



The Effects of Mercury Exposure on the Surface Morphology of Gill Filaments in *Perna perna* (Mollusca: Bivalvia)

M. A. GREGORY†*, R. C. GEORGE†, D. J. MARSHALL†, A. ANANDRAJ† and T. P. MCCLURG‡

†Electron Microscope Unit and Department of Zoology, University of Durban-Westville and Division of Water, Environment and Forestry Technology, Durban Natal 4000, South Africa

‡CSIR, Durban, South Africa

This study investigated the possibility that changes in the surface morphology of mussel (*Perna perna*) gill filaments may be used to indicate the relative toxicity of pollutants in the marine environment. Healthy, adult *P. perna* were collected and immersed in 2 free-flow tanks. Mercury was added to seawater as it entered tank 1 to achieve a constant level of 50 µg/l⁻¹ over 24 days. Uncontaminated seawater was circulated over the mussels in tank 2 (control) for the same period. A 25 mm² area of gill filament was removed from each of the 5 specimens before and after 24 days immersion in tank 2, and after immersion for 1, 2, 4, 8, 16 and 24 days in tank 1. These were examined using a scanning electron microscope. The remaining soft tissues from each animal were analysed for mercury using an atomic absorption spectrometer. Mercury concentration increased from 0.13 (pre-immersion) to 87 µg/g after day 24. Surface morphology remained normal for all animals in tank 2 and for those exposed to Hg for up to 8 days. However, from 16 to 24 days exposure there was a gradual increase in the diameters of microvilli, a depletion of abfrontal cilia, an increase in abnormal, perhaps necrotic cells and an unusual increase in the number of cilia on the lateral surfaces. These results confirm that *P. perna* is an efficient bio-accumulator and suggest that their gill pathomorphology may be a useful indicator of toxicity. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Mussels are commonly used in monitoring the effects of pollutants on marine ecosystems (Kraak *et al.*, 1992; Grace and Ganey, 1987). Being long-lived and sedentary, they are particularly useful in integrating pollution impact at specific locations over extended time periods. Studies have shown that mussels exposed to effluent-containing heavy metals sequester such metals in both hard and soft tissues (Phillips, 1976). The fact that

mussels accumulate heavy metals that may transiently or permanently be present in their environment has been widely used in marine pollution impact assessments (Beliaeff *et al.*, 1997; Odzak *et al.*, 1994; Stronkhorst, 1992). Indeed, the importance of bivalve molluscs in pollution impact studies is shown by the magnitude and longevity of the International Mussel Watch (Calow, 1994), a programme that originated in the late 1960s and continues to maintain momentum today.

Pollution monitoring techniques using mussels include measurement of physiological rates (Watling and Watling, 1982), determining the presence and amount of heavy metals in tissues (Regolli and Orlando, 1994) and recording heavy metal-mediated changes in soft tissues, especially those associated with gill filament morphology (Sunila, 1986, 1987, 1988). Although interest has been increasing recently in the Asia-Pacific region, Australia and South America, most studies employing bivalves as bio-indicators of marine pollution have been concentrated in Europe and North America. These studies have principally centred around their indigenous northern hemisphere species (*Mytilus californicus*, *M. edulis* and *M. galloprovincialis*), while the dominant southern hemisphere species, such as *P. perna*, have received relatively little attention.

P. perna is prolific on the rocky shores of the Indian Ocean coast of southern Africa and is widely distributed in the tropical and subtropical regions of both the Indian and Atlantic Oceans and Mediterranean sea. It occurs in dense aggregations attached to a rock substrate, but may also colonize other hard materials such as wood, concrete or steel (Berry, 1978). Compared with certain species of the closely related genus *Mytilus*, and particularly *M. edulis*, where numerous studies describe the normal and abnormal appearance of gill filaments and other soft tissues before and after exposure to heavy metals, little was known of these structures in *P. perna*.

