Chapter 4: Light and electron microscope assessment of the lytic activity of bacteria on *Microcystis*

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CHAPTER 4

LIGHT AND ELECTRON MICROSCOPE ASSESSMENT OF THE LYTIC ACTIVITY OF BACTERIA ON *MICROCYSTIS*

Abstract

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. Maybe these bacteria utilized the cyanobacteria cell contents as their nutrient source. In contrast the control areas the cyanobacteria cells were healthy and did not show any visible distortion of cell structure. The presence and possible role of free-bacteria, i.e., bacteria that was 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. Isolates B2 and B16 were found to have a lytic effect on *Microcystis* cells. SEM images of isolate B2 and B16 did not reveal any unique attachments that may allow 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 appear to reveal at least two mechanisms of *Microcystis* lysis i.e. contact and parasitism. The TEM observations did not reveal any endoparasitism of *B. mycoides* B16 or *Bdellovibrio*-like behaviour. There were ultrastructural changes that occurred in bacteria treated *Microcystis* cells. SEM images showed swollen *Microcystis* cells, either due to osmosis and or the presence and multiplication of *Bdellovibrio*-like bacteria inside the *Microcystis* cell. The *Bdellovibrio*-like behaviour of *B. mycoides* B16 was indirectly shown by the presence of bdelloplasts that were present during the last stages of *Microcystis* lysis.

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

4.1. INTRODUCTION

Harmful algal blooms (HABs) in freshwater resources, are often dominated by *Microcystis* species, 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 are microbial agents such as bacteria and viruses that have been 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: (a) competition for available organic matter; (b) provision of extracellular substances by cyanobacteria which are beneficial to bacteria and vice versa; (d) antagonistic behaviour whereby the bacteria feeds on cyanobacteria and (d) production of cyanobacteria exudates which inhibit 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 harmful algal bloom dynamics (Bates et al., 2004). There is a close spatial and temporal 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 in 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). Scanning electron micrographs revealed the attachment of *Flexibacter flexilis* to the sheaths of filamentous

Oscillatoria willimasii, which resulted in excretion of lysozyme and then lysed the cyanobacterium (Sallal, 1994). The *F. flexilis* bacterium benefits from the cyanobacterium nutrients after lysis and helps 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 recognition sites to allow for interactions between bacteria and cyanobacteria. In the natural environment, the BLB are selective only invading *M. aeruginosa* but not *M. 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 indicate 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 have 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 studies (Chapter 3), it was observed that bacterial agents were responsible for plaque developments on *Microcystis* lawns. The main objectives of this study were

to assess which bacterial morphotypes were present in the plaque zones and to explore the relationships between the bacterial agents and *Microcystis* cells during the lytic process. Scanning electron microscopy (SEM) was used to assess the morphological changes of the *Microcystis* cells. Transmission electron microscopy (TEM) was used to determine ultrastructural changes that occurred during the interactions between *Microcystis* and predatory bacteria. Light microscopy was used to observe the interactions between predator and prey (wet mounts). The light and electron microscopy studies formed the basis for the development of a model describing the mechanism of *Microcystis* lysis.

4.2. MATERIALS AND METHODS

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

Agar sections were cut from the plaques (Chapter 3: 3.3.2.) on BG11 agar plates and were examined with SEM and TEM. Areas with green *Microcystis* lawns indicating the absence of plaques served as controls.

4.2.1.1. Scanning Electron Microscopy

The agar sections were fixed with 2.5% v/v gluteraldehyde in 0.075M-phosphate buffer (30 min) and then filtered through a 0.22µm membrane. The membrane filter was washed three times with 0.075M 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 5.0kV.

4.2.1.2. Transmission Electron Microscopy

Thin sections of agar were made with a stainless blade and then immersed in gluteraldehyde solution for 30 minutes. This was washed three times with 0.075M phosphate buffer (15 min) and fixed with osmium tetroxide (1 hour). The osmium tetroxide was removed through repeated washings (three times with 0.075M phosphate buffer (15 min)) and embedded overnight in Quetol resin. Sections were

cut on an ultramicrotome (Reicher-Jung Ultracut E), stained in uranyl acetate and lead acetate before being examined and photographed using a transmission electron microscope (Philips EM301).

4.2.2. Evaluations of cyanobacteria-bacteria interactions in liquid phases

4.2.2.1. Experimental set up

Culture suspensions of cyanobacteria (20 mℓ) (Section 3.2.4.1) and bacteria (20 mℓ) (Section 3.2.4.3) were mixed in a 250 mℓ Erlenmeyer flask. The incubation procedure in Section 3.2.4.1 was followed. On d 4 samples were taken for microscopy analysis. All the experiments and controls were done in duplicate.

4.2.2.2. Light microscopy

One drop of the suspension (control and treated samples) was placed onto a microscopic slide and then 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.

4.2.2.3. Scanning electron microscopy

A suspension (control and treated) was filtered through a 0.22µm membrane filter and fixed with 2.5% v/v gluteraldehyde in 0.075M phosphate buffer (30 min). The same procedure in Section 4.2.1.1 was followed.

4.2.3. Algicide disruption of *Microcystis* cell membranes

Aliquots of copper sulphate (10 mg/mℓ) and (0.01%) Triton X-100 were added to *Microcystis* suspension (Section 3.2.4.1). The experimental design is shown in Figure 4.1 was incubated as in Section 4.2.2.1 for 24 h. SEM was performed as described above.

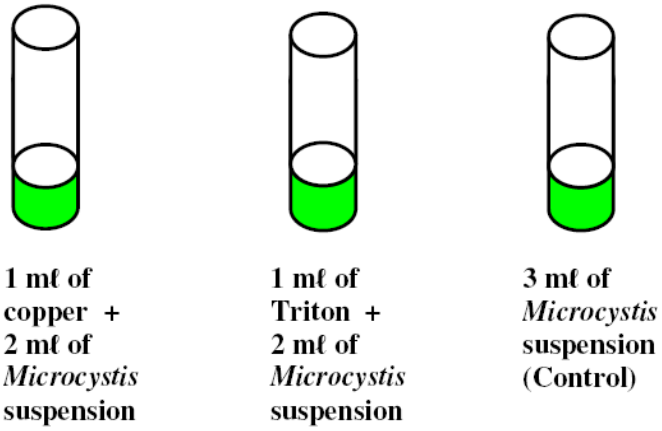

Figure 4.1: Experimental design for testing of algicides

4.2.4. Ultrastructural changes in *Microcystis* cells during lysis after exposure to *B. mycoides* B16

4.2.4.1 Preparation of freeze dried *B. mycoides* B16

An inoculum of the *B. mycoides* B16 was cultured in 100 mℓ of one-tenth of tryptic soy broth (TSB) in a 250 mℓ Erlenmeyer flask under shaking incubation (128 rpm, 25^{o}C) for 24 h (Di Franco et al., 2002). After cultivation the bacterial cell suspensions were harvested by centrifugation (10,000rpm, 15 min, 25^{o}C) and freeze dried (Edwards freeze dryer: minus 50^{o}C, 2.8mbar, 72 h). The lyophilized powder was stored at 4^{o}C until further use.

4.2.4.2. Experimental set up

To a 20 mℓ *Microcystis* suspension (Section 3.2.4.1), 198.3 mg of lyophilized powder (Section 4.2.4.1) was added and subjected to shake incubation (78 rpm; 25^{o}C; 2000 lux continuous light illumination) for 20 h. For a control, 20 mℓ *Microcystis* suspension was used and incubated as above. At hourly intervals 1 mℓ of treated and control samples were sampled and centrifuged (10,000 rpm, 10 min, 25^{o}C). The pellet was re-suspended and fixed in 1 mℓ of 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer at pH 6.65 and stored at 4^{o}C until further use for TEM (Section 4.2.4.3) and SEM (Section 4.2.2.3).

