Full Length Research Paper

Light and electron microscope assessment of the lytic activity of *Bacillus* on *Microcystis aeruginosa*

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During the screening of lytic bacteria, plaques were obtained on *Microcystis* lawns. In the plaques, at least five distinct morphotypes of bacteria were found. The plumb rod-shaped *bacilli* were the most abundant and were found aggregated around unhealthy *Microcystis* cells and were the probable cause of deflation and lysis. These bacteria may have utilized the cyanobacteria cell contents as their nutrient source. In contrast to the control areas, the cyanobacteria cells were healthy and did not show any visible distortion of cell structure. The presence and possible role of the free-bacteria, that is, bacteria that were not attached or associated with the cyanobacteria in the plaque is not clear. Maybe their function is to scavenge the skeletal remains of *Microcystis* cells. *Bacillus mycoides* B16 were found to have a lytic effect on *Microcystis* cells. Scanning electron micrographs (SEM) images of *B. mycoides* B16 did not reveal any unique attachments that may have allowed them to adhere to *Microcystis* cells. The *Microcystis* cells were exposed to copper, *B. mycoides* B16 and Triton X-100, in order to ascertain the level of cell membrane damage. The membrane cell damage was most severe with copper stripping the entire *Microcystis* cell membrane leaving a honeycomb skeletal structure and *B. mycoides* B16, leaving perforations on the cell membrane. The electron microscopy observations appeared to reveal at least two mechanisms of *Microcystis* lysis (contact and parasitism). The light and electron microscope (LEM) observations did not reveal any endoparasitism of *B. mycoides* B16 or *Bdellovibrio*-like behaviour.

Key words: *Microcystis*, mechanism of lysis, *Bacillus mycoides* B16, photosynthesis, copper.

INTRODUCTION

Harmful algal blooms (HABs) in freshwater resources are often dominated by *Microcystis* species and are on the increase worldwide including South Africa and can cause a wide range of social, economic and environmental problems. The HABs are associated with the production of microcystins that affect water quality with adverse effects on lake ecology, livestock, human water supply and recreational amenities (Codd et al., 1997; Nakamura et al., 2003b; Choi et al., 2005). During the bloom period, there were microbial agents such as bacteria and viruses that have were found to have commensalistic and antagonistic relationships with the cyanobacteria (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005).

The interactions of bacteria and cyanobacteria in aquatic environments are numerous, ranging from: (1) competition for available organic matter, (2) provision of extracellular substances by cyanobacteria which are beneficial to bacteria and vice versa, (3) antagonistic behaviour whereby the bacteria feeds on cyanobacteria, and (4) production of cyanobacteria exudates which inhibit the growth of bacteria and vice versa (Bates et al., 2004).

The relationships between these microbial agents and cyanobacteria are becoming increasingly important for the better understanding of harmful algal bloom dynamics (Bates et al., 2004). There is a close spatial and temporal

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coupling of microbial agents and cyanobacteria and both groups tend to synthesise metabolites that can be beneficial (Brunberg, 1999) or even harmful to one another (Grossart, 1999; Bates et al., 2004). Different types of bacteria with specialized extracellular substances are associated with the initiation, maintenance and termination phases of algal blooms (Riemann et al., 2000). Specific bacteria may also be attracted to the phycosphere, the region surrounding the algae cells, where their growth may be stimulated by algal exudates (Bates et al., 2004). Some bacteria are found attached to live or dead algal cells indicating the possibility of an antagonistic relationship, which may be explored for biological control (Maruyama et al., 2003; Gumbo et al., 2008). Scanning electron micrographs (SEM) revealed the attachment of *Flexibacter flexilis* to the sheaths of filamentous *Oscillatoria willimasii*, which resulted in the excretion of lysozyme which then lysed the cyanobacterium (Sallal, 1994). The *F. flexilis* bacterium benefited from the cyanobacterium nutrients after lysis and helped regulate population levels of *O. willimasii* in raw sewage aeration tanks. The cyanobacterium, *O. willimasii* is known to produce exudates that contribute to the biodeterioration of raw sewage settling tanks. Thus, the management control of this cyanobacterium is important.

Microcystis cells were lysed by a *Bdellovibrio*-like bacterium (BLB) after penetration (Caiola and Pellegrini, 1984). Once the host was penetrated, the BLB was localised between the cell wall and cytoplasm membrane, which appeared thickened on TEM negative staining. The cell wall appeared broken at many sites and this was attributed to the breakdown of the cell wall leading to cell lysis and death. The BLB adhered to the *Microcystis* cell plasmalema by means of tubular structures. These membranous extensions may possibly represent re-cognition sites to allow for interactions between bacteria and cyanobacteria. In the natural environment, the BLB are selective only invading *Microcystis aeruginosa* but not *Microcystis wesenbergii* (Caiola and Pellegrini, 1984). This was attributed to the fibrous glycocalyx that function as a recognition site between the bacteria and its prey. In an earlier study involving a BLB, *Bdellovibrio bacteriovorus* lysed the cyanobacterium *Phormidium luridum* without penetrating the prey, which indicates a close physical relationship (Burnham et al., 1976). These studies indicated that, there are two types of BLB, which are parasitic towards cyanobacteria in the natural environment.

Microbial agents such as bacteria and viruses may play a major role in the prevention, regulation and termination of harmful algal blooms (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005). Some of these microorganisms have been isolated from eutrophic waters and had a lytic effect on the growth of cyanobacterial species (Nakamura et al., 2003a). Often, predatory bacteria are in abundance during the decline of

a harmful algal bloom (HAB) and may be involved in the collapse of blooms in nature (Bird and Rashidan, 2001).