While South Africa has never formally adopted a mussel watch program, it subscribes to the principles of such a program by the use of bivalve molluscs in its

*Corresponding author. E-mail: mrgregory@pixie.udw.ac.za

national marine pollution surveys (Gardner *et al.*, 1983; Hennig, 1985). For South Africa to establish a local assessment program or to contribute to an already established international mussel watch, it is desirable that the characteristics of indigenous species such as *P. perna* be firmly established. To this end, we have already characterized the normal morphology of *P. perna* gill filaments by light, transmission (Gregory *et al.*, 1996) and scanning electron microscopy (Gregory *et al.*, 1999). This study investigates the rate and extent of mercury accumulation in the soft tissues of *P. perna* and employs scanning electron microscopy to determine the effect of sub-lethal concentrations of mercury on gill filament morphology.

Methods

Collection and maintenance of mussels

Specimens of *P. perna* (43–60 mm; $n=400$) were collected at spring low tide from an unpolluted rocky shore near Isipingo Beach, south of Durban (29° 05' S 30° 56' E) (McClurg *et al.*, 1999). The animals were kept in well-oxygenated, flow-through, plastic seawater aquaria (75.5 cm × 40.5 cm × 21 cm) and a glass aquarium (75 cm × 45 cm × 13 cm). Using a sharp blade, the surfaces of shells were scraped clean of all epiphytes before being placed in the aquaria. The animals were kept for 5 days prior to the commencement of experiments. While in the aquaria, the animals were supplied with a suspension (7000–27 000 cells/ml) of unicellular alga, *Chlorella* (12 µm). Each of the 2 aquaria containing 100 animals was cleaned every 2 days, by siphoning the fecal matter. Ambient room and seawater temperatures were recorded daily and found to remain at 23°C ± 1°C, even without temperature control mechanisms. An algae concentration of ± 4800 cells/ml (500 ml concentrated algae suspension into 100 l seawater) was maintained in both tanks for the duration of the experiment.

Tank system

A system was designed that allowed a constant supply of high quality seawater (drawn from the intake of the Durban aquarium and transported to the laboratory in a stainless steel tanker) to each of the 2 aquaria. Seawater was fed by gravity from 2 reservoir tanks (> 1000 l) to the holding tanks (100 l each; 2 per aquaria). The holding tanks were manually filled with fresh seawater on a daily basis. This supplied each aquarium with seawater at a flow rate of 12 l/h (288 l/day). Two separate delivery systems were created to run simultaneously: Tank 1 delivered fresh seawater (controls), Tank 2 delivered Hg. Mercuric chloride (HgCl₂ – 0.007 g), was dissolved in 2 ml of distilled water and added to tank 2 each day to provide an estimated final concentration of 50 µg/l. The experiment and control were run for 24 days. Using atomic absorption spectrometry (AA), the concentration of Hg in the water of tank 2 was

found to be 44 µg/l on day 2, 46 µg/l on day 8 and 45 µg/l on day 24.

Preparation of tissue for SEM

Gill tissue (25 mm²) was acquired by dissection through the full thickness (ascending and descending lamellae) of the central regions of each gill from each specimen. This was placed in fresh 2% glutaraldehyde in seawater at pH 7.2 for 1 h. The tissue was then washed in fresh seawater, post-fixed in a solution of 1% osmium tetroxide in seawater (pH 7.2), dehydrated through graded ethanols and critical-point dried in carbon dioxide. Each specimen was trimmed and mounted on brass stubs using carbon tape and individually orientated to show either filament inter-lamellar connections, the frontal or abfrontal aspects of rows of filaments or the lateral aspects of individual filaments. Each stub was sputter-coated with approximately 10 nm of gold using a Polaron SC500 coater. Specimens were examined using a Jeol 6100 scanning electron microscope (SEM) from 10 to 15 kV.

Preparation of tissue for mercury analysis

After removal of the specimens for SEM, the remaining fresh, soft tissues from each of the 10 bivalves were obtained after 1, 2, 4, 8, 16 and 24 days exposure to Hg. The tissues from all 10 animals in each group were pooled, ground and 2.5 g (wet weight) placed in 100 ml stoppered flasks. 10 ml of 5% K₂S₂O₈ and 10 ml H₂SO₄ were added to each flask which were then left to stand for 2 h. This was followed by heating for 4–6 h (60°C) in a water bath and allowing to stand overnight at RTP. Thereafter, 15 ml 50 g/l KMnO₄ was added and after standing overnight at RTP, 1 ml conc. HNO₃ was added and the solution allowed to stand for a further 12 hours. The subsequent solutions were analysed by AA. Three blanks containing no tissue were prepared simultaneously to serve as negative controls. A single reading was made of each pooled sample using a Varian Spectra 30 model AA Spectrometer. All readings were converted from µg/l to µg/g dry tissue weight.