4.2.4.3. Transmission electron microscopy

The samples were then fixed with 1mℓ of 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer supplemented with 1% alcian blue (previously filtered through a Cameo acetate 0.22 µm syringe filter) for 2 h at 4°C. After primary fixation, the samples were centrifuged (10,000 rpm, 15 min, 25°C) and the pellet re-suspended in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer. This process was repeated three times and the samples were then post-fixed for 1 h in 1% OsO_4. The samples were then rinsed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer and dehydrated in an ascending ethanol series [50, 70, 90 and 100% (thrice)].

4.3. RESULTS AND DISCUSSION

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

After incubation of an aliquot of the eutrophic waters on BG11 agar, plaques were formed. 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 4.2 and 4.3) and free-living (Figure 4.4c); (2) a long rod-shaped bacillus with one end sharpened, not attached (3 to 6µm) (Figure 4.4b); (3) a plumb rod-shaped bacillus with fimbriae, not attached (1.5 to 3 µm)(Figure 4.4d); (4) vibrio shaped rods, not attached (Figure 4.4a) and (5) coccoid shaped bacteria (0.6 µm) (Figure 4.4a & c). SEM micrographs showed the presence of plumb shaped bacillus aggregated around unhealthy *Microcystis* cells (Figure 4.2). The *Microcystis* cells appeared distorted or deflated wherever these bacillus rods were present.

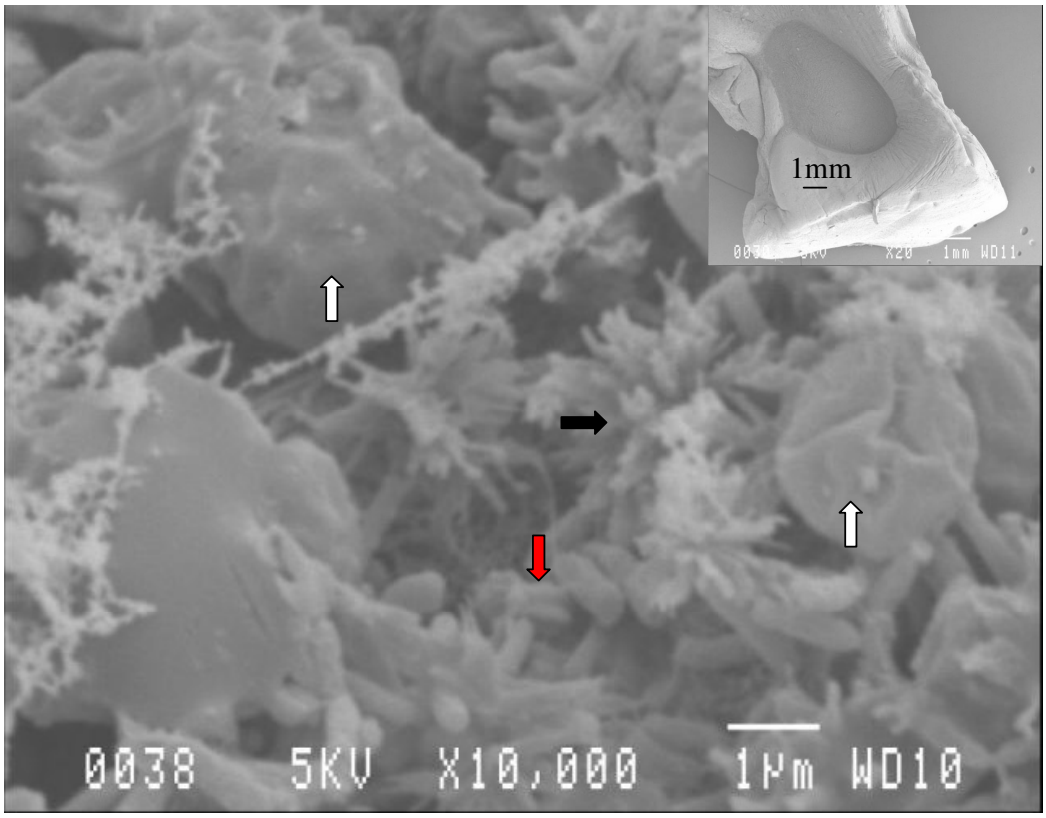

Figure 4.2: SEM micrograph of plaque zone (insert) showing interactions of plumb rod-shaped bacillus (red arrow) and *Microcystis* cells. In the background some of the *Microcystis* cells are 'deflated' (white arrow). The 'star-like' items (black arrow) are sections of agar material.

These results show similar bacteria flora that were observed and consisted of 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 (Robarts and Zohary, 1986). 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 agar material (Figure 4.2). 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 4.2) that were closely associated with unhealthy *Microcystis* cells (Figure 4.3a).

In the control areas the cyanobacteria cells were healthy and did not show any visible distortion of cell structure (Figure 4.3b). At this stage the unhealthy *Microcystis* cells appeared to be associated with plumb rod-shaped bacillus that were probably the cause of deflation. Maybe these 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 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 (Figures 4.5). Possibly these bacteria were scavenging the skeletal remains of *Microcystis* (Figures 4.5 e-f).

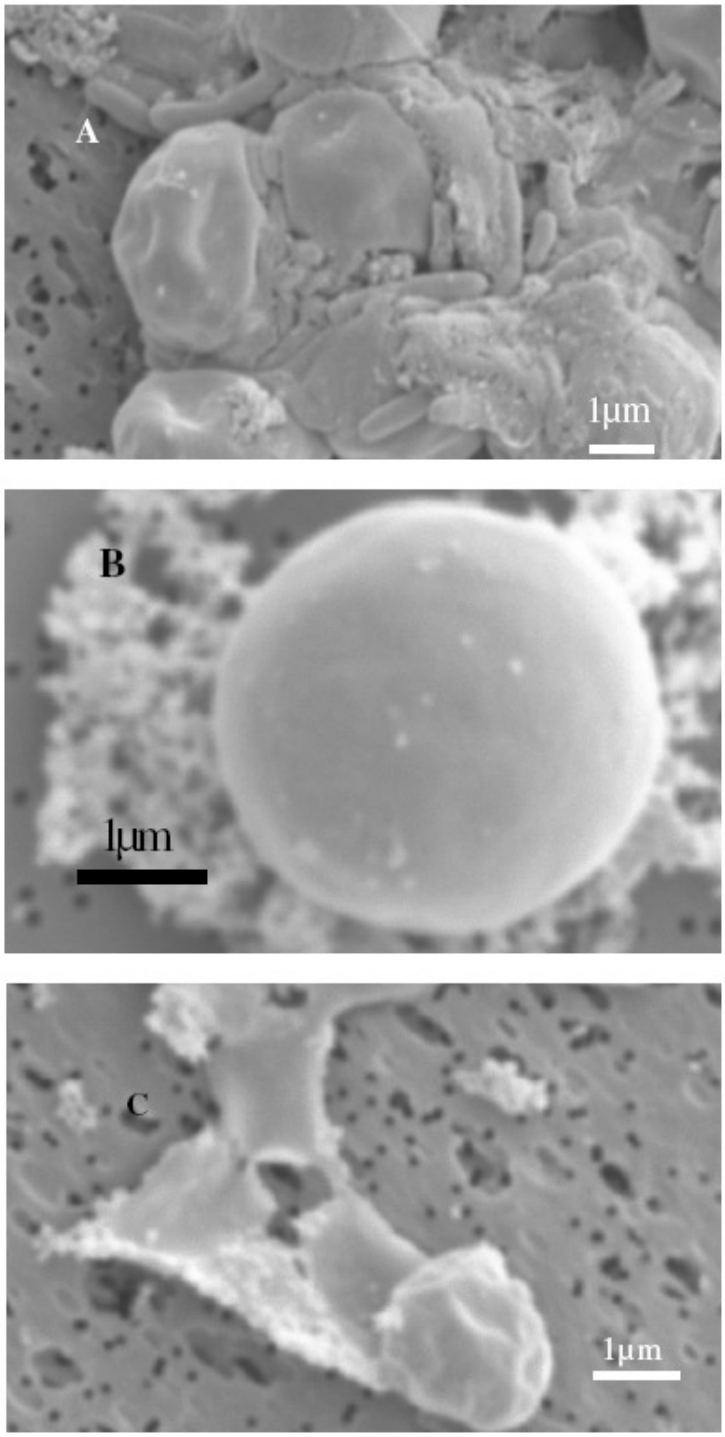

Figure 4.3: (A) Plumb rod-shaped bacillus bacteria were abundant and were found aggregated around *Microcystis* cells, which were deflated and unhealthy. (B) A healthy looking *Microcystis* cell from the control area. Note the absence of any distortion on the cell structure or 'deflation'. (C) Disintegration of scrolls from a *Microcystis* cell wall.

