following formula (Equation 2) was used to calculate the cyanobacterial cell concentration (www.superior.de):

$$\frac{\text{Number of cells}}{\text{Counted area (mm}^2\text{) x chamber depth (mm) x dilution}} = \text{cells.}\mu\text{l}^{-1} \quad ..(2)$$

The initial cyanobacterial cell count in each of the flasks was 1.08 x 10^5 cells.ml^{-1}. The bacteria was grown on nutrient agar plates as described previously for the predator-prey experiment. In this case, the bacterial colonies were washed off the plates using sterile ringers, but the resulting solution was not diluted, in order to give a higher concentration of bacteria. The original cell count of the bacterial solution was 2.73 x 10^9 cfu.ml^{-1} when a serial dilution was plated on nutrient agar. To the first flask containing 200ml cyanobacteria culture, 1g of Phoslock® was added to give a 1% (w/v) solution. To the second flask, 10ml of the bacterial solution was added, resulting in a bacterial concentration of 1.3 x 10^8 cells.ml^{-1}, which was approximately 1000 times the amount of cyanobacteria. 1g of Phoslock® and 10ml of bacterial solution were added to the third flask. The final flask was left untreated to act as a control. Cyanobacterial cell counts were performed every three days, and bacterial cell counts were determined every seven days by performing serial dilutions as described previously. All experiments and counts were performed in duplicate.

3. Results

3.1. Bacterial identification

The unknown culture was found to be a Gram negative rod following Gram staining and microscopic analysis (Figure 1). The Gram positive culture sporulated after 6h incubation, so Gram staining following 5h incubation was performed; once sporulation began the cells appeared to stain Gram negative. The Gram positive culture showed no colour change in either the aerobic or anaerobic Hugh-Liefson test when an overnight culture was used as inoculum. The test was repeated using 5h old cultures to avoid the effect of sporulation. The tube without paraffin turned yellow after 2d incubation indicating that the organism is oxidative. The culture was also found to be oxidase and catalase positive. As *Bacillus* is the only genus of Gram positive rods to be catalase positive (Cullimore, 2000), the unknown species appeared to be a *Bacillus* sp. The sequence is presented in Appendix A. When a BLAST analysis was performed, the unknown bacterial sequence had a 100% match to *Bacillus cereus* (Table 2).

Table 2: BLAST results for the sequence of the unknown bacteria, showing the species with the highest percentage match and coverage

Matching GenBank accession number	Species	Percentage match (Query coverage)	Description
AY826631.1	*Bacillus cereus*	100% (99%)	Isolate 4.5 MW-5 16S rRNA gene, partial sequence
AY425946.1	*Bacillus cereus*	100% (99%)	Strain BGSC 6A5 *rrnM* operon, complete sequence
AE016877.1	*Bacillus cereus*	100% (99%)	ATCC 14579, complete genome

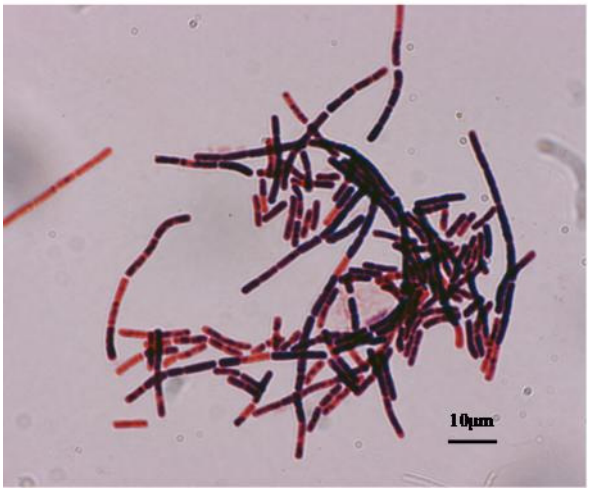

Figure 1: Gram stain of the Gram positive rod at 1000X magnification.

3.2. Critical predator-prey ratio

The cyanobacterial cell counts (Figure 2), indicated that a predator-prey ratio of 1:1 caused a decrease in algal growth by almost 50% by day 12 from 2.09 x10^7 cells.ml^{-1} to 1.25 x 10^7 cells.ml^{-1}. 1:10 and 1:100 ratios showed steady cyanobacterial populations, where 1:1000 and 1:10000 ratios showed an increase in growth of *M. aeruginosa* up to day 12, as did the control. After 12 days, the cell numbers decreased in all cultures indicating that the nutrients were depleted from the BG-11 media. The critical predator-prey ratio was therefore 1:1. For the first 3 days, counts were performed daily and no change was observed in the cell numbers of treated flasks, but when counts were

performed only every 3 days after that, more dramatic results were seen. This indicates that it may be necessary for the bacteria to be in contact with the cyanobacteria.

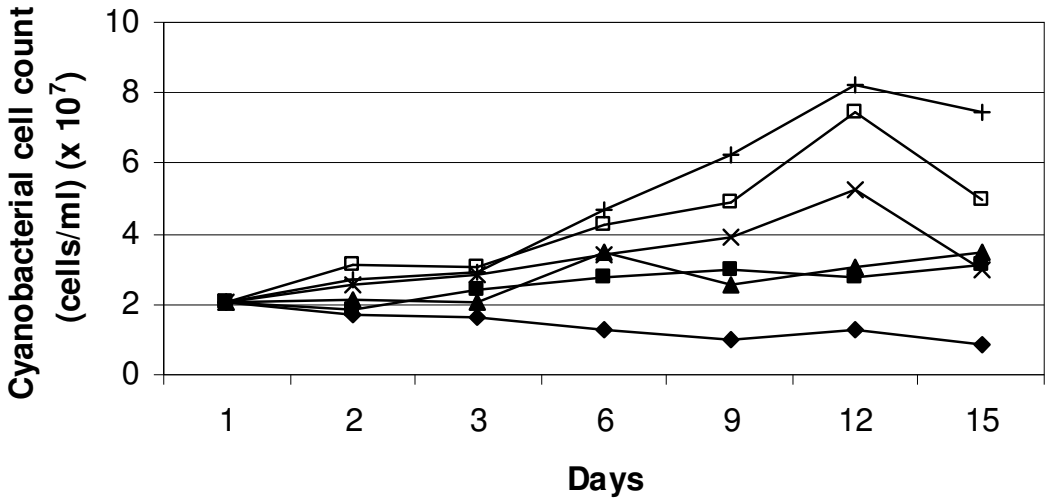

Figure 2: Effect of different predator-prey ratios on the growth of *Microcystis aeruginosa* (♦) 1:1 (▲) 1:10 (■) 1:100 (×) 1:1000 (□) 1:10 000 (+) Control

Bacterial cell numbers more than doubled in the 1:1 ratio flask, stayed approximately constant in the 1:10 ratio flask, and decreased in the other flasks indicating once again that a 1:1 predator-prey ratio is required (Table 3). These results reinforce the fact that contact is needed between the bacteria and algae, as bacterial numbers only started increasing in the 1:1 flask after 3d. The fact that bacterial numbers increased while cyanobacterial numbers decreased indicates that the bacteria were able to use the algae as their only nutrient source, as no bacterial nutrients were added to the medium.

It was observed in the 1:1 and 1:10 flasks that no algae adhered to the flask bottom, whereas adherence was apparent in the other flasks, especially the control. Colony formation also appeared to be reduced in these flasks when compared with the control (Figure 3), indicating that the presence of the predatory bacteria may prevent *M. aeruginosa* cell aggregation and may affect attachment capabilities.

Table 3: Bacterial cell counts taken at days 1, 3, 6 and 12

Ratio	Original Count (cfu/ml)	Count at Day 3 (cfu/ml)	Count at Day 6 (cfu/ml)	Count at Day 12 (cfu/ml)
1:1	2.5×10^7	2.2×10^7	4.8×10^7	5.2×10^7
1:10	2.5×10^6	2.1×10^6	5.2×10^6	4.8×10^6
1:100	2.5×10^5	5.8×10^5	1.39×10^5	2.1×10^5
1:1000	2.5×10^4	2.0×10^4	3.0×10^3	2.8×10^3
1:10000	2.5×10^3	2.4×10^3	1.8×10^3	1.2×10^3

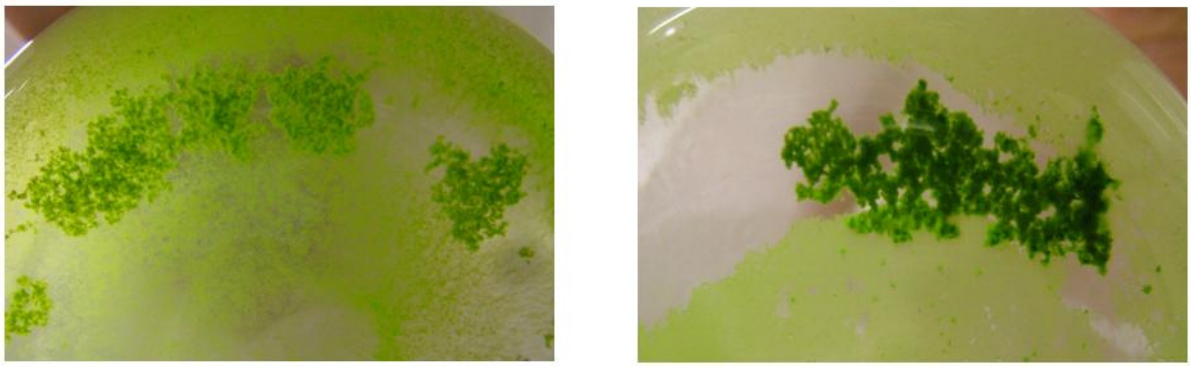

Figure 3: Colony formation in flask treated with bacteria in a 1:1 ratio of bacteria to algae (left) and control (right)

3.3. Effects of Phoslock® on bacterial growth

Bacillus cereus cell counts in the untreated (control) and Phoslock® treated flasks showed slower growth in the Phoslock® treated flask after 6h when compared with the control. However, after 12h the cell count was nearly identical in the two flasks (Table 5). This indicated that Phoslock® did not affect the growth potential of the bacteria, although the initial growth rate was lower.

Table 5: Bacterial cell counts in untreated and Phoslock® treated cultures

Incubation time (h)	Control (cfu/ml)	Phoslock® Treated (cfu/ml)
6	2.30×10^8	6.10×10^7
24	3.30×10^8	3.24×10^8

3.4. Combined Phoslock® and bacteria treatment

The cyanobacterial numbers in the control increased steadily over a 14d period, and then decreased in the final seven days, perhaps due to the depletion of nutrients (Figure 7). Numbers of cyanobacteria decreased 2.2-fold after 14d when treated with the bacteria, and 3.5-fold when treated with 0.5% (w/v) Phoslock®. The combination of Phoslock® and bacteria showed the same reduction in cyanobacterial numbers as the bacterial treatment alone. There was therefore no synergistic effect observed when these treatments were combined. Bacterial cell numbers doubled in the bacteria treated flask, and increased to nearly four times their original amount in the flask treated with Phoslock® and bacteria (Table 6).

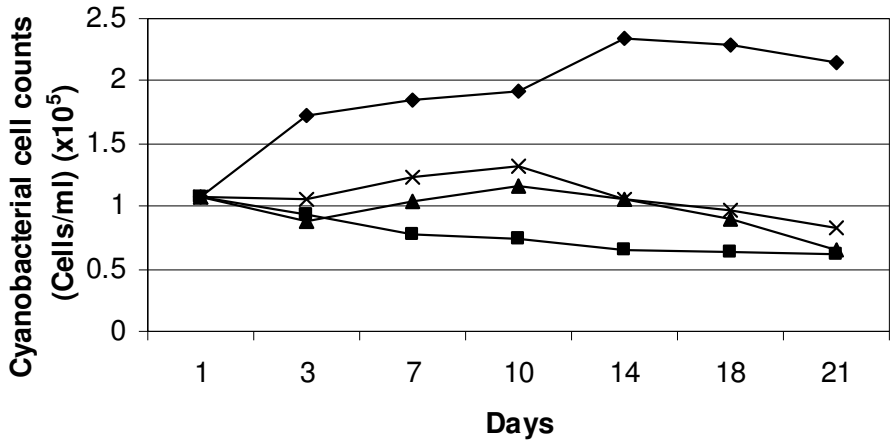

Figure 7: Effects of treatment with bacteria and Phoslock® on *M. aeruginosa* cell numbers (♦) Control (×) 0.5% Phoslock® (w/v) and bacteria (▲) Bacteria (1000:1) (■) 0.5% Phoslock® (w/v)

Table 6: Bacterial cell counts in bacteria treated flask and flask treated with both Phoslock® and bacteria:

	Bacterial cell count (cfu/ml)	
	Bacteria treated	**0.5% Phoslock® + bacteria**
Day 1	1.01×10^8	1.03×10^8
Day 7	1.72×10^8	2.67×10^8
Day 14	1.83×10^8	3.2×10^8
Day 21	2.18×10^8	4.1×10^8

4. Discussion

Bacillus cereus has previously been documented to have cyanobacteriolytic activity. Nakamura *et al*. (2003) found that *B. cereus* had a high degree of lytic activity towards *Microcystis aeruginosa,* and the substance responsible for the lytic activity, produced in the stationary phase of growth, was non-proteinaceous, hydrophilic and heat stable, with a molecular weight less than 2kDa. The bacteria attached to the surface of the cyanobacteria to first cause aggregation of cyanobacterial cells before lysis with extracellular products. Shunyu *et al*. (2006) isolated a strain of *Bacillus cereus* from Lake Dianchi, China, which was capable of rapidly lysing the bloom-forming cyanobacterium *Aphanizomenon flos-aquae* through cell-to-cell contact, and also showed lytic activity towards *Microcystis aeruginosa*. Other *Bacillus* species, namely *Bacillus pumilis, B. megaterium, B. subtilis* and *B. licheniformes* also produced cyanobacteriolytic volatile substances (Wright *et al.*, 1991; Wright & Thompson, 1985). Reim *et al*. (1974) showed that *B. brevis* displayed cyanobacteriolytic behaviour in its stationary phase of growth, with the production of a non-volatile lytic substance coinciding with sporulation. The bacteria used in this study required contact for lysis, as with *B. cereus* in the study performed by Nakamura *et al*. (2003), but aggregation of the cyanobacteria was reduced in treated flasks. This may indicate that the strains are different, with the lytic substance and mechanism of lysis differing between these two organisms.

The critical predator-prey ratio was 1:1, as lower ratios of bacteria to *M. aeruginosa* did not cause the cyanobacterial population to decrease, although ratios of 1:10 and 1:100 kept the cyanobacterial population steady. An initial 1:1 ratio was only capable of reducing the population by 50% over a 14 day period, even though the bacterial

population was seen to double in this time. It may therefore be beneficial to increase the initial bacterial numbers to induce higher rates of lysis. The fact that the bacteria were able to lyse the algal cells and increase in number in the absence of other nutrient sources indicated that *Bacillus cereus* can utilize *Microcystis aeruginosa* as its sole nutrient source. This is of great importance in terms of the formation of a biological control product, as no addition nutrients will need to be supplied to the bacteria.

Phoslock® had no effect on the final cell count of bacteria cultured for 12 hours when compared with the control, reinforcing the potential use of a combination of these two agents to create a novel biological control product. However, when the two agents were combined to assess the possibility of synergism, treatment with both Phoslock® and bacteria was no more effective than bacteria alone, and Phoslock® alone was more effective than either treatment with bacteria or with a combination of Phoslock® and bacteria. There is therefore no synergistic effect when these agents are used in combination, and Phoslock® is the most effective treatment method. The fact that the bacterial numbers increased to four times their original number in the combination treatment, compared with only a doubling in number in the bacteria treated flask, may be due to the increased surface area for growth provided by the Phoslock®. The 2.2-fold reduction in cyanobacterial numbers observed with a 1000:1 ratio of bacteria to cyanobacteria confirms the earlier assumption that an increase in the predator-prey ratio from the critical ratio of 1:1 will increase the degree of cyanobacterial cell lysis.

5. Conclusion

Bacillus cereus had previously been documented in laboratory studies as an effective control agent against *Microcystis aeruginosa*. As with most other bacteria that have shown predatory activity towards cyanobacteria, there have not been many field scale trials to determine the effectiveness of this organism on a large scale, and laboratory tests cannot simply be extrapolated, especially because the predator-prey ratio appears to be important. The undertaking of field trials is therefore essential to determine the success of this organism as a biological control agent. Phoslock® could possibly provide a vehicle for a biological control agent, as it does not affect the growth of the bacteria, and in fact promotes growth by providing a surface area for attachment.

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CHAPTER 7:

THE PHYSICAL AND CHEMICAL CHARACTERISATION OF FLY ASH

1. Introduction

Fly ash is a waste material predominantly produced during the combustion of coal in the process of electricity generation. Fly ash can be described as a crystalline skeleton, for example quartz and mullite, enshrouded in a glass phase of varying composition (Kruger, 1996). The composition of a specific fly ash depends on the geological age and composition of the coal used, which in turn is dependant on the geology of the environment surrounding the coal deposits. As the mineral matter in coal is passed through the combustion process, the mineral phases undergo thermal alteration into different forms, many of which are chemically reactive or which can be chemically activated. The resultant physical properties of ash, such as moisture content, particle mass, glass composition, and the portion of unburned carbon, will depend on the combustion temperature at which the coal was fired, the air:fuel ratio, coal pulverization size and the rate of combustion (Scheetz & Earle, 1998).

On a macro scale, fly ash appears homogeneous, but microscopically the individual particles vary in size, morphology, mineralogy and chemical composition (Kruger, 1996). The surface of fly ash is highly porous, and the particle size is the most important characteristic in terms of reactivity. Smaller fly ash particles tend to be more reactive as they have a larger surface area, and small particles cool down faster after exiting the combustor, resulting in a more disordered structure (Iyer & Scott, 2001). The primary components of power station fly ash are SiO_2, AlO_3 and Fe_2O_3, with varying amounts of carbon, calcium (as lime or gypsum) magnesium and sulphur. The type of coal burned affects the percentage composition of each of these major components. Generally, fly ash can be categorised into one of two major groups: Type F and Type C. Type F is produced when anthracite, bituminous or sub-bituminous coal is burned, and is low in lime. Type C comes from lignite coal and contains more lime. The amounts of the major oxides of Type C and Type F ash are presented in Table 1 (Iyer, 2002).

Table 1: The average composition of the major oxides found in Type F and Type C fly ash

Oxide (%)	Type F	Type C
SiO_2	49.90	53.79
AlO_3	16.25	16.42
Fe_2O_3	22.31	5.00
TiO_2	1.09	1.55
CaO (Lime)	4.48	18.00

Fly ash has a characteristic microscopic structure that consists mainly of small hollow spherical particles, known as cenospheres. Fisher *et al*. (1976) examined the structure of fly ash using scanning electron microscopy, and found that the cenospheres contained smaller spheres (named plerospheres), which were themselves packed with spheres 1µm in diameter or less. Microcrystals were also present in some samples. It was suggested that the spherical nature of the cenospheres was a result of pressure and surface tension on the molten inorganic particle as it is forced upwards against gravity and cooled rapidly.

Besides the major constituents mentioned above, fly ash may contain As, B, Be, Ca, Cd, Cr, Fe, Hg, Mg, Mo, Na, Ni, Pb, Ra, Se, Th, U, V, and Zn either on the surfaces of the ash particles and/or in the aluminosilicate matrix, and these can be leached from fly ash depending on the conditions (Iyer, 2002; Ram, 2007). A low pH facilitates the leaching of metals (Wang *et al*., 2006). The fly ash itself also influences the pH of a solution, the final pH of the leachate being mainly dependent on the concentration of calcium leached from the fly ash (Iwashita *et al*., 2005). Toxic elements in the leachate like Pb, Cd, Cr, As and Hg may have detrimental effects on human health and aquatic life (Ram *et al*., 2007).

The aims of this study were to examine the physical and chemical structures of seven different fly ash samples, and to determine the effect of these ashes on the pH of water as well as the leaching characteristics of the ashes, in both distilled water at pH 7 and in acidified distilled water. From the leaching data, the potential effects of fly ash on the water quality of aquatic ecosystems were assessed.

2. Materials and Methods

2.1. Fly ash samples

The fly ash used in this study was provided by Eskom. Six samples from different power stations using coal from different mines (Table 2), were evaluated in terms of their chemical and physical properties.

Table 2: Fly ash samples

Sample Number	Power station	Coal Mine
1	Thutuka	Newdenmark
2	Arnot	Arnot Coal
3	Duvha	Middelburg mine BHP Biliton
4	Hendrina	Optimum
5	Kendal	Khutala
6	Matla	Matla Coal
7	Lethabo	Newvaal

2.2. X-ray diffraction (XRD)

After addition of 20% Si (Aldrich 99% pure) for determination of amorphous content and milling in a McCrone micronizing mill, the 7 fly ash samples were prepared for XRD analysis using a back loading preparation method. They were analysed using a PANalytical X'Pert Pro powder diffractometer with X'Celerator detector and variable divergence- and receiving slits with Fe filtered Co-Kα radiation. The phases were identified using X'Pert Highscore plus software. The relative phase amounts (weight %) were estimated using the Rietveld method (Autoquan Program).