Results

It is important to note that animals in tank 1 and those exposed to Hg in tank 2, all survived the 24 day experimental period.

AA data

The data showed a gradual increase in the amount of Hg in soft tissues over the 24 day experimental period (Fig. 1). From day 2 to day 8, Hg in tissues increased at the rate of 0.05 µg/g per hour, from day 8 to day 16, 0.15 µg/g per hour and from day 16 to 24, 0.2 µg/g per hour dry weight. These data show that the concentration of mercury in soft tissues increased 30 fold from day 2 to day 24.

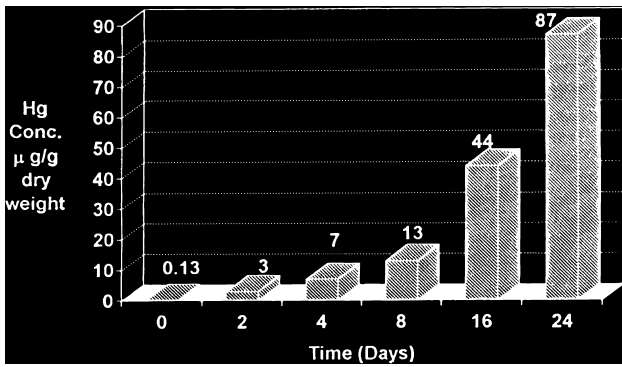


Fig. 1 Mercury concentrations ($\mu\text{g/g}$) wet weight in tissue over a 24 day exposure (day 2, 4, 8, 16 and 24) period and a control (day 0). Total tissue weight of 10 animals per day.

SEM data

There were no obvious gross differences in the ultra-structure of filaments derived from control animals kept from 1 to 24 days in tank 1. In brief, each lamella is comprised of numerous parallel filaments approximately 20 μm apart, inter-connected by ciliary disks composed of numerous densely packed, interlocking cilia. Individual filaments are characterized by the presence of unique abfrontal, lateral and frontal cilia and sheets of lateral-frontal cirri (Figs. 2 and 3). The entire lateral surface is covered by a mat of microvilli between which projects occasional groups of up to 4 elongated cilia (Fig. 4).

Other than perhaps a moderate reduction in the number of cilia projecting from lateral frontal cells, there were no obvious surface morphological abnormalities of gill filaments in animals exposed to Hg for up to 4 days. After 8 days, however, occasional areas devoid of microvilli were present on some lateral surfaces. Progressively more severe abnormalities were seen from sixteen to 24 days after exposure. There were extensive areas where abfrontal surfaces were denuded of cilia (Fig. 5). In some instances, abfrontal cells appeared

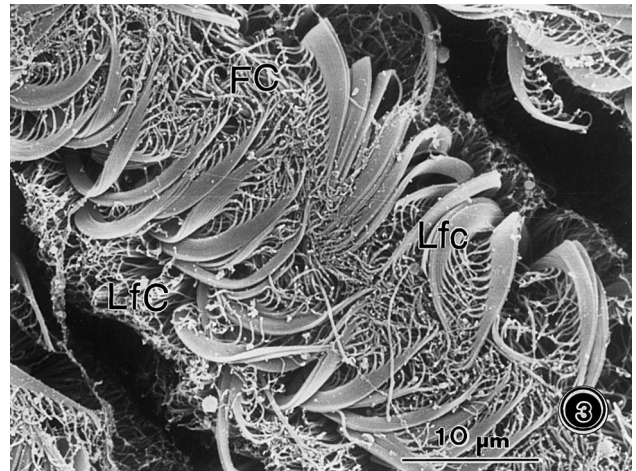


Fig. 3 SEM: Frontal aspect of (control) gill filament showing frontal cilia (FC), lateral frontal cirri (Lfc) and lateral frontal cilia (Lfc).

