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Active biomonitoring in freshwater environments: early warning signals from biomarkers in assessing biological effects of diffuse sources of pollutants

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Abstract

Effluents are a main source of direct and continuous input of pollutants in aquatic ecosystems. Relating observed effects to specific pollutants or even classes of pollutants remains a very difficult task due to the usually unknown, complex and often highly variable composition of effluents. It is recognized that toxicants interfere with organism integrity at the biochemical level and give rise to effects at the individual level and is manifested in reduced ecologically relevant characteristics such as growth, reproduction and survival, and ultimately at the ecosystem level. By integrating multiple endpoints at different ecologically relevant levels of organization within one test organism, it should be possible to gain understanding in how different levels of organization within this organism respond to toxic exposure and how responses at these different levels are interrelated. This paper presents results from a field study in the Rietvlei Wetland system, Gauteng, South Africa using the freshwater mollusk (*Melanoides tuberculata*) and freshwater fish (*Oreochromis mossambicus*) as bioindicator organisms. Active biomonitoring (ABM) exposures were conducted where organisms were exposed for 28 days in an effluent dominated river during high flow conditions in April 2003. The river receives effluent from a wastewater treatment plant and an industrial complex, so that up to 75% of the total flow of the river is effluent-based. Effects of field exposure were determined using cellular biomarkers e.g. DNA damage, HSP 70, metallothionein, acetylcholine esterase, lactate dehydrogenase and ethoxyresorufin-o-deethylase activity. The results clearly indicate that although the traditional mortality-based whole effluent toxicity testing did not indicate any toxicity, the *in situ* exposed organisms were stressed. A multivariate statistical approach was particularly useful for integrating the biomarker responses and highlighting sites at which more detailed analysis of chemical contamination would be useful. Based on the individual biomarker results' contributing towards the distinct groupings it is possible to conclude that Site 1 is subjected to organic pollutants, whereas Sites 2 and 3 undergo a combination of metallic and organic pollutant stress. However, it is essential that a rapid and sensitive biomarker that is representative of the responses of a suite of biomarkers be tested before ABM can be implemented as a routine biomonitoring practice in water resource management.

Keywords: Active biomonitoring, biomarkers; Mollusks; Fish; Multivariate statistical analysis

1. Introduction

The development of whole effluent toxicity (WET) methodologies has become an important part of applied ecotoxicology in recent years (Smolders et al., 2002). Because effluents are a main source of direct and continuous input of pollutants in aquatic ecosystems, the study of the effects of effluent exposure on organisms has a high ecological relevance (Ausley, 2000; Burgess et al., 2000; Chapman, 2000). However due to the unknown and complex nature of effluents it is virtually impossible to relate

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observed effects to specific pollutants or even a class of pollutants (De Maagd, 2000; Dyer et al., 2000; Guerra, 2000). The traditional WET test has always been mortality based (Slabbert et al., 1998) but the inherent characteristics of effluents imply that WET should provide a holistic and integrative overview of the effects chronic exposure has on different biological processes at different levels of biological organisation within the test organism(s) (De Coen et al., 2000; Munkittrick and McCarthy, 1995). Hence the need for more effect orientated studies, which include *in situ* bioassays such as active biomonitoring (AMB) techniques (Smolders et al., 2003).

Previous studies showed that transplanting mollusks and fish from a reference site to a polluted area can be a feasible strategy for biomonitoring the effects of environmental changes in an aquatic system (Smolders et al., 2004a). This form of biomonitoring (ABM) is based on the comparison of chemical and/or biological properties of samples which have been collected from one population and which have, after randomisation and translocation, been exposed to different environmental conditions at monitoring sites. The response of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (Van der Oost et al., 2003). The most compelling reason for using biomarkers is that they can give information on biological effects to pollutants rather than a mere quantification of their environmental levels. Lagadic et al. (2000) underlined the interest in measuring several biomarkers at the same time in the same animals, which allows a pertinent approach to evaluate the effects of pollutants on individuals. Various biochemical parameters have been tested for their response to toxic substances and tested for their use as biomarkers of exposure and effect (Van der Oost et al., 2003). Most biomarker studies evaluate the responses individually without an integrated assessment. It is only recently that a multivariate approach has been used to assess multiple biomarkers in marine (Astley et al., 1999) and freshwater (Smolders et al., 2004b) environments.