2.3. X-ray flourescence (XRF)

The fly ash samples were ground to <75m in a Tungsten Carbide milling vessel, roasted at 1000ºC to determine Loss On Ignition value and after adding 1g sample to 9g $Li_2B_4O_7$ fused into a glass bead. Major element analyses were executed on the fused bead using the ARL9400XP+ spectrometer. Another aliquot of the sample was pressed in a powder briquette for trace element analyses.

2.4. The effect of fly ash on the pH of water

A 1% (wt/vol) concentration of each fly ash sample (1-7) was added to distilled water with an initial pH of 6.1. The solutions were stirred continuously for 6h using a magnetic stirrer, after which time the pH was measured. The same amount of each fly ash was then added to water taken from the Hartbeespoort Dam with an initial pH of 7.16, and once again stirred continuously for 6h before measuring the pH.

2.5. Leaching of fly ash

In order to determine the leaching characteristics of the various fly ash samples, samples were leached in distilled water at an initial pH of 7, as well as in distilled water acidified with H_2SO_4 to an initial pH of 2. 50g of each fly ash sample was added to 1000ml water at pH 7 and pH 2 respectively, and stirred continuously using a magnetic stirrer for 24h. For the ash leached in acidified water the pH was readjusted to 2 after 1h. The mixtures were filtered through 0.45µm pore membranes and the supernatant submitted to Waterlab (PTY) LTD, Persequor Park, Pretoria for cation analysis by ICP-MS.

2.6. Scanning electron microscopy (SEM) of fly ash samples

In order to determine differences in size and/or structure of the different fly ash samples, the samples were mounted, coated with gold and viewed under a scanning electron microscope (JOEL JSM-840 SEM). Pictures of each fly ash sample were taken at 250x, 1000x, 2500x and 10 000x magnification.

2.7. Particle sizing

Partical sizing of each fly ash sample was performed on the Malverne Mastersizer 2000. Fly ash was mixed to a paste using distilled water before adding it slowly to a beaker of distilled water until the obscuration was in range. Samples were treated with ultrasound during particle size measurement to break up any particle clusters that may lead to an over-estimation of the average particle size, or a skewed particle size distribution.

3. Results

3.1. X-ray diffraction (XRD)

The XRD results are presented in Table 3. The ash samples were all low in lime, and could thus be classified as Type F ashes (Iyer, 2002). Mullite ($Al_6Si_2O_{13}$) and quartz (SiO_2) comprised the largest weight percentage in all the ash samples.

3.2. X-ray flourescence (XRF)

The XRF results for the major elements are presented in Table 4, and the trace elements in Table 5. SiO_2, Al_2O_3, Fe_2O_3, CaO and MgO were the major molecules present in all the fly ash samples, with the other major elements all comprising less than 1% of the mass. There were no striking differences between the major element compositions of the different ashes; all had a SiO_2 content between 50 and 55%, and an Al_2O_3 content between 24 and 31%. The CaO content was also similar (4-7%), with the exception of Sample 3 (Duvha) which was below 4%.

With the exception of As, Mo, Nb, U, W, Cl, Sc, and Cs, all the other trace elements tested were above 50ppm in the ash samples tested. Sr, S and Ba had the highest concentration (above 1000ppm) in all the ashes.

Table 3: XRD Results (weight %)

	Thutuka	Arnot	Duvha	Hendrina	Kendal	Matla	Lethabo
Amorphous	58.22 ± 0.78	62.92 ± 0.69	48.4 ± 0.78	46.59 ± 0.93	56.44 ± 0.72	64.99 ± 0.78	64.99 ± 0.57
Lime (CaO)	0.77 ± 0.08	0.43 ± 0.08	0.2 ± 0.07	0.47 ± 0.08	0.3 ± 0.07	1.14 ± 0.1	0.05 ± 0.03
Hematite (Fe_2O_3)	1.2 ± 0.15	0.7 ± 0.14	0.88 ± 0.14	0.99 ± 0.16	0.69 ± 0.14	0.94 ± 0.15	0.26 ± 0.11
Magnetite (Fe_3O_4)	2.13 ± 0.14	2.07 ± 0.12	1.8 4 ± 0.12	2.44 ± 0.14	1.39 ± 0.11	1.25 ± 0.13	0.44 ± 0.09
Mullite ($Al_6Si_2O_{13}$)	22.28 ± 0.66	20.89 ± 0.57	32.09 ± 0.72	27.41 ± 0.78	30.01 ± 0.63	25.5 ± 0.69	25.22 ± 0.51
Pyrite (FeS_2)	0	0	0.14 ± 0.1	0.03 ± 0.08	0.04 ± 0.09	0	0
Quartz (SiO_2)	14.99 ± 0.36	12.52 ± 0.33	15.89 ± 0.36	21.56 ± 0.39	10.84 ± 0.33	6.06 ± 0.36	8.64 ± 0.28
Sillimanite (Al_2SiO_5)	0.41 ± 0.2	0.47± 0.18	0.57 ± 0.33	0.51 ± 0.2	0.3 ± 0.19	0.12 ± 0.19	0.41 ± 0.18

Concentration reported as mean ± SD (σ =3)

Table 4: XRF Results for major elements (weight %)

	Sample 1 Thutuka	Sample 2 Arnot	Sample 3 Duvha	Sample 4 Hendrina	Sample 5 Kendal	Sample 6 Matla	Sample 7 Lethabo
SiO_2	54.60	51.47	52.19	54.20	53.13	51.78	55.55
TiO_2	1.52	1.47	1.69	1.33	1.60	1.91	1.55
Al_2O_3	26.98	24.94	27.72	24.42	31.17	30.48	30.38
Fe_2O_3	5.15	4.49	4.05	4.65	3.80	3.28	3.75
MnO	0.05	0.06	0.03	0.04	0.03	0.03	0.03
MgO	1.80	1.90	0.90	1.46	1.63	1.93	1.11
CaO	6.88	6.58	3.41	5.04	5.15	6.90	4.37
Na_2O	0.42	0.08	0.04	0.08	0.17	0.42	0.21
K_2O	0.82	0.59	0.61	0.66	0.81	0.74	0.70
P_2O_5	0.46	0.41	0.66	0.51	0.67	1.15	0.46
Cr_2O_3	0.03	0.03	0.02	0.04	0.02	0.03	0.03
NiO	0.01	0.01	0.01	0.02	0.01	0.01	0.01
V_2O_5	0.03	0.02	0.02	0.02	0.02	0.03	0.03
ZrO_2	0.04	0.05	0.06	0.05	0.05	0.02	0.05
LOI	1.04	5.96	7.76	6.02	1.20	1.43	0.63
TOTAL	99.83	98.07	99.17	98.54	99.48	100.15	98.86

Table 5: XRF Results for trace elements (ppm)

	Thutuka	Arnot	Duvha	Hendrina	Kendal	Matla	Lethabo
As	3	3	17	3	3	4	7
Cu	51	47	52	46	47	62	54
Ga	56	43	53	36	47	78	48
Mo	8	6	9	6	6	12	4
Nb	36	35	40	29	41	48	36
Ni	82	72	80	66	47	57	62
Pb	62	65	96	56	48	95	72
Rb	46	36	43	39	55	58	43
Sr	1988	1215	1038	1239	1615	2450	983
Th	52	51	58	45	53	66	44
U	26	19	18	15	22	31	15
W*	16	13	16	14	14	14	10
Y	98	80	84	80	81	91	71
Zn	50	58	74	101	35	65	58
Zr	385	421	477	392	434	467	403
Cl*	8	8	8	8	8	8	8
Co	28	30	31	28	18	23	16
Cr	209	219	203	230	186	206	257
F*	719	671	409	602	455	716	364
S*	2085	1526	1656	1519	1233	2837	1158
Sc	21	17	19	17	16	18	19
V	153	124	124	139	120	160	147
Cs	9	9	9	9	9	9	9
Ba	1300	974	1129	1342	1654	2234	964
La	77	80	84	88	87	87	65
Ce	192	219	237	243	244	240	185

Results for elements indicated with an * should be considered semi-quantitative

3.3. The effect of fly ash on the pH of water

When a 1% (wt/vol) concentration of each fly ash was added to distilled water with an initial pH of 6.1, the pH increased to above 9 for all the samples after 6h. The greatest increase in pH was observed for fly ash samples 1, 2 and 4 (Table 6). When the same amount of fly ash was added to water taken from the Hartbeespoort dam with an initial pH of 7.16 (Table 7), a smaller increase in pH was observed when compared to distilled water, although addition of fly ash samples 1, 2 and 4 once again resulted in a greater pH increase than the other samples.

Table 6: Effect of fly ash samples on the pH of distilled water with initial pH of 6.1

Sample	pH	pH increase
1	11.16	5.06
2	10.94	4.84
3	9.80	3.70
4	11.16	5.06
5	9.94	3.84
6	9.39	3.29
7	9.98	3.88

Table 7: Effect of fly ash on the pH of water from the Hartbeespoort Dam with initial pH of 7.16

Sample	pH	pH increase
1	8.78	1.73
2	9.11	2.06
3	7.82	0.77
4	9.92	2.87
5	8.08	1.03
6	8.67	1.62
7	7.75	0.70

3.4. Chemical leaching of fly ash in distilled water

The results for the leaching of the fly ash samples in distilled water with the initial pH of 7 and in acidified distilled water (pH 2) are presented in Tables 8 and 10 respectively. In distilled water, the following elements were leached: Al (especially high in samples 5 and 7), B (>3ppm in sample 7), Ba, Ca (>100ppm in all samples), Cr (<1ppm in all samples), Fe, Ga, K, Mo, Na, Se, Si (high in sample 3 and 7), Sr, Ti, V, and W. Table 9 shows the percentage of the toxic elements that were leached from the total amount present in each fly ash sample, with the exception of B as this element was not included in the XRF analysis. Less than 0.4% of the total As was leached, and less than 0.1% of the total Cr, Ni and Zn.

In acidic water there was a large increase in the number of metals leached into solution, as well as the quantities leached. Elements that were leached at a high concentration included Al (>70ppm for all ash samples), B and Ca (>550ppm for all samples). The concentrations of Mg leached from the ashes in acid water were approximately 1000% higher than when leached in distilled water. The Mn concentrations leached in acid water were between 2 and 6ppm, whereas Mn was below detection in distilled water. Concentrations of K, Na, Si, and Sr were higher when fly ash was leached in acid water than in distilled water. Concentrations of the toxic elements Cr, Ni and Zn were less than 1ppm in the leachate for all the ash samples leached in acid water. The percentages of the toxic elements that were leached from the total amount present in each fly ash sample are presented in Table 11. Less than 3% of the total amounts were leached.

Table 8: Leaching results (ppm) for samples in distilled water (pH_i 7). Detectable limit <0.1

	Sample 1 Thutuka	Sample 2 Arnot	Sample 3 Duvha	Sample 4 Hendrina	Sample 5 Kendal	Sample 6 Matla	Sample 7 Lethabo
Ag	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Al	0.14	0.27	2.51	0.18	9.91	0.07	7.67
As	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Au	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
B	0.07	0.02	0.08	0.05	1.09	0.13	3.11
Ba	1.22	0.78	0.54	1.71	0.45	2.31	0.24
Be	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Bi	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ca	371	288	145	358	158	367	106
Ce	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Co	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cr	0.24	0.12	0.07	0.07	0.08	0.27	0.09
Cs	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cu	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Dy	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Er	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Eu	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Fe	0.06	0.01	0.02	0.03	0.01	0.08	0.06
Ga	0.02	0.05	0.03	0.02	0.06	0.01	0.07
Gd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ge	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Hf	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Hg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ho	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
In	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ir	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
K	1.51	0.73	1.53	1.2	1.71	1.18	0.8
La	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Li	0.33	0.14	0.28	0.19	0.44	0.52	0.25
Lu	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mg	0.12	0.12	0.16	0.11	0.14	0.12	0.18
Mn	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mo	0.08	0.06	0.05	0.09	0.09	0.17	0.06
Na	5.77	1.72	1.94	1.83	2.5	4.35	1.77
Nb	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Nd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ni	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Os	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
P	<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
Pd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pr	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pt	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Rb	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01
Re	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ru	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Sb	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	0.01
Sc	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Se	0.01	0.01	0.02	0.01	0.03	0.01	0.03
Si	0.91	2.13	6.91	1.11	3.97	1.64	5.46
Sm	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sn	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sr	7.91	3.77	1.73	4.39	1.99	8.14	1.35
Ta	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tb	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Te	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Th	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ti	0.53	0.46	0.25	0.54	0.25	0.61	0.18
Tl	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tm	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
U	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
V	0.01	0.01	0.07	0.01	0.06	0.02	0.13
W	0.04	0.03	0.05	0.03	0.06	0.05	0.05
Y	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Yb	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zn	0.01	<0.01	0.01	0.01	0.01	<0.01	<0.01
Zr	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 9: Percentage of toxic elements leached from the fly ash samples at an initial pH of 7

%	**1**	**2**	**3**	**4**	**5**	**6**	**7**
As	0.33	0.33	0.06	0.33	0.33	0.25	0.14
Cr	0.11	0.05	0.03	0.03	0.04	0.13	0.04
Ni	0.01	0.01	0.01	0.02	0.02	0.02	0.02
Zn	0.02	0.02	0.01	0.01	0.03	0.02	0.02

Table 10: Leaching results (ppm) for samples in acidified distilled water (pH 2) Detectable limit <0.1

	Sample 1 Thutuka	Sample 2 Arnot	Sample 3 Duvha	Sample 4 Hendrina	Sample 5 Kendal	Sample 6 Matla	Sample 7 Lethabo
Ag	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Al	84.3	95.5	97.1	95.1	73.5	92.4	149
As	<0.08	<0.09	0.16	0.03	0.01	0.03	0.03
Au	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
B	8.29	8.4	4.37	5.02	6.41	15.1	12.3
Ba	0.11	0.1	0.08	0.08	0.11	0.09	0.08
Be	0.05	0.04	0.03	0.04	0.02	0.04	0.03
Bi	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ca	568	568	633	558	603	574	641
Ce	0.33	0.54	0.52	0.53	0.38	0.44	0.54
Co	0.06	0.1	0.08	0.08	0.03	0.07	0.03
Cr	0.4	0.24	0.13	0.17	0.07	0.55	0.25
Cs	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cu	1.3	0.42	0.36	0.26	0.16	0.3	0.2
Dy	0.05	0.07	0.05	0.05	0.05	0.08	0.05
Er	0.03	0.04	0.03	0.03	0.02	0.05	0.03
Eu	0.01	0.01	0.01	0.01	0.01	0.02	0.01
Fe	0.35	0.75	0.24	0.61	0.91	0.25	0.58
Ga	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01
Gd	0.06	0.08	0.06	0.06	0.05	0.1	0.06
Ge	0.2	0.2	0.28	0.16	0.13	0.33	0.16
Hf	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Hg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ho	0.01	0.01	0.01	0.01	0.01	0.01	0.01
In	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ir	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
K	7.36	2.98	3.49	4.46	4.94	13.7	3.04
La	0.24	0.35	0.27	0.28	0.2	0.31	0.3
Li	0.55	0.35	0.57	0.48	0.56	0.9	0.52
Lu	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
Mg	196	273	114	213	158	334	77.5
Mn	4.69	5.6	2.95	4.4	2.14	4.5	1.66
Mo	0.01	0.01	0.03	0.02	0.01	0.03	0.01
Na	7.85	4.68	2.66	3.7	3.98	7.19	3.93
Nb	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Nd	0.19	0.27	0.22	0.22	0.17	0.29	0.24
Ni	0.22	0.3	0.24	0.23	0.12	0.25	0.16
Os	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
P	<0.01	<0.01	5.42	0.77	<0.01	1.9	1.67
Pb	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pr	0.05	0.07	0.06	0.06	0.04	0.07	0.07
Pt	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Rb	<0.01	0.01	0.01	0.01	0.01	0.01	0.01
Re	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ru	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

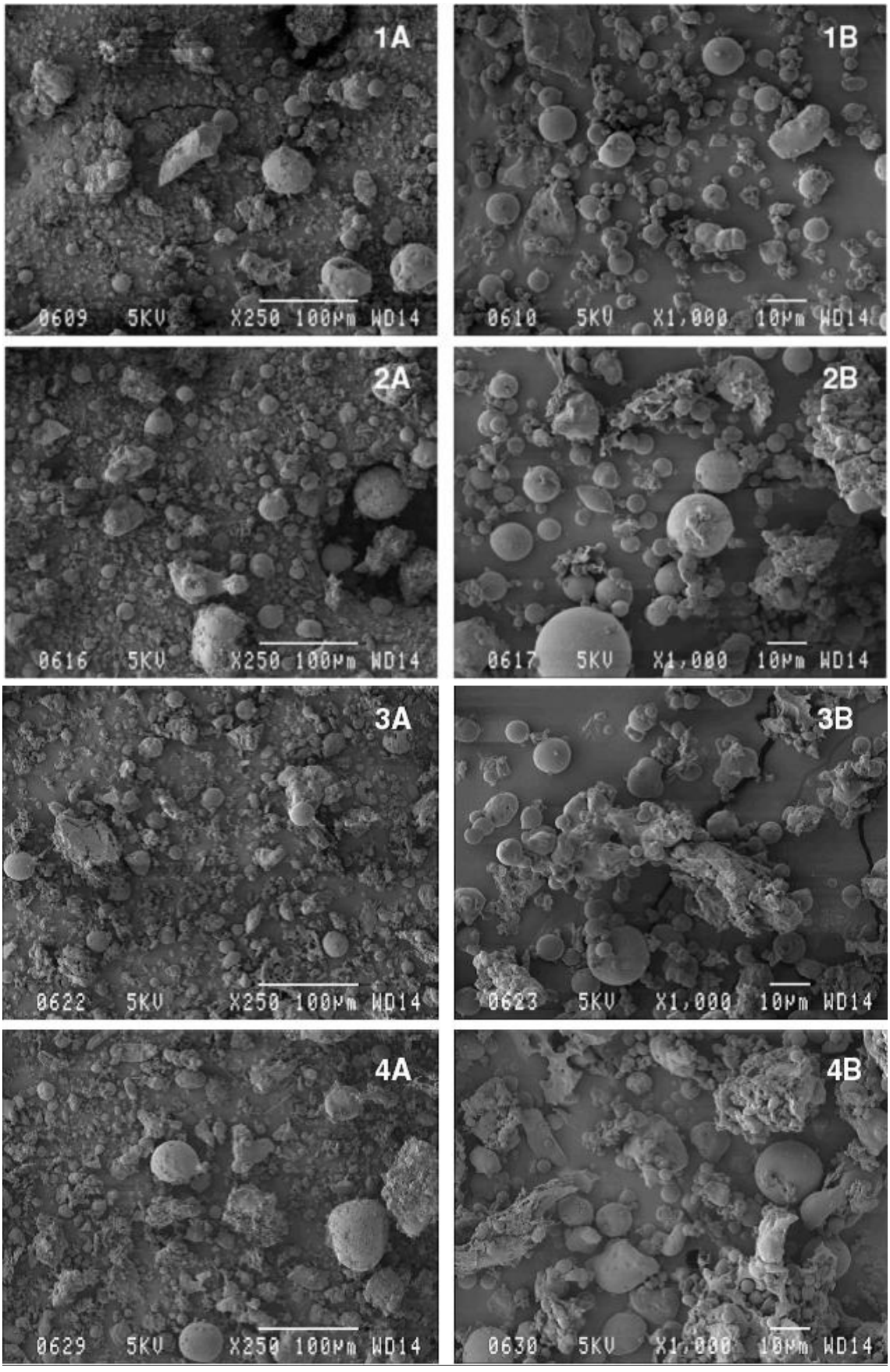
Sb	<0.01	<0.01	0.01	0.01	0.01	0.01	0.01
Sc	0.05	0.05	0.04	0.04	0.05	0.07	0.05
Se	0.02	0.02	0.02	0.01	0.01	0.02	0.01
Si	200	233	212	212	202	306	216
Sm	0.04	0.06	0.05	0.05	0.04	0.07	0.05
Sn	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sr	16.9	11.3	9.36	11.9	8.34	16.7	8.7
Ta	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tb	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Te	0.02	0.01	0.01	0.01	0.01	0.01	0.01
Th	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ti	1.43	1.55	1.53	1.57	1.48	1.56	1.67
Tl	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tm	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
U	0.28	0.18	0.12	0.16	0.04	0.01	0.15
V	0.16	0.12	0.65	0.3	0.23	1.21	0.47
W	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Y	0.3	0.43	0.33	0.32	0.27	0.53	0.29
Yb	0.02	0.03	0.02	0.02	0.02	0.04	0.02
Zn	0.54	0.52	0.37	0.51	0.19	0.45	0.47
Zr	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 11: Percentage of toxic elements leached from the fly ash samples at pH 2

%	1	2	3	4	5	6	7
As	2.7	3	0.93	1	0.33	0.82	0.41
Cr	0.19	0.11	0.10	0.10	0.04	0.27	0.10
Ni	0.27	0.42	0.30	0.35	0.25	0.44	0.26
Zn	1.07	0.90	0.49	0.51	0.55	0.70	0.81

3.5. SEM of fly ash samples

The particle structure of the fly ash samples is presented in Figure 1. All of the samples contained particles varying in size from smaller than 1µm to larger than 100µm. Fly ash sample 6 appeared to be the finest, with a greater percentage of small particles, and fly ash 5 the coarsest, with a small percentage of small particles. In all the samples, the fly ash particles appeared predominantly spherical, especially the particles smaller than 10µm. The larger particles varied in shape.