In previous study, it was observed that the bacteria *B. mycoides* were responsible for plaque developments on *M. aeruginosa* lawns (Gumbo et al., 2010). The main objectives of this study were to assess which other bacterial morphotypes were present in the plaque zones and to explore the relationships between the bacterial agents and the *Microcystis* cells during the lytic process. SEM was used to assess the morphological changes of the *Microcystis* cells. Transmission electron microscopy (TEM) was used to assess the ultrastructural changes that occurred between the *Microcystis* cells and predator bacteria. Light microscopy was used to observe the interactions between predators and prey (wet mounts).

MATERIALS AND METHODS

Evaluations of cyanobacteria-bacteria interactions in solid media/phases (plaques)

Agar sections were cut from the plaques (Gumbo et al., 2010) on BG11 agar plates and were examined with SEM and TEM. The areas with green *Microcystis* lawns indicating the absence of plaques served as controls.

Scanning electron microscopy (SEM)

The agar sections were fixed with 2.5% (v/v) gluteraldehyde in 0.075 M phosphate buffer (30 min) and then were filtered through a 0.22 µm membrane. The membrane filter was washed three times with 0.075 M phosphate buffer (15 min), dehydrated with 50% ethanol (15 min), 70% ethanol (15 min), 90% ethanol (15 min) and three times with 100% ethanol (15 min). This was followed by critical point drying (Bio-Rad E3000) and gold coating process (Polaron E5200C). The material was then examined in a Joel JSM 840 scanning electron microscope operating at 5.0 kV.

Transmission electron microscopy (TEM)

Thin sections of agar were made with a stainless blade and then were immersed in gluteraldehyde solution for 30 min. This was washed three times with 0.075 M phosphate buffer (15 min) and fixed with osmium tetroxide (1 h). The osmium tetroxide was removed through repeated washings [three times with 0.075 M phosphate buffer (15 min)] and was embedded overnight in Quetol resin. Sections were cut on an ultramicrotome (Reicher-Jung Ultracut E), stained in uranyl acetate and lead acetate before been examined and photographed using a transmission electron microscope (Philips EM301).

Evaluations of cyanobacteria-bacteria interactions in liquid phases

Culture of organisms

M. aeruginosa PCC7806 was cultured in 500 ml Erlenmeyer flasks using modified BG11 medium (Krüger and Eloff, 1977) under shaking incubation (78 rpm, 25°C) for 8 days under continuous light. Two 18 W cool white florescent lamps (Lohuis FT18W/T8 1200LM) that were suspended above the flasks provided
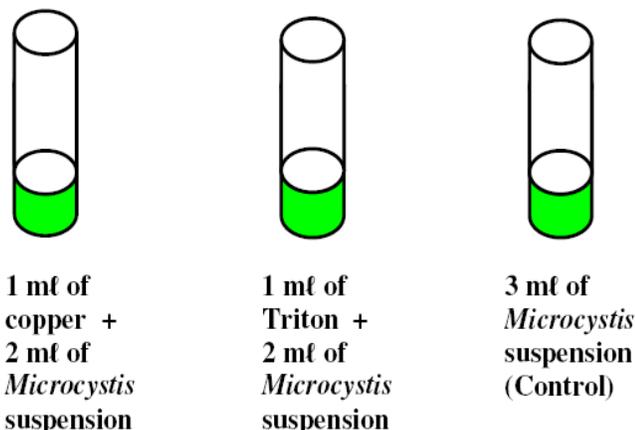
1 mℓ of copper + 2 mℓ of *Microcystis* suspension

1 mℓ of Triton + 2 mℓ of *Microcystis* suspension

3 mℓ of *Microcystis* suspension (Control)

Figure 1. The experimental design for testing of algicides.

continuous lighting (2000 lux). The *M. aeruginosa* cell suspensions were used as prey.

The bacteria *B. mycoides* B16 was used as a 24 h culture. Fresh bacteria were prepared by inoculation into 100 ml of one-tenth tryptic soy broth (TSB) in a 250 ml Erlenmeyer flask and was shake incubated (128 rpm, 25°C) for 24 h (Di Franco et al., 2002).

Experimental set up

Culture suspensions of cyanobacteria (20 ml) and bacteria (20 ml) (as previously described) were mixed in a 250 ml Erlenmeyer flask. The mixture was incubated in a Labcon shaker (78 rpm, 25°C) for 8 days under continuous light provided by two 18 W cool white florescent lamps (Lohuis FT18W/T8 1200LM) that were suspended above the flasks (2000 lux). All the experiments and controls were done in duplicate. Then on day 4 under sterile conditions, an aliquot was obtained from the Erlenmeyer flasks and was subjected to microscopy analysis.

Light microscopy

One drop of the suspension (control and treated samples) was placed onto a microscopic slide and then was covered with a cover slip. The material was examined using a Nikon optiphot light microscope fitted with appropriate illumination sources and filters and pictures were captured with a Nikon digital camera DXM1200.

Scanning electron microscopy (SEM)

The suspension (control and treated) was filtered through a 0.22 μm membrane filter and was fixed with 2.5% (v/v) gluteraldehyde in 0.075 M phosphate buffer (30 min). The same procedure for the culture of organism was followed.

Algicide disruption of *Microcystis* cell membranes

Aliquots of copper sulphate (10 mg/ml) and 0.01% Triton X-100 were added to the *Microcystis* suspension (as previously described). The experimental design shown in Figure 1 was incubated as in the previous section for 24 h. SEM was performed as described earlier.