This study evaluated the use of ABM and biomarker methodologies as an early warning system of aquatic pollution. The Rietvlei Wetland, Gauteng, South Africa, was chosen because it is highly polluted with large amounts of effluent from a variety of industries. Pollution in the Rietvlei Wetland System has also led to feminisation in male fish due to exposure to endocrine disrupting chemicals (Barnhoorn et al., 2003). In this study, the biomarker results following a four week *in situ* exposure experiment using the mollusk, *Melanoides tuberculata*, and fish species, *Oreochromis mossambicus*, are presented for a high flow period (summer) in the Rietvlei Wetland System. The biomarkers that were selected as endpoints of exposure and effect in this study were: DNA damage, HSP 70 concentration, lactate dehydrogenase (LDH) activity, Acetycholine esterase (AChE) activity, Ethoxyresorufin-O-deethylase (EROD) activity and metallothionein (MT) concentration. This suite of multiple biomarker assays using fish and mollusks was subjected to multivariate analysis to provide an integrated biomarker response assessment of the system. A concomitant laboratory-based WET assessment of the raw water was conducted to assess the toxicity of the water at the selected sampling sites using standard algal, invertebrate and fish bioassays.

2. Material and methods

2.1. Study area

The study was conducted in a 10-km stretch of the of the Sesmylspruit (Rietvlei Nature Reserve, Gauteng, South Africa), which includes the impoundment portions of Rietvlei Dam and Marais Dam. Three sites including the reference site were under investigation for whole effluent toxicity testing and active biomonitoring. The Rietvlei reserve acts as a catchment area that provides 15% of the water demand of the Metropolitan Municipality of Tswane (Pretoria, South Africa). Water in the Rietvlei system receives effluent from industries, agriculture, informal settlements and municipal sewage treatment plants. Three sampling sites (Site 1, Site 2, and Site 3) (Fig. 1) were selected for the ABM exposure. Marais Dam acts as a sedimentation pond before the water passes into Rietvlei Dam, where the water is abstracted for purification and domestic use.

2.2. Water sampling and analysis

Raw water was collected from each of the three sites for WET. The reference site (control) was the UJ Aquarium, where the bioindicator organisms were cultured. Grab water samples of 15 liters were collected at each sampling site during the late high-flow period (April, 2003). The following physico-chemical water quality properties were determined *in situ* at all three site: pH, temperature, conductivity/total dissolved solids (TDS) and dissolved oxygen using Eutech Cyberscan Instruments water proof series meters (oxygen: DO300 Series, pH: 300 Series and conductivity: 410 Series).

2.3. Active biomonitoring techniques

Indigenous fish species (*O. mossambicus*) and fresh water gastropods (*M. tuberculata*) species were selected for the ABM exposures and were cultured in the aquarium facilities of the Zoology Department, UJ. These organisms were transferred from the laboratory and deployed into the Rietvlei Wetland System at the three sampling sites (Sites 1–3 in Fig. 1) during the high flow period (April 2003). The bioindicator organisms were deployed in non-toxic polyethylene cages and allowed to be suspended in the water column at about 50 cm below the water surface.