1A
0609 5KV X250 100µm WD14
1B
0610 5KV X1,000 10µm WD14
2A
0616 5KV X250 100µm WD14
2B
0617 5KV X1,000 10µm WD14
3A
0622 5KV X250 100µm WD14
3B
0623 5KV X1,000 10µm WD14
4A
0629 5KV X250 100µm WD14
4B
0630 5KV X1,000 10µm WD14

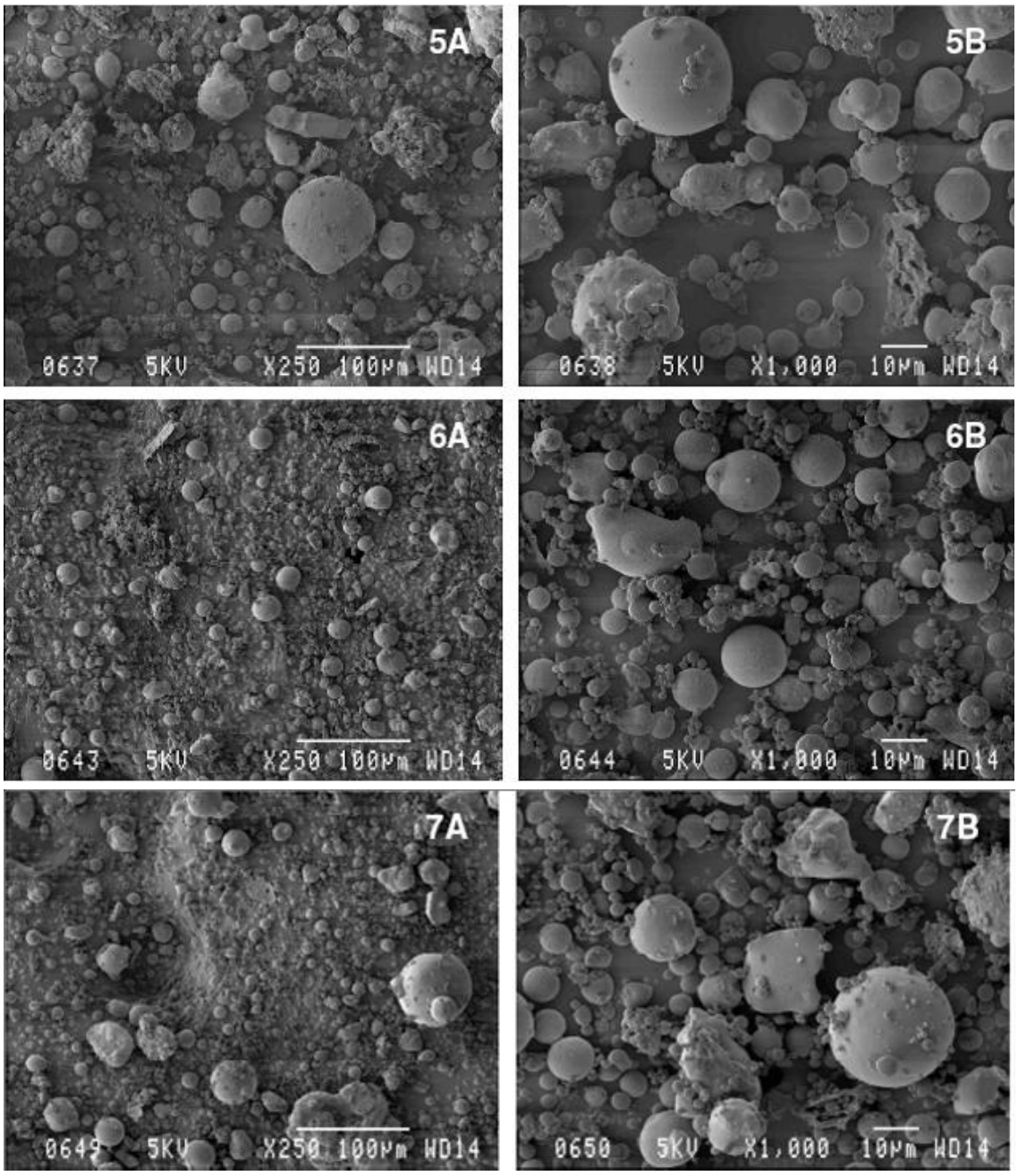


Figure 1: Scanning electron microscopy of the fly ash samples (1-7) at 250x (A) and 1000x (B) magnification

3.6. Particle sizing

The particle size distributions for fly ash samples 1-7 are presented in Figure 2, and the particle diameters below which 10%, 50% and 90% of the particle volume is contained respectively for each fly ash sample is shown in Table 12. It is clear from the distributions that sample 6 had a higher percentage of small particles than the other ashes. Sample 7 also had a high percentage of particles <1µm in size, but had a higher

percentage of particles above 100µm in size than sample 6. Sample 5 had the largest particles, with a d(0.9) of 202.113µm and the smallest quantity of particles below 0.1µm.

Table 12: Particle diameters (µm) below which 10%, 50% and 90% of the particle volume is contained respectively for each fly ash sample

Sample	d(0.1)	d(0.5)	d(0.9)
1	3.192	47.139	193.198
2	3.204	36.965	179.825
3	2.357	27.272	119.225
4	4.767	39.242	199.396
5	5.145	51.719	202.113
6	0.188	8.84	47.757
7	0.275	19.336	144.701

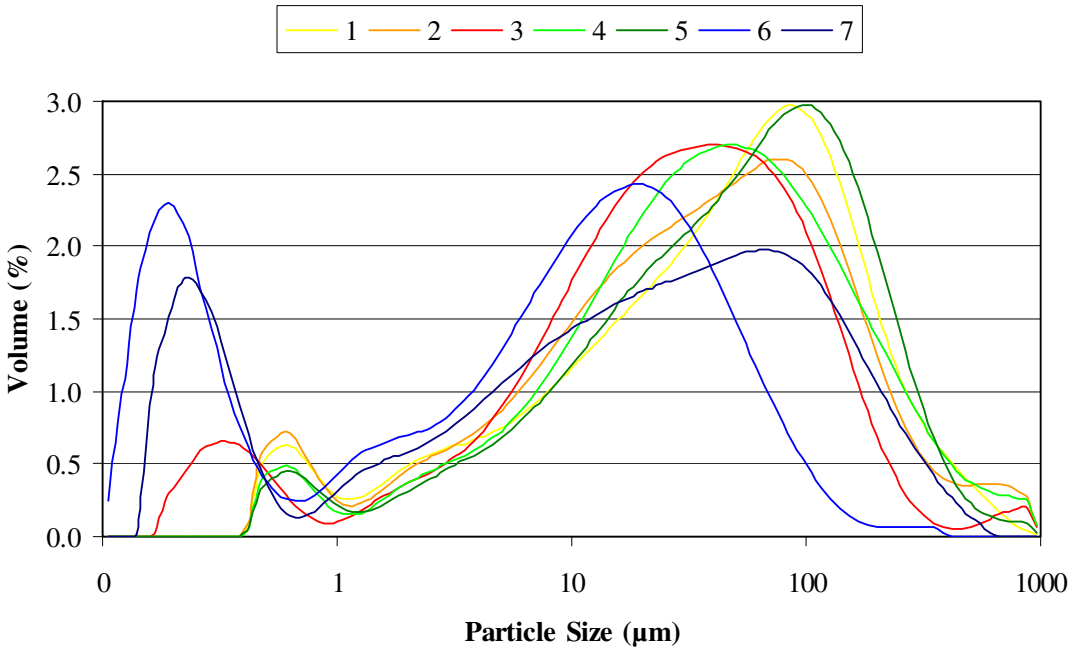


Figure 2: Particle size distributions for the 7 fly ash samples

4. Discussion

According to the XRD and XRF results, the ash samples used in this study were all low in lime (0.05-0.3%), and could therefore be classified as Type F ashes (Iyer, 2002). Mullite ($Al_6Si_2O_{13}$) and quartz (SiO_2) comprised the largest weight percentage in all the

ash samples. The XRF results revealed that SiO_2, Al_2O_3, Fe_2O_3, CaO and MgO were the major molecules present in all the fly ash samples, with the other major elements all comprising less than 1% of the mass. There were no striking differences between the major element compositions of the different ashes; all had a SiO_2 content between 50 and 55%, and an Al_2O_3 content between 24 and 32%. The CaO content was also similar (4-7%), with the exception of Sample 3 (Duvha) which was below 4%. With the exception of As, Mo, Nb, U, W, Cl, Sc, and Cs, all the other trace elements tested comprised more than 50ppm of the ash samples tested. Sr, S and Ba had the highest concentration (above 1000ppm) in all the ash samples tested.

When a 1% (wt/vol) concentration of each fly ash was added to distilled water, the pH increased to above 9 for all the samples with the greatest pH increase observed for fly ash samples 1 (increase of 5.06), 2 (increase of 4.84) and 4 (increase of 5.06). According to Iwashita *et al*. (2005), the pH of the fly ash leachate increased with the amount of calcium leached, as the main species of calcium in fly ash are alkaline species such as CaO. They found the final pH of the leachate to be almost independent of the leaching amount of other alkaline salt elements such as K, Na and Mg. This was because the amount of Ca in fly ash was much greater than these elements. When the amount of calcium leached from ash samples in distilled water in this study was examined, samples 1, 2 and 4 did indeed have a higher concentration of calcium (371ppm, 288ppm and 358ppm respectively) when compared with the other samples, although the relationship between the amount of calcium and pH increase did not appear to be linear as described by Iwashita *et al*. (2005). However, the amount of calcium leached from fly ash 6 (367ppm) was greater than that from sample 4, but showed a smaller increase in pH (3.29).

When the same amount of fly ash was added to water taken from the Hartbeespoort Dam with an initial pH of 7.16, a smaller increase in pH was observed when compared to distilled water, although addition of fly ash samples 1, 2 and 4 once again displayed a greater pH increase than the other samples. These results indicated that there were natural pH buffers in the water that were able to minimise the pH increase.

In distilled water, the following elements were leached: Al (especially high in samples 5 and 7), B (>3ppm in sample 7), Ba, Ca (>100ppm in all samples), Cr (<1ppm in all

samples), Fe, Ga, K, Mo, Na, Se, Si (high in sample 3 and 7), Sr, Ti, V, and W. In terms of the toxic elements, less than 0.4% of the total As was leached from the ash samples, and less than 0.1% of the total Cr, Ni and Zn.

In acidic water there was an increase in the number of metals leached into solution, as well as the quantities leached. Elements that were leached at a high concentration included Al (>70ppm for all ash samples), B and Ca (>550ppm for all samples). The concentrations of Mg leached from the ashes in acid water were approximately 1000% higher than when leached in distilled water. The Mn concentrations leached in acid water were between 2 and 6ppm, whereas Mn was below detection in distilled water. Concentrations of K, Na, Si, and Sr were higher when fly ash was leached in acid water than in distilled water. Concentrations of the toxic elements Cr, Ni and Zn were less than 1ppm in the leachate for all the ash samples leached in acid water. The percentage of the toxic elements that were leached from the total amount present in each fly ash sample is presented in Table 11. Less than 3% of the total amounts were leached.

The Department of Water Affaris and Forestry (DWAF) has set water quality guidelines for South Africa for aquatic ecosystems (DWAF, 1996b) and human consumption (DWAF, 1996a) (Table 13). In the case of aquatic ecosystems, it is seldom possible to mitigate the effects of poor water quality to the same degree as for domestic, agricultural and industrial water uses, these being predominantly off stream. Hence, for the purpose of protecting and maintaining aquatic ecosystems, prevention, rather than mitigation, of the effects of poor water quality has to be given even greater emphasis than would be the case for other water uses. For this reason, the criteria for aquatic ecosystems provide stricter levels of protection when compared to other water uses (Dallas & Day, 1993).

When the ash samples were leached in distilled water, the concentrations of Al, Cr, and Ca in the leachates exceeded the target water quality range (TWQR) for both human consumption and aquatic ecosystems. The Fe concentration for some of the leachates exceeded the TWQR for human consumption; the guideline concentration for aquatic ecosystems was not available. The Zn concentration was within the limits for human consumption, but exceeded the TWQR for aquatic ecosystems. Se was above the TWQR for aquatic ecosystems in all ash leachates, but only leachates from samples 5

and 7 exceeded the guideline concentration for human consumption. The concentration of fly ash used for leaching was 5% (wt/vol). These results indicate that a lower dosage of fly ash leached in distilled water may produce a leachate with the concentration of toxic elements below recommended limits.

When the ash samples were leached in acid water, concentrations of Al, As, Ca, Cr, Cu, Fe, Mg and Mn exceeded the TWQR for both human consumption and aquatic ecosystems. Concentrations of Se and Zn were within the guideline concentrations for human consumption, but exceeded the TWQR for aquatic ecosystems.

Table 13: DWAF water quality guidelines for South Africa for aquatic ecosystems and human consumption (DWAF, 1996a; DWAF, 1996b)

	TWQR[a] (mg.l^{-1})			
Element	**Aquatic ecosystems**	**CEV[b]**	**AEV[c]**	**Human consumption**
Al	0.005	0.01	0.02	0.015
As	0.01	0.2	0.13	0.01
Ca	NA[d]			32
Cr	0.012	0.024	0.34	0.05
Cu	0.0003	0.00053	0.0016	1
Fe	NA			0.01
Pb	0.0002	0.0005	0.004	0.01
Mg	NA			30
Mn	0.18	0.37	1.3	0.05
Hg	0.04	0.08	1.7	0.001
Ni	NA			NA
Se	0.002	0.005	0.03	0.02
Si	NA			NA
Zn	0.002	0.0036	0.036	3

[a] **Target Water Quality Range**: This is the range of concentrations or levels within which no measurable adverse effects are expected on human health or the health of aquatic ecosystems, and should therefore ensure their protection

[b] **The Chronic Effect Value** is defined as that concentration or level of a constituent at which there is expected to be a significant probability of measurable chronic effects in up to 5% of the species in the aquatic community.

[c] **The Acute Effect Value** is defined as that concentration or level of a constituent above which there is expected to be a significant probability of acute toxic effects in up to 5% of the species in the aquatic community

[d] **Not available**

Elevated concentrations of bio-available aluminium in water are toxic to a wide variety of organisms. The toxic effects are dependent on the species and life stage of the organism, the concentration of calcium in the water, and the pH. The pH may not only affect the chemistry of aluminium but may also determine how the organism responds to dissolved aluminium. In acidic waters, aluminium is generally more toxic over the pH range of 4.4 - 5.4, with maximum toxicity occurring at pH 5.0 - 5.2. The mechanism of toxicity in fish seems to be related to interference with ionic and osmotic balance and with respiratory problems resulting from coagulation of mucus on the gills. It has also been suggested that aluminium interferes with calcium metabolism and ion exchange sites, in particular those involved in sodium homeostasis. This in turn may lead to neuromuscular dysfunction (DWAF, 1996b).

Arsenic has a variety of adverse effects on both vertebrate and invertebrate aquatic organisms; the type and severity of the effects being dependent on the life stages of the organisms concerned. Exposure to arsenic results in reduced growth and reproduction in both fish and invertebrate populations and causes behavioural changes such as reduced migration in fish. The response of organisms to arsenic is reduced by pre-exposure, and organisms may become gradually acclimated to high concentrations of arsenic in aquatic ecosystems (DWAF, 1996b).

Copper is a micronutrient, and an essential component of the enzymes involved in redox reactions. It is rapidly accumulated by plants and animals, and is toxic at low concentrations in water. The early life stages of organisms appear to be more sensitive than adults to copper pollution. Metabolically, copper interacts with zinc, molybdenum, arsenic and selenium. A high concentration of copper in the water causes brain damage in mammals. Nitrogen fixation by blue-green algae is reduced by the addition of trace amounts of copper (DWAF, 1996b).

Chromium exerts a toxic effect at different concentrations in different groups of aquatic organisms. Fish are the most resistant, although a temporarily reduced growth phase has been reported for young fish at low chromium concentrations. Invertebrates are usually at least an order of magnitude more sensitive than vertebrates, with *Daphnia* spp. showing the greatest sensitivity to chromium. Green algae are also more sensitive than fish, whilst bacterial responses to chromium are variable (DWAF, 1996b).

Lead is a toxic trace metal which readily accumulates in living tissue. Metabolically, lead interacts with iron and therefore interferes with haemoglobin synthesis. It also affects membrane permeability by displacing calcium at functional sites, and inhibits some of the enzymes involved in energy metabolism. Lead absorbed by vertebrate organisms is largely deposited in the bony skeleton, where it does not usually exhibit toxic effects. However, stress may result in decalcification or deossification, whereupon symptoms of toxicity may appear. Rainbow trout develop spinal deformities after exposure to lead in soft water. In fish, low concentrations of lead in the water results in the formation of a film of coagulated mucous over the gills and subsequently over the entire body. This has been attributed to a reaction between lead and an organic constituent of the mucous, and leads to death by suffocation. Lead is bio-accumulated by benthic bacteria, freshwater plants, invertebrates and fish (DWAF, 1996b).

Because these are chemical similarities between selenium and sulphur, selenium can replace sulphur in some organic molecules and thereby cause toxic effects. In fish, selenium toxicity includes changes in feeding behaviour and equilibrium, pathological changes, deformities, haematological changes and death. Fish are generally less sensitive to selenium than invertebrates. Toxic effects of selenium that have been recorded in invertebrates include immobilisation, reduced survival and reduced reproduction. Selenium is passed through the aquatic food chain and accumulates in the liver of mammals and fish. Selenium undergoes biological methylation in sediments, and selenomethionine is ten times more toxic than inorganic selenium (DWAF, 1996b).

Zinc is a trace metal, and is also an essential micronutrient in all organisms. The lethal effect of zinc on fish is thought to be due to the formation of insoluble compounds in the mucus covering the gills. Sub-lethal concentrations at which toxic effects are evident depend on the concentration ratio of zinc to copper, since zinc interferes with copper absorption. Observed symptoms of sub-lethal toxicity include depressed white blood cell counts, oedema and liver necrosis. Invertebrate responses to zinc toxicity vary, although molluscs are generally more resilient than are other organisms. Sub-lethal effects include reduced rates of shell growth, oxygen uptake and larval development. Algal photosynthesis can be inhibited by zinc (DWAF, 1996b).

From the toxicity information above, it is clear that care must be taken to ensure that highly concentrated fly ash leachates, especially those leached under acid conditions, do not reach underground water sources or natural water bodies, as they contain concentrations of toxic elements that are above the recommended limits.

In terms of the physical structure of the ash samples, all of the samples contained particles varying in size from smaller than 1µm to larger than 100µm when viewed with SEM. Fly ash sample 6 had the highest percentage of small particles, whereas fly ash 5 was the coarsest, with a small percentage of small particles. In all the samples, the fly ash particles appeared predominantly spherical, especially the particles smaller than 10µm. When particle size analysis was performed, the results reflected the observations made with SEM; sample 6 (Matla fly ash) had the greatest percentage of small particles below 1µm.

5. Conclusion

SiO_2, Al_2O_3, Fe_2O_3, CaO and MgO were the major molecules present in all the fly ash samples, and there were no striking differences between different ashes in terms of their major elemental compositions. All the fly ash samples caused an increase in pH when added to distilled water, although a larger pH increase was observed for samples 1, 2 and 4. When the fly ash samples were added to dam water, a smaller increase in pH was observed, indicating that the water had a buffering effect. In terms of physical properties, fly ash sample 6 was the finest, with the greatest portion of particles below 1µm and sample 5 was the coarsest

Leaching of the fly ash samples in acid water resulted in a higher amount of metals being leached, and at higher concentrations that in neutral distilled water. However, although lower, in some cases the concentrations of toxic metals leached in distilled water were above the recommended guidelines for human consumption as well as aquatic ecosystems. In acid water the concentrations of Al, As, Ca, Cr, Cu, Pb, Mg, Mn, Se and Zn greatly exceeded the recommended concentrations for aquatic ecosystems.

