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

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:

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

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 5.1×10^9cfu.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.

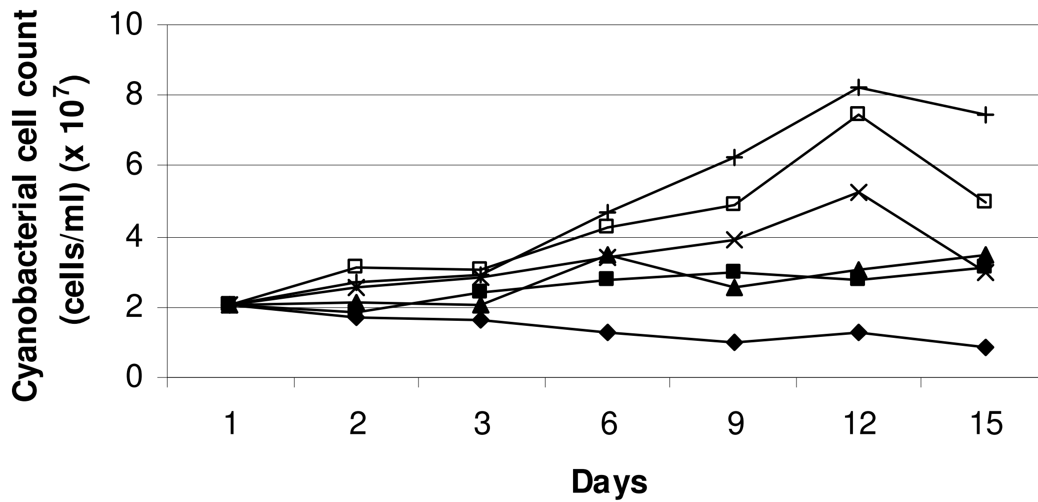


Figure 2: Effect of different predator-prey ratios on the growth of *Microcystis aeruginosa* (♦) 1:1 (▲) 1:10 (■) 1:100 (×) 1:1000 (□) 1:10 000 (+) Control

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

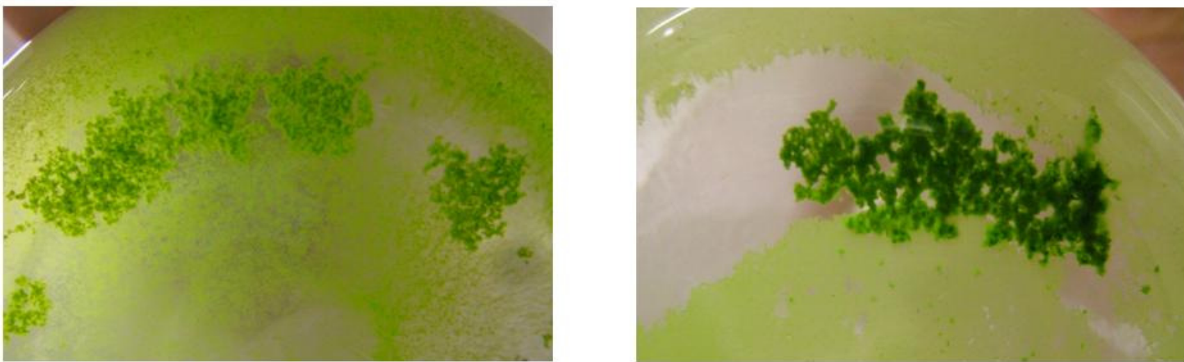


Figure 3: Colony formation in flask treated with bacteria in a 1:1 ratio of bacteria to algae (left) and control (right)

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).