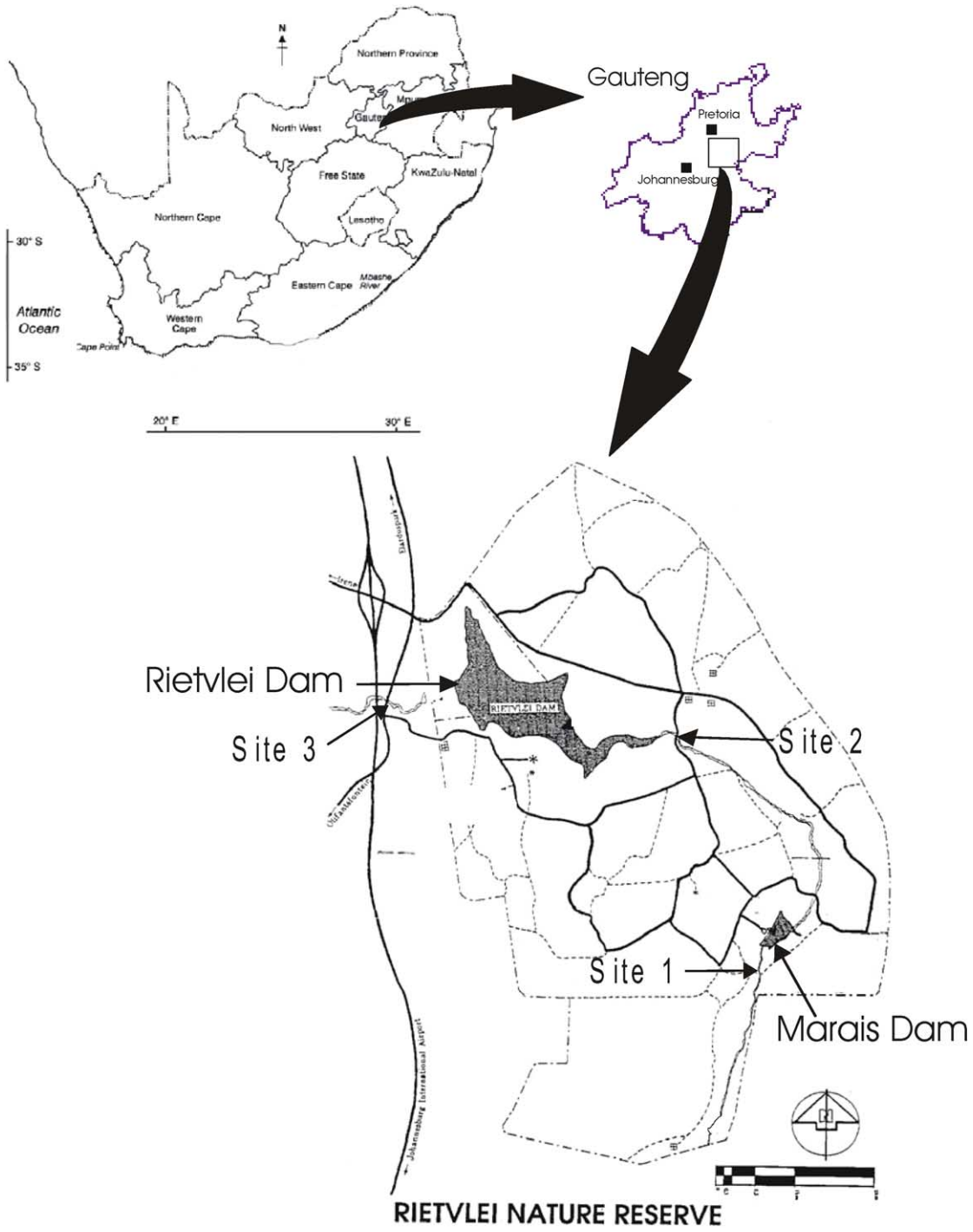

Fig. 1. Map of Rietvlei Wetland System indicating sampling sites where ABM exposures were carried out during April 2003 (high flow).

Snails were fed on lettuce for the first week of the exposure to the water and thereafter sufficient algae had developed in the cage to allow for normal feeding of both the snails and fish. Both the fish and the molluscs were collected after four weeks exposure. The fish were immediately transferred to liquid nitrogen until further analyses in the laboratory. The snails were transported live to the laboratory where they were de-shelled and weighed. Both the snail and fish tissue was stored in Hendrikson storage buffer at −80°C prior to biomarker assays.

2.4. Biomarker assays

The biomarkers were analysed in the ABM exposed and control organisms obtained from the hatching facilities at UJ. The organisms were rinsed in double distilled water and homogenised in a general homogenising buffer (50 mM Tris base, 0.25 M sucrose pH 7.4) to make up a final concentration of 100 mg/ml. If sample mass was too low to allow for analyses (i.e. less than 200 μg), the organisms were pooled as required before homogenisation.

2.4.1. DNA damage assay

DNA damage was determined using the method described by De Coen (1999). Tissue samples were gently homogenised using a glass rod in DNA extraction buffer containing 250 mM NaCl, 100 mM Tris base and 100mM ethylenediaminetetraacetic acid (EDTA). Cells in the homogenate were then lysed by adding 10% sarcosyl solution. Ribonuclease (3 μl) was added followed by incubation on ice for 10 min. Protein kinase (5 μl) was added and then incubated in a warm water bath for 30 min. Adding PCI and centrifuging for 7 min at 3000 rpm twice and repeating the procedure using chloroform purified the extracts. Purified extracts were loaded on a horizontal electrophoresis unit along with a DNA molecular weight marker Hind II (0.12–23.1 kbp) as a standard on a non-denaturing gel containing 0.5% agarose in TBE buffer (65 mM Tris HCL, 22.5 mM boric acid and 0.25 mM EDTA) at 70 V for ±31/2 h. Bromophenol blue was loaded in the first well of each gel to follow the migration of the samples. The gels were stained for at least 40 min in the dark in 200 ml TBE buffer with 20 μl SYBR Green I. The stained gels were analysed using Ultraviolet transilluminator (UVP). Photos were taken using the annotated image capture system, Grab-IT (version 2.5) and by using SigmaScan. The image was quantified using OptiQuant image analyses software.