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CHAPTER 8:

THE FLOCCULATION OF CYANOBACTERIA USING FLY ASH

1. Introduction

The removal of surface algae using flocculants has proven successful. Tenney *et al.* (1969) investigated the use of synthetic organic polyelectrolytes for the flocculation of algae. Algal cells form stable microbial suspensions, possess a chemically reactive cellular surface, and possess a net negative surface charge due to the ionisation of functional ionogenic groups. The stability of algal suspensions depends on the forces acting between the particles themselves as well as on the forces interacting between the particles and the water; hence algae can be classified as hydrophilic bio-colloids. Addition of a cationic synthetic organic polyelectrolyte induced algal flocculation, but anionic and non-ionic polymers were not effective. The mechanism of chemically induced flocculation was described in terms of a bridging phenomenon between the discreet algal cells and the linearly extended polymer chains, which formed a three-dimensional matrix capable of subsiding. The flocculation efficiency was directly related to the extent of polymer coverage of the active sites on the algal cell surface; the algal surface charge needed to be reduced to a level which allowed the extended polymer chains to bridge the separation distance established by electrostatic repulsion. Optimal algal flocculation occurred at 50% coverage of the algal surface. Flocculation was most effective at the low pH levels of 2 to 3 due to reduced electrostatic repulsion between the algal cells and improved polymeric bridging because of a greater extension of polymer chains. Flocculation efficiency was affected by the algal growth phase, with the least amount of flocculant required in the late log and early declining log phase. High molecular weight extracellular metabolites produced by algae accumulate rapidly during the late log phase. These polymeric molecules comprise of long chain polysaccharides, proteins and nucleic acids and are of sufficient length to form bridges between algal particles, hence enhancing flocculation. In later growth stages the accumulation of this material could have acted as a protective colloid.

The potential use of clays to control harmful algal blooms has been investigated in East Asia, Australia, the U.S.A. and Sweden. Minerals such as montmorillonite and montmorillonite-containing sediments such as phosphatic clay, kaolinite and yellow loess have been used effectively. Cell removal occurs through the flocculation of algal particles leading to the formation of larger aggregates which settle rapidly (Sengco & Anderson, 2004). Pan *et al.* (2006) investigated the algal removal abilities of 26 clays

and minerals, and found that sepiolite, talc, ferric oxide, and kaolinite were the most effective, with an 8h equilibrium removal efficiency >90% at a clay loading of 0.7g.L^{-1}. When the clay loading was reduced to 0.2g.L^{-1}, the removal efficiency for 25 of the materials decreased to below 60%, except for sepiolite which remained about 97%. The high efficiency for sepiolite to flocculate *Microcystis aeruginosa* cells in freshwaters was due to the mechanism of netting and bridging.

Divakaran & Pillai (2002) investigated the use of chitosan to flocculate three freshwater species of algae, and one brackish alga. Chitosan is obtained by the deacetylation of chitin and is a cationic polyelectrolyte, thus is expected to coagulate negatively charged suspended particles found in natural waters. The flocculation efficiency was sensitive to pH, with the optimal flocculation of the freshwater algae occurring at pH 7, which differs to the findings of Tenney *et al*. (1969). Microscopy showed that the cells were intact after flocculation, but stuck together in clumps. Culturing of the flocculated clumps showed growth as usual, indicating that the cells were alive, but the clumps remained settled and fresh cells took longer to surface. Zou *et al*. (2006) found that clay particles could be turned into highly efficient flocculants to remove *Microcystis aeruginosa* cells in freshwaters when they were modified by chitosan. As yet no assessment of the use of fly ash as a potential algal flocculant has been published.

Of the millions of metric tones of fly ash that are produced word-wide every year, only a portion (10-20%) is reused for productive purposes, primarily in cementitious (concrete and cement) products and in construction, such as highway road bases, grout mixes and for stabilising clay-based building materials (Iyer & Scott, 2001). The remaining amount of fly ash produced annually must either be disposed in controlled landfills or waste containment facilities, or stockpiled in slag heaps (Shackelford, 2000) all of which can be regarded as unsightly and environmentally undesirable. With competition for limited space and stricter regulations on surface and ground water discharge, water originating from fly ash disposal sites must be well managed. The long-term maintenance of ash disposal sites and the necessary water management involved can pose a significant financial burden. The development of other means of commercial exploitation of fly ash beyond the cement and construction industries is therefore a priority.

The use of fly ash in wastewater treatment has been studied extensively, and the results of laboratory tests showed that fly ash is a good sorbent for the removal of heavy metals (Ayala *et al*., 1998; Héquet *et al*., 2001). Estivinho *et al*., (2007) used fly ash to adsorb chlorophenols, which are highly toxic and mutagenic. They found that fly ash was a good alternative to activated carbon, with the reduced sorption capacity of fly ash when compared to activated carbon not being significant considering the lower costs of the fly ash.

Oguz (2005) assessed the use of Yatagan fly ash to remove phosphate, an essential macronutrient that spurs the growth of photosynthetic algae and cyanobacteria, from aqueous solutions. Fly ash was a highly successful adsorbant, with a phosphate removal efficiency in excess of 99%, and a phosphate adsorption capacity of 71.87mg.g^{-1}. According to the X-ray spectra obtained, it was thought that there was an electrostatic attraction on the solid/liquid interface between the phosphate salts and the fly ash particles, which led to ion exchange and weak physical interactions. Agyei *et al*. (2002) examined the phosphate ion removal from solution using fly ash, slag and ordinary Portland cement (OPC). The rate and efficiency of phosphate adsorption increased in the order fly ash, slag, OPC, which was the same order as the increasing percentage CaO in the adsorbants. This led to the conclusion that the extent of phosphate removal was related to the percentage CaO or Ca^{2+} ions released into the solution via hydration and dissolution. This was confirmed by the research of Chen *et al*. (2006), who concluded that phosphate immobilization by fly ash was governed by the amount of Ca in the ash, especially CaO and $CaSO_4$. They also attributed a portion of the phosphate removal to the presence of Fe_2O_3. The greatest removal of phosphate occurred in alkaline conditions for high calcium fly ash.

The aims of this study were to evaluate the ability of various fly ash samples to flocculate algae, determine which sample was the most effective, and investigate the possible mechanism of flocculation. The fly ash samples were also tested for their ability to adsorb phosphate from aqueous solutions.

2. Material and Methods

2.1 Fly ash samples

The fly ash used in this study was provided by Eskom. Six samples from different power stations using coal from different mines (Table 1), with varying physical and chemical characteristics (Chapter 7), were tested for their ability to flocculate cyanobacteria.

Table 1: Fly ash samples

Sample Number	Power station	Coal Mine
1	Thutuka	Newdenmark
2	Arnot	Arnot Coal
3	Duvha	Middelburg mine BHP Biliton
4	Hendrina	Optimum
5	Kendal	Khutala
6	Matla	Matla Coal
7	Lethabo	Newvaal

2.2. Cyanobacteria samples

Water samples were taken from the eutrophied Hartbeespoort Dam during June and July 2007. The water had a high concentration of cyanobacteria at the time of sampling.

2.3. Flocculation experiments

2L beakers (surface area ≈ 133cm^2) were filled with 600ml of cyanobacteria-containing water from the Hartbeespoort Dam. The beakers were allowed to stand until all the cyanobacteria had floated to the surface to form a definitive layer. In order to determine which fly ash had the greatest flocculating ability, varying quantities of each fly ash were spread evenly over the surface using a sieve, and the beakers were allowed to

stand for 6h to allow the flocculated cyanobacteria to settle and form a layer on the bottom of the beaker. The top layer was carefully skimmed off, and the clear water pored off the bottom layer. The volumes and the chlorophyll-a concentrations of the top and bottom layers were measured to determine the flocculation efficiency. Chlorophyll-a was measured using the methanol extraction method (Lorenzen, 1967; Golterman & Clymo, 1970; Holm-hansen, 1978). 100ml of sample was filtered through a membrane filter (GF/C 0.45µm pore size, 47 mm diameter, Whatman) and the filter was placed into a 50ml Greiner tube, filled with 10ml methanol and wrapped in aluminium foil to avoid degradation by light. Following homogenisation, the tube was centrifuged for 10 min at 3200 rpm. The absorbance of the supernatant was measured at 665nm and 750nm against pure methanol (Spectronic® 20 Genesis™, Spectronic Instruments), using a 1cm cuvette. The following formula was then used to calculate the chlorophyll-a concentration:

$$\text{Chl a } (\mu g.l^{-1}) = (Abs_{665nm} - Abs_{750nm}) \times A \times Vm/V \times L$$

Where:
A = absorbance coefficient of Chl a in methanol (12.63)
Vm = volume of methanol used (mL)
V = volume of water filtered (L)
L = path length of cuvette (cm)

Once it was established which fly ash was the most effective, this ash was used to determine the amount of fly ash needed for optimal flocculation by investigating the effect of varying the amount of ash used and the thickness of the cyanobacterial layer. During the experiments, samples for re-growth experiments and electron microscopy were taken and the pH value was measured. Water samples to which no fly ash was added were used as negative controls.

2.4. Re-growth experiments

Samples from the bottom layer containing flocculated cyanobacteria and fly ash were taken at 6h and 36h after treatment. Modified Allen's BG-11 medium (Table 2), (Krüger & Eloff, 1977) was inoculated with these samples to test for re-growth of the

cyanobacteria. The cultures were grown in 250ml cotton plugged sterile Erlenmeyer flasks at ambient temperatures (24-26°C) with shaking to allow for aeration. Continuous lighting of 2000lux (Extech instruments Datalogging lightmeter model 401036) was provided by 18W cool white fluorescent lamps (Lohuis FT 18W/T8 1200LM) suspended above the flasks. The concentration of chlorophyll-a was measured immediately after inoculation and again after approximately two weeks. Growth media was inoculated with a sample of the floating layer from the control beaker (without addition of fly ash) as a positive control.

Table 2: Mineral composition of modified BG-11 medium

Component	Concentration
$NaNO_3$	$1.500g.l^{-1}$
K_2HPO_4	$0.040g.l^{-1}$
$MgSO_4.7H_2O$	$0.075g.l^{-1}$
$CaCl_2.2H_2O$	$0.036g.l^{-1}$
Na_2CO_3	$0.020g.L^{-1}$
$FeSO_4$	$0.006g.L^{-1}$
$EDTA.Na_2H_2O$	$0.001g.L^{-1}$
Citric acid	$0.0112g.L^{-1}$
Trace metal solution (Table 2.1)	$1ml.l^{-1}$

Table 2.1: Trace metal solution for modified BG-11 media

Trace metal solution component	Concentration ($g.l^{-1}$)
H_3BO_3	2.8600
$MnCl_2.4H_2O$	1.8100
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.5H_2O$	0.300
$CO(NO_3)_2.H_2O$	0.0494
$CuSO_4.5H_2O$	0.0790

2.5. Scanning electron microscopy (SEM) of flocculated cyanobacteria

SEM was performed on flocculated cyanobacterial samples as well as samples from the negative control. Samples were concentrated by centrifugation and, following removal of the supernatant, fixed in a solution of 2.5% gluteraldehyde in 0.075M sodium phosphate buffer (pH 7.4) overnight at 4^{o}C. The samples were then rinsed 3 times in 0.075M sodium phosphate buffer (10min per rinse), centrifuging between each rinse. After rinsing with the buffer the material was fixed in 1% aqueous OsO_4 for 1.5h, then rinsed again in distilled water. The samples were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 3x 100%; 10 min each), and were then dried to critical drying point. The dried samples were mounted on SEM slides, gold coated and viewed with a JOEL JSM-840 SEM. After the initial viewing, the samples mounted on the slides were covered with sticky tape, and the tape removed in an attempt to break up cell clusters so that the interior cyanobacteria could be seen. The samples were recoated and viewed again.

2.6. SEM of etched samples

In order to investigate the structure of the flocculated cyanobacterial colonies, samples were fixed and dehydrated as described for SEM, and were then embedded in epoxy resin (Quetol). The material was treated with half-concentrated resin for 1 h, followed by concentrated resin for 4h. The resin was allowed to polymerise for 48h at 60°C, and was then dried. Finally, the samples were sectioned with a diamond knife and contrasted with 4% aqueous uranyll acetate (15min) and lead citrate (5min). The resin blocks were then sectioned to produce smooth surfaces using a glass knife, and were then etched in NaOH dissolved in methanol (saturated solution) for 7min to remove the resin. Finally they were rinsed in methanol and dried. Slices of the resin blocks were then mounted on SEM slides, coated with gold and viewed using a Zeiss Ultra^{TM} 55.

2.7. Phosphate adsorption study

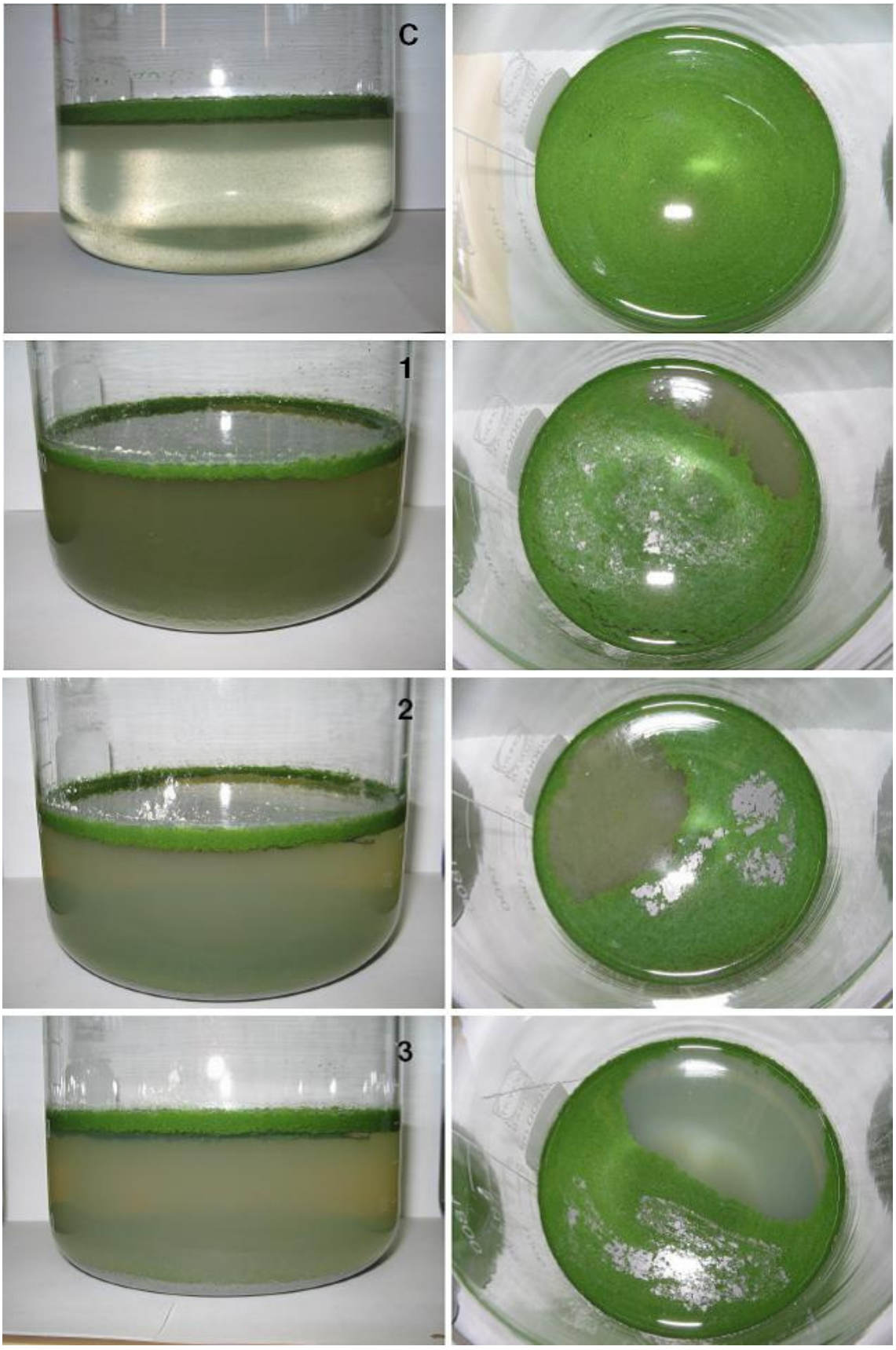
All glassware was prepared by rinsing once with 1M HCl and three times with distilled water to remove any residual phosphates from the glass surface. KH_2PO_4 was added to distilled water to make a 200mg.l^{-1} stock solution, and 50ml of this was added to 950ml

of distilled water in each beaker to give a final concentration of 10mg.l^{-1} PO_4-P (31.25mg.l^{-1} PO_4^{3-}). A 20:1 ratio of fly ash to PO_4^{3-} was used for treatment according to Oguz (2005), which was 625mg of ash per 1000ml. The samples were stirred continuously, and samples were taken at various time intervals after the addition of fly ash. 10ml was drawn up with a syringe and filtered through a 0.22µm filter disk into test tubes for PO_4-P testing. The phosphorus concentration of each sample was measured with the Spectroquant Phosphortest (PMB) 1.14848.001 (Merck), according to the manufacturer's instructions using the Photometer SQ118. The experiment was repeated with a 40:1 dosage of fly ash (1250mg ash per 1000ml), as well as with a higher initial PO_4-P concentration (20mg.l^{-1}) and 625mg of ash (10:1 treatment ratio).

3. Results

3.1. Flocculation experiments

The entire floating layer of cyanobacteria flocculated after the application of all 7 fly ash samples, but the fly-ash-cyanobacteria mixture separated into two layers after a few hours to form a floating top layer and a bottom layer. The top floating layer consisted of cyanobacteria and fly ash with low density, and the bottom layer of the fly ash particles more dense than water and the flocculated cyanobacteria. The results of flocculation with 5g (approximately 37.6mg.cm^{-2} surface area) of fly ash samples 1-7 48h after treatment are presented in Figure 1.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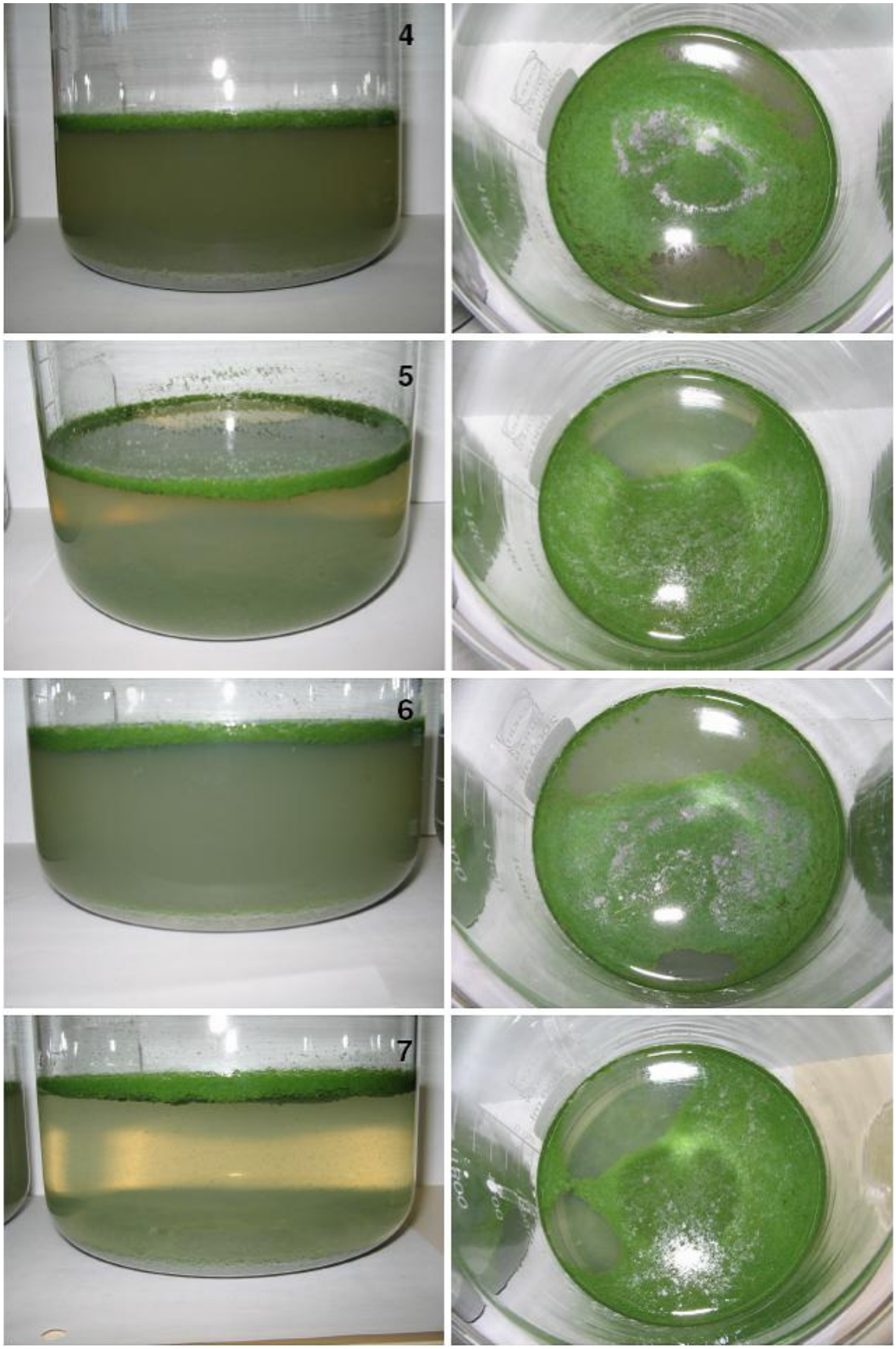


Figure 1: Results of flocculation tests 48h after addition of 5g of fly ash samples 1-7 (37.6mg.cm^{-2}). C is the negative control. The pictures on the left represent the view from above.