RESULTS AND DISCUSSION

Evaluations of cyanobacteria-bacteria interactions in solid media

The incubation of an aliquot of the eutrophic waters on BG11 agar resulted in the formation of plaques (Gumbo et al., 2010). Epiphytic and free-living bacteria were observed in the plaque areas. At least five distinct morphotypes of bacteria were found in the plaque zones: (1) plumb rod-shaped bacillus that was attached (1 to 1.5 μm) (Figures 2 and 3) and free-living (Figure 4c); (2) a long rod-shaped *Bacillus* with one end sharpened and not attached (3 to 6 μm) (Figure 4b); (3) a plumb rod-shaped *Bacillus* with fimbriae and not attached (1.5 to 3 μm) (Figure 4d); (4) vibrio shaped rods and not attached (Figure 4a) and (5) coccoid shaped bacteria (0.6 μm) (Figure 4a, c). SEM micrographs showed the presence of plumb shaped *Bacillus* aggregated around the unhealthy *Microcystis* cells (Figure 2). The *Microcystis* cells appeared distorted or deflated wherever these *Bacillus* rods were present.

These results showed similar bacteria flora that were observed by Robarts and Zohary (1986) which consisted extremely small cocci (0.1 to 0.2 μm), large rods (~1 μm), presumably *Bacilli*, that were mostly attached to *Microcystis* cells (in hyperscums) and filamentous bacteria. In the same study, the researchers observed that when hyperscum reached its peak mass, it was accompanied by an increased bacterial heterotrophic activity that was followed by a breakdown (decline) of the hyperscum. These findings may suggest that the bacteria were responsible for the termination of the hyperscums.

The direct examination of the plaques did not reveal a clear association between bacteria and cyanobacteria due to interference of the agar material (Figure 2). To eliminate the interference, material from plaque zones was scrapped and suspended in minimum Ringer's solution and then processed for SEM. The SEM micrographs showed that, the bacteria flora was mainly composed of plumb rod-shaped *Bacillus* (Figure 2) that were closely associated with unhealthy *Microcystis* cells (Figure 3a).

In the control areas, the cyanobacteria cells were healthy and did not show any visible distortion of cell structure (Figure 3b). At this stage, the unhealthy *Microcystis* cells appeared to be associated with the plumb rod-shaped *Bacillus* that were probably the cause of deflation. It may be that the bacteria caused the cyanobacteria cells to leak out their cell contents and the bacteria benefited nutritionally. This supports Stewart et al. (1973) and Burnham et al. (1984) who also concluded that, the plaque formation was attributed to a single predatory bacterium that had multiplied and caused cyanobacterial lysis.

During bloom conditions, bacteria are known to exist embedded within the *Microcystis* mucilage with their
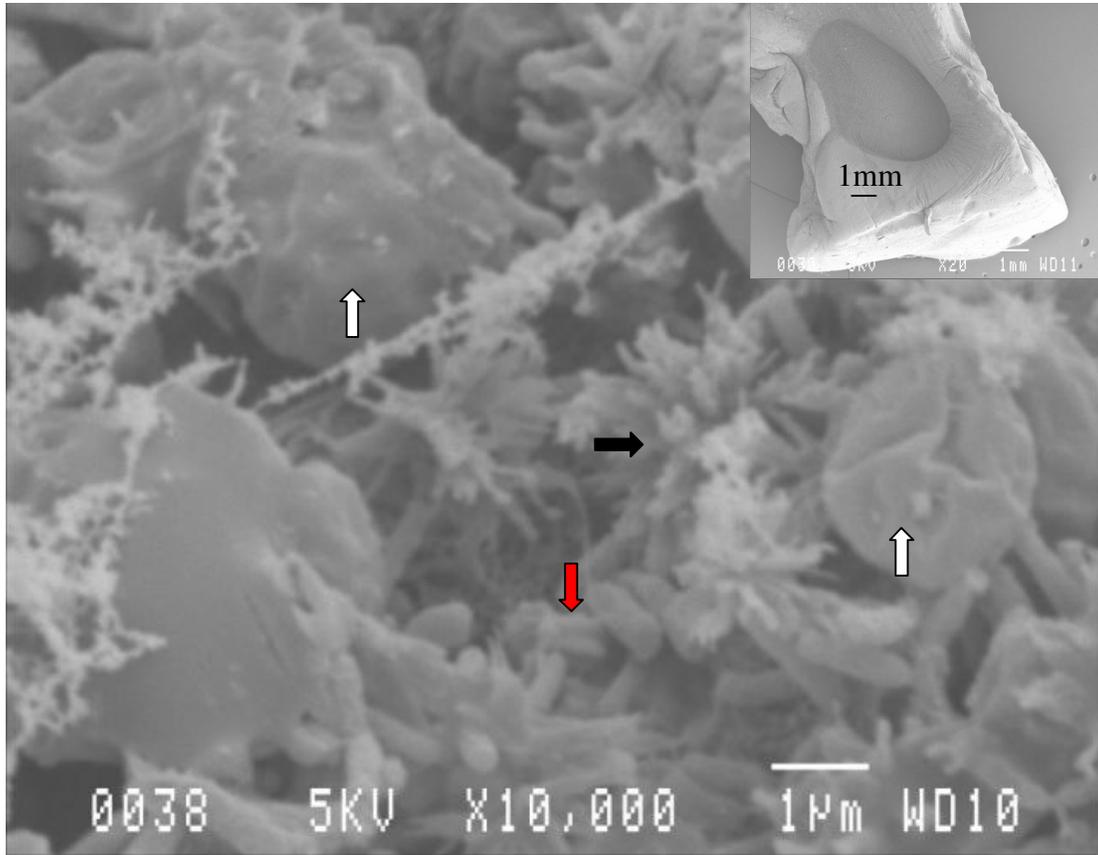
Figure 2. SEM micrograph of plaque zone showing interactions of plumb rod-shaped *Bacillus* (red arrow) and *Microcystis* cells. In the background, some of the *Microcystis* cells are 'deflated' (white arrow). The 'star-like' items (black arrow) are sections of agar material.

abundance and community structure composition differing according to *Microcystis* species (Maruyama et al., 2003). The researchers illustrated the possible role of free and attached bacteria found in the mucilages of *Microcystis* colonies as degrading the microcystins.