2.4.2. HSP 70 assay

HSP 70 expression was determined in the gills of *O. mossambicus* only because there were no suitable antibodies sensitive enough to detect the HSP 70 in *M. tuberculata*. The gills were chosen for HSP 70 determination because they are the first organs to be affected by contaminants via water exposure. The gills were homogenised in sucrose buffer (0.25 M sucrose; 50 mM Tris base; pH 7.4) and centrifuged at 15 000 rpm for 15 min. The HSP 70 concentration was determined in the supernatant, which was kept at −80 °C until analysis. The protein concentration for the samples was determined using the Bradford method (Bradford, 1976) to get the amount of sample needed to obtain 15 μg protein per well. The proteins were separated using sodium dodecysulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane by western blotting at 30 V for 16 h at 4 °C. The transferred proteins were stained in Ponceau S for 15 min. The nitrocellulose membrane was blocked for $3\frac{1}{2}$ h in non-fat dried milk containing 0.02% sodium azide then incubated in HSP 70 primary antibody for 20 h. The membrane was washed three times in phosphate buffer saline and once in Tris buffer saline for 10 min each then incubated in non-fat azide free blocking solution with secondary HSP 70 antibody for 3 h. The membrane was then washed in four changes of Tris buffer saline before Enhanced Chemiluminescence (ECL) detection. ECL-Western blotting reagents (Amersham Pharmacia Biotech) were used to detect the HSP 70 proteins. The HSP 70 bands were quantified as relative intensities using UVP GRAB IT (image analyser) and Gene Tools from Sygene both from the same hyperfilm and were expressed as arbitrary units.

2.4.3. Lactate dehydrogenase assay

The activity of LDH was determined using the UV assay with pyruvate and NADH (Bergmeyer and Bernt, 1974) by determining of the amount of NADH oxidation at 340 nm. Phosphate/pyruvate solution (3 ml) (50 mM phosphate, pH 7.5, 0.63 mM pyruvate) was pipetted into cuvettes and 50 μl NADH solution (11.3 mM β-NADH) added then 100 μl of sample homogenate was added and mixed immediately and the extinction was read after every minute interval for a period of 4 min. The LDH activity was calculated using the following formula: Volume of activity = $5056 \times \Delta E/\text{min}$ [U/l].

2.4.4. Protein assay

The Bradford method (Bradford, 1976) was used to determine protein concentration and involves the binding of Coomassie Brilliant Blue G250 to protein.

2.4.5. Acetylcholine esterase assay

The protocol of Ellman et al. (1961) was followed to carry out the AChE assay. This is a calorimetric method that measures the enzyme activity in terms of ACh breakdown. A reaction mixture, consisting of the buffer (2.1 ml), substrate (100 μl, s-acetylthiocholine iodide) and Ellman's reagent (100 μl, 2,2′-dinitro-5,5-dithiobenzoic acid) was incubated for 2 min at room temperature. After incubation, 50 μl of sample was added and the reaction was recorded every 60 s for 6 min at 405 nm. Ellman's reagent interacts with thiocholine, an enzyme breakdown product, to produce 2-nitro-5-thiobenzoic acids, which has a yellow colour. Sample and reagent volumes were adjusted to conduct the test in a 24-well microplate. This allowed the simultaneous analyses of multiple samples and automated absorbance measurements with an ELx 800 Universal microplate reader. The enzyme activity (absorbance/minute) was determined using a standard curve. Calculations were carried out as follows: AChE (Abs/min/wet weight in mg) = (Abs/min of sample/20) × (Total volume of homogenate/wet weight of organism in mg).

2.4.6. Ethoxyresorufin-O-Deethylase (EROD) assay

The direct flourometric method of Burke and Mayer (1974), measuring the O-deethylation of ethoxyresorufin to resorufin was used. Buffer (2 ml), sample (20 μl) and ethoxyresorufin (10 μl) were mixed in a test tube. Ten microliters of, β-nicotinamide adenine dinucleotide phosphate (NADPH) was mixed to start the reaction. The progressive increase in fluorescence (formation of resorufin) was recorded at 10-s intervals for 1 min (room temperature) in a flourometer at extinctions of 510 and 586 nm. Activity of EROD (nM/min) was determined using a standard curve. Calculations were carried out as follows: EROD (nM/min/wet weight in mg) = (EROD nM/min of

sample/20) × (Total volume of homogenate/wet weight of organism in mg).

2.4.7. Metallothionein (MT) assay

The chloroform extraction of MT followed a modified procedure of Viarengo et al. (1997). Tissue was homogenised in 20 mM Tris–HCl, 0.5 M sucrose buffer pH 8, to which phenylmethylsulphonylflouride (PMSF) and leupeptine were added and centrifuged at 30,000 rpm at 4 °C for 20 min to obtain the cytosolic fraction. The supernatant was stored at −80° until further analyses. Absolute ethanol (500 µl at −20 °C and 40 µl absolute chloroform were added to the supernatant and centrifuged at 7000 rpm for 10 min. Ethanol (3 ml at −20 °C) was added and the mixture and frozen for 1 hour at −20°C after which it was centrifuged at 3,000 rpm for 20 min at 4 °C. The supernatant was decanted and pellet washed twice in 95% absolute ethanol, 95% absolute chloroform and 20 mM Tris–sucrose buffer (pH 8), mixed and centrifuged at 3,000 rpm for 20 min at 4 °C. Pellets obtained were dissolved in 300 µl 5 mM Tris base and 1 mM EDTA buffer. A solution of 0.43 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (4.2 ml) was added to 0.3 ml of standards blank and sample. Sample (300 µl) was dispensed into 96-well microplate and incubated at room temperature for 15 min. Sample absorbencies were read on a ELx Universal microplate reader at 405 nm. A calibration curve was prepared from reduced glutathione (GSH) stock solution. The standard curve was used to determine metallothionein concentrations expressed as nM/wet weight (Viarengo et al., 1997). The content of MT was calculated as follows: 1 µM SH = 1 µM GSH = 0.055 µM MT.