Water treated with fly ash samples 1, 2, 4 and 6 became turbid, darkened in colour and began to develop a strong odour within 6 hours after the treatment. Samples 3 and 5 only became turbid and darkened after 36h, and not to the same degree as samples 1, 4 and 6. Sample 7 became only slightly turbid and appeared to show an improvement after 60h. The water in the negative control remained clear with a floating cyanobacteria layer during the experimental period.

When the beakers were shaken after flocculation, some of the flocculated cyanobacteria floated to the surface again. This indicated that the attachment of the fly ash particles to the cyanobacteria was reversible in some cases.

The flocculation efficiency of the fly ash samples is presented in Table 3 and Figure 2. 5g, 6g and 8g of each fly ash were added to 600ml of water containing a similar amount of algae in order to determine which ash had the best flocculation efficiency. The treatment dosage was expressed in $mg.cm^{-2}$ surface area as well as $mg.cm^{-3}$ volume of algal layer.

Table 3: Cyanobacterial flocculation efficiency (%) after the addition of fly ash samples 1-7 at different dosages.

	5g 37.6mg.cm^{-2} 47mg.cm^{-3}	**6g 45.1mg.cm^{-2} 50.1 mg.cm^{-3}**	**8g 60.2 mg.cm^{-2} 57.3 mg.cm^{-3}**	**Average**	**Standard Deviation**
Control	22.6	7.3	5.3	11.7	9.5
1	50.5	53.5	50.1	51.4	1.9
2	57.9	79	53.2	63.4	13.7
3	49.7	51.7	57.2	53	3.9
4	60.3	54.8	58.3	57.8	2.8
5	55.3	62.2	66.8	61.4	5.8
6	59.8	80.8	86.3	75.6	13.98
7	53.8	48.7	66.5	56.3	9.2

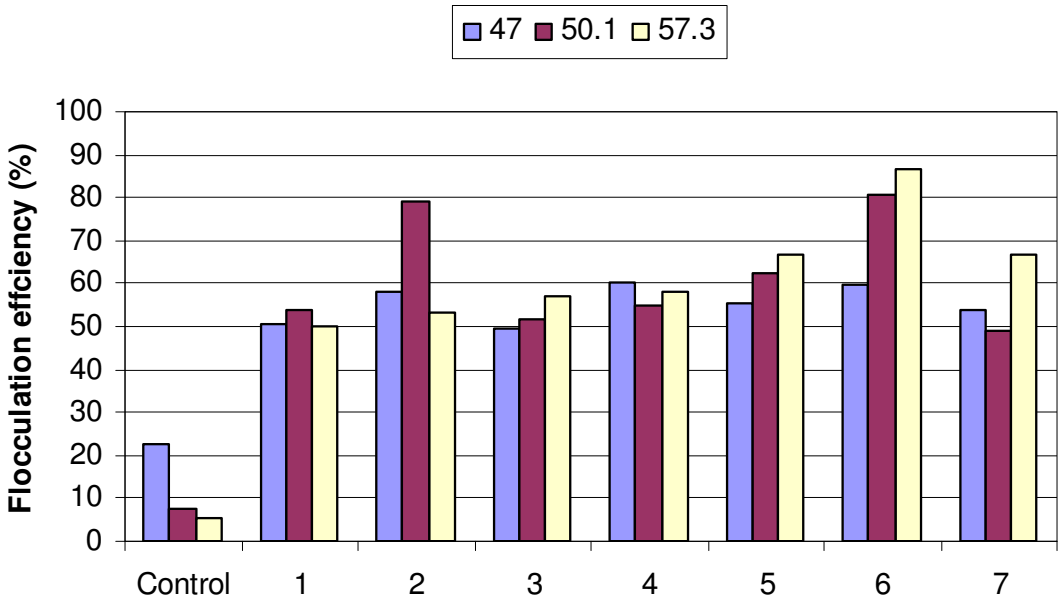


Figure 2: Flocculation efficiency of fly ash samples (1-7) at different dosages (mg.cm^{-3})

Sample 6 (Matla) showed the highest average flocculation efficiency for the dosages tested, although it also had the highest standard deviation. This ash was chosen to investigate the optimal fly ash dosage for optimal flocculation by varying the fly ash amount as well as the thickness of the cyanobacterial layer. Figure 3 shows the flocculation efficiency of the ash compared with the dosage, at two different algal layer thicknesses (3mm and 9mm). Increasing the fly ash dosage only increased the flocculating efficiency to a certain point, after which further addition of fly ash had no effect. The flocculating efficiency was greater for the thinner layer of cyanobacteria when compared with the thicker layer at the same dosage. The maximum flocculation of the 3mm layer was 95%, whereas that of the 9mm layer was approximately 65%. The optimal amount of fly ash was between 40mg.cm^{-3} and 50mg.cm^{-3}, depending on the thickness of the cyanobacterial layer.

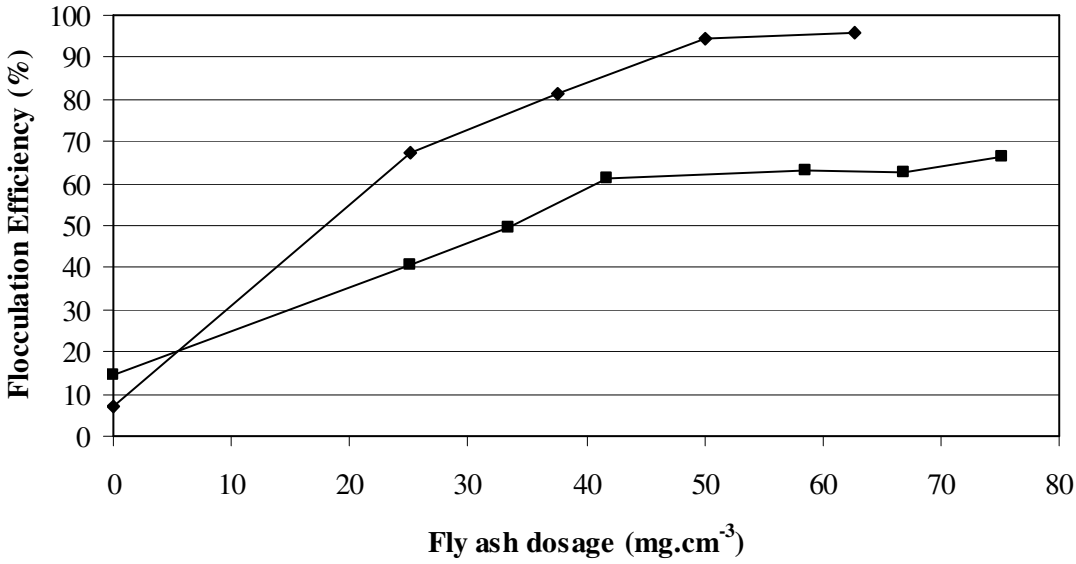


Figure 3: Flocculation efficiency of fly ash sample 6 at increasing concentration at two different cyanobacterial layer thicknesses (♦) 3mm thick (■) 9mm thick

3.2. Re-growth experiments

When BG-11 media was inoculated with cyanobacteria flocculated with fly ash samples 1, 2, 4 and 6, the media appeared pale green with few floating cells, whereas those inoculated with water treated with fly ash samples 3, 5 and 7 contained more floating cells the same colour as the control (Figure 4). The results of the re-growth experiments are shown in Table 4. After 6h, the cyanobacteria flocculated by all 7 ash samples were still alive as they showed growth in BG-11 media. However, after 36h, only cyanobacteria flocculated with fly ash samples 3, 5 and 7 were sufficiently viable to show growth.

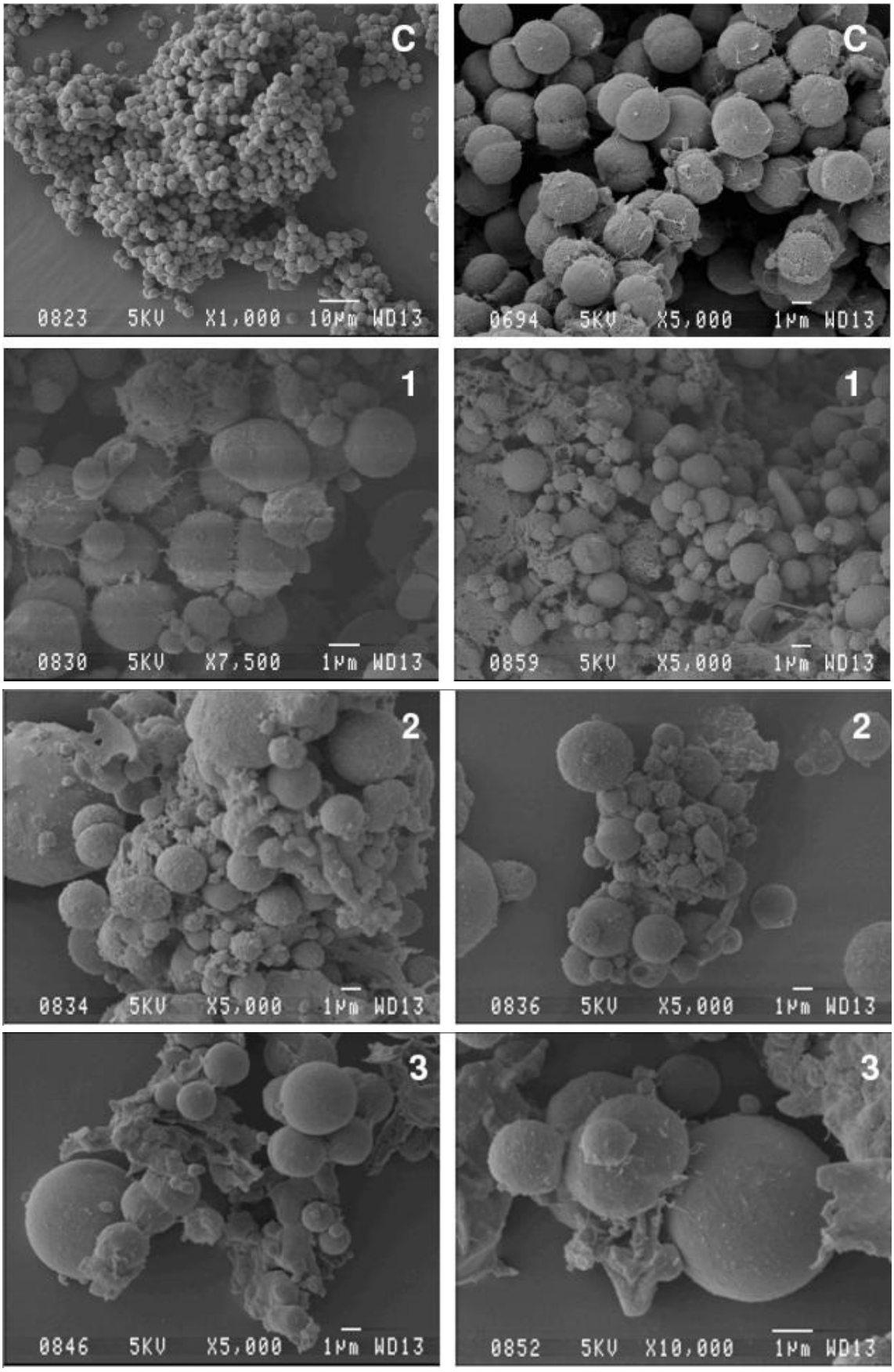
Figure 4: Cultures for re-growth experiment immediately after inoculation with cyanobacteria taken from the bottom of the beakers 36h after flocculation (a) inoculated with untreated cyanobacteria (Control); (b) inoculated with cyanobacteria treated with fly ash sample 5; (c) inoculated with cyanobacteria treated with fly ash 6.

Table 4: Re-growth of cyanobacterial samples taken 6h and 36h after flocculation with fly ash samples 1-7; (+) poor growth; + growth; □ no growth

	6h	36h
Control	+	+
1	+	□
2	+	□
3	+	+
4	+	(+)
5	+	+
6	+	□
7	+	+

3.3. SEM of flocculated cyanobacteria

SEM was used to observe the binding of the fly ash particles to the flocculated cyanobacterial cells (Figure 5). The floating cyanobacteria sampled from the untreated control were in large clusters, with cells in various stages of cell division. Extracellular polymers were visible on the cluster surfaces (Figure 5C). Cells sampled from the bottom of the untreated control that had sunk of their own accord (not shown) had a larger amount of extracellular material than the floating cells. Clusters were also observed in the SEM pictures of the flocculated cyanobacteria, but the cluster surfaces were composed mainly of fly ash. A few cyanobacteria were visible on the surfaces, distinguished by their surface properties and by the fact that some of the cells were in the process of dividing. The amount of cyanobacteria visible in the pictures was much less than expected, considering the volume ratio of fly ash to cyanobacteria.


C
0823 5KV X1,000 10µm WD13
C
0694 5KV X5,000 1µm WD13
1
0830 5KV X7,500 1µm WD13
1
0859 5KV X5,000 1µm WD13
2
0834 5KV X5,000 1µm WD13
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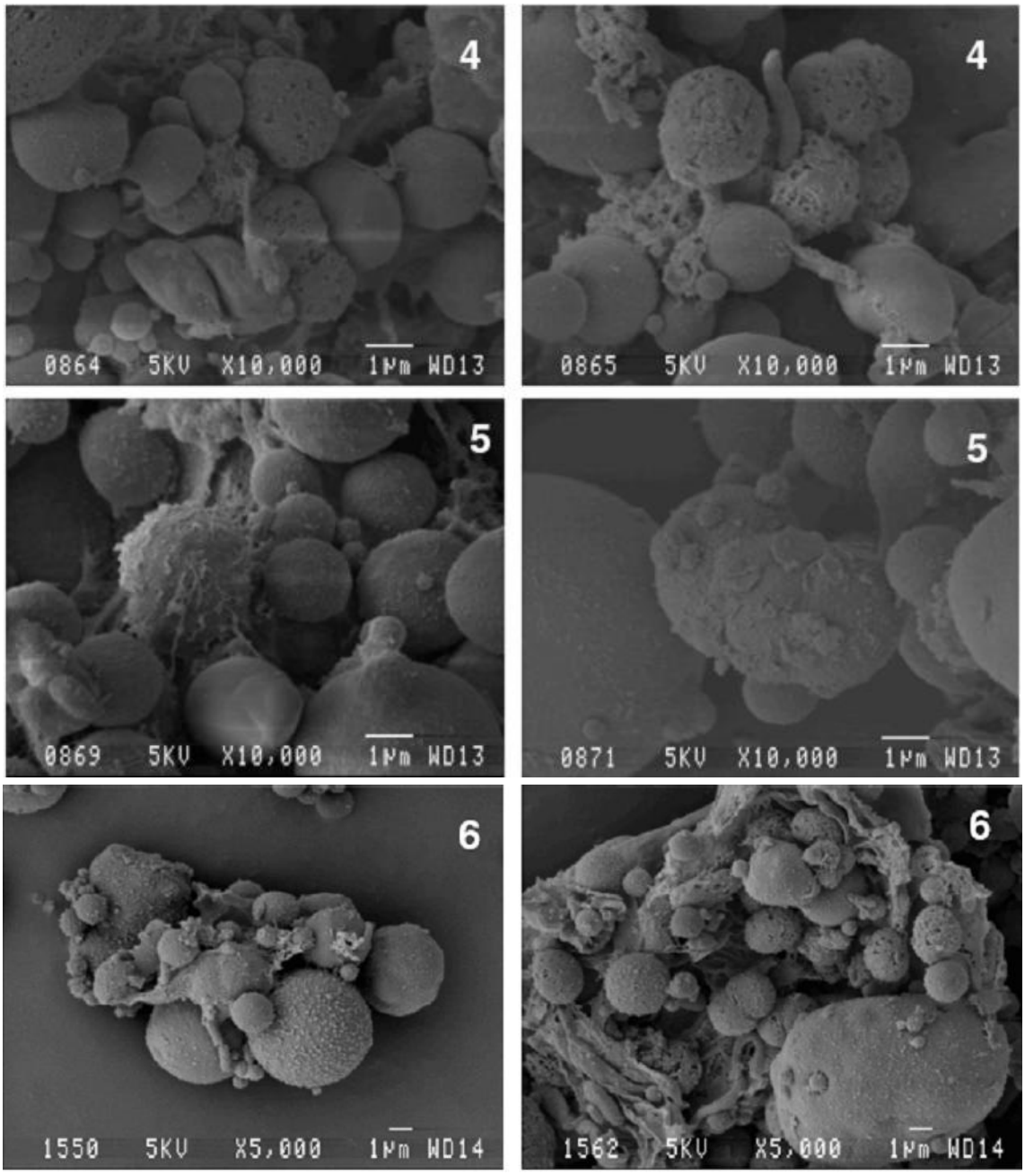
3
0852 5KV X10,000 1µm WD13


4
0864 5KV X10,000 1µm WD13
4
0865 5KV X10,000 1µm WD13
5
0869 5KV X10,000 1µm WD13
5
0871 5KV X10,000 1µm WD13
6
1550 5KV X5,000 1µm WD14
6
1562 5KV X5,000 1µm WD14

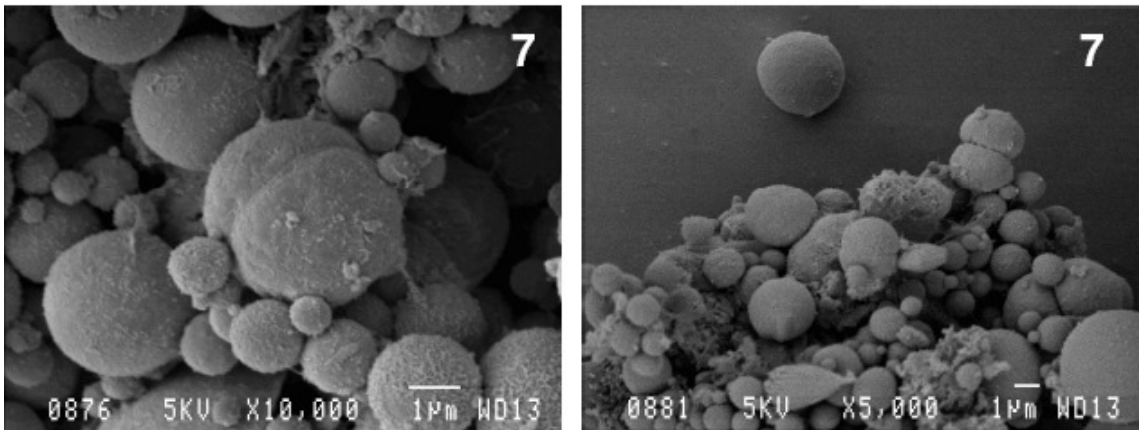


Figure 5: Scanning electron microscopy of the flocculated cyanobacteria and fly ash at various magnifications. There are two examples for each ash treatment, as well as for the untreated control (C).

Because it seemed likely that the cyanobacterial cell clusters were encapsulated by the fly ash particles, sticky tape was fixed to and removed from the mounted SEM slides in an attempt to pull the clusters apart to remove the outer fly ash layer and reveal the cyanobacteria cells. Figure 6 presents the results of the slides from fly ash 6 and fly ash 7. These pictures show that the cyanobacterial clusters were indeed surrounded by the fly ash particles, as more cyanobacteria were visible in the centre of each cluster. It was observed in these pictures that the cyanobacterial cells appeared healthier in the sample flocculated with fly ash 7 than that flocculated with fly ash 6.