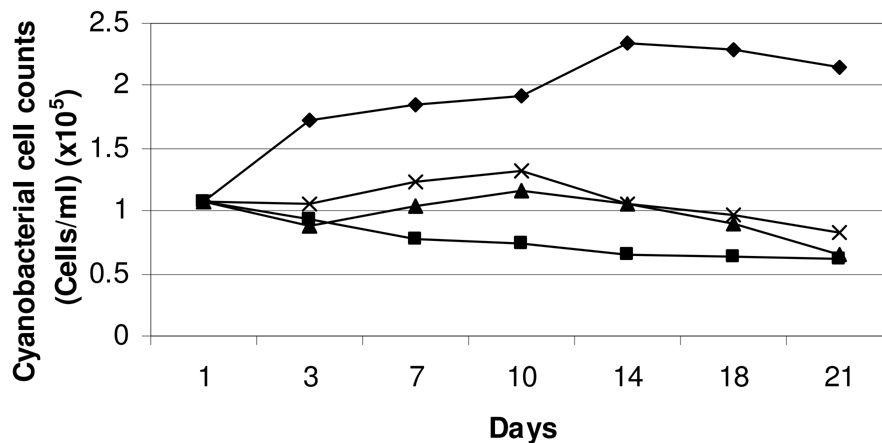


Figure 7: Effects of treatment with bacteria and Phoslock® on *M. aeruginosa* cell numbers (♦) Control (×) 0.5% Phoslock® (w/v) and bacteria (▲) Bacteria (1000:1) (■) 0.5% Phoslock® (w/v)

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.

6. References

Botha-Oberholster, A.M., 2004. Assessing genetic diversity and identification of toxic cyanobacterial strains in selected dams in the Gauteng/North West Metropolitan areas through PCR based marker technology. *WRC project.* No. K5/1502.

Burnam, J.C., Collart, S.A., & Highison, B.W., 1981. Entrapment and lysis of the cyanobacterium *Phormidum luridum* by aqueous colonies of *Myxoccus xanthus* PCO2. *Arch. Microbiol.* 129:285-294.

Burnam, J.C., Collart, S.A. & Daft, M.J., 1984. Myxococcal predation of the cyanobacterium *Phormidium luridum* in aqueous environments. *Arch. Microbiol.* 137:220-225.

Burnam, J.C., Stetak, T. & Boulger, J., 1973. An improved method of cell enumeration for filamentous algae and bacteria. *J. Phycol.* 9:346-349.

Coveny, M.F. & Wetzel R.G., 1992. Effects of nutrients on specific growth rate of bacterioplankton in oligotrophic lake water cultures. *Appl. Environ. Microbiol.* 58(1):150-156.

Cullimore, D.R., 2000. A practical atlas for bacterial identification. Lewis Publishers. pp 85-87.

Fjellbirkeland, A., Torsvik. V. & Øvreås, L., 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF* and 16S rDNA sequences. *Antonie van Leeuwenhoek*. 79:209-217.

Greenop, B. & Robb, M., 2001. Phosphorus in the Canning: 1999-2000 Phoslock™ trials. *River Science.* 17:1-7

Hoeger, S.J., Shaw, G., Hitzfeld, B.C. & Dietrich, D.R., 2004. Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. *Toxicon.* 43:639-649.

Krüger, G.H.J & Eloff, J.N., 1977. The influence of light intensity on the growth of different *Microcystis* isolates. *J. Limnol. Soc. Sth. Afr.* 3(1):21-25.

Lam, A.K., Prepas, E.E., Spink, D. & Hrudrey, S.E., 1995. Chemical control of hepatotoxic phytoplankton blooms: implications for human health. *Water Res.* 29(8):1845-1854.

Marienfield laboratory glassware: Info on counting chamber. (www.superior.de). Accessed 2005/08/29

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin Microbiol*. 2:317-322.

Nakamura, N., Nakano, K., Sugiura, N. & Matsumura, M., 2003. A novel cyanobacteriolytic bacterium, *Bacillus cereus*, isolated from a eutrophic lake. *J. Biosci. Bioeng.* 95(2):179-184.

Procedures- Bacterial Counting, 2000 http://whitewolf.newcastle.edu.au/techinfo/proc_bacto_counts.html Accessed 2005/05/03.

Rashidan, K.K., & Bird, D.F., 2001. Role of predatory bacteria in the termination of a cyanobacterial bloom. *Microbial Ecol.* 91:97-105.

Reim, R.M., Shane, M.S. & Cannon, R.E., 1974. The characterization of a *Bacillus* capable of blue-green bactericidal activity. *Can. J. Microbiol.* 20:981-986.