2.4.8. Whole effluent toxicity testing

The following tests were used: 96 h fish (*Poecilia reticulata*) lethality test, a 48 h *Daphnia pulex* lethality test and a 72 h algal (*Selenastrum capricornutum*) growth inhibition test. These tests were conducted according to the procedures established at Environmentek, CSIR and described in the guidelines for toxicity bioassays of waters in South Africa (Slabbert et al., 1998). The fish and daphnid tests are based on USEPA (USEPA, 1991) methodology, while the algal test (Slabbert and Hilner, 1990) is a modification of the standard USEPA flask test (USEPA, 1998).

2.5. Statistical analyses

The graphical presentations were performed using the GraphPad Prism Programme and the data reported as mean + SE (standard error of the mean). The variations in each biomarker were tested by one-way analysis of variance (ANOVA), considering sites as variables. Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests, respectively. When the ANOVA revealed significant differences, post-hoc multiple comparisons between sites, and between flow periods, were made using the appropriate Scheffé (parametric) or Dunnette-T3 (non-parametric) test to determine which values differed significantly. The significance of results was ascertained at $p < 0.05$. Multivariate analysis based on Bray–Curtis similarity coefficients and group averaged sorting was performed on the data using the PRIMER (Plymouth Routines in Marine Environmental Research) program v4.0, (Plymouth Marine Laboratory). The biomarker data from the control and deployed bioindicator organisms were analysed separately (Astley et al., 1999). Cluster analysis and multi-dimensional scaling (MDS) were performed on the averaged data of each individual biomarker test for every site.

3. Results

3.1. Physico-chemical parameters of water

Some physico-chemical parameters of water are given in Table 1. The pH was stable around neutral at all three sites. Site 2 displayed the highest total dissolved solid concentrations and Site 3 the lowest oxygen saturation. In general the physico-chemical parameters did not indicate the potential for any drastic water quality-related stress of aquatic biota.

3.2. WET assessment

No toxicity (*viz*. mortality greater than 10% in *D. pulex* and *P. reticulata* or a change in growth by more than 20% in *S. capricornutum*) was recorded for all three sites in all three toxicity bioassays.

3.3. Biomarker responses during ABM

The DNA strand lengths in *M. tuberculata* were different ($p < 0.05$) from the controls at all the sites following 4 weeks exposure (Fig. 2). There were no differences in the DNA strand lengths between the three sites in the Rietvlei System. There were no differences in the DNA strand lengths between the control fish and fish from all three sites in the Rietvlei system (Fig. 3).

Table 1
Physico-chemical analysis of water at the three sampling sites in the Rietvlei Wetland System

Site	% Oxygen saturation	pH	Redox Potential (mV)	Conductivity (µS/cm)	TDS (mg/l)	Temperature (°C)
1	83.2	7.46	−61.3	345	153	12.6
2	65.5	7.54	−68.8	671	341	12.7
3	58.3	7.62	−70.3	535	263	15.5