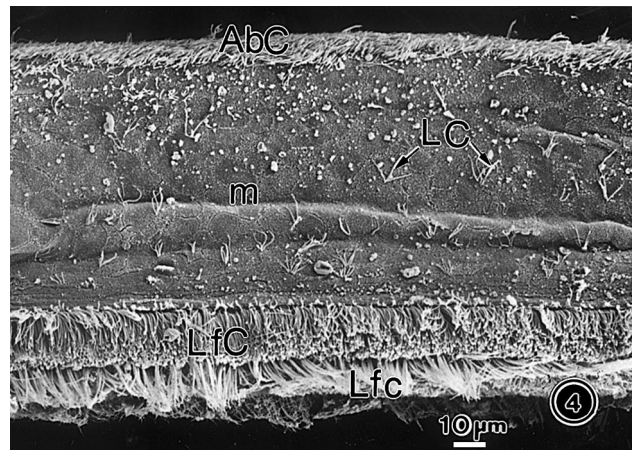


Fig. 4 SEM: Lateral aspect of (control) gill filament showing abfrontal cilia (Abc), lateral cilia (LC), microvilli (m), lateral frontal cilia (Lfc) and lateral frontal cirri (Lfc).

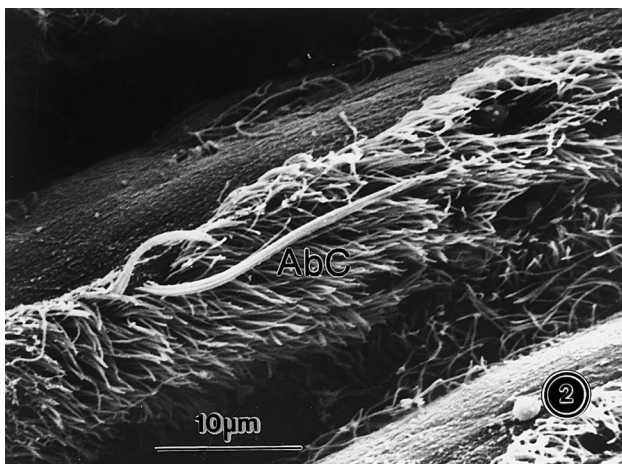


Fig. 2 SEM: Abfrontal aspect of (control) gill filament showing abfrontal cilia (Abc).

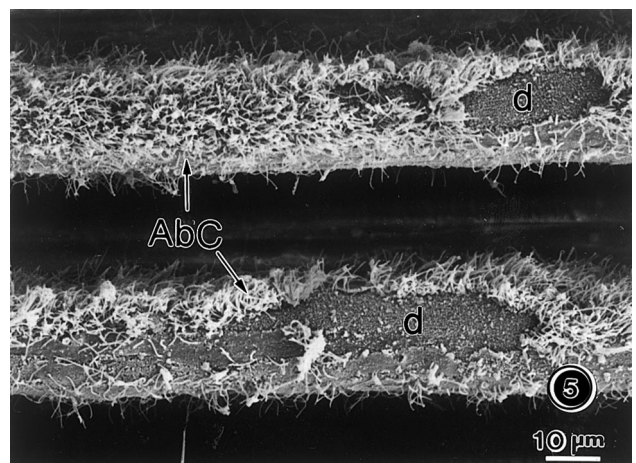


Fig. 5 SEM: Abfrontal aspect of two (16 day Hg, experimental) gill filaments showing abfrontal cilia (Abc) and areas of denuded cilia (d).

necrotic, their internal organelles spilling into the extracellular space (Fig. 6).

Lateral squamous cell microvilli had become more irregular in shape and had increased in diameter from 140 nm (control $n=20$; SD 17 nm) to 170 nm ($n=20$; SD 30 nm) after 24 days exposure to mercury. The most obvious alteration in filament morphology was the remarkable increase in the number of cilia projecting from squamous lateral cells. Cilia projecting from these cells on filaments from control specimens and those exposed to Hg for up to 8 days were quite sparse. However, at 16 and 24 days after exposure to Hg, much of the lateral surfaces were covered with cilia (Figs. 7 and 8). In some instances the cilia appeared to project in clumps from lateral surfaces (Fig. 8). At 24 days after exposure to Hg, other than frontal-lateral cirri appearing to have lost most of their 'curl' (Fig. 9), there were no obvious changes in either lateral frontal or frontal cilia numbers or length.