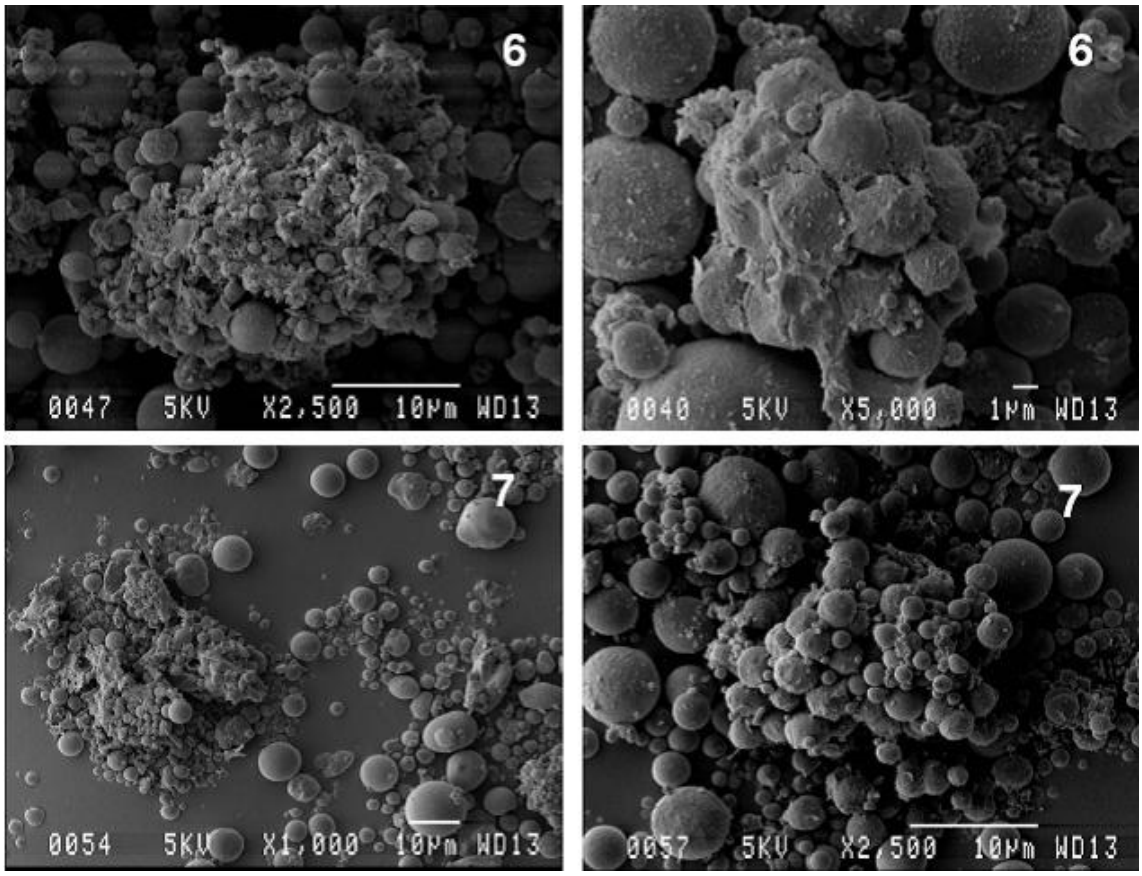


Figure 6: SEM pictures of flocculated cyanobacterial clusters from samples 6 and 7 broken up with tape

To further confirm the assumption that the fly ash particles enclosed the cyanobacterial clusters, new samples were embedded in resin which was cut to produce a smooth surface, and the resin was then etched away. When viewed under SEM, cell colonies in the untreated control displayed smooth edges, indicating that they were encapsulated in a layer of extracellular polymers (Figure 7: C1 and C2). In the pictures of the flocculated cyanobacterial cell clusters, spherical fly ash particles were visible on the edges as well as many broken pieces that appeared to be fly ash particles damaged during sample preparation (Figure 7: 6a and 6b).

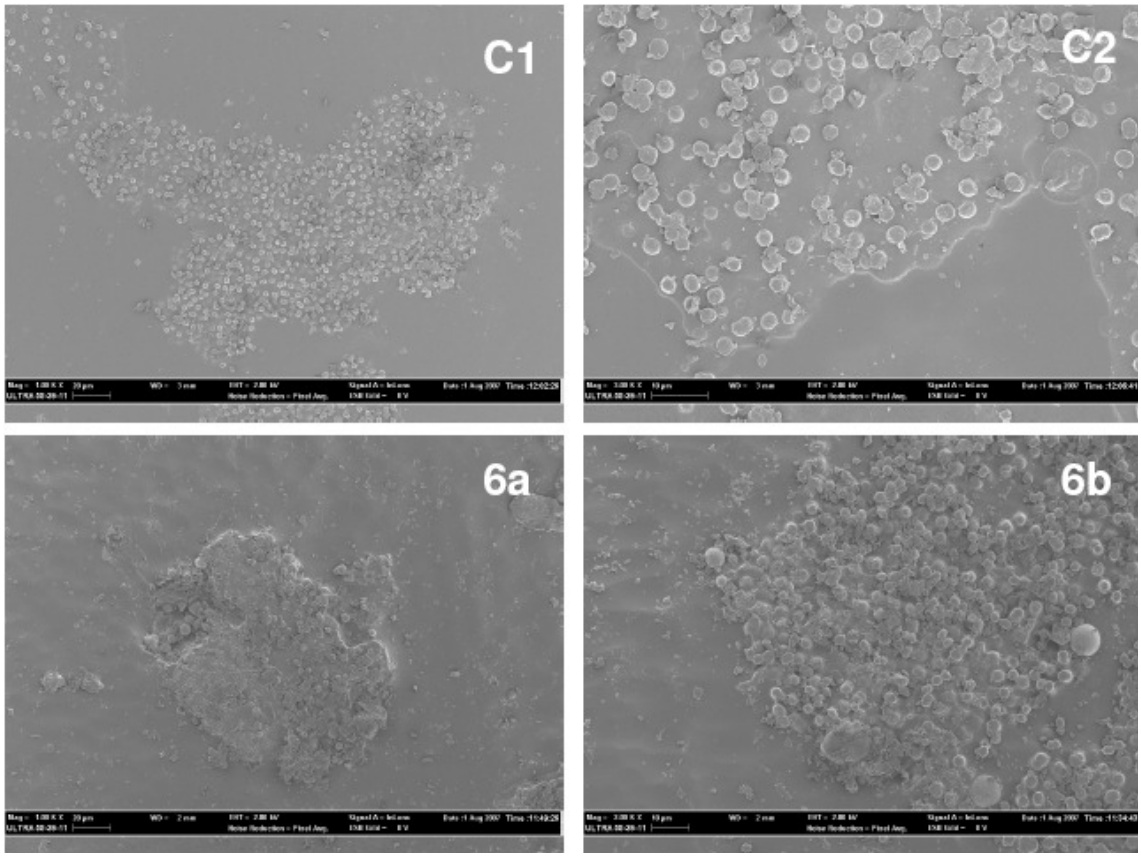


Figure 7: Scanning electron microscopy of the untreated control (C), and cyanobacterial colonies flocculated by fly ash sample 6 (6a and 6b). Samples were prepared by etching, and the magnification is as follows: C1: 1000x; C2: 3000x; 6a:1000x; 6b:2000x

3.4. Phosphate adsorption study

A 20:1 ratio of fly ash to PO_4^{3-} was used for treatment according to Oguz (2005), which was 625mg of ash per 1000ml water with a final concentration of 10mg.l^{-1} PO_4-P. The PO_4-P concentration was measured at various time intervals after the addition of the ash, but after 6h of continuous stirring there was no reduction in the PO_4-P. The experiment was then repeated with a 40:1 dosage of fly ash (1250mg ash per 1000ml water with a final concentration of 10mg.l^{-1} PO_4-P), and again no adsorption was apparent. Finally, in an attempt to increase the adsorption capacity of the ash, 625mg of the fly ash samples were added to a solution with a higher initial PO_4-P concentration of 20mg.l^{-1} (10:1 treatment ratio), and once again the PO_4-P concentration remained constant. These results were unexpected.

4. Discussion

All of the ash samples tested were able to flocculate the cyanobacteria to some degree, although sample 6 (Matla) had the greatest flocculation efficiency. The flocculation efficiency of this ash increased in a linear fashion with the amount of fly ash applied up to a point of maximum flocculation, after which further addition had no effect. For the Matla ash, the optimum amount of ash for maximum flocculating efficiency was approximately 45mg ash per 1cm^3 cyanobacterial biomass. This translates to 45g of fly ash per m^2 of surface area and 1mm thickness of the cyanobacterial layer.

Matla fly ash had the greatest percentage of small particles below 1µm (Chapter 7). According to the results from the XRD and XRF, there did not seem to be a significant difference between the samples in terms of their chemical properties. Thus, the flocculating efficiency is most likely directly related to the particle size, with the ash samples with the smallest particles being the most effective.

When the fly ash samples were added to water from the Hartbeespoort Dam (Chapter 7) there was a smaller increase in pH than when the ashes were added to distilled water, indicating that the dam water had a buffering effect.

The portion of the fly ash that was less dense than water remained floating on the surface, which would not be desirable in the treatment of a natural water body. This portion of the ash played no role in the flocculation of the cyanobacteria. In order to solve this problem, fly ash could be separated into two phases; that which is more dense than water and that which is less dense by first floating off the less dense phase and removing it (Kruger, 1996). The dense ash could then be filtered out and dried, and this phase used as a cyanobacterial flocculant.

The attachment of fly ash to some of the cyanobacteria was reversible when the beakers were shaken. This may pose a problem in a naturally occurring water body, as the normal mixing due to wind and fish activity could also cause detachment. In this case the cyanobacteria would not be retained at the bottom of the lake long enough to be killed by a lack of light or by the fly ash itself.

As can be seen by the SEM pictures, the cyanobacteria (*Microcystis aeruginosa*) form large colonies of cells. These colonies are enveloped in extracellular polymers, forming a protective layer. The mechanism of flocculation seemed to be related to this slime layer, as the fly ash particles appeared to stick to the outer surface of the colonies. Once sufficient fly ash had become attached to the outer surface of the colony it became too dense to remain floating, sinking to the bottom. The cyanobacteria may be able to overcome this by producing more gas vacuoles to increase buoyancy (Oliver, 1994) but the density of the fly ash may be too great to overcome. This appears to have been the case, as the cyanobacteria did not return to the surface, even after 48h. Vigorous shaking did release some cell colonies to the surface; these may have had less fly ash particles attached to them.

The re-growth experiments indicated that four of the seven fly ash samples (1: Tutuka; 2: Arnot; 5: Kendal and 6: Matla) caused cyanobacterial cell mortality within 36h of flocculation. Samples from these flocculation tests did not show re-growth in cyanobacterial growth media. However, the remaining samples (3: Duvha; 4: Hendrina; and 7: Lethabo) showed growth comparable to the media inoculated with the untreated control. The same samples that did not re-grow had a greater degree of turbidity, colouring and odour than the samples that were capable of growth. Furthermore, when examined with SEM, many of the cyanobacterial cells flocculated with fly ash sample 6 appeared to have damaged cell walls, when those flocculated with sample 7 (which showed re-growth) appeared to be healthy (smooth surfaces in various stages of cell division) and comparable to the control. It is possible that fly ash samples 3, 4 and 7 were capable of causing cyanobacterial cell mortality, but required more time than the 36h of the experiment.

One possible explanation for the killing effect seen with some ashes was the potential leaching of elements toxic to cyanobacteria. The pH of the water for the flocculation tests was above pH 7, therefore the results obtained for leaching in distilled water (Chapter 7) were expected to be similar to the leaching in the flocculation tests. Of the toxic elements (As, B, Cr, Hg, Ni, Pb and Zn), only B, Cr and Zn were present in solution, all others were below the detection limit of 0.01ppm. B was below 0.2ppm for all samples except for sample 5 (Kendal) at 1.09ppm and sample 7 (Lethabo) at 3.11ppm. The Cr concentration was the highest for samples 1 (Tutuka), 2 (Arnot), and 6

(Matla) at 0.24ppm, 0.12ppm and 0.27ppm respectively. None of the samples showed a Zn concentration above 0.1ppm in solution. These results correlate partially to the mortality results, in that samples 1, 2, and 6 leached the highest amount of Cr, and these did not show growth. However, the Cr concentration was low in sample 5, and this sample did not show growth either. The B concentration was high in this sample, but was higher in sample 7, which showed healthy re-growth. Palumbo *et al.* (2007) investigated the toxicity of several fly ash leachates using the Microtox© system, which is a standard biosensor based measurement technique for toxicity testing of water and soil. The method makes use of the luminescent bacterium *Vibrio fischeri* NRRL-11177. The luminescent bacteria were added to the leachates and the toxicity was measured by the decreased luminescence compared to a negative control. Of 8 leachates tested, which were leached at various pHs and contained both B and Cr, only one highly alkaline (12.4) leachate exhibited toxicity. This may also have been caused by the high pH, as the toxic effect was reduced when the leachate was neutralised. Therefore, although it is possible that the high concentration of Cr in the samples that did not show re-growth may have caused cell morbidity, it is unlikely when comparing the results from this study with those of Palumbo *et al.* (2007). However, cyanobacteria may be more sensitive to a high Cr concentration than *Vibrio fischeri.*

When the fly ash samples were leached in water at pH 2 (Chapter 7), more metals were leached than in distilled water, and a higher concentration of toxic elements was leached. However, the percentage of each toxic element that was leached from the fly ash samples was below 3% for all the elements. No Hg or Pb was leached, even at this low pH.

The concentrations of toxic metals leached in distilled water (Chapter 7) were above the DWAF target water quality range (TWQR) for human consumption as well as aquatic ecosystems. In acid water the concentrations of Al, As, Ca, Cr, Cu, Pb, Mg, Mn, Se and Zn greatly exceeded the TWQR for aquatic ecosystems. The amount of fly ash used in the leaching experiments was 50g per 1000ml (5% wt/vol). However, when 6g of the Matla fly ash was added to 600ml of water containing cyanobacteria, 81% of the cyanobacteria were flocculated. This translates to a 1% leaching solution, and the concentrations of toxic elements in the water can be expected to be less than those leached at high concentrations of ash.

Therefore, fly ash can potentially be used to flocculate cyanobacteria from a natural water body. The amount needed to achieve sufficient flocculation will have a negligible effect on the water chemistry because the elements leached will be highly diluted. The pH values of the sediment are seldom below pH 2, and the fly ash itself would have a neutralising effect on acidic sediments. A sediment pH of 2 is a "worst case scenario", and at the low relative dosages of fly ash needed for flocculation it is unlikely that the DWAF TWQRs would be exceeded.

When Agyei *et al*. (2002) and Chen *et al*. (2006) examined phosphate ion removal from solution using fly ash; they concluded that the extent of phosphate removal was related to the percentage CaO or Ca^{2+} ions in the ash. Oguz *et al*. (2005) used a 20:1 ratio of Yatagan fly ash (11.57% CaO) to PO_4^{3-}, and found that the phosphate removal efficiency was 99%, and the phosphate adsorption capacity 71.87mg.g^{-1}. The ash used by Agyei *et al*. (2002) consisted of 4.1% CaO, and more than 85% of the PO_4^{3-} was adsorbed from solution at a dosage ratio of 25:1. The fly ash samples used in this study had CaO concentrations which ranged from 3.41% to 6.9%. Although these concentrations were less than half the amount of CaO found in the Yatagan fly ash used by Oguz *et al*. (2005), no PO_4^{3-} was adsorbed by any of the fly ash samples tested, even at a treatment ratio of 40:1. This was unexpected, especially for samples 1, 2 and 6 which had CaO concentrations above 6.5%. Furthermore, the CaO concentrations of the ash samples were comparable to that of the ash used by Agyei *et al*. (2002), which consisted of 4.1% CaO. Even when the treatment ratio was more than double that used by Agyei *et al*. (2002), no adsorption was observed. Chen *et al*. (2006) also attributed a portion of the phosphate removal to the presence of Fe_2O_3. The ash samples 1-7 had a high Fe_2O_3 content ranging from 3.28% to 5.15%. It was not clear why the fly ash samples tested were not capable of adsorbing PO_4^{3-} from solution.

Activated carbon is often used to remove the toxins produced by cyanobacteria, as well as taste and odour compounds such as geosmin (Cook & Newcombe, 2004). Fly ash is capable of adsorbing toxic compounds, and so has potential for use in water treatment as well as in natural water bodies where the toxin level is above the recommended health standards as a result of a severe algal bloom.

5. Conclusion

Fly ash was generally an effective flocculant of cyanobacteria. Fly ash with a large amount of small particles was the most effective; in this study the ash from the Matla power station had the highest flocculation efficiency. The optimal dosage of Matla fly ash was 45g per m^2 of surface area and 1mm algal layer. The mechanism of flocculation appeared to involve the binding of the fly ash to the extracellular polymers on the surface of the cyanobacterial cell colonies, causing them to become too dense to remain afloat. Only the fly ash particles that were more dense than water were involved in the flocculation process, as the less dense particles remained floating on the surface. Fly ash added to water from the Hartbeespoort dam had a smaller pH increase than in distilled water. Four out of the seven fly ash samples tested caused cyanobacterial cell death after 36h. This was possibly related to the leaching of toxic elements, although only a small percentage of the total amount of trace elements were leached into solution, even at the low pH value of 2. This implies that the addition of fly ash to natural water bodies may not be hazardous, especially considering the added benefits of toxin removal from the water. None of the fly ash samples tested were capable of adsorbing phosphate from solution, despite the fact that the percentage of CaO in the samples was camparable to other ashes that showed a high phosphate adsorption efficiency

The results of this study cannot simply be extrapolated to a large scale treatment of a natural system. Future research questions should include the following:

- What causes cyanobacterial cell death, and would this affect other aquatic organisms?
- Would the concentrations of toxic elements leached into solution in a natural water body be high enough to affect other organisms (ie. be above the DWAF TWQR)?
- How would the natural mixing of a water body affect the permanence of cyanobacterial flocculation?

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CHAPTER 9:

GENERAL DISCUSSION

In this study, phosporus limitation was examined as a possible means for cyanobacterial bloom control. The methods that were investigated focused on phosphorus reduction and the effect of this reduction on cyanobacterial and eubacterial community structures in a natural water body, as well on the treatment and removal of cyanobacterial blooms.

Phoslock®, a lanthanum-modified bentonite clay capable of removing phosphorus by adsorption, was first characterised in the laboratory in terms of its kinetics and the effect of initial pH and phosphorus concentration on the adsorption capacity. The product was also tested in cyanobacteria-containing lake water with a high pH value under laboratory conditions in order to gain understanding of the behaviour of Phoslock® in a natural water body. Phoslock® was most effective between pH 5 and pH 8, with a decrease in the adsorption capacity above pH 9. This was attributed to the formation of the hydroxyl species of the lanthanum ions on the clay surface above pH 8.35, which decreased the number of phosphorus binding sites available. Phoslock® settled more rapidly at higher pH values, which also reduced the contact time with the phosphorus in solution. The negative effects of high pH could not be overcome by increasing the Phoslock® dosage, however in a natural eutrophic water body, the pH of the sediment is lower than the overlying waterbody, so Phoslock® is expected to reach equilibrium in the sediment. Phosphorus remained bound to Phoslock® under anoxic conditions.

Phoslock® was then tested under natural conditions in a field trial at Hartbeespoort Dam from January to December 2006. The FRP (filterable reactive phosphorus) was reduced by more than 50% in the 24h following Phoslock® application. There was no change in the control area over this period, so it can be concluded that Phoslock® was responsible for removing the phosphorus from the water, despite the high pH of the surface waters. Phoslock® had no effect on the pH or nitrate concentration of the treated area, as the results were similar to those of the control throughout the trial. From August 2006 the water temperature increased, but the phosphorus concentration remained low in the treated site compared to the control, even after a large amount of nutrient containing water entered the site after the first rains. The cyanobacterial growth was visible from much earlier in summer in the control area, and the bloom was more severe throughout the summer months. The low phosphorus concentration in the water body and the reduced concentration in the sediment therefore effectively reduced the incidence and severity of the algal bloom in the treated site.