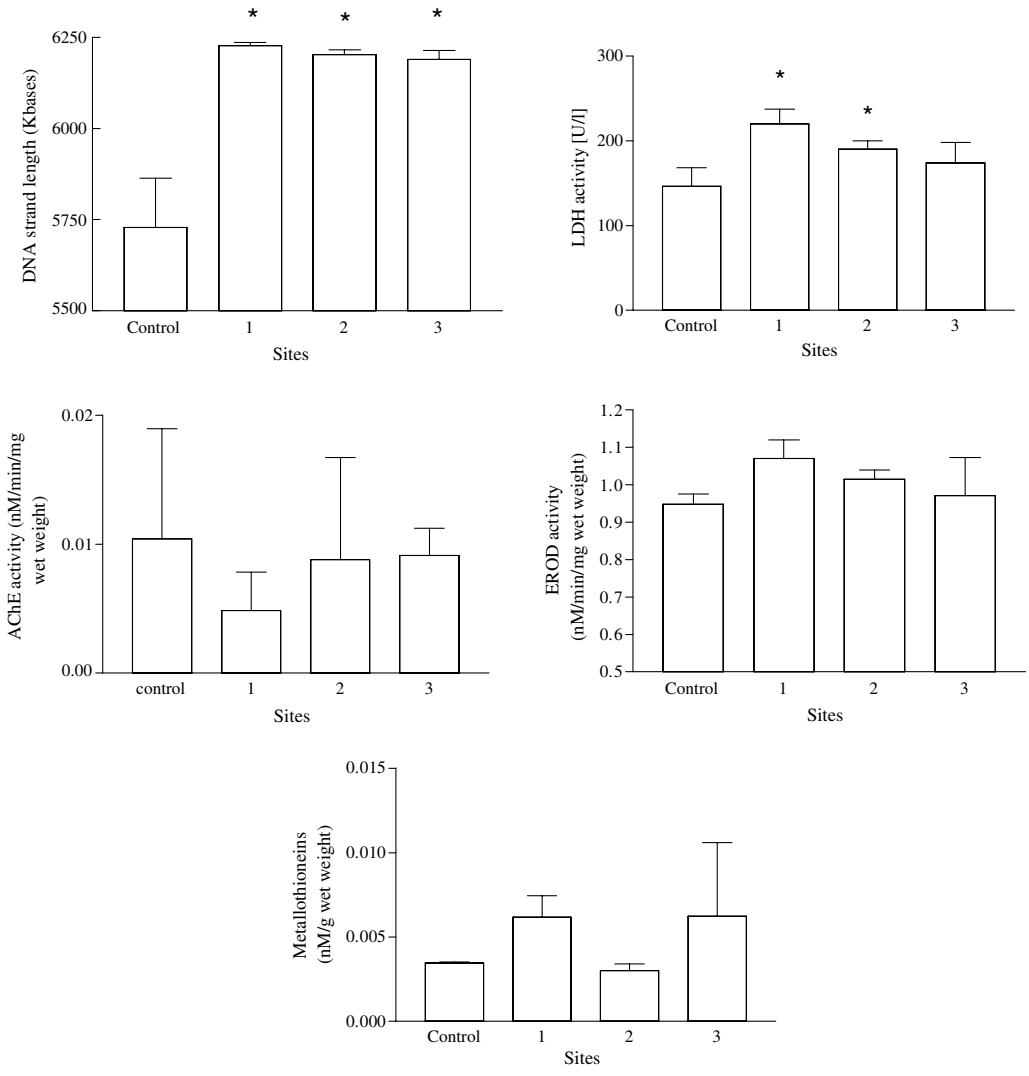

Fig. 2. Spatial biomarker responses of *M. tuberculata* at three sites in the Rietvlei Wetland System following a four week exposure period during high flow (April 2003) conditions. Data for DNA damage, LDH activity, AChE, EROD and MT are presented as means ± SE ($n = 10$). Asterisks indicate values significantly different from the control values ($p < 0.05$).

Only *O. mossambicus* HSP 70 from the high-flow four weeks were available due to the unsuitability of the antibodies used in the assay for mollusk tissue. HSP 70 expression in *O. mossambicus* during the high-flow exposure regime was highest Site 1 decreasing downstream at Site 2 and with no difference from the control at Site 3 (Fig. 3). Fish exposed at Sites 1 and 2 showed significant differences from the control fish ($p < 0.05$).

Activity of LDH in *M. tuberculata* at all the three sites and between the sites did not differ from the controls (Fig. 2). The activity of LDH in *O. mossambicus* was lower ($P < 0.05$) at Site 1 compared to sites 2, 3 and the controls (Fig. 3). Activity in fish exposed at sites 2 and 3 were not different from the control fish or between the two sites.

Acetylcholine and EROD (Fig. 2) activities in *M. tuberculata* did not differ significantly between the three sites. However the MT content was significantly higher at Site 1 than Site 2. Fig. 3 shows that there were no significant differences in AChE activity, EROD activity and MT content of *O. mossambicus* between the different sites or between the exposures and control groups ($p < 0.05$). In general the AChE activity of ABM exposed organisms were lower than the control organisms (Fig. 3), with the lowest AChE activity at Site 1 and increasing to Site 3. Metallothionein content also increased from Site 1 to Site 3. The activity of EROD was similar in all three sites.

The dendrogram resulting from performing cluster analysis and the MDS plot on the averaged biomarker responses for the control and deployed organisms are shown in Fig. 4. The stress value of 0.00, which was obtained, indicates that it is an excellent representation of the data (Clarke and Warwick, 1994). The dendrogram and MDS ordination places the biomarker responses of the two bioindicator organisms into two discrete groupings. However, within both the fish and mollusk groupings there are distinct groupings with Sites 2 and 3 showing similar biomarker responses, which are distinctly different from Site 1.