TEM micrographs of the plaque showed intermingled bacteria and *Microcystis* cells in various stages of degradation (Figure 5). Possibly these bacteria were scavenging the skeletal remains of *Microcystis* (Figures 5e to f).

Burnham et al. (1981) showed that, the colonial spherule of *Myxococcus xanthus* PCO2 entrapped the filamentous cyanobacterium *P. luridum* which then proceeded to degrade the cyanobacterium. Their studies indicated that, *M. xanthus* PCO2 released an extracellular substance that dissolved the cyanobacteria cell wall at the point of contact. It was therefore, speculated based on the Burnham studies (1981) that, there is a possibility of release of exoenzymes during the physical contact between bacteria and *Microcystis* cells used in this study (Figure 5a). The result is damage to the cyanobacteria cell wall, indicated by a number of sites that had ruptured (Figures 5c to d). The lysed *Microcystis* cells are shown at various stages of degradation, some

are deflated and some with damaged outer membranes (scroll like structures) (Figures 3c and 5f). These findings agree with the research work of Daft et al. (1973), who pointed out that the 'scroll-like structures' originate from a cyanobacteria cell wall layer. Bacteria were also observed inside the *Microcystis* skeletal remains (Figure 5d). There are a number of theories that may be advanced. Maybe the bacteria behaved like a *Bdellovibrio* and entered the cyanobacteria or other bacteria came in at later stage to scavenge the *Microcystis* remains.