Robb. M., Greenop, B., Goss, Z., Douglas, G. & Adeney, J., 2003. Application of Phoslock™, an innovative phosphorus binding clay, to two Western Australian waterways: preliminary findings. *Hydrobiologia.* 494:237-243

Shunyu, S., Yongding, L., Yinwu S., Genbao, L, Dunhai, L., 2006. Lysis of *Aphanizomenon flos-aquae* (Cyanobacterium) by a bacterium *Bacillus cereus. Biological Control.* 39:345-351.

Strydom, R., 2004. The development and evaluation of new South African ozoniser technology for removal of enteric viruses and tastes and odours present in Hartbeespoort dam water. *WRC report.* No.1127/1/04.

Walker, H.L. & Higginbotham, L.R., 2000. An aquatic bacterium that lyses cyanobacteria associated with off flavour of channel catfish (*Ictalurus punctatus*). *Biological control.* 18:71-78.

Wright, S.J.L., Linton, C.J., Edwards, R.A. & Drury, E., 1991. Isoamyl alcohol (3-*methyl*-1-butanol), a volatile anti-cyanobacterial and phytotoxic product of some *Bacillus* spp. *Lett. Appl. Microbiol.* 13:130-132.

Wright, S.J.L. & Thompson, R.J., 1985. *Bacillus* volatiles antagonise cyanobacteria. *FEMS Microbiol Lett.* 30:263-26.