Discussion

Many pollution monitoring surveys rely on the measurement of metals in water and sediment samples to evaluate the impact of an effluent discharge. The vagaries of tide, current and weather, however, may cause the concentration of metals in such samples to vary widely over relatively short periods of time (Phillips, 1976). Earlier studies have shown that mussels imbibe pathogens, heavy metals and other toxic substances from their environment and store them in their soft tissues (Goldberg, 1975; Phillips, 1976; Roesijadi and Fellingham, 1987; Lakshmanan and Nambisan, 1989). Indeed, the amount of metal in bivalve tissues has been reported to be 10^3 - 10^6 times higher than that in water (Phillips, 1976). By examining mussels living near marine outfalls, this phenomenon has been utilized by various mussel watch agencies to detect and quantify the amount of toxic substances, especially heavy metals, in

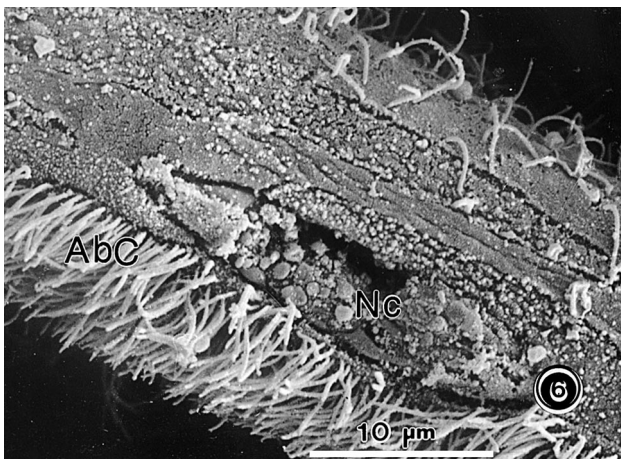


Fig. 6 SEM: Abfrontal aspect of (24 day Hg, Experimental) gill filament showing abfrontal cilia (Abc) and necrotic cell (Nc).

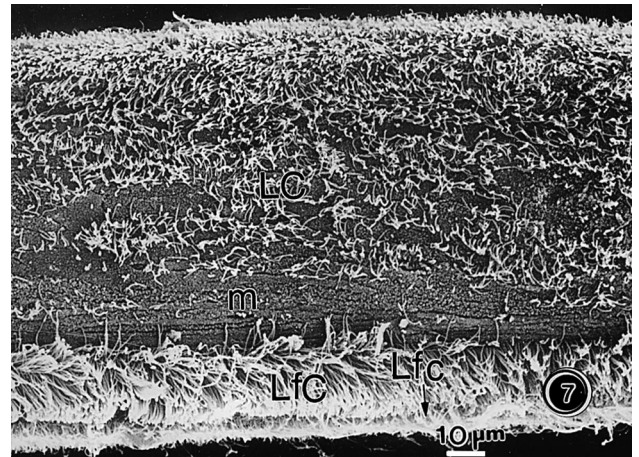


Fig. 7 SEM: Lateral aspect of (16 day Hg, Experimental) gill filament showing microvilli (m), lateral frontal cilia (Lfc), lateral frontal cirri (Lfc) and an increased number of lateral cilia (LC).

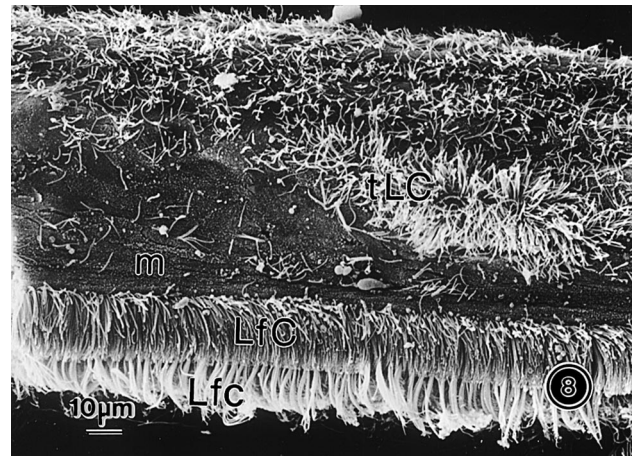


Fig. 8 SEM: Lateral aspect of (24 day Hg, Experimental) gill filaments showing microvilli (m), lateral frontal cilia (Lfc), lateral frontal cirri (Lfc) and a tuft of lateral cilia (tLC).