Evaluations of cyanobacteria-bacteria interactions in liquid phases

Light and electron microscopy were used to assess the morphological changes that occurred on the *Microcystis* cell membrane after exposure to *B. mycoides* B16. The micrographs revealed that, the morphological details of *Microcystis* cells (treated with *B. mycoides* B16) were different from the control. The results of the control were normal and healthy *Microcystis* cells (Figures 6a, b) and bacterial presence resulted in swollen *Microcystis* cells (Figures 6c-d).
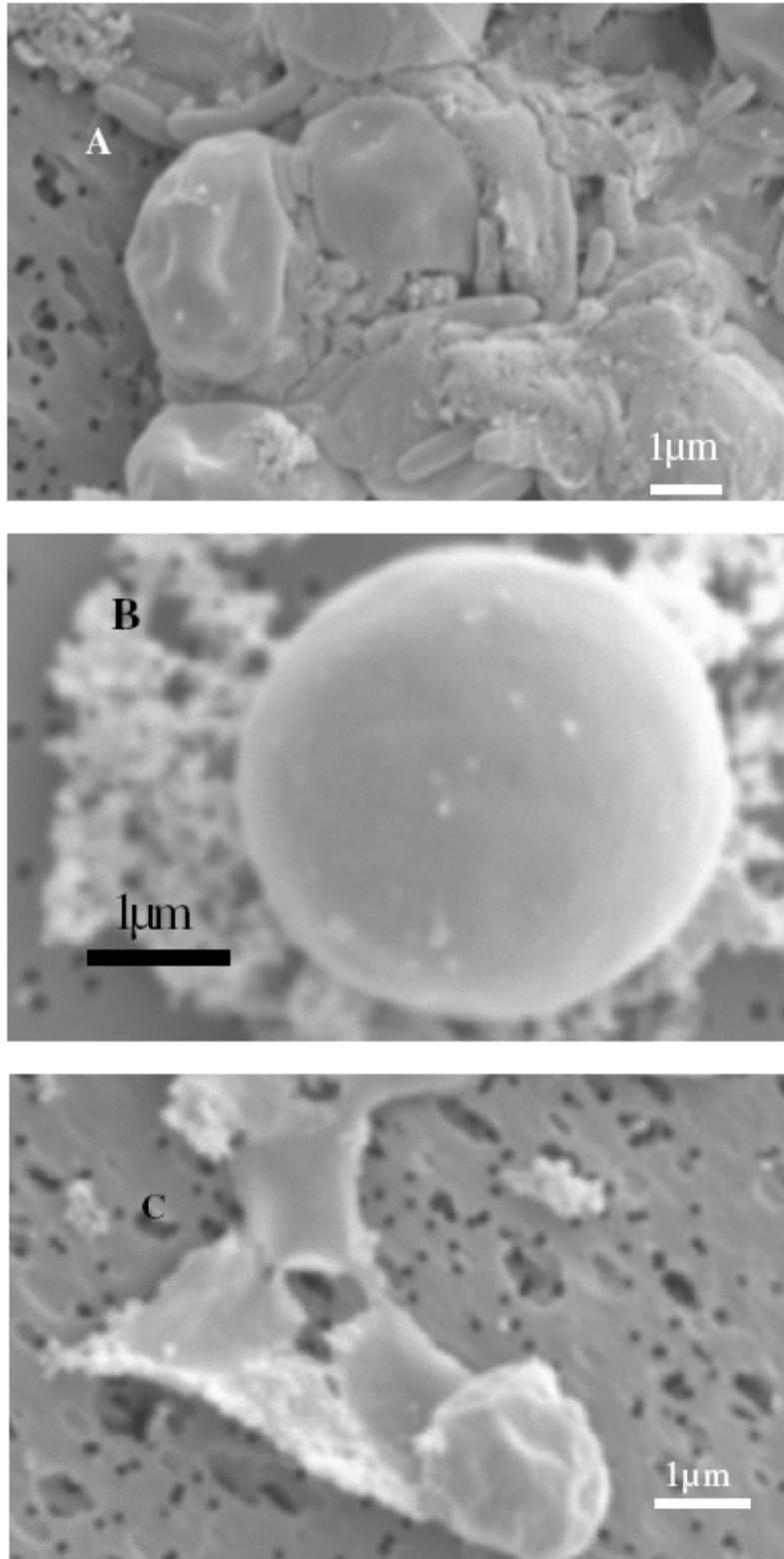
Figure 3. Distribution of bacteria within the plaque area and the control area. (A), Plumb rod-shaped *Bacillus* bacteria were abundant and were found aggregated around the *Microcystis* cells, which were deflated and unhealthy; (B), a healthy looking *Microcystis* cell from the control area (Note the absence of any distortion on the cell structure or 'deflation'); (C,) disintegration of scrolls from a *Microcystis* cell wall.
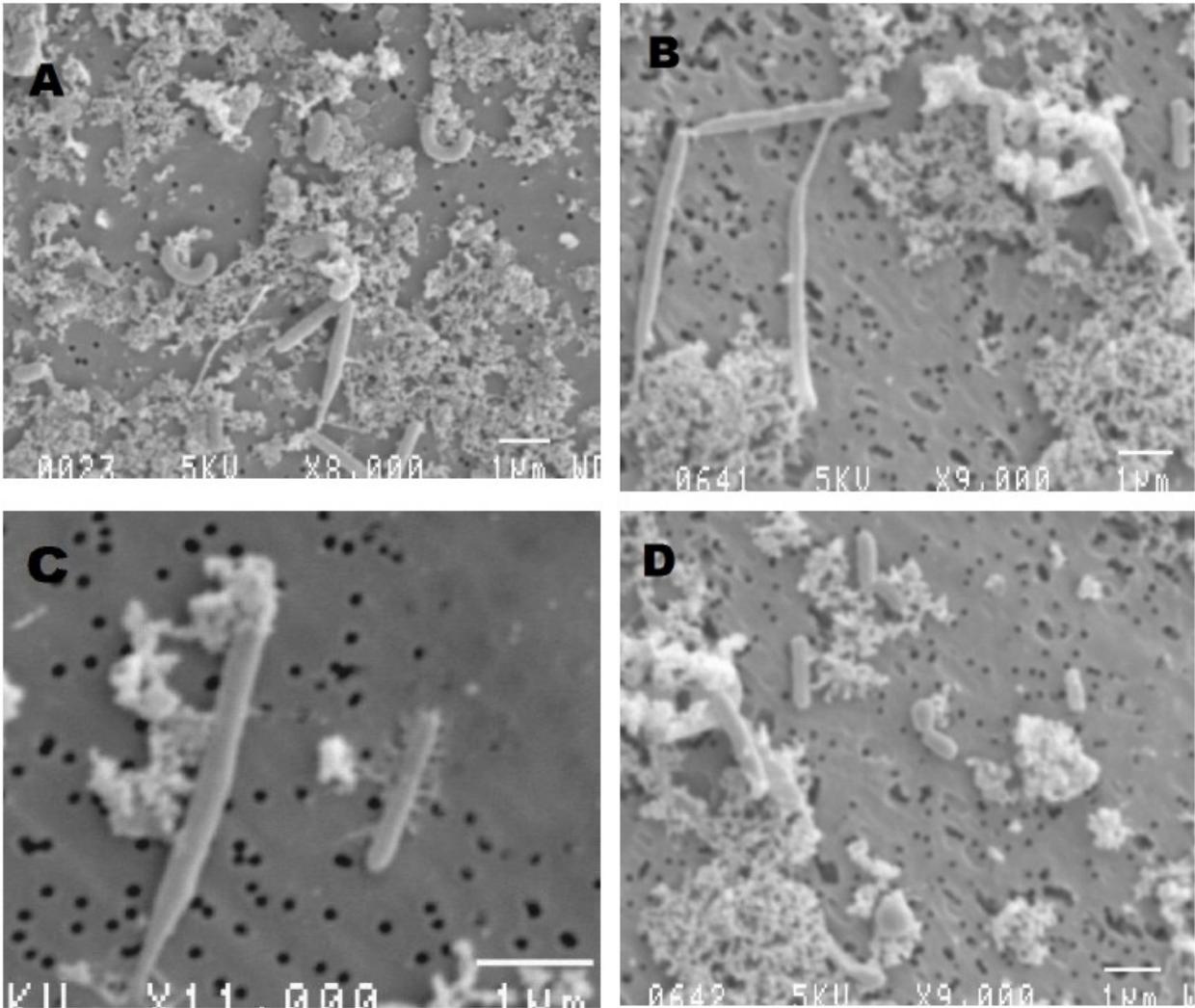
Figure 4. Distribution of unattached bacteria within the plaque area. (A), Vibrio shaped, long and short rod-shaped *Bacillus* coccoid shaped bacteria; (B), long rod-shaped *Bacillus* with sharp ends; (C), long rod-shaped *Bacillus* with prominent fimbriae; (D), short rod-shaped *Bacillus*, coccoid shaped bacteria.

SEM images of swollen *Microcystis* cells were presumably due to osmosis or the presence and multiplication of *Bdellovibrio*-like bacteria inside the *Microcystis* cell. The later is more plausible since bacterial movements were observed (wet mounts) inside swollen *Microcystis* cells. Reim et al. (1974) and Burnham et al. (1981) also reported the existence of swollen cyanobacteria cells prior to cell lysis, but did not account for what may have caused the swelling phenomenon. These findings suggest that, the bacteria penetrated the *Microcystis* cell and replicated producing progeny that caused the *Microcystis* cells to swell. The bdelloplasts then (*Bdellovibrio* progeny) feed on the host nutrients such that, the end result was distorted *Microcystis* cells. These progeny became part of the normal bacterial population. *Bdellovibrio*-like bacteria have been observed in field water samples of *Microcystis* cells and were localized within the cell wall and cytoplasm membrane (Caiola and Pellegrini, 1984). However, the studies did not indicate the life cycle of the *Bdellovibrio*-like bacteria or the presence of bdelloplast and these results were therefore difficult to compare with our studies.