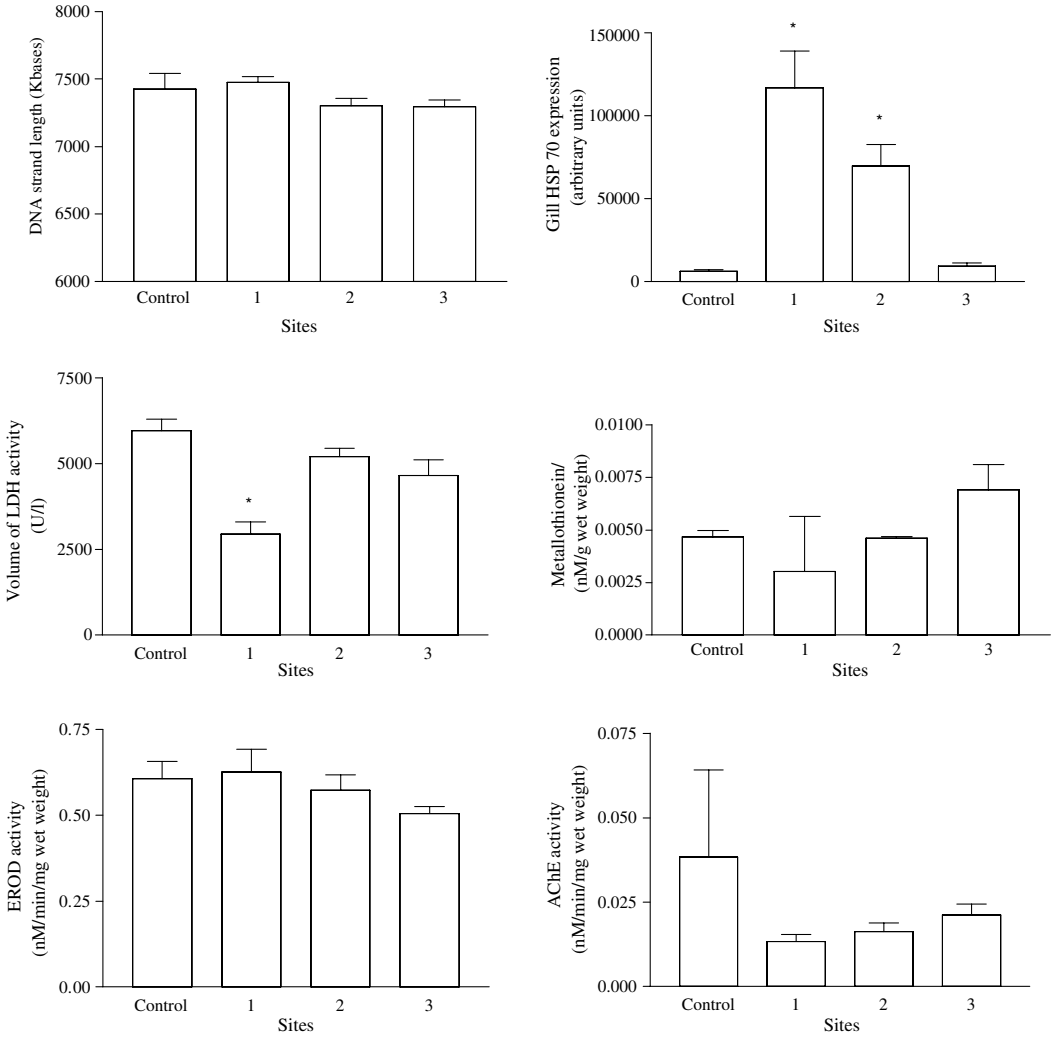

Fig. 3. Spatial biomarker responses of *O. mossambicus* at three sites in the Rietvlei Wetland System following a four week exposure period during high flow (April 2003) conditions. Data for DNA damage, HSP70 concentrations, LDH activity, AChE, EROD and MT are presented as means ± SE ($n = 10$). Asterisks indicate values significantly different from the control values ($p < 0.05$).

3.4. Discussion

The WET assessment did not indicate any toxicity at the three sites assessed. Whole effluent toxicity tests based on daphnids and fish can only express toxicity WET tests only indicate toxicity at a particular site in terms of mortality and does not give information on the sublethal responses or the nature of toxicant responsible for causing these responses, which it inherently should provide. Therefore more effect orientated assessments, which include *in situ* bioassays such as AMB techniques (Smolders et al., 2003) should be implemented in routine biomonitoring.