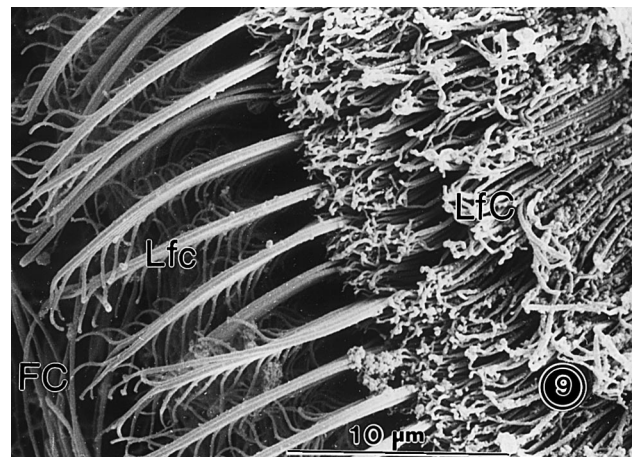


Fig. 9 SEM: Frontal aspect of (24 Day Hg, Experimental) gill filament showing frontal cilia (FC), lateral frontal cirri (Lfc) and lateral frontal cilia (Lfc) that have lost their 'curl'.

effluent deposited into the sea (Manly *et al.*, 1996; Hamilton, 1989).

Heavy metals ingested by bivalves are either excreted, or after binding to metallothienin proteins, stored in an inert form (Clark, 1996). When stored, they gradually accumulate in the soft tissues, especially the mantle, gills, kidney, foot and digestive gland (Odzak *et al.*, 1994; Regolli and Orlando, 1994; Ward, 1990; Lakshmanan and Nambisan, 1989; Lobel, 1987). Numerous authors have reported that mercury accumulates in the tissues of bivalves (Micallef and Tyler, 1990; Clark, 1996), the highest concentration (22 µg/g after 4 days exposure to 200 µg/l) in *P. viridis* being found in the gills (Roesijadi and Fellingham, 1987).

This study has clearly shown that in common with other mussel species, *P. perna* is an efficient accumulator of mercury. In spite of a substantial increase of mercury in tissues, there was, however, no mortality during the experimental period. Indeed, a measure of their resilience to toxic substances was the observation that until 8 days after exposure to mercury, other than perhaps a reduction in the numbers of cilia projecting from lateral-frontal cells, no pathological changes were evident in gill filament morphology. After 16 days exposure, however, there was a progressive increase in surface pathomorphology.

From 16 to 24 days exposure to mercury, there was a gradual increase in the number of ruptured cells on abfrontal and lateral surfaces. Lateral cell microvilli became distended and there were large areas where abfrontal cells were denuded of cilia. By 24 days after exposure to mercury, the cilia comprising the lateral-frontal cirri appeared to be separating. Further, although only a subjective observation, unlike those in controls, the cirri did not curl over the frontal cilia, but hung in a 'languid' fashion beyond them. The most obvious and perhaps significant change in morphology was the increase in the number of cilia projecting from lateral squamous cells.

Gill filaments from control animals were populated with occasional lateral squamous cells from which projected 2 to 4 elongated cilia. While control animal surface morphology remained normal over the experimental period, 16 days after exposure to Hg, there was a marked increase in the number of cilia projecting from the lateral regions of experimental animals. By 24 days after exposure to Hg, in some instances, the whole lateral surfaces were covered with cilia and there was no apparent division between abfrontal and lateral cilia. In these filaments, abfrontal cilia appeared to continue down lateral surfaces. These observations pose some intriguing questions – has there been a hyperplasia of the normally quite rare ciliated lateral squamous cells or has there been a metaplastic change of cell type from lateral squamous to abfrontal cells?