In order to assess the effect of the Phoslock® treatment on the cyanobacterial and bacterial species composition, a 16S PCR-DGGE analysis was performed on the field trial site. Samples were taken monthly from the treated and control areas from July 2006 until February 2007. Cyanobacterial specific 16S rDNA primers were used to amplify cyanobacterial DNA (Nübel *et al*., 1997), and general bacterial 16S rDNA primers (Muyzer *et al*., 1993; Fjellbirkeland *et al*., 2001) were used to amplify DNA from the entire bacterial population, including the cyanobacteria. DGGE profiles of each of the monthly samples were generated and analysed. It could be seen from the results that it was necessary to use cyanobacterial specific primers to analyse the cyanobacterial community composition by DGGE, as general bacterial primers did not give a detailed picture of the cyanobacterial species present in a sample. Using the 16S rRNA gene as a target was practical, as this sequence database is the largest. However, for the *Microcystis* spp., the resolution was low with this gene region, so it was concluded that DGGE of the rRNA-ITS region should be considered if a more detailed *Microcystis* profile is required (Janse *et al*., 2003). The lower phosphorus concentration in the treated area of the field trial encouraged the presence of diatoms, which are indicators of healthy species diversity. Unicellular cyanobacteria were present in both the treated and control areas, but there appeared to be a lag in the appearance of these species in the treated area. The different trophic levels of the treated and control areas affected the filamentous cyanobacterial population. Filamentous species were more prevalent in the treated area during the summer months than in the control area, and the treated area had a higher species diversity. The cyanobacterial species composition was thus affected by the Phoslock® treatment. As the cyanobacteria became more dominant in the treated and control areas from October, there appeared to be a shift in the bacterioplankton population. Species of Actinobacteria and Bacteroidetes were present in both the treated and control areas only until October, with one species of Actinobacteria only being present in the treated area. From November, the bacterioplankton population was dominated by β- and δ-proteobacteria. The Phoslock® treatment itself did not appear to affect the bacterial population, as the treated and control areas displayed similar patterns. For both the cyanobacteria and the bacterioplankton, the greatest effect on the species composition was in fact the seasonal change from winter to summer, as expected.

A bacterial species that was isolated from Hartbeespoort Dam that appeared to have cyanobacteriolytic activity was identified as *Bacillus cereus.* The cyanobacteriolytic nature of this species against *Microcystis aeruginosa* has previously been documented in the literature. Nakamura *et al*. (2003) found that the substance responsible for the lytic activity was produced in the stationary phase of growth, was non-proteinaceous, hydrophilic and heat stable, with a molecular weight less than 2kDa. It was thought that the bacteria attached to the surface of the cyanobacteria to first cause aggregation of cyanobacterial cells before lysis with extracellular products. The bacteria used in this study required contact for lysis, as with *B. cereus* in the studies performed by Nakamura *et al*. (2003) and Shunyu *et al.* (2006), but aggregation of the cyanobacteria was reduced in treated flasks. This may indicate that the strains were different, with the lytic substance and mechanism of lysis differing between these two organisms. The critical predator-prey ratio was 1:1 (cyanobacteria to predatory bacteria), as lower ratios of bacteria to *M. aeruginosa* did not cause the cyanobacterial population to decrease, although ratios of 1:10 and 1:100 kept the cyanobacterial population steady. A 1:1 ratio reduced the cyanobacterial population by 50% over a 14 day period, even though the bacterial population was seen to double in this time. A higher initial dosage may result in a higher degree of cyanobacterial cell death. *Bacillus cereus* was able to use *Microcystis aeruginosa* as its only nutrient source. This is of great importance in terms of the formation of a biological control product, as no addition nutrients will need to be supplied to the bacteria. No field trials have been performed to determine the effectiveness of this organism on a large scale, and laboratory tests cannot simply be extrapolated, especially because the predator-prey ratio appears to be important. The undertaking of field trials is therefore essential to determine the success of this organism as a biological control agent.

When Phoslock® and the cyanobacteriolytic bacteria were combined in a bacterial culture, Phoslock® had no effect growth rate of the bacteria. However, when the two agents were combined to assess the possibility of synergism, treatment with both Phoslock® and bacteria was no more effective than bacteria alone, and Phoslock® alone was more effective than either treatment with bacteria or with a combination of Phoslock® and bacteria. There is therefore no synergistic effect when these agents are used in combination, and Phoslock® was the most effective treatment method. The fact that the bacterial numbers increased to four times their original number in the

combination treatment, compared with only a doubling in number in the bacteria treated flask, may be due to the increased surface area for growth provided by the Phoslock®. Phoslock® could therefore be the vehicle for the bacteria as it does not affect the growth of the bacteria, and in fact promotes growth by providing a surface area for attachment. In addition, when used in combination, the phosphates released from the lysed cyanobacterial cells would be immediately adsorbed by Phoslock®, thus minimising any increase in the soluble phosphorus concentration in the water body and preventing further blooms.

Fly ash was an effective flocculant of cyanobacteria. Fly ash with a large amount of small particles was the most effective; in this study the ash from the Matla power station had the highest flocculation efficiency, and had an optimal dosage of 45g per m^2 of surface area and 1mm algal layer. The fly ash particles attached to the extracellular metabolites on the surface of the cyanobacterial cell colonies, causing them to become too dense to remain afloat. Four out of the seven fly ash samples tested caused cyanobacterial cell death after 36h. This was possibly related to the leaching of toxic elements, although only a small percentage of the total amount of trace elements were leached into solution, even at pH 2. The addition of fly ash to natural water bodies may not be hazardous, especially considering the added benefits of potential toxin removal from the water. As with the cyanobacteriolytic bacteria, field trials are necessary with the fly ash in order to determine the effect on a large body of water as well as whether the flocculation would be permanent in the turbulent conditions of a natural water body.

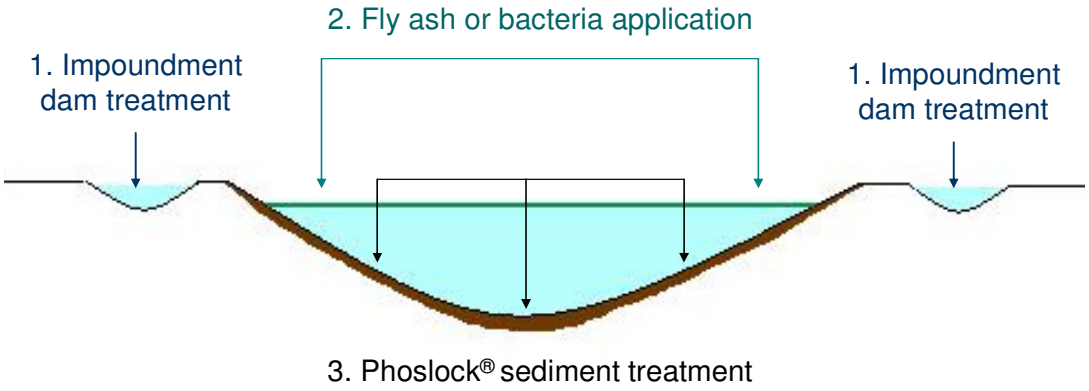
Phosphorus limitation using Phoslock® is a valuable tool for cyanobacterial bloom control. However, to treat a dam such as Hartbeespoort Dam with Phoslock® would be costly and logistically challenging. Combining Phoslock® with other control methods in an integrated manner may present a viable solution. The following treatment plan is therefore recommended for Hartbeespoort Dam:

1. Impoundment dams should be constructed at the mouths of each of the three rivers flowing into the dam, and the water in these dams treated with Phoslock® before being allowed to enter the dam. This will help to minimise further phosphorus input and prevent the problem from worstening.
2. Cyanobacteriolytic bacteria or fly ash should be applied to the surface cyanobacterial bloom in order to cause cell lysis, which will release the

phoshorus stored in the cyanobacterial cells. The maximum amount of phosphorus will therefore be available to be adsorbed during a Phoslock® application.

3. Ideally, a full Phoslock® dosage should be applied at this point, to remove the soluble phosphorus from the water body as well as to form a sediment cap to prevent future recycling of phosporus from the sediment. However, in order to minimise cost, it is recommended that Phoslock® be applied to the sediment only, by means of submerged pipes at sediment level. Although this will not be as effective as treating both the water and the sediment, recycling of phosphorus will be minimised. This strategy, combined with the prevention of new phosphorus inflow using impoundment dams, should cause the dam to move towards a more mesotrophic state.

This remediation plan can be illustrated schematically:



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CHAPTER 10:

CONCLUSION

The rehabilitation of a eutrophic water body cannot involve the use of a single strategy. Instead, an integrated rehabilitation plan is essential in order to ensure that the immediate problem of high nutrient concentrations and toxic algal blooms are dealt with as well as the long term goal of limiting nutrient input. The methods of eutrophication control that were discussed in this study target various stages of such an integrated eutrophication management plan.

Phoslock® treatment of a eutrophic water body should form an integral part of rehabilitation, as it resets the ecological clock by returning a water body to its natural, mesotrophic state. This is essential, as limiting point sources is simply not enough; it will take many years for a highly eutrophic water body to improve even if all incoming nutrient sources are stopped completely, as the sediment itself acts a source. Phoslock® removes soluble phosphorus from the water body, and forms a layer on the sediment preventing release of phosphorus back into the overlying water. The positive effect of Phoslock® was demonstrated in the field trial at Hartbeespoort Dam, where the soluble phosphorus remained significantly lower in the treated area than in the control. The reduced phosphorus availability also affected the cyanobacterial growth, which was reduced in the treated area and began later in the summer.

The DGGE results confirm that the nutrient status of the Phoslock® treated area versus that of the control affected the cyanobacterial population composition. More filamentous species were present in the treated area than in the control area, where only unicellular species were present. The lower phosphorus concentration in the treated area encouraged the presence of diatoms, which are indicators of a healthy ecosystem as they are sensitive to the N:P ratio.

Biological control with cyanobacteriolytic bacteria and flocculating the cyanobacteria with fly ash are methods that focus on treating the symptoms of eutrophication. Neither of these treatments are capable of completely removing an algal bloom, but both provide possible solutions to the immediate aesthetic problem. Fly ash can potentially adsorb the toxins produced by many bloom-forming cyanobacteria, and therefore may have the added benefit of improving the water quality while removing the algae. Fly ash may also be used in waste water treatment for this purpose.

Treatment of a cyanobacterial bloom with cyanobacteriolytic bacteria or fly ash will cause lysis of the cyanobacterial cells, resulting in the release of stored phosphorus. Therefore, it would be useful to apply one or both of these agents to a cyanobacterial boom before a Phoslock® treatment, to ensure that the maximum amount of phosphorus in available in solution for adsorption onto the Phoslock® surface.

Future research goals arising from this research include the following:

- The reason for cyanobacterial death when treated with certain fly ash samples needs to be determined.
- Field trials need to be performed with the cyanobacteriolytic bacteria as well as with the fly ash, as laboratory data cannot safely be extrapolated to large scale conditions.
- The effect of fly ash leaching in a large water body needs to be clarified.
- The effect of fly ash on the cyanobacteriolytic bacteria should be examined if these two agents are to be used in conjuction, as the fly ash may have a killing effect on the bacteria as observed in the cyanobacteria treatments.
- The potential use of Phoslock® as a vehicle for the biological control agent should be investigated.
- The potential ability of fly ash to adsorb cyanobacterial toxins such as microcystin must be tested, as this may be a promising alternative to activated carbon in water treatment.

RESUMÉ

Of the problems currently being experienced with natural and man-made water bodies, eutrophication is one of the most important. Eutrophication is the enhancement of the natural process of biological production in rivers, lakes and reservoirs, caused by an increase in nutrient levels, usually phosphorus and nitrogen compounds. These increased nutrient levels usually result in an increased phytoplankton biomass, which is often dominated by toxic cyanobacterial species. Eutrophication has a severe impact on the water quality and impairs the use of water for drinking, industry, agriculture and recreation.

The management of a eutrophic water body usually involves treating toxic algal blooms, as well as controlling nutrient input. However, reducing nutrient input as well as the internal source is the only feasible means of long term eutrophication management, as in many shallow lakes the phosphorus accumulated in the sediment may be many times greater than that in solution. In this study, Phoslock®, a lanthanum-modified bentonite clay capable of removing phosphorus by adsorption, was characterised in the laboratory in terms of its kinetics and the effect of initial pH and phosphorus concentration on the adsorption capacity. The product was also tested in cyanobacteria-containing lake water with a high pH value under laboratory conditions in order to gain understanding of the behaviour of Phoslock® in a natural water body. Phoslock® was most effective between pH 5 and pH 8, with a decrease in the adsorption capacity above pH 9. Furthermore, phosphorus was not released under anoxic conditions. Phoslock® was then tested in a field trial at Hartbeespoort Dam, and the soluble phosphorus concentration was successfully reduced from 0.2mg.l^{-1} to below 0.05mg.l^{-1}, the threshold for cyanobacterial bloom formation. Cyanobacterial growth was visible from much earlier in summer in the control area, and the bloom was more severe throughout the summer months. The low phosphorus concentration in the water body and the reduced concentration in the sediment therefore effectively reduced the incidence and severity of the algal bloom in the treated site.

Limiting the amount of phosphorus in a water body, and thus increasing the N:P ratio, was likely to affect the entire microbial community composition, not only that of the

cyanobacteria and algae. Samples were taken monthly from the Phoslock® field trial site between July and February, and the effect of reduced phosphorus concentration on the cyanobacterial and eubacterial community composition was examined using denaturing gradient gel electrophoresis (DGGE). Unicellular cyanobacteria were present in both the treated and control areas, but there was a lag in the appearance of these species in the treated area. The different trophic levels of the treated and control areas affected the filamentous cyanobacterial population, as filamentous species were more prevalent in the treated area during the summer months than in the control area, and the treated area had a higher species diversity. As the cyanobacteria became more dominant in the treated and control areas from October, there appeared to be a shift in the bacterioplankton population. Species of Actinobacteria and Bacteroidetes were present in both the treated and control areas only until October, with one species of Actinobacteria only being present in the treated area. From November, the bacterioplankton population was dominated by β- and δ-proteobacteria. The Phoslock® treatment itself did not appear to affect the bacterial population, as the treated and control areas displayed similar patterns. For both the cyanobacteria and the bacterioplankton, the greatest effect on the species composition was in fact the seasonal change from winter to summer, as expected.

A bacterial species that was isolated from Hartbeespoort Dam that appeared to have cyanobacteriolytic activity was identified as *Bacillus cereus.* The cyanobacteriolytic nature of this species against *Microcystis aeruginosa* has previously been documented in the literature. The bacteria used in this study required contact for lysis, as in previous studies, but aggregation of the cyanobacteria was reduced in treated flasks. This may indicate that the strains were different, with the lytic substance and mechanism of lysis differing between these two organisms. The critical predator-prey ratio was 1:1 (cyanobacteria to predatory bacteria), as lower ratios of bacteria to *M. aeruginosa* did not cause the cyanobacterial population to decrease, although ratios of 1:10 and 1:100 kept the cyanobacterial population steady. A 1:1 ratio reduced the cyanobacterial population by 50% over a 14 day period, even though the bacterial population was seen to double in this time. *Bacillus cereus* was able to use *Microcystis aeruginosa* as its only nutrient source. This is of great importance in terms of the formation of a biological control product, as no addition nutrients will need to be supplied to the bacteria.

The combination of this potential biological control agent with Phoslock® was investigated in order to determine whether the two agents could be used together to treat both the cause and symptoms of eutrophication simultaneously. When Phoslock® and the cyanobacteriolytic bacteria were combined in a bacterial culture, Phoslock® had no effect on the growth rate of the bacteria. However, when the two agents were combined to assess the possibility of synergism, treatment with both Phoslock® and bacteria was no more effective than bacteria alone, and Phoslock® alone was more effective than either treatment with bacteria or with a combination of Phoslock® and bacteria. There is therefore no synergistic effect when these agents are used in combination, and Phoslock® was the most effective treatment method.

Various flocculants have been investigated for cyanobacterial removal in wastewater treatment as well as in natural water bodies. These include synthetic organic polyelectrolytes, chitosan, and various clays. In this study, fly ash, a waste product in the burning of coal for electricity generation, was investigated as a potential cyanobacterial flocculant. Samples from seven different power stations were tested, and it was found that the ash with the smallest particle size had the highest flocculation efficiency; between 65 and 95% depending on the thickness of the algal layer. Four out of the seven fly ash samples tested caused cyanobacterial cell death after 36h. This was possibly related to the leaching of toxic elements, although only a small percentage of the total amount of trace elements were leached into solution, even at pH 2. The addition of fly ash to natural water bodies may not be hazardous, especially considering the added benefits of potential toxin removal from the water. As with the cyanobacteriolytic bacteria, field trials are necessary with the fly ash in order to determine the effect on a large body of water as well as whether the flocculation would be permanent in the turbulent conditions of a natural water body.

The various methods for remediating both the causes and symptoms of eutrophication that were investigated in this study can all potentially reduce the impact of eutrophication on natural water bodies. However, it is unlikely that any single technique used in isolation would allow a eutrophic water body to return to its natural mesotrophic state. Instead, the combination of techniques addressing both the cause and the result of eutrophication will increase the likelihood of successful remediation.

Appendix A

1. Sequences obtained from DGGE bands in Chapter 5

1.1 Partial 16S rDNA sequences obtained from from bands in the cyanobacterial specific DGGE gels, and their accession numbers in GenBank

1a (EU94509)

CAGCCAACCGCTTCGCAATGGGGTTCTTTTAAAGCCACAATTTCACGCTCCC
TGGNAATTCCCTTTACTTTCTATACTCTAGTCTAATAGTTTCGACTGCGATTT
TGAAGTTGAGCTTCAAGATTTAACAGTTGACTTATTAAACCACCTACAGACG
CTTTACGCCCAGTGATTCCGGATAACACTTGCATCTTCCGTCTTACCGCGGC
TGCTGGGACGGAGTTAGCCGATGCTTATTCTCCAGGTACACGTCCTTTTGTT
CCTCCCTGAAAAAAGAGGTTTACAACGCATAGGCCGGTATCCCTCAGGCGA
GATTGCTCCGTCANTTTTCAAACAATGCGGAAGTTCCCCCGGGCGAGTCGGC
CTGCCGCCGG

2a (EU94510)

GTTCGGCCCAGTACCCACGTTTCGCTATGGGGTTCTTTTCANNNATACCAAT
TTCACCGCTACACTGGGAATTCCTGCNTCTTCTACTGCTCTCTAGTCTGCCAG
TTTCCACTGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCAGACTTGGCT
GACCACCTGCGGACGCTTTACGCCCAATAATTCCGGGTAACGCTTGCCTCCC
CCGTCTTACCGCGGCTGCGGGGACGGAGTTAGCCGAGGCTTATTCCTCAGGT
ACCGTCAGAACTTCTTCCTTGAGAAAAGAGGTTTAAAATCCAAAGACCTTCC
CCCCCTCACGCGGTGTTTCCCCATCAGGTTTTCGCCCATTGCGCAAAAATCC
CCCCGGGGGG

3a (EU94511)

CAGTTCGGCCCCTACACGCTTTCGCACTGAGGATCTTNNNCNCTAGGCATTT
CACCGCTACACTGGGAATTCCTGTTACCCCTAGTGCTCTCTAGTCTGCCAGT
TTCCACTGCCTTTAGGTCGTTAAGCATCCTGATTTGACGGCAGACTTCGTTG
ACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCCC
CGTATTACCGCGGCTGCTGGCACGGATTTAGCCGAGGCTTATTCCTCAGGTA

CCGTCAGAACTTCTCCTTTGAGAAAAAAGGTTACAATCCAAAGCTCTTCCTC
CCTCACGCGGTGGTTCTCCCTCAGGTTTTCCCCCATTGCG

4a (EU94512)

ATTTCGCCACTGGGGAAAGNAANCNCTACCCATTTCACCGCTACACTGGGA
ATTCCGGCTACCCATACTGTTTTTTAGTCTGCAAGTTTCCACCGCCTTTAGGT
CGTTAAGCAACCTGATACTTGTCTGACCACCTGCGGACGCTTTACGCCCAAT
AATTCCGGATAGCGTTTGCCTCCCCCGTATTACCGCGGCTGCTGGAACGAAT
TTAGACAAGGCTGATTCCTCAAGTACCGTCANAACTTCTTCCTTGAGAAAAG
AGGGGACAATCCAAACTCCTTCCTACCGACGAAATGTTTCTCGAACAGGAA
TAACCCCATTGCGGAAAGTTCCCCCGGGCGGGGGCGG

5a (EU94513)

TTTCGCATGAGTTCTNNAACCNACGAATTTACCCTCCTGGGAATTCCTGCTA
CCCTTACTGCTCTCTAGTCTGCCAGTTTCCACCGCCTTTAGGTGGTTAAGCA
ACCTGATTTGACGGCAGACTTGGCTGACCACCTGCGGACGCTTTACGCCCAA
TAATTCCGGATAACGCTTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGA
GTTAGCCGAGGCTGATTCCTCAAGTACCGTCAGAACTTCTTCCTTGAGAAAA
GAGGTTACAATCCAAAGACCTTCCTCCCTCACGCGGCGTTGCTCCGTCGGGT
TTTCCCCCATTGCGAAAAATTCCCCCGGGCGGGGGCTGT