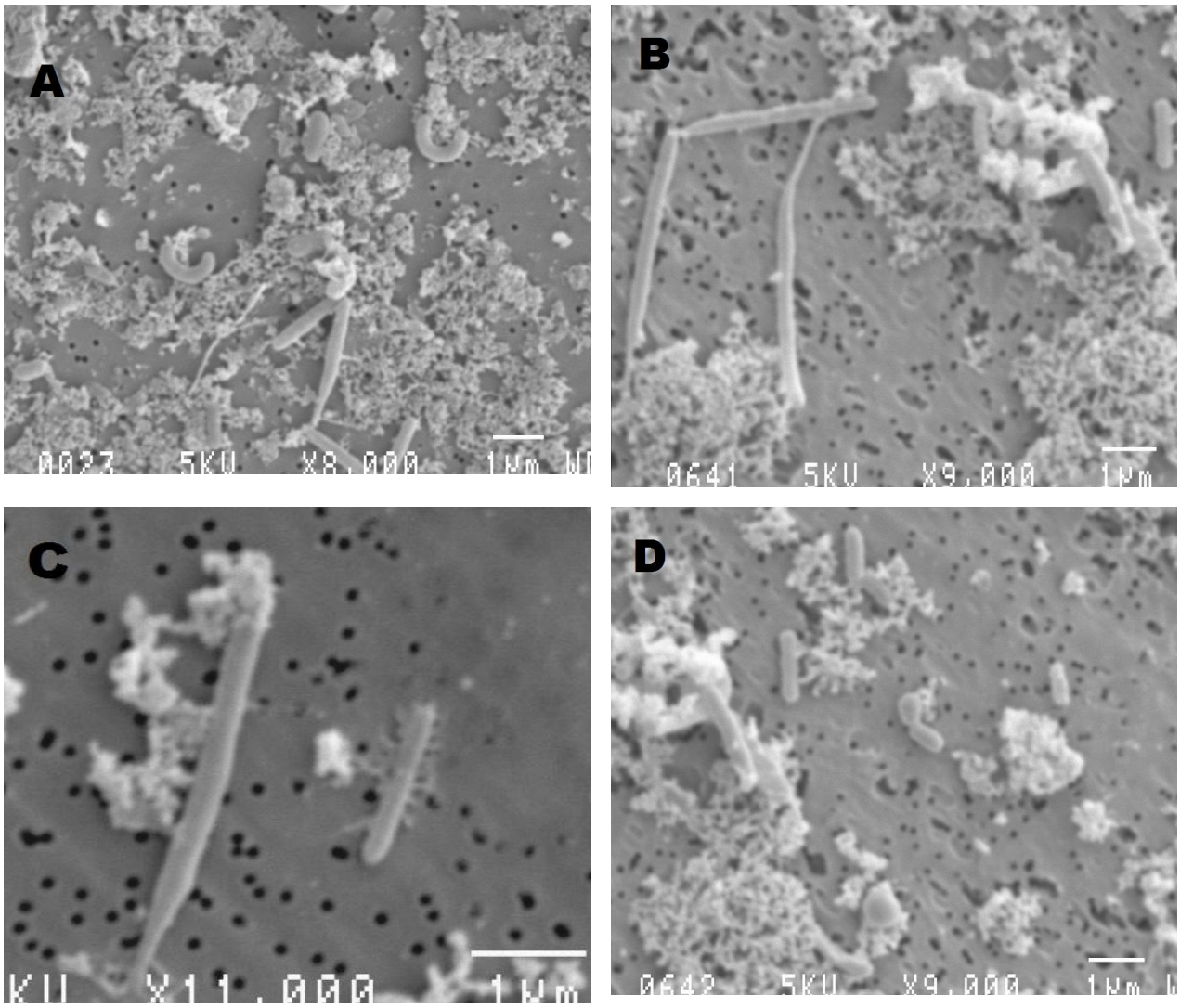

Figure 4.4: (A) Vibrio shaped, long and short rod-shaped bacillus, coccoid shaped bacteria; (B) Long rod-shaped bacillus with sharp ends (C) Long rod-shaped bacillus with prominent fimbriae and (D) Short rod-shaped bacillus, coccoid shaped bacteria.

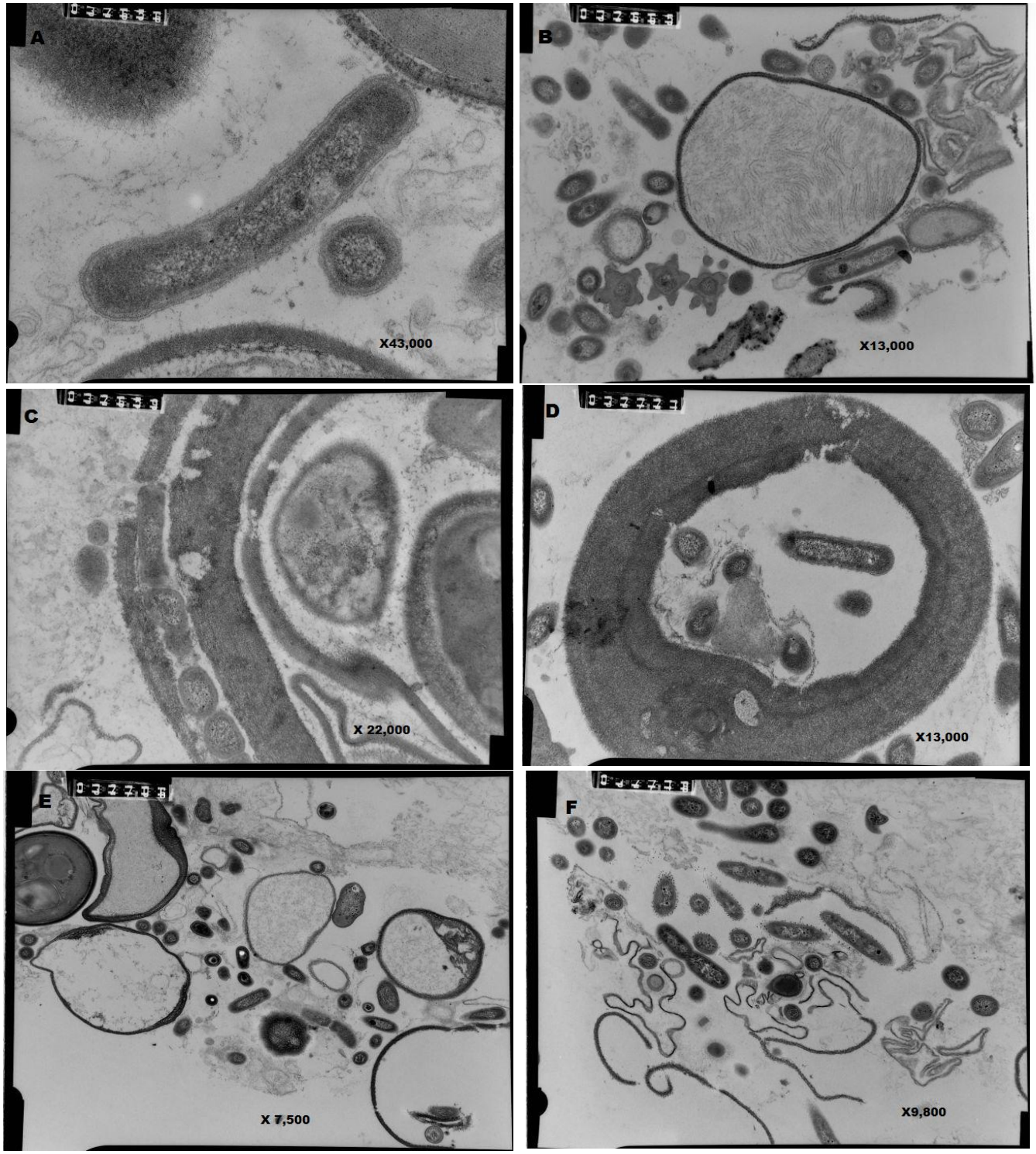

Figure 4.5: TEM micrographs showing interactions between bacteria and *Microcystis* cells. (A) Physical contact between bacteria and *Microcystis* cell; (B) Bacillus rod shaped bacteria around a skeleton *Microcystis* cell; (C) damage on *Microcystis* cell membrane may be entry point for bacteria; (D) some of the bacteria are inside a *Microcystis* cell and or skeleton. (E and F) Bacteria amongst ghost *Microcystis* cells and cell debris.