Earlier transmission electron microscopic analyses (TEM) of bivalve gill filaments have shown that cilia projecting from various cell types have a unique mor-

phology (Good *et al.*, 1990; Stephens and Good, 1990; Gregory *et al.*, 1996). In the case of *P. perna*, frontal cilia have a well-defined neck while cilia projecting from lateral squamous cells do not (Gregory *et al.*, 1996). It was considered possible that a careful examination of abnormal lateral cilia may enable the cell type to be determined. Unfortunately, the "undergrowth" of lateral cilia made it impossible to either conclusively prove or disprove the presence of a ciliary neck. The answer to these and other questions regarding heavy metal-mediated pathomorphology await the results of current light and transmission electron microscopical studies being carried out by this group.

While microvillous swelling, cell necrosis and other pathomorphological alterations might be expected as a consequence of toxic insult, it is more difficult to explain the reason for ciliary hyperplasia in lateral cells. Under normal conditions, the few cilia projecting from lateral squamous cells probably help with the circulation of oxygenated water to the epithelium. We postulate that extended exposure to mercury may deleteriously affect respiration. If this is the case, then the observed increase in lateral cilia may be a response by the animal to increase flow of oxygenated water over the epithelium thereby improving respiration.

It may be concluded that *P. perna* is similar to other bivalves in that it efficiently accumulates mercury in its soft tissues. In addition, it would appear that chronic exposure to increased mercury induces significant morphological changes in its gill tissues. While exposure to other pollutants, either singly or in combination may produce other responses, it is hoped that these data will provide an initial comparative baseline for future studies.

The authors would like to thank the Foundation for Research Development (FRD) for funding this project and the Council for Scientific and Industrial Research (CSIR) for the use of their facilities during the experimental stages of this study. Also Mr G. Parsons of the CSIR for his help in undertaking AA analyses.

- Beliaeff, B., O'Connor, T. P., Daskalakis, D. K. and Smith, P. J. (1997) US mussel watch data from 1986–1994. Temporal trend detection at large spatial scales. *American Journal of Chemistry and Society* **31** (5), 1411–1415.
- Berry, P. F. (1978) Reproduction, growth and production in the mussel, *P. perna* (Linnaeus), on the East coast of South Africa. O.R.I. Investigational report No. 48, 1–28.
- Calow, P. (1994) Heavy metals. In *Handbook of Ecotoxicology*, vol. 2, pp. 79–97. Oxford Blackwell Scientific Publ., London.
- Clark, R. B. (1996) Metals. In *Marine Pollution*, Ch. 5, pp. 64–82. Oxford University Press, Oxford, New York.
- Gardner, B. D., Connell, A. D., Eagle, G. A., Moldan, A. G. S., Oliff, W. D., Orren, M. J. and Watling, R. J. (1983) South African National Scientific Programmes report No. 73. CSIR, Pretoria, p. 105.
- Goldberg, E. D. (1975) The mussel watch – A first step in global marine monitoring. *Marine Pollution Bulletin* **6**, 111.
- Good, M. W., Stommel, E. W. and Stephens R. (1990) Mechanical sensitivity and cell coupling in the ciliated epithelial cells of *M. edulis* gill. *Cell Tissue Research* **259**, 51–60.
- Grace A. L., Ganey, L. F. Jr. (1987) The effects of copper on the heart rate and filtration rate of *M. edulis*. *Marine Pollution Bulletin* **18**, 87–91.