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

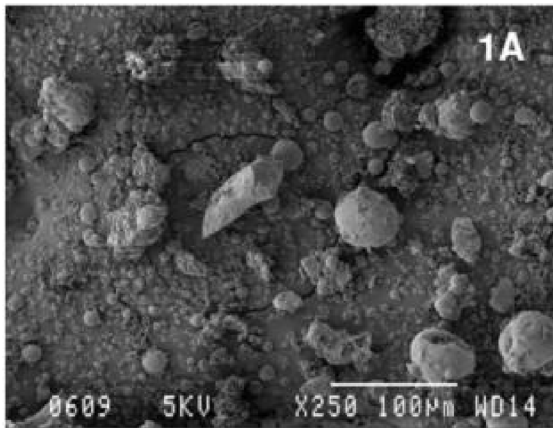
Sb	<0.01	<0.01	0.01	0.01	0.01	0.01	0.01
Sc	0.05	0.05	0.04	0.04	0.05	0.07	0.05
Se	0.02	0.02	0.02	0.01	0.01	0.02	0.01
Si	200	233	212	212	202	306	216
Sm	0.04	0.06	0.05	0.05	0.04	0.07	0.05
Sn	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sr	16.9	11.3	9.36	11.9	8.34	16.7	8.7
Ta	<0.01	<0.01	<0.01	<0.01	<0.01	<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.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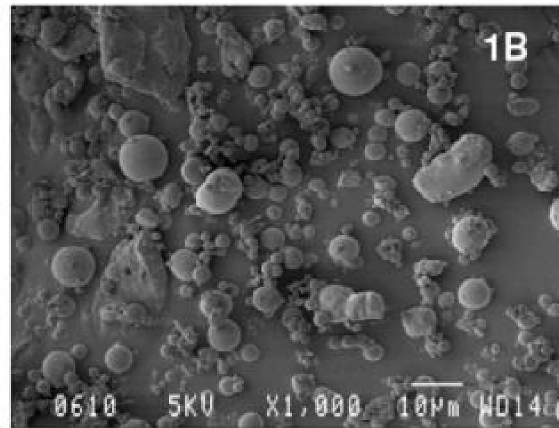
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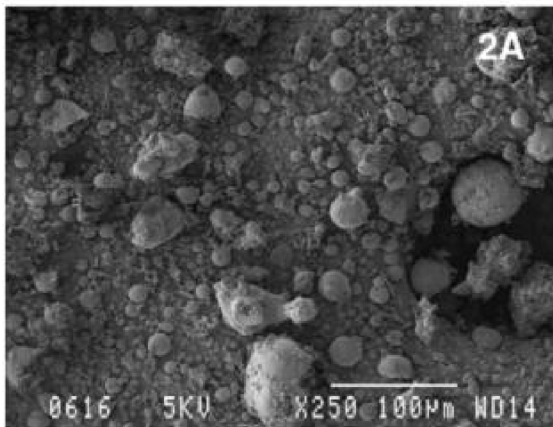