Burnham et al. (1981) showed that the colonial spherule of *Myxococcus xanthus* PCO2 entrapped the filamentous cyanobacterium *Phormidium 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 4.5a). The result is damage to the cyanobacteria cell wall, indicated by a number of sites that had ruptured (Figures 4.5c-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 4.4d and 4.5f). 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 4.5d). 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 remains.

4.3.2. 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 4.6a-b) and bacterial presence resulted in swollen *Microcystis* cells (Figures 4.6c-d).

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 localised 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.

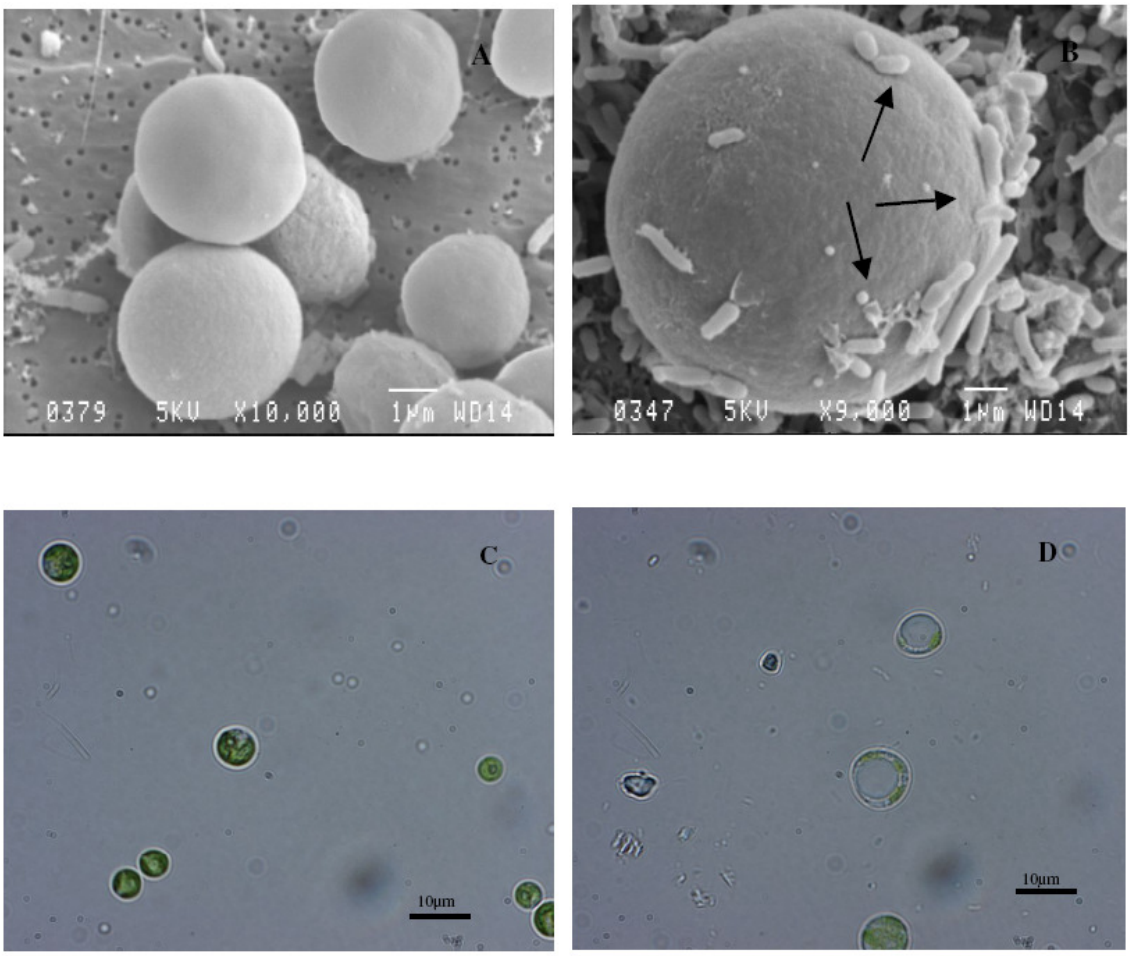

Figure 4.6: (A & C) Control *Microcystis* cells which are normal and healthy cells and (B & D) *B. mycoides* B16 treated *Microcystis* cells showing the size of the swollen cells. Note the presence of plumb rod-shaped bacillus bacteria attached to the *Microcystis* cell (arrows).

Scanning electron microscopy observations showed bacteria that were attached to *Microcystis* cells (Figure 4.7). 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 samples disturbed the physical contact process between the cyanobacteria and bacteria.