Scanning electron microscopy observations showed bacteria that were attached to *Microcystis* cells (Figure 7). The bacterial rods appear to bind onto the surface of the *Microcystis* cell. The bacterial attachment appears to be related to either fimbriae (Dobson and McCurdy, 1979) and/or through the use of exopolymers (Cloete and Oosthuizen, 2001). The use of fimbriae as an attachment may either be temporary or irreversible. If it is temporary then, any agitation of liquid cultures is bound to disrupt the attachment. This in turn delays or even disrupts the *Microcystis* lysis process. Earlier on, Shilo (1970) and Daft and Stewart (1971) pointed out that, agitation of
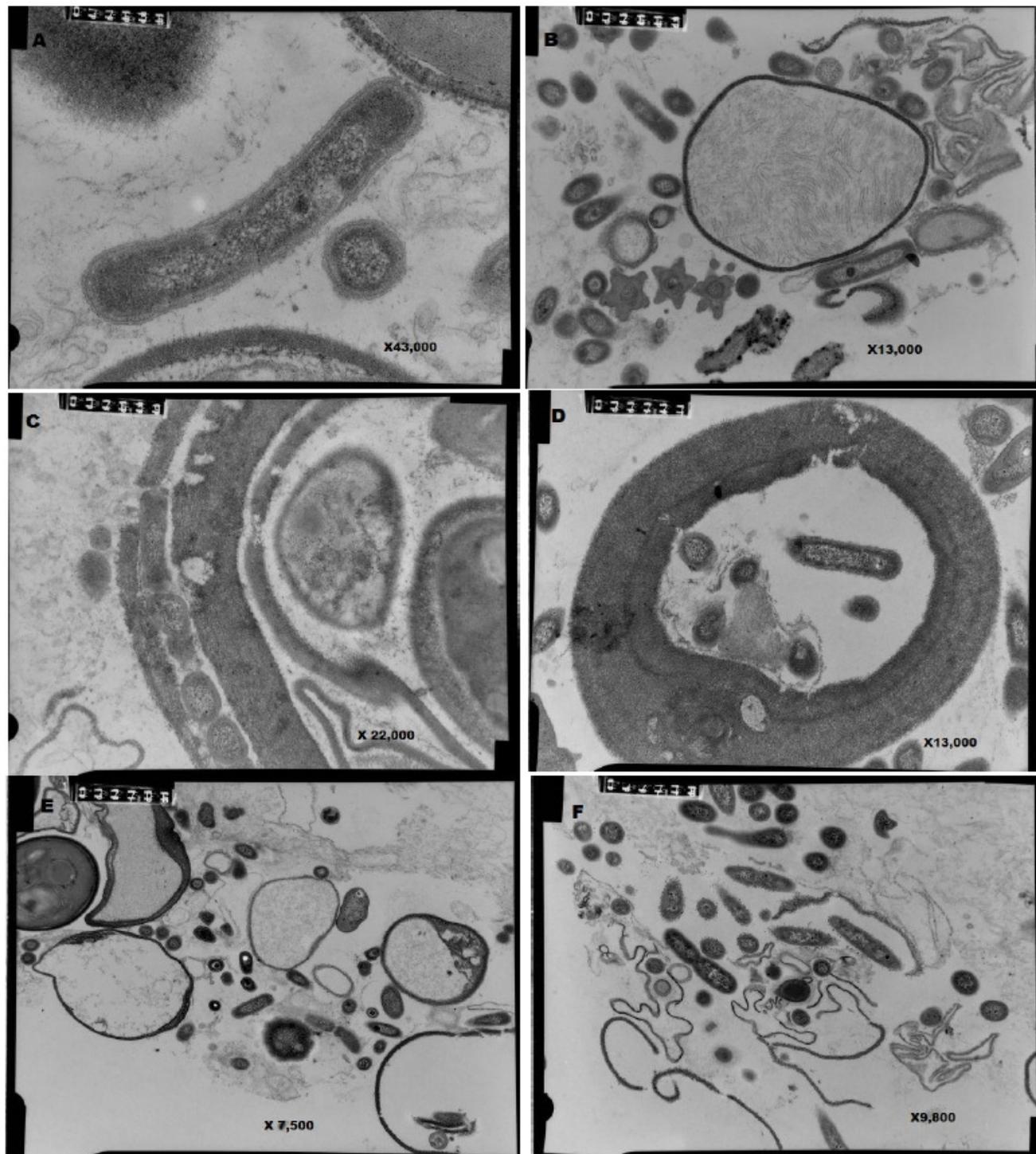
Figure 5. TEM micrographs showing interactions between bacteria and *Microcystis* cells. (A), Physical contact between bacteria and *Microcystis* cell; (B), *Bacillus* rod shaped bacteria around a skeleton *Microcystis* cell; (C), the damage on *Microcystis* cell membrane may be entry point for the bacteria; (D), some of the bacteria inside a *Microcystis* cell and/ or skeleton; (E and F), bacteria amongst the ghost *Microcystis* cells and cell debris.

samples disturbed the physical contact process between the cyanobacteria and bacteria.