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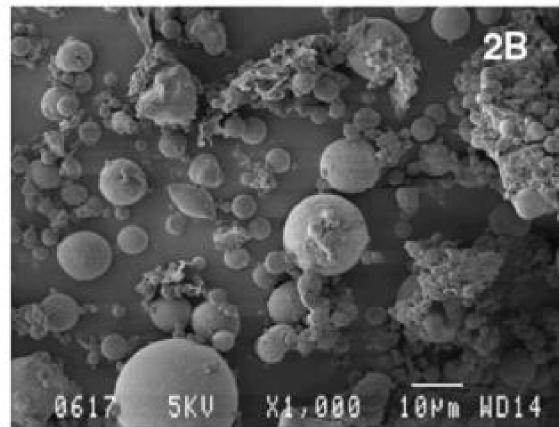
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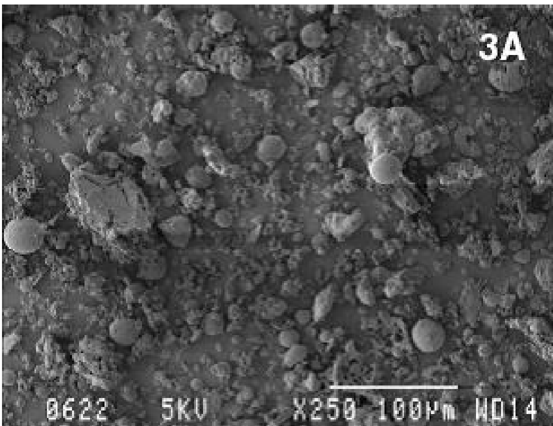
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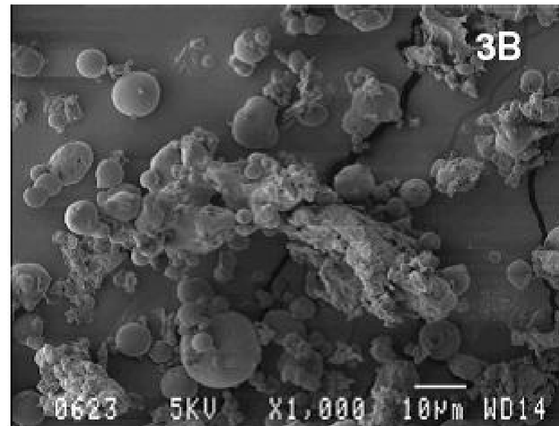
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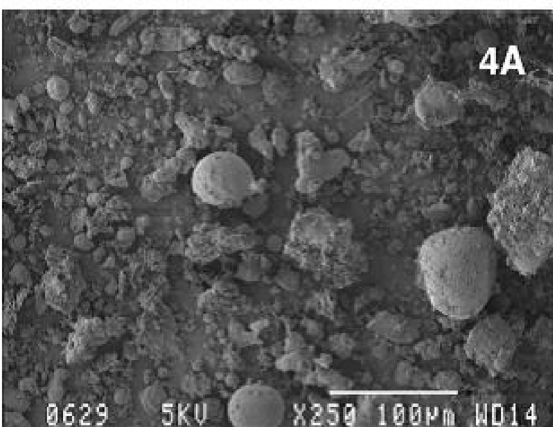
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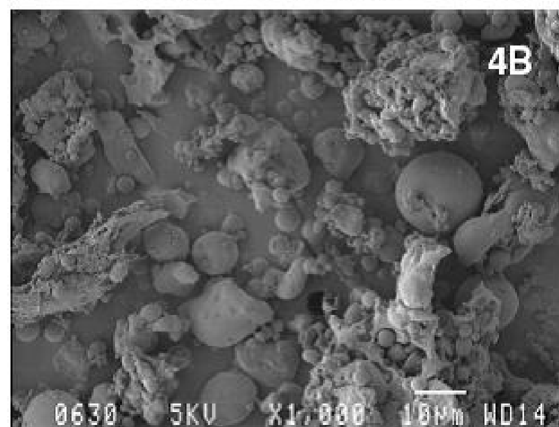
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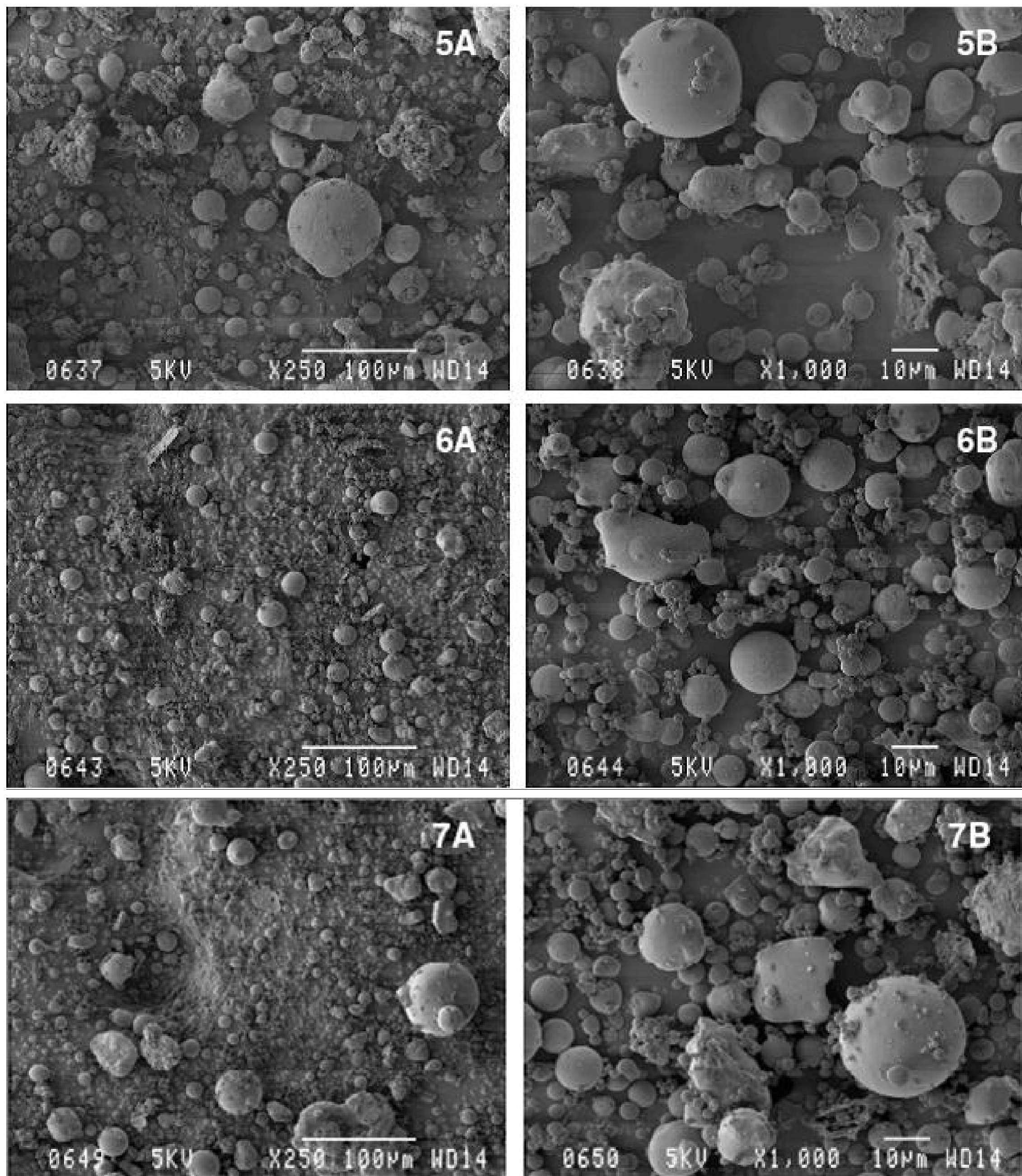