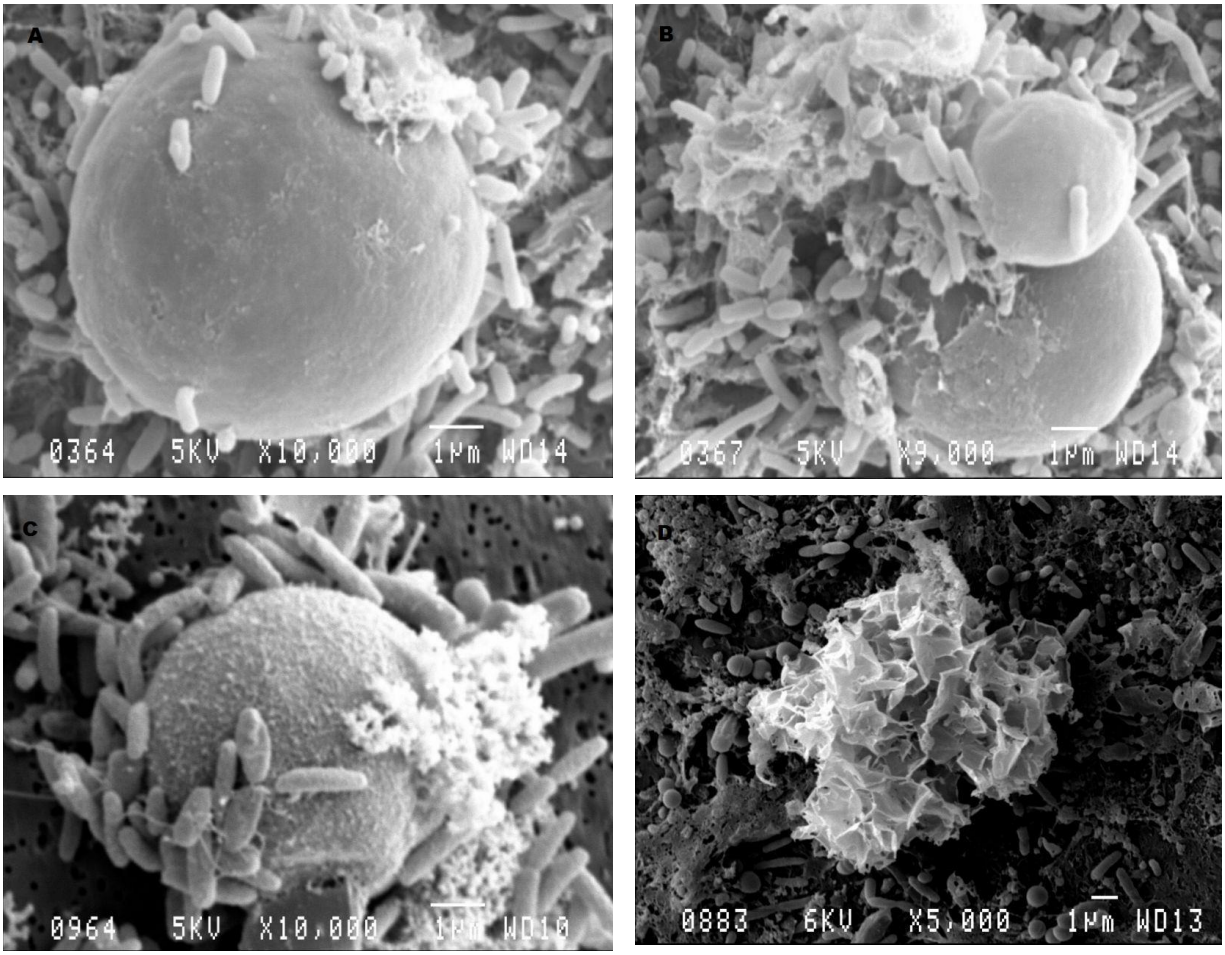

Figure 4.7: SEM micrographs showing the *Microcystis* interaction with *B. mycoides* B16; (a) bacterial attachment on cell; (b) damage on cell membrane; (c) perforations on cell membrane (c) and (d) skeletal remains.

Thus the exposure of *Microcystis* cells to *B. mycoides* B16 resulted in complete lysis as indicated by the skeletal remains (Figure 4.7d). These findings indicate the potential use of *B. mycoides* B16 in the management of *Microcystis* algal blooms.

4.3.3. Algicide 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 a permeabilising agent that causes damage of the cell membrane such that fluorescent dyes are able enter to 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 4.8). A normal and healthy *Microcystis* cell has a spherical shape with a smooth exterior surface and showed no visible damage (Figure 4.8a). *Microcystis* cells that were treated with copper were stripped of their entire cell membrane leaving behind a skeleton structure (Figure 4.8b).

Triton X-100 caused lesions on the *Microcystis* cell membrane structure (Figure 4.8c). 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.

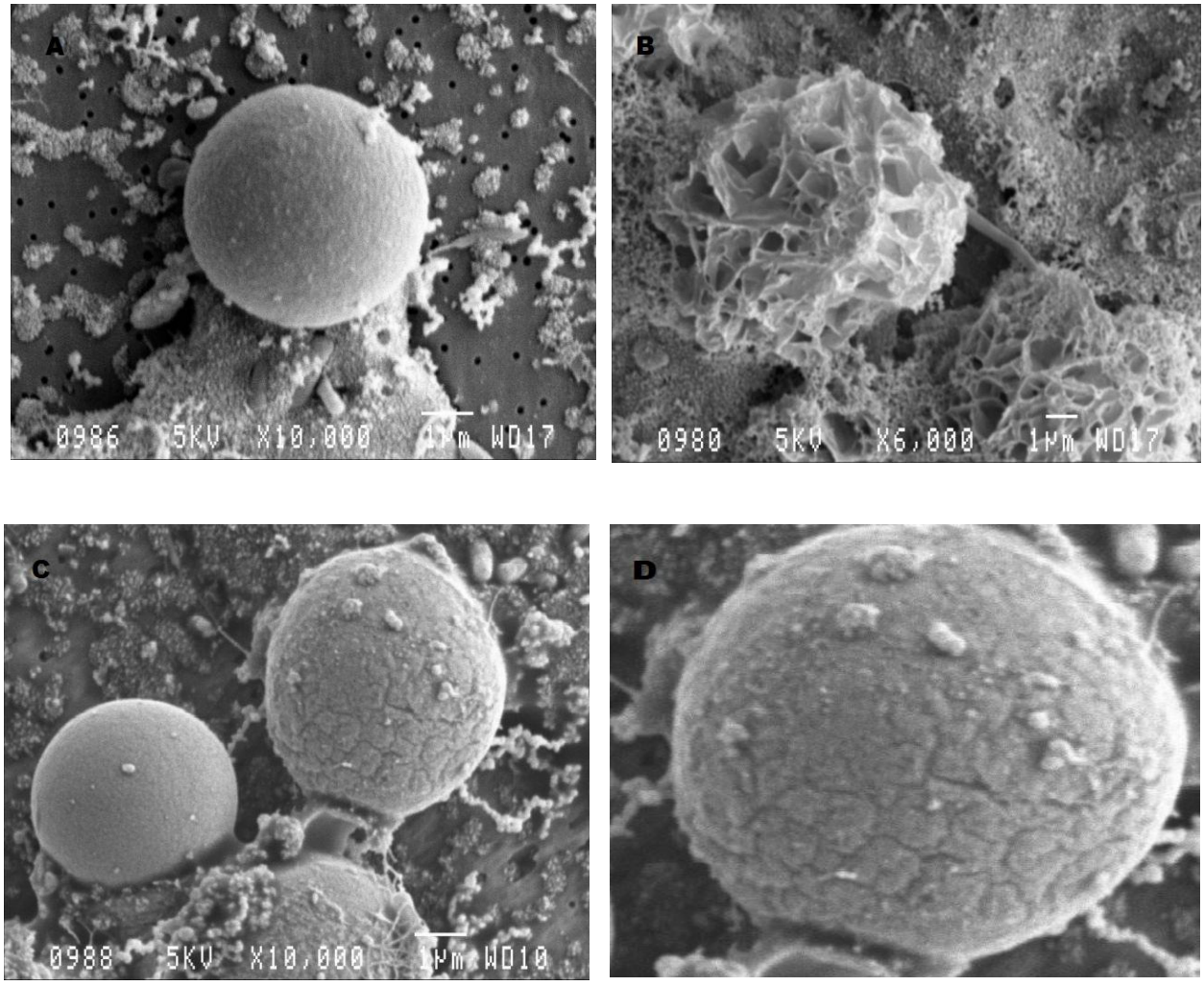

Figure 4.8: SEM indicating the morphological changes to *Microcystis* cell membrane (A) control sample showing smooth cell structure, no visible damage; (B) copper treated *Microcystis* showing the remains of a skeleton; (C) Triton X showing damage to cell membrane and (D) enlargement of (C) showing the 'cracks' on the cell membrane.

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

4.3.4. Ultrastructural changes in *Microcystis* cells during lysis after exposure to *B. mycoides* B16

Transmission electron microscopy observations revealed that the ultrastructural details of *Microcystis* cells treated with *B. mycoides* B16 were different from the control. The ultrastructural characteristics of a healthy and normal *Microcystis* cell are shown in Figure 4.9(a) and confirmed that of previous studies (Reynolds et al., 1981; Zohary, 1987). The internal structure of a healthy *Microcystis* consisted of a multilayered cell wall (CW), plasmalemma (P) and storage granules made up of cyanophycin (C) and polyphosphate bodies (P.B.) and parallel arrays of thylakoid membranes (TH).

After 2 h of incubation with *B. mycoides* B16, the internal structure of *Microcystis* was changed with a distorted cell wall (CW) enclosing the distorted plasmalemma (P) surrounding large glycogen granules (G), large polyphosphate bodies (P.B.) and large thylakoid membranes (TH) (Figure 4.9b). The *Microcystis* cells (bacteria treated) shrunk in size in comparison to the controls and scattered intracellular material presumably polyphosphate bodies were found in the external media (evidence of lysed *Microcystis* cells) (Figure 4.9c). Between 4 h to 8 h a similar lytic action was observed, i.e., distorted *Microcystis* cell walls and damage of the cell wall membrane and disoriented thylakoid membranes (Figure 4.10b). After 23 h, the *Microcystis* control showed evidence of growth as indicated by cell division (Figures 4.10e), whilst the *Microcystis* (bacteria treated) showed a distorted cell wall with bacteria attached (Figure 4.10d) and lysed *Microcystis* cell debris (Figure 4.10f).