Thus, the exposure of *Microcystis* cells to *B. mycoides*

B16 resulted in complete lysis as indicated by the skeletal remains (Figure 7d). These findings indicate the potential use of *B. mycoides* B16 in the management of
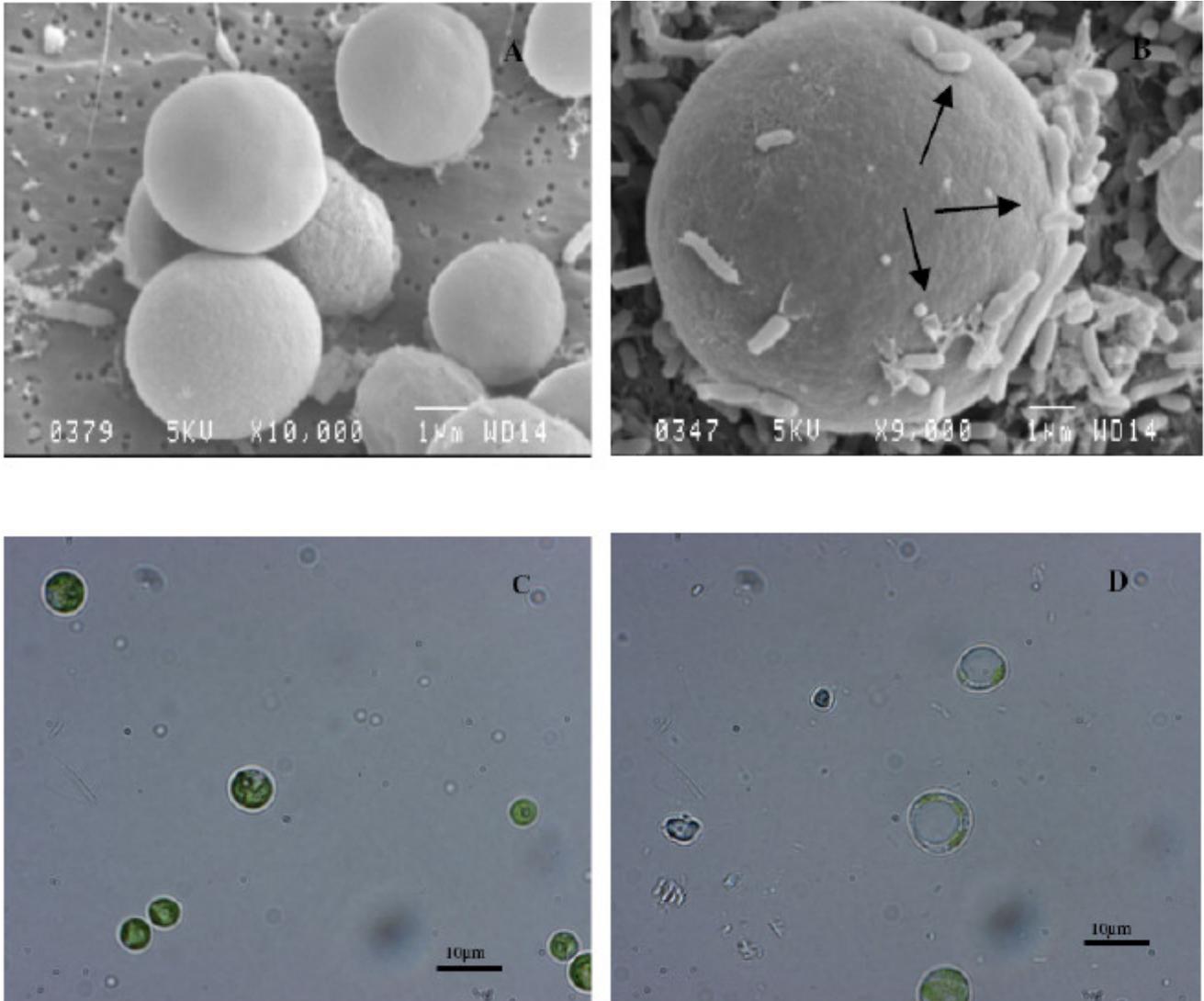
Figure 6. Light and electron micrographs of the treated and control samples. (A and C); Control *Microcystis* cells which are normal and healthy cells; (B and D), *B. mycoides* B16 treated *Microcystis* cells showing the size of the swollen cells. Note the presence of the plumb rod-shaped *Bacillus* bacteria attached to the *Microcystis* cell (arrows).

Microcystis algal blooms.

Algicides disruption of *Microcystis* cell membranes

Microcystis cells were exposed to copper and Triton X-100 to ascertain the level of damage to the cell membranes. Copper sulphate is a well-known algicide that is used to treat *Microcystis* algal blooms (Liam et al., 1995; García-Villada et al., 2004). Triton X-100 is used as permeabilising agent that causes damage of the cell membrane such that fluorescent dyes are able to enter into cell and stain a specific cell function during flow cytometric analysis (Hayden et al., 1988).

SEM images showed variations in the degree of damage on the *Microcystis* cell membrane (Figure 8). A normal and healthy *Microcystis* cell has a spherical shape with a smooth exterior surface and showed no visible damage (Figure 8a). *Microcystis* cells that were treated with copper were stripped of their entire cell membrane leaving behind a skeleton structure (Figure 8b).

Triton X-100 caused lesions on the *Microcystis* cell membrane structure (Figure 8c). Copper in the form of cupric ions (Cu^{2+}) lysed the *Microcystis* cell in the following ways: inhibition of carbon dioxide fixation and PSII activity, inhibition of nitrate uptake and synthesis of nitrate reductase and changes in cell volume (García-Villada et al., 2004). The consequences of copper use results in stripping of the *Microcystis* cell membrane and the release of intracellular contents including microcystins into the water.