Figure 1: Scanning electron microscopy of the fly ash samples (1-7) at 250x (A) and 1000x (B) magnification

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 <1 μm 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

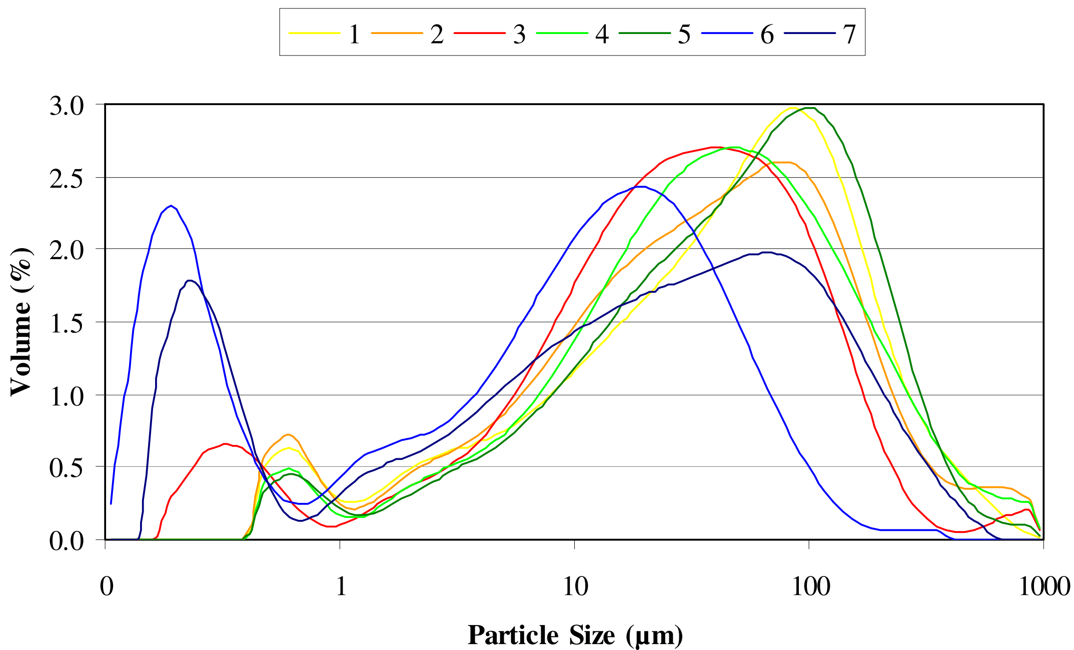

Figure 2: Particle size distributions for the 7 fly ash samples

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.

6. References

Dallas. H.F. & Day, J.A., 1993. The effect of water quality variables on riverine ecosystems: A review. Water Research Commission Report No. TT 61/93.

Department of Water Affairs and Forestry, 1996a. South African Water Quality Guidelines (second edition). Volume 1: Domestic Use.

Department of Water Affairs and Forestry, 1996b. South African Water Quality Guidelines. Volume 7: Aquatic Ecosystems.

Fisher, G.L., Chang, D.P.Y. & Brummer, M., 1976. Fly ash collected from electrostatic precipitators: Microcrystalline structures and the mystery of the spheres. *Science.* 192:553-555.

Iyer, R., 2002. The surface chemistry of leaching coal fly ash. *J. Hazard. Mater*. 93:321-329.

Iyer, R.S. & Scott, J.A., 2001. Power station fly ash- a review of value-added utilisation outside of the construction industry. *Resour. Conser. Recycl*. 31:217-228.

Kruger, R.A., 1996. Fly ash beneficiation in South Africa: creating new opportunities in the market place. *Fuel*. 76:777-779.

Ram, L.C., Srivastava, N.K., Tripathi, R.C., Thakur, S.K, Sinha, A.K., Jha, S.K., Masto, R.E. & Mitra, S., 2007. Leaching behaviour of lignite fly ash with shake and column tests. *Environ. Geol*. 51:1119-1132.

Scheetz, B.E. & Earle, R., 1998. Utilization of fly ash. Current Opinion in Solid State *Material Science*. 3:510-520.

Wang, J., Ban, H., Teng, X., Wang, H &Ladwig, K., 2006. Impacts of pH and ammonia on the leaching of Cu(II) and Cd(II) from coal fly ash. *Chemosphere*. 64:1892-1898.