- Gregory, M. A., George, R. C. and McClurg, (1996) The architecture and fine structure of gill filaments in the brown mussel, *P. perna*. *South African Journal of Zoology* **31**, 193–206.
- Gregory, M. A., George, R. C. and McClurg. (1999) The surface morphology of gill filaments in *P. perna*. *South African Journal of Zoology*, February 1999, submitted.
- Hamilton, E. I. (1989) Chemical Contamination of French Coasts—the results of a ten year Mussel Watch. *Marine Pollution Bulletin* **20**, 523–528.
- Hennig, H. F. (1985) Review of metal concentrations in Southern African coastal waters, sediments and organisms. South African National Scientific Programmes Report No. 108. FRD, CSIR, Pretoria. p. 40.
- Kraak, M. H. S., Lavy, D., Peeters, W. H. M. and Davids, C. (1992) Chronic exotoxicity of copper and cadmium to the Zebra Mussel *Dreissena polymorpha*. *Archives of Environmental and Contamination Toxicology* **23**, 363–369.
- Lakshmanan, P. T. and Nambisan, P. N. K. (1989) Bioaccumulation and depuration of some trace metals in the mussel, *P. viridis* (Linnaeus). *Bulletin of the Environmental and Contamination Toxicology* **43**, 131–138.
- Lobel, P. B. (1987) Intersite, intrasite and inherent variability of the whole soft tissue zinc concentrations of individual mussels *M. edulis*: importance of the kidney. *Marine Environmental Research* **21**, 59–71.
- Manly, R., Blundell, S. P., Fifield, F. W. and McCabe, P. J. (1996) Trace metal concentrations in *M. edulis* L. From the Laguna San Rafael, Southern Chile. *Marine Pollution Bulletin* **32**, 444–448.
- McClurg, T. P., Rathbone, P. -A., Parsons, G. A. and Chetty, J. (1999) A baseline environmental survey in the vicinity of the proposed sea-outfall at Prospecton. In *Sea Disposal of Sewage: Environmental Surveys in the Durban Outfall Region*. Report No. 17. Surveys made in 1998, pp. 199–223.
- Micallef, S. and Tyler, P. A. (1990) Effect of Mercury and selenium on the gill function of *M. edulis*. *Marine Pollution Bulletin* **21**, 288–292.
- Odzak, N., Martinic, D., Zvonaric, T. and Branica, M. (1994) Bioaccumulation rate of Cd and Pb in *M. galloprovincialis* foot and gills. *Marine Chemistry* **46**, 119–131.
- Phillips, D. J. H. (1976) The common mussel *M. edulis* as an indicator of pollution by zinc, cadmium, lead and copper 11. Relationship of metals in the mussel to those discharged by industry. *Marine Biology* **38**, 71–80.
- Regolli, F. and Orlando, E. (1994) Accumulation and subcellular distribution of metals (Cu, Fe, Mn, Pb and Zn) in the Mediterranean mussel *M. galloprovincialis* during a field transplant experiment. *Marine Pollution Bulletin* **28**, 592–600.
- Roesijadi, G. and Fellingham, G. W. (1987) Influence of Cu, Cd, and Zn pre-exposure on Hg toxicity in the mussel *M. edulis*. *Canadian Journal of Fisheries and Aquatic Science* **44**, 680–684.
- Stephens, R. E. and Good, M. J. (1990) Filipin-sterol complexes in molluscan gill ciliated epithelial membranes: intercalation into ciliary necklaces and induction of gap junctional particle arrays. *Cell Tissue Research* **262**, 301–306.
- Stronkhorst, J. (1992) Trends in pollutants in Blue Mussel *M. edulis* and Flounder *Platichthys flesus* from two Dutch estuaries, 1985–1990. *Marine Pollution Bulletin* **24**, 250–258.
- Sunila, I. (1987) Histopathology of mussels (*M. edulis* L.) from Tvärminne area, the Gulf of Finland (Baltic Sea). *Ann. Zool. Fennici* **24**, 55–69.
- Sunila, I. (1988) Acute histological responses of the gill of the mussel, *Mytilus edulis*, to exposure by environmental pollutants. *Journal of Invertebrate Pathology* **52**, 137–141.
- Sunila, I. (1986) Chronic histopathological effects of short-term copper and cadmium exposure on the gill of the mussel, *M. edulis*. *Journal of Invertebrate Pathology* **47**, 125–142.
- Ward, R. E. (1990) Metal concentrations and digestive gland lysosomal stability in mussels from Halifax Inlet, Canada. *Marine Pollution Bulletin* **21**, 237–240.
- Watling, H. R. and Watling, R. J. (1982) Comparative effects of metals on the filtering rate of the brown mussel (*P. perna*). *Bulletin of the Environmental and Contamination Toxicology* **29**, 651–657.
-