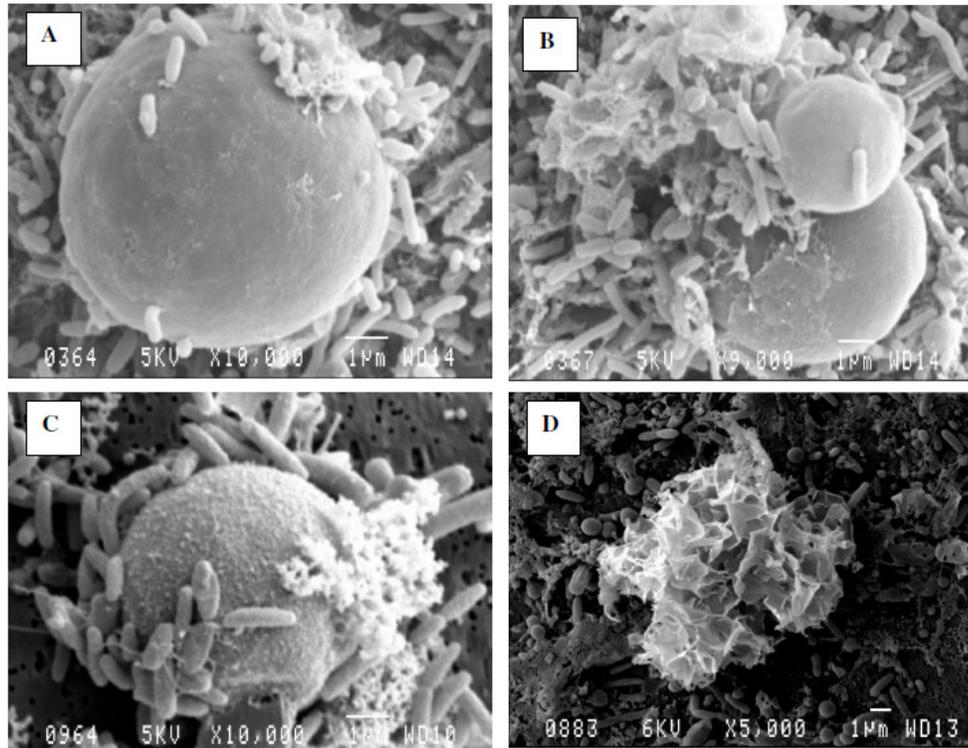The results showed that, the exposure of *Microcystis* to

Figure 7. SEM micrographs showing the *Microcystis* interaction with *B. mycoides* B16; (A), Bacterial attachment on cell; (B) damage on cell membrane; (C) perforations on cell membrane; (D) skeletal remains.
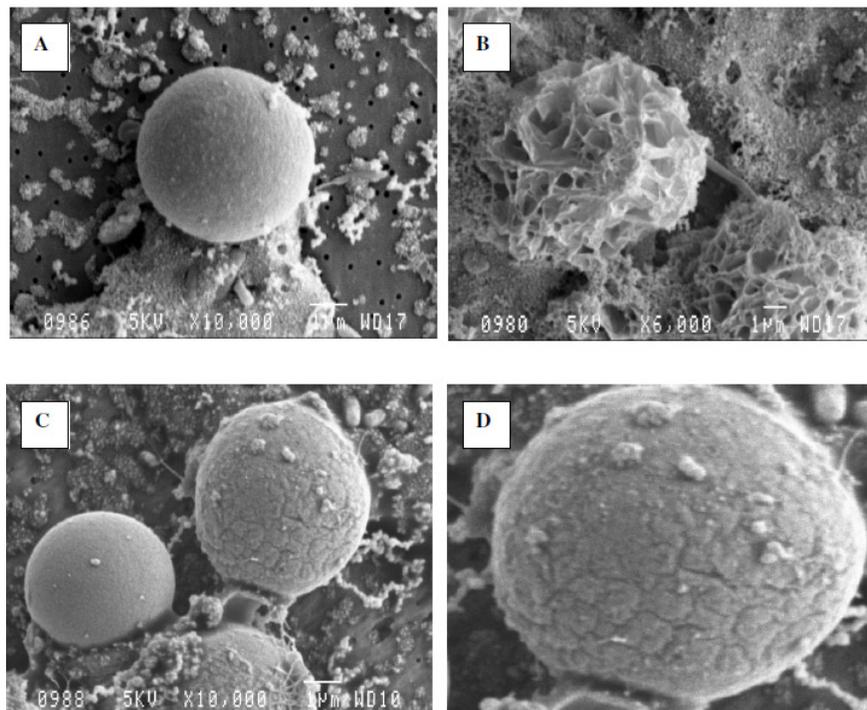

Figure 8. SEM indicating the morphological changes to *Microcystis* cell membrane. (A), Control sample showing smooth cell structure no visible damage; (B) copper treated *Microcystis* showing the remains of a skeleton; (C) Triton X showing damage to cell membrane; (D) enlargement of (C) showing the 'cracks' on the cell membrane.

copper and Triton X-100 caused cell membrane damage with copper stripping the entire cell. These findings also confirm that, the extensive use of copper in the management of *Microcystis* algal blooms.

Conclusions

Electron microscope studies confirmed that, there were at least five distinct morphotypes of bacteria found in the plaques: (1) plumb rod-shaped *Bacillus* that was attached and free-living; (2) a long rod-shaped *Bacillus* with one end sharpened, not attached; (3) a plumb rod-shaped *Bacillus* with fimbriae, not attached; (4) vibrio shaped rods, not attached and (5) coccoid shaped bacteria, the plumb rod-shaped *Bacilli* were abundant and were found aggregated around unhealthy *Microcystis* cells and were probably the cause of deflation and lysis of the algae, *B, mycoides* B16 were capable of causing damage of the *Microcystis* cell membrane and electron microscope studies showed that, the extent of *Microcystis* membrane damage was most severe with copper, followed by *B. mycoides* B16 and Triton X-100.

ACKNOWLEDGEMENTS

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