6a (EU94514)

ACTGGGGTCCTAATCCCTTGTTCCCCGGGGTTTTCTTNAAANCNNAGGCTTT
ACCGCTACACCTGGATTCCTCCTGNNCTATCNCTCTCTAGTCTCACAGTTTCC
ATTGCCGATCCAAGGTTGAGCCTCGGGCTTTGACAACAGACTTATCAAACA
GCCTACGTACGCTTTACGCCCAATAATTCGGGATAACGCTTGCATCCTCCGT
CTTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTTATTCGTCAGGTACCG
TCATTACCTCCCCTAACAAAAAAGGTTTACAACCCACCGGCCCTCGTCCCTC
CAACGGTTTTGTCCCCCCAGGGGTTTGCCCCTTNCGAAAATTCCCCC

7a (EU94515)

CCCAGTTGCACCTTGGTGTTCTGANGNGNCTCCGCATTTCACCGCTACACCG
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CAAGGTTGAGCCTCGTGCTTTGACAACAGACTTATCAAACAGCCTACGTAC

GCTTTACGCCCAATAATTCCGGAATAACGCTTGCATCCTCCGTCTTACCGCG
GCTGCTGGCACGGAGTTAGCCGATGCTTATTGTCAGGTACCGTCATTATCTT
CCTTAACAAAAAAGGGGTACAACCCACAGGCCTTCTTCCCTCACGCGGTATT
GCTCCGTCAGAGTTTCGC

8a (EU94516)

AGTTCAGTCCAGCACCCGCTTTCACCACTGGTGTTCTTGTAGAGNATACGCA
TTTCACGCTACACCGGGAATTCCTCCTGGCCTATCTATCTCTAGTCTNACAG
TTTCCATTGCCGATCTAAGGTTGAGCCTCGGGCTTTGACAACAGACTTATCA
AACAGCCTACGTACGCTTTACGCCCAATAATTCCGGATAACGGTTGCTTCCT
CCGTCTTACCGCGGCTGCTGGGACGGAGTTAGCCGATGCTTATTCGTCAGGT
ACCGTCATTATCTTCTCTAAAAAAAAGAGAGAACAACCGACAGGGCTTCGT
CCCTCACGCGGGATTGCTCCCTCAGGGTTTTGCCAATAGCCCAAAATTCCCC
CGGGCGGGGGGGGTGTGACCTGAGCGTGGCGCCCCGGGGAAGTTTCG

9b (EU94517)

AGTGTTAGTNATAGCCCAGTAAAGTGCCTTCGCCATCGGTGTTCTTTNNANA
NCTACGCATTTCACCGCTCCACTGGAAATTCCCTTTACCCCTACTATACTCTA
GTCTAATAGTTTCGACTGCTGTTTTGAGGTTAAGCCTCAAGATTTAACAGTT
GACTTATTAAACCACCTACAGACGCTTTACGCCCAGTGATTCCGGATAACAC
TTGCATCCTCCGTCTTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTTAT
TTTTCAGGTACACGTCATTTTTTTCCTCCCTGAAAAAAGAGGTTTACAACCA
GGGGGGTTTTCTCCCCACGGGGGTTTTCCCCCC

10b (EU94518)

GTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCNNNNCN
CTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTA
GTCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCA
GACTTGGCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGC
TTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGAT
TCCTCAAGTACCGTCAGAACTTCTTCCTTGAGAAAAGAGGTTTACAATCCAA
AGACCTTCCTCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCACATTGCGG
AAAATTCCCC

11b (EU94519)

AGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCCNANN
CNCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTC
TAGTCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACAg
CAGACTTGGCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAAC
GCTTGcCTCCCCCGTATTACcGCGGCTGctGGcACGgAGTTAGccgAgGcTgATTC
ctCAaGTACCGtCaGAaCTTCTTCCtTGAGAAAAGAGGtTTACAATCCAAAGACC
TTCcTCCCTCCcGcGGCGTTGCTCCGTCAGgcTTTCGCccATTGCGGAAAATTCC
CCCGGGcGGG

12b (EU94520)

GTCAGATACAGCTCAGTAGCAGCTTTCGCCACCGATGTTCTTCNAANCTCTA
CCATTTTACCGCTACCTGGGAATTCTGCTATCCTACTGCTCTCTAGTCTGCCA
GTTTCCACCGCCTTTAGGTGGTTAAGCCACCTGATTTGACAGCAGACTTGGC
TGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCC
CCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTATTCCTCAAG
TACCGTCAGAACTTCTTCCTTGAGAAAAGAGGTTTACAATCCAAAGACCTTC
CTCCCTCACGCGGCGTTGCTCCGTCAGGTTTTCGCCCATGCGGAA

13b (EU94521)

GTGTCAGATACAGCCCAGCAGGACGCTTTCGCCACTGGTGTTCTTCCCAATA
TCTACGCATTTCACCGCTACACTGGGAATTCCTGCTGCCCCTACTGCTCTCTA
GTCTGCCAGTTTCCACTGCCTTTAGGAGGTTAAGCATCCTGATTTGACAGCA
GACTTGTCTGACCGCCTACGGACGCTTTACGCCCAATAATTCCGGATAACGC
TTGCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGAT
TCCTCAGGTACCGTCAGAATTTTTTCTTTGAGAAAAGAGGTTTACAATCCAG
AGATCTTTCTCCCTCACGCGGTGGTGCTCCCTGAGGTTTTCCCCTAT

14b (EU94522)

GTCAGATACAGCCCAGTAGGACGCTTTCGCCACTGGTGTTCTTCNGAAANCT
ACGCATTTCACCGCTACACTGGGAATTCCTGCTGCCCCTACTGCTCTCTAGT
CTGACAGTTTCCACTGCCTTTAGGAGGTTAAGCCTCCTGATTTGACAGCAGA
CTTATCAAACCGCCTACGGACGCTTTACGCCCAATAATTCCGGATAACGCTT
GCCTCCTCCGTCTTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTATTC

CTCAGGTACCGTCAGAATTTCTTCCTTGAGAAAAGAGGTTTACAATACAAA
GACTTTCCTCTCTCACGCGGTGGTTCTCCCTGGGGTTTTCC

15b (EU94523)

GTCAGATACAGCCCAGCAGGACGCTTTCGCCACTGGTGTTCTTCCAGAATCT
ACGCATTTCACCGCTACACTGGGAATTCCTGCTNCCCCTACTGCTCTCTAGT
CTGACAGTTTCCACTGCCTTTAGGAGGTTAAGCATCCTGATTTGACAGCAGA
CTTATCAAACCACCTACGGACGCTTTACGCCCAATAATTCCGGATAACGCTT
GCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTATTC
CTCAGGTACCGTCAGAATTTTTTCTTTGAGAAAAGAGGTTTACAATACAAAG
ATCTTCCCCTCTCACGCGGTGGTTCTCCCTGAGGTTTTCCC

16b (EU94524)

GTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCCCAATC
TCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTA
GTCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCA
GACTTGGCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGC
TTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGAT
TCCTCAAGTACCGTCAGAACTTCTTCCTTGAGAAAAGAGGTTTACAATCCAA
AGACCTTCCTCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGG
AANATTCCCCCGGGCGGGG

1.2. Partial 16S rDNA sequences obtained from from bands in the general bacterial DGGE gel

1. (EU94525)

TACAGCGGCTGCTGGCCATGGTGAGCATGTATTACCGCGGCTGCTGGCCAA
TGGTGAGCATGTATTACCGCG

2 (EU94526)

ATGGCAGCGGCGGACGGGTGCGTNANNNNNNNNNNNNNNTGAGGTGGGG
GACAACCCTGGAAANGGGGCTAATACCGCATATGGGCTGAGGCCCAAAGCC
GAGAGGGGNNTTAGGAGCGGCCTGCGTCCGATTAGCTAGNNGGNGGGGAA
GGCCTACCAAGGCTCCGATCGGNAGCTGGTCTGAGAGGCGATCAGCCACAC

TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGGGGTAAGAAGGCCT
TTCGGATCGAAAGCCCTTCGACAGGGACGATAATGACGAACTGTATAGTGC
CCCGGTAATTCNGGGC

3 (EU94527)

ATTTGCGGCGANNNNNNNNNNNNNNNNNNNTCTGCCTTCAACNCTGGGN
NNNNNNNNNNAAACCGGGGNTAATACCGGATATGAGCCTTCGCGATCNTCC
GCNTNNNNGTTTTCGGCCTGAGTGATCTCCGGCTTCACCTTGTTGGTGGGTA
AGGCTCCCAAGGCACGCCCGCACCCGCCTGGAGGGGACGNCCCCCCGGGGC
TGAGACACGCCCAATCCCTACGGAGGCACCGTGGGGAAAATGGGNAATGA
GGAAACTTGACCCACCACCCCCTTGCGCATGAGGCCTTGGGTTTTAACCCCT
TCTTAGGTATTTAGCGCAATAAGGTACCTCCGAAGAGGAGGAGGTNACTAT
TTCCACCGCGCGCTAAAAA

4 (EU94528)

GGTGAGCATGTATTACCGCGGCTGATGTCCCAAAGGCTTAAGNACTAACGC
GGCAGAAGGCCTTCAGGCTGGCGCGGTANGGCAGGATTAGGCTTGGCTNCA
TTGCGTAAAATTCCCCACTGCTGTCTCCCGTANGAGCGGGGAGTGTCTCGCA
GACCATCTACCGGTCCGTCCTCTCAGACCAGCTGGACCTCGCAACTATGTTA
TCCCTTTACCCCACTAACTACCTAATCTGACATCGTTTNGCCCAACAGCACT
AGGCCTTATGGTCCCCCGCTTTCACACGTAGTTCGTATGCGGTATTACTCCG
GTTCTCGCCGCGCTATCCCCCACTGTTGCGCACGTTNCGATGCATTACTCAC
CCGTTTTTNACTCGCCGCCGGGTTGNCCCTTGAGTACGGTGGGGCTTGTCAG
TGTAATGCATGCCGCCAGCGTTCAACCTGAGCAAGGATCAAACTCTCAGA

5 (EU94529)

TGCACGTCGAGCGGCAGCGNGAAAGTAGCTTGCTACTTTTGCCGGGAGNGG
CGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGNNGAGGGGGATAACTAC
TGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGAC
CTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTG

AAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTA
GCTAATAACTGCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACT
CCGTGCCAGCAGCCNGCGGTAA

6 (EU94530)

TTAGCATGTATTACAGCGACTGCTGTTCCAANGGGAGTAGNNCTTCCCCGGC
GGTGCGNCATCGGGNTGGGGTTGATNGNTTTGGACNANATTCNNCACTGTT
GCGTACCATAGTGGTCTGGGCCGTATCTCAGGTGNNGTGNGTCCTTCTCTCC
TCTCAGGTCCGCTACCCGNCGNTGCCATGGTGTGGCGTTACCACCCAAACTA
NCTGATAGGCCGCGATCCCATCCTAAACCGAAATTTTTTCCCCACCCNAAGA
TGCCCTAAAGGTTCGTATCTGGNATTAGGTCCCGTTACCCGGAGTTATCCCC
AAGTGCAGGGCAGATTGCTCACGTGTAACCCACCCGTACCCCACTAATTTGC
CCGGATTTTGCTCCNNNTTCGTCGTTCGCTGGGGTGTGGTTGGGGGGCCCCA
NCAGCGTTCGTCCTGAGCCAGGATCANACACTCAA

7 (EU94531)

GCTCGGCGGCGTGCCTAACACATGCAAGTCGAACGGGCATCTTCGGTGCGG
GGGGCGGGGGGGGTGAGTCACGCGAAAGAGTCTTCCTTCGCGGCAGGAACCA
CTGTTGGTAGCGACTGCACATACCCTGTATGTCGGAGGGAGGAACCTAATC
GGCCTAGAGACGCCCTGGCGTCTGATCGACTTGTTGGGGGGGAAAGAGCCT
ACCAAGGCCACGATTAATAGGTGGTCTAAGAGGATGAGCAG

8 (EU94532)

GCCTAACACATGCAAGTCGAACGGGAATCTGCGGCAATGGTGGCGGAGGG
GTGACTAACGGGTAAAAATCTAGCGTCGGGACCCGTCCTGCGGTATGTAGC
GATAGCTACTACCCTTTTCTTCGTAAATGGCATGTATTAGCTGTGAAAGGGC
TGGCGTCTGAT

9 (EU94533)

TAACACATGCAAGTCGAACGATAAAATTGTTTGCGAGGGTCAGAGGTGATG
ACGGACGTGAAAGCTATTGGTCTCCCCAGTAACAAGTCTTTAAAGAGATAT
TGAAAAGCCAATAAGACTGTA

10 (EU94534)

GGCGGCGTGCCTAACACATGCAAGTCGAACGGTAATGTGGGTTAACAGCGG
CGGAGGGGTGAGTAGGGGGAAAGAGTAGAAATACGGGCGGGGTTGGTGGG
TTAGTAACCGGTGAAAAA

11 (EU94535)

TCGANCGGGAGTATTCGGNTTCTCGTGGCAGANGGGTGNNNNNNNNNNNN
NNNNTNNCTTCANNTCCGGNATNCNGTTGGAAACAAGAGCAANTCCCCNAT
ATNCCGCNAGGCGAAACCTAATTGCNCTGGCGAAGAGCTTGTTTCTGTATNT
TCAGTTGGGGGGNTAAGACCTTACCAAGGCNACTATCAGAAGCTGGNCTGA
GAGGATGAGCAGCCACACTGGGACTGAGACACGGCCCACACTCCTACGGGA
GCCAGCANTGGGGAATTTTCCCCAATGGGGGAAACCCTGACGGANCAACGC
CGCGGGAGGGAGGAAGGCCTTTGGGTTGGAAACCTCTTTTCTCAGGGAAGA
AGTTCTGNCNNTCCTTGATGGATTATCCTCGGNTAACTCCGTGCCAGCCNGC
CGGCGGNAATAGGGGCAAACCACCCCCCCCANANNCCGNTGCACCCCGCCC
CGGGGAATANANAGAGANNGGGNGACNANNCCN

12 (EU94536)

ACGGNATCTTCGTATTCTAGTGGCGGACGGGTGANTNNNNNNNNNNGTCTN
NCTTCNGGACNTGNNCCNCGGTTGAAAACANGGGCAACTACCCGATATGCC
GCAAGGTGAAACCTAATTGGCCTGAAGAAGAGCTTGCGTCTGATTTTTTAGT
TGGTGGGGTAAGAGCCTACCAAGGCGACGATCAGTGGCTGGCCTGAGAGGA
TGAGCAGCCCCCCTGGGACTGAGACACGGCCCACACTCCTACGGGAGGAAG
CNGTGGGGAATTTTCCGCAATGGGCGAAAGCNTGACGGAGCAACGCCGCGT
GAGGGAGGAAGGNCTTTGGATTGTAAACCTCTTTTCTCAAGGAAGAAGTTC
TGACGGTACTTTGAGGAATTTGCCTCGGCTAACTCCGTGCCAGCAGCCGCGG
GAATACNTGCAAA

13 (EU94537)

TGGTGAGCAT GTATTACAGC GGCTGCTGGC CAAAGGTGAG TNNNANTACC
GCGGCTGTTGGTCTCGAGGNTTTCTCTTTTGCGAAAAATTCCCTACTGGTGT
CGTCGTAATTCTTGGTCCGTCTCTCAGTCCCAGTGTGGGTGATCATCCTCTCA
GAAGGTGTACTGCTCTTCGCCGTGATGAGCTTTTACCCCCTGCTATGTGATA
ACCTGACGCCAGCCTCNATTTTACCGGANNTCTCTTTCCCCCACAGCATATT

GGTATTAAAGCAATTTTCCAACTGGTGTCTCCGCCGNCAAGATAAAATTTCA
CGCGGGNNCCCCCCCCCCCCCCAATAAAATACGAANATCTTGNTACAACTTG
AATGAATGAGTCACTCCGGCGTGTTTCATCCGGAGCCAGGANAAATCCTCG
AAAGAGGGNCTCNNGCTCACATCN

14 (EU94538)

TGGTGAGCCCGTATTACCGCGACTGCTGGCCCNAAAGNCTTNNNNNNNACG
CGGCAGTTGTGCCTCAGGGTTTCTTCCATNGNGCAAAATTTCCCACTGGTGC
CTCCCGTAGGAGTGCGGGCCGTGGCTCAGNCCCANTGGGGNTGGCCATTCT
CTTAAACCAACTAACGGTCATCGCCATGGTAGGCCCTTGTCCGACCANCTAG
CTAATCATACGCACGCTCTTCTTACCCCAACAAATCTTTCATGCTAAACGTC
ATATTCTAGCACCTATGCGGTATCCGAACGGGGTTCCAGATGTGATCCCCCA
GTGTAAGGGAGATTACCCCCGCGTTACTCACCCATCCGAAAATGATGNATC
TCCGAAGATACCTTATTGACCCACTTGGATGTCTTCGGCGGTC

15 (EU94539)

GCCTAACACATGCAAGTCGAACGGTAAAGTGGGTTAGAGAGTGTTCTGGGG
GCGAACGGGGGCGAATCTGTTACGACACTCCCTTCTACACAGGGAAAGCAT
TGGGAAACCGGTGCTAATCCCGCATATTGAAGCTTAATTGACATGGGGAAC
ATCTATTCAAAGAAAAGTGAATTAGTTTCAAACGCCCAAC

16 (EU94540)

GCTTAATACATGCAAGTCGAACGGGAAAGTTGGCAGAGAGGGATGAGGGC
GCTGGATGGGACGATCTGTGTCGACCATCCCTTTCGTACAGTGAAAGAGGC
GCGAAAACGGTATAAACACTTAATGTTAAAGATTAAATGCCATAAAAGACG
TGAGTATAT

17 (EU94541)

GCTCGAACGCTCGGCGGCAGGCCTAACACATGCAAGTCGAACGGAAAGCTT
ACAGAGTGGGTGACGGGTGAGTAACATGCGGGAATCCGCCTTGTGGTTCCG
GTCAACATTGGGATACCGGTGCTAAAACTAGATAAATCCTCACGGGGAAAG
TTTTAATGCCATAAGATGAGCCCGGATTCGATTAGTTAGTTGGGGAGGGAA
AGACTCTCCAAGACAATGATTAATAGCTGATCTGAGAGGATGAACC

18 (EU94542)

GCCTAACACATGCAAGTCGAACGGTAATGTTCGTATGCTAGCGGCGGACGG
GTGAGTAACGTGTAAGAATCTATCTTCACTACGTTTACAACGGTTGGAAACG
ACAGCAAATACTCGATATGCCGCAAGGTGAAACCTAATTGGCCTGGAGAAC
AGCTTGCGTCTGATTA GCTAGTTGGGGGGGTAA

2. Sequence of unknown bacteria (Chapter 6), 100% match to *Bacillus cereus*

GCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACCGATAATTACGAC
CCGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT
GCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT
CCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTG
AGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGA
CAGCCGAAGCCGCCTTTCAATTTCGAACCATGCGGTTCAAAATGTTATCCGG
TATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCAC
GTGTTACTCACCCGTCCGCCGCTAACTTCATAAGAGCAAGCTCTTAATCCAT
TCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGG
ATCAAACTCTC

Appendix B

Presentations and Publications Arising From This Research

G. Ross & T.E. Cloete, 2006. The control of cyanobacterial blooms using predatory bacteria and Phoslock. The 14th Biennial Congress of the South African Society for Microbiology, 9-12 April 2006.

G. Ross & T.E. Cloete, 2006. The use of Phoslock® for the control of eutrophication. IWA International Conference, Beijing, September 2006.

T.E. Cloete & G. Ross, 2006. The control of cyanobacterial blooms using predatory bacteria and Phoslock®. International Conference and Exhibition on Water in the Environment. 20-22 February 2006, Stellenbosch, South Africa.

Gumbo J.R., G. Ross & T.E. Cloete, 2007. The biological control of *Microcystis* dominated harmful algal blooms. Submitted to *Harmful Algae.*

G. Ross, F. Haghserecht & T.E. Cloete, 2008. The effect of pH and anoxia on the performance of Phoslock®, a phosphorus binding clay. *Harmful Algae*. 7(4):545-550.

G. Ross, A.K.J. Surridge & T.E. Cloete, 2008. Analysis of the microbial community diversity in Phoslock® treated and control areas of Hartbeespoort Dam using PCR-denaturing gradient gel electrophoresis. Submitted to *Water Research.*

G. Ross J.R. Gumbo & T.E. Cloete, 2008. The mechanism of *Microcystis aeruginosa* cell death upon exposure to *Bacillus mycoides.* IWA International Conference, Vienna, September 2008.