These findings suggest that during physical contact the bacteria released extracellular substances (Daft and Stewart, 1971; Burnham et al., 1981) that damaged the *Microcystis* cell membrane. Nakamura et al. (2003a) isolated an extracellular substance from *B. cereus* N14, closely related to *B. mycoides* B16 (von Wintzingerode et al., 1997) that showed lytic activity towards *Microcystis*. It is possible that a similar extracellular substance produced by *B. mycoides* B16 may have resulted in cell lysis during this study.

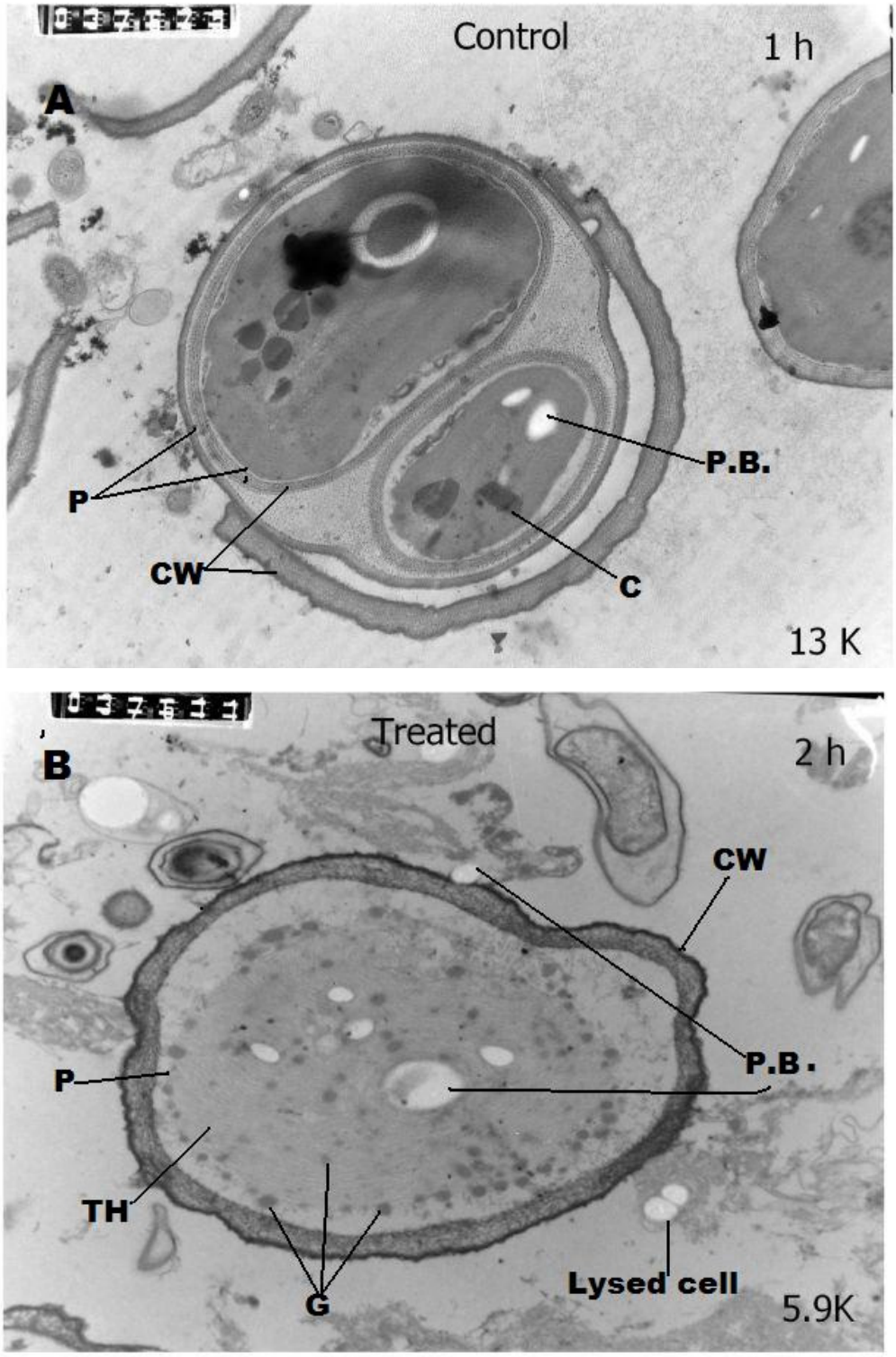

Figure 4.9: (A) Control: Healthy *Microcystis* cell in the process of division, cell wall (CW) and plasmalemma (P), storage granules cyanophycin (C) and polyphosphate bodies (P.B.); (B) *Microcystis* (bacteria treated); large glycogen granules (G); polyphosphate bodies (P.B.), large thylakoids (TH); (C) After 4 h incubation, *Microcystis* cell was smaller due to loss of internal contents and lysis.

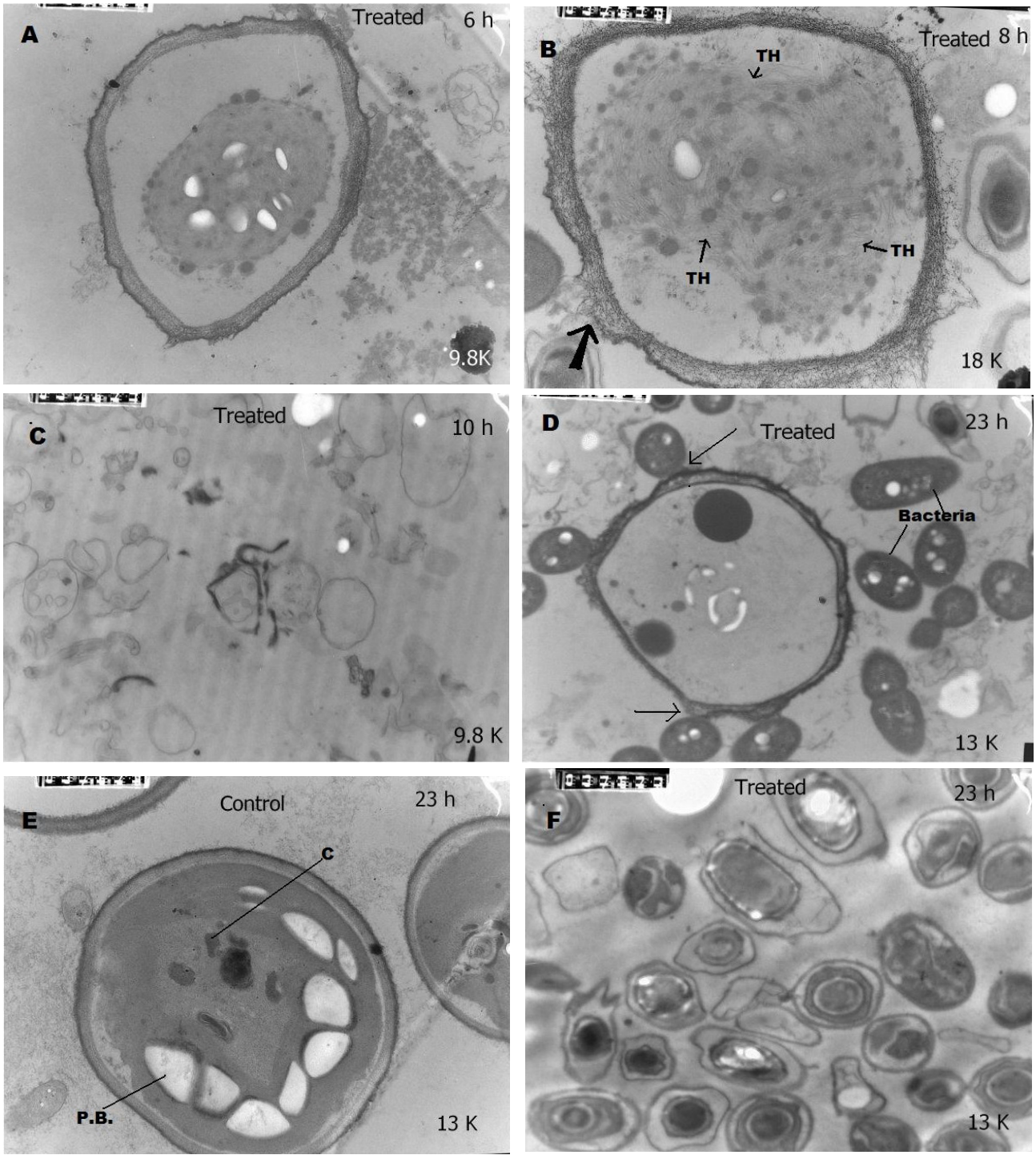

Figure 4.10: (A-B) *Microcystis* cell showing signs of distortion after 6h and 8h in the presence of bacteria. Note damage on cell wall membrane (arrow) and distorted thylakoids (arrow). (C) Lysed *Microcystis* cells. (D) Bacteria surrounding the *Microcystis* cell and attacking the cell (arrow); (E) Normal and healthy *Microcystis* cell after 24 h and (F) Lysed *Microcystis* cells after 24 h after exposure to bacteria.

Previous studies have shown that under experimental conditions of low light irradiance, *Microcystis* cells had large cyanophycin granules, numerous thylakoid membranes and a large nucleoplasmic area (Canini et al., 2001). On switching to high light conditions, the *Microcystis* produced superoxides to minimise damage to the cell and thylakoid systems and internal organelles were normal. Borbéy et al. (1990) reported similar results, except that the cyanobacteria, when subjected to an adverse external environment such as the presence of predatory bacteria, the cyanobacteria responded by accumulating of storage granules. Polyphosphate bodies, function as a store of phosphorus and appear as spherical inclusions of varying diameters located in the centre of the cell (Jensen, 1968). In our study numerous *B. mycoides* B16 numbers formed a 'cover or shade' over the *Microcystis* cells creating conditions of low light irradiance. It was hypothesized that the cyanobacteria then resorted to the production of storage granules like polyphosphates and glycogen. These are for the maintenance of essential processes instead of growth. The other evidence is the expansion of the thylakoid system to capture as much light as possible (Stewart and Alexander, 1971; Schneegurt et al., 1994; Moezelaar and Stal, 1997). The bacteria surrounding to the *Microcystis*, presumably also released extracellular substances that interfered with the *Microcystis* photosynthesis system as indicated by the collapse in the plasmalemma and thylakoid membranes that was more evident after 24 h of bacteria treatment (Figures 4.10b and f).

Veldhuis et al. (2001) noted that during automortality the last lysis stage involved the degradation of photosynthetic pigments and the reduction in photosynthesis of the phytoplankton. Nakamura et al. (2003a) also observed that during the lytic action by *B. cereus* N14 on *Microcystis* led to the exudation of photosynthetic pigments to the external media. At the same time the *B. mycoides* B16 may have derived nutrition from cell contents that leaked out of the *Microcystis* cells due to the damage of cell wall making it permeable (Figure 4.10d).

4.3.5. Behavioural changes in *B. mycoides* B16 during the lysis of *Microcystis*

Burnham et al. (1984) and Daft et al. (1985b) showed that *Myxococcus fulvus* BG02 exhibited polymorphism: spherules and myxospores, which showed lytic activity against *Phormidium luridum* and only resorted to the vegetative state when the

external environment was nutrient limited. Their mechanism of cyanobacteria lysis was based on the process of spore formation that led to the successful entrapment of cyanobacteria and release of extracellular substances (antibiotics) that targeted the peptidoglycan layer thus enabling *M. fulvus* BG02 to obtain nutrients. *B. mycoides* B16 displayed similar characteristics changing from single cells to chains during the lysis of *Microcystis* (Figure 4.11).

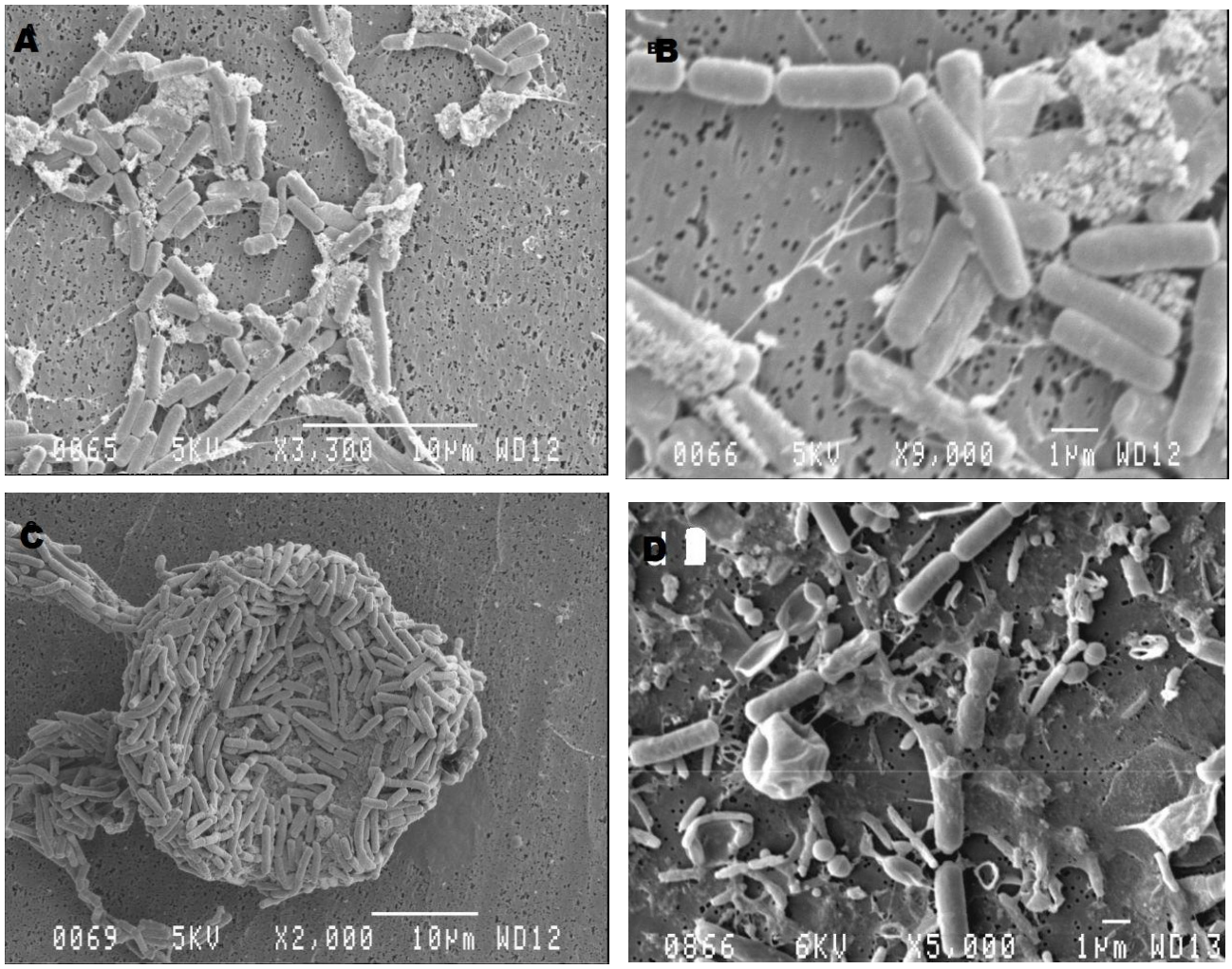

Figure 4.11**:** SEM images of *Bacillus mycoides* B16: (A) individual cells and chains; (B) in chains; (C) multicellular forms and (D) in chains during the *Microcystis* lysis stage.

B. mycoides B16 switched from single cell to a multicellularity phenotypes which is a characteristics of rhizodal phenotypes of *B. mycoides* species (Villain et al., 2006). The rhizoidal growth of *B. mycoides* through chain elongation over long distance may

be viewed as translocation through the *Microcystis* algal blooms. Thus the bacteria were able to derive nutrients from the lysis of *Microcystis* cells during the translocation process. Also the *B. mycoides* species formed clumps, possible as a survival strategy in the event of nutrient limitations (Villain et al., 2006). It was very difficult to break these clumps except through ultrasonication. Some *Bacillus* species were reported to revert to spore formation, only germinating and becoming active once the external environment was conducive for growth (Villain et al., 2006). The spore formation state was not observed with *B. mycoides* B16 through the malachite test.

4.3.6. The mechanism of lytic action of *B. mycoides* B16 on *Microcystis*

The results of this study on the interactions of *Microcystis aeruginosa* and *Bacillus mycoides* B16 indicated a series of morphological and ultrastructural changes with the cyanobacteria cell leading to its death. These are summarised in a conceptual model that was developed (Figure 4.12).

We have hypothesised that the first phase in the lysis process is the bacterial attachment onto the *Microcystis*. At this encounter the large bacteria numbers create conditions of 'shading' over the *Microcystis* cells, thus creating an environment of low light irradiance. Within 2 h with insufficient light the following features were observed: accumulation of glycogen, polybetahydroxybutyrate, cyanophycin and expansion of parallel thylakoids membranes. The controls showed normal and healthy *Microcystis* cells. After 6 h the cyanobacteria cell wall was distorted, in some cases broken. The thylakoids, light gathering apparatus, was in disarray and probably not functioning as well. The cyanobacteria resorted to use of accumulated granules for survival and not for growth.

The second and third phases are the bacterial release of lytic substances that damage the cyanobacteria cell membrane. In a separate study of *Bacillus cereus*, closely related to *Bacillus mycoides*, was found to release an unidentified lytic substance that lysed *Microcystis* cells (Nakamura et al., 2003b). The study of algicides showed that this was possible as the evidence of *Microcystis* cell membrane damage was similar to that of the bacterial attack. Swollen cyanobacterial cells were observed during this

stage. This was presumably due to osmosis or the presence of *Bdellovibrio*-like bacteria that multiplied within the cyanobacteria cell. The ultrastructural evidence presented does not support the later hypothesis despite light microscopy studies (wet mounts) detected bacterial movements within the cyanobacteria cell.

The last phase involved the leaking of cyanobacteria cell contents to the external environment indicating cell death. This aspect is the basis for the next study (Chapter 5) on the assessment of cell viability using flow cytometry. The *B. mycoides* B16 derived nutrition from the cell contents that leaked out of the *Microcystis*. An interesting point was the formation of chains by *B. mycoides* B16 cells. It was suggested that the rhizoidal growth of *B. mycoides* through chain elongation over a long distance could be viewed as a translocation through lysed *Microcystis* algal blooms in search of new prey.

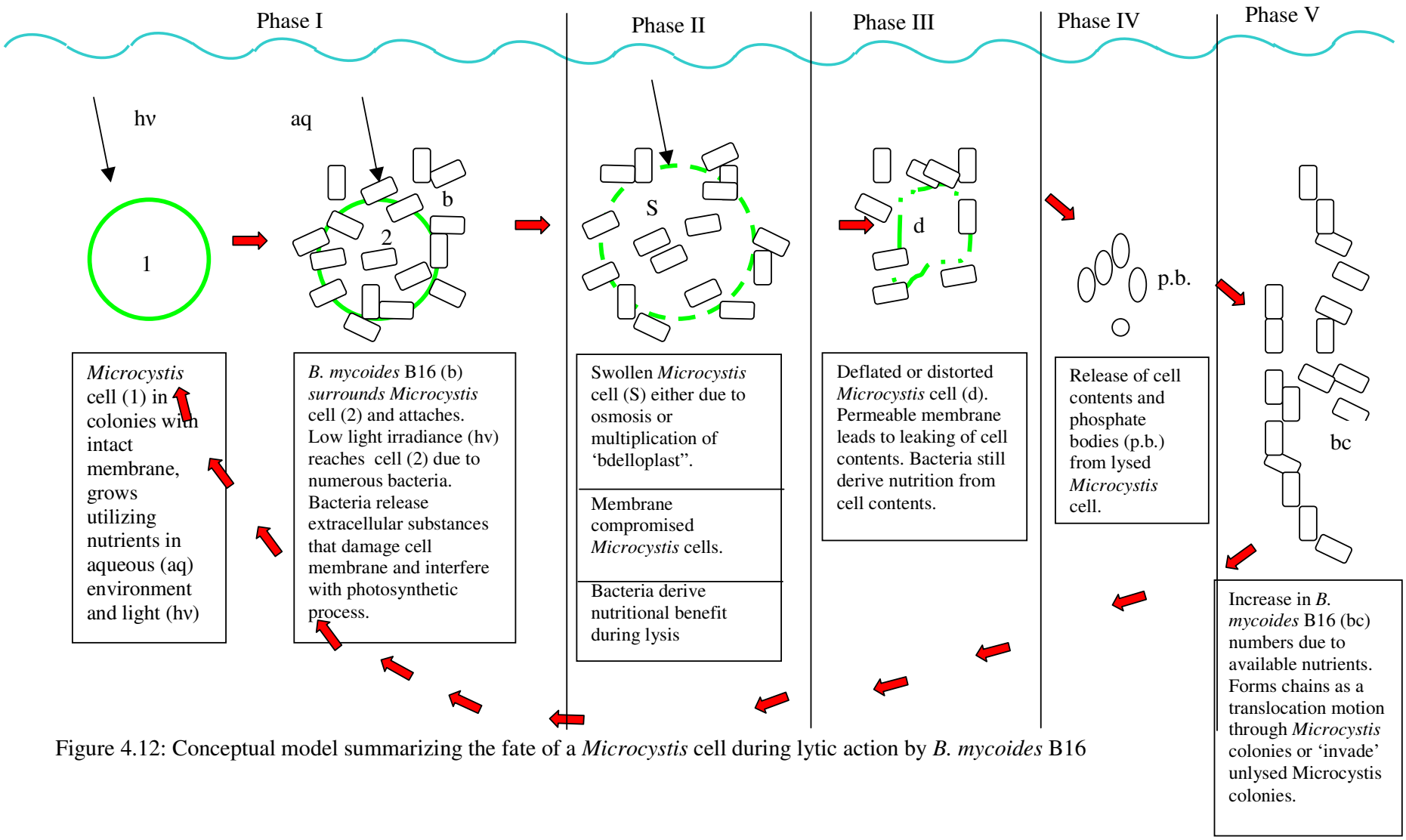

Figure 4.12: Conceptual model summarizing the fate of a *Microcystis* cell during lytic action by *B. mycoides* B16

4.4. 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 the probably the cause of deflation and lysis of the algae.
- Isolates B2 and B16 were capable of causing damage of the *Microcystis* cell membrane.
- Electron microscope studies showed the extent of *Microcystis* membrane damage, most severe with copper, followed by *B. mycoides* B16 and Triton X-100.
- There is evidence, which showed that *Bacillus mycoides* B16 might be an ectoparasite during the lysis of *Microcystis* cells and exhibit multicellularity forms that are *Bdellovibrio*-like bacteria during the last stages lysis of *Microcystis* cells in order to survive an adverse external environment that was nutrient limited.
- The mechanism of cyanobacterial lysis may involve changes in ultrastructure of *Microcystis aeruginosa*, possibly affecting energy sources and the photosynthetic system after exposure to bacteria. This may lead to the death of the cyanobacteria after exhaustion of energy sources and loss of nutrients to the predator bacteria, *B. mycoides* B16.