The extent of genotoxic stress is assessed through an evaluation of the DNA strands. Highly fragmented low molecular weight DNA strands migrate farther than non-damaged high molecular weight DNA strands. The average DNA strand lengths in *M. tuberculata* in the Rietvlei System were larger than the controls although there were no differences between the sites. This shows that there are genotoxic contaminants in the Rietvlei System. Concentration dependent differences in electrophoretic mobility of DNA was also found by Black et al. (1996) in mussels exposed to lead where 50 μg/l lead showed large zones of smaller DNA fragments which migrated farther than larger fragments. Since there is probably a large dilution capacity of the river during the high-flow period, it can be expected that the lower levels of the contaminants may have induced repair processes for the double stranded DNA so that DNA damage was not detected thus average strand lengths were actually above the controls. Increased average base pair lengths above the controls due to induction of DNA repair was also found by Hoff et al. (2003) in *Cyprinus carpio* exposed for 5 days in 16, 270 and 864 ng/g perfluorooctane sulfonic acid. This is also similar to findings by Black et al. (1996) although in their case increased lead concentrations caused no detectable DNA damage after exposure of mussels to different concentrations of lead for 28 days. Pruski and Dixon (2003) found that mussels from the shallowest and less active vents showed greatest amount of DNA damage as compared to two deeper and

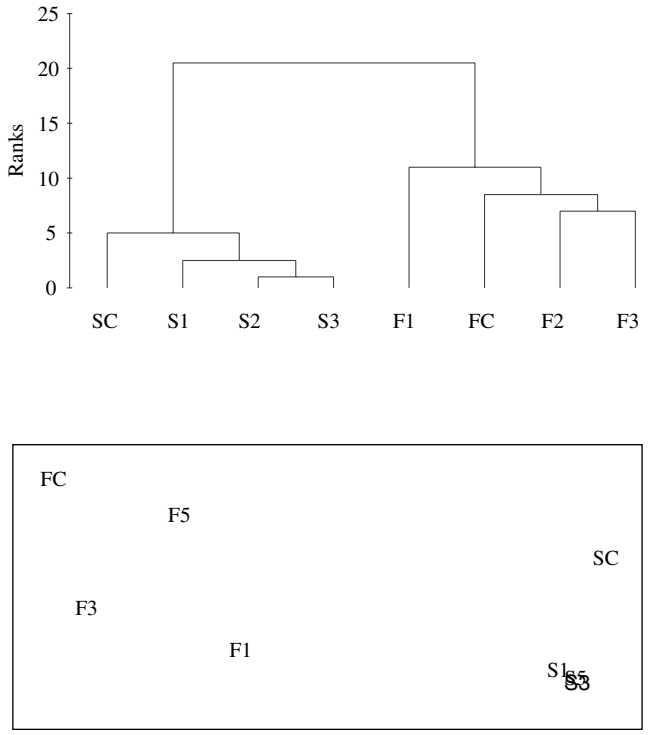

Fig. 4. MDS plots and dendrograms for the control and deployed biomarker responses in *M. tuberculata* (indicated by S) and *O. mossambicus* (indicated by F). Control animals are indicated by C and the sites in the Rietvlei System by 1-3. The 2-dimensional stress after 99 iterations was 0.00.

putatively more toxic sites. Although it was expected that the effects of the contaminants on DNA strand length would differ significantly from Site 1 to Site 2 because of the presence of Marais Dam that acts as a sludge dam and a wetland that filters the contaminants, the sites were not different. DNA damage can persist as a result of various physical and chemical reactions resulting in complex interactions (De Coen, 1999) thus the reasons for the lack of differences between Site 1 and Site 2 are not clear. Metals notably inhibit DNA repair enzymes and enhance the production of highly toxic hydroxyl radicals (Hartwig, 1998). The complex reactions that result in DNA damage makes it difficult to deduce the type of effluent, which is causing DNA damage. Although it is known that the Rietvlei System receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants (Barnhoorn et al., 2003) the complexity of the reactions between a genotoxic compound and the DNA molecule makes it difficult to link the type of effluent with DNA damage in the system. Some compounds react directly while others cause damage indirectly or only after metabolism (De Coen, 1999).

There were no differences in the amount of DNA damage in *O. mossambicus* exposed for four weeks in the Rietvlei System at all the sites. This may once again be as a result of induced DNA repair processes so that DNA damage was not detectable by the end of our exposure period or simply because the contaminants were diluted to such a degree that they did not cause any DNA damage. It has been demonstrated that contaminant exposure does not necessarily lead to corresponding increases in DNA damage (Steinert, 1999). Thus it can be deduced that the level of contaminants during high-flow was too low to induce DNA damage.

In the unstressed cell, stress proteins have constitutive functions that are essential in protein metabolism. The HSP 70 family is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone and mediate the repair and degradation of altered or denatured proteins (Basu et al., 2002). The increased expression of HSP 70 in *O. mossambicus* at Sites 1 and 2 indicates that these proteins are induced by contaminants in the system. The expression of HSP 70 in the control fish shows that HSP 70 is expressed constitutively in *O. mossambicus*. HSPs can be up regulated in cells that are exposed to a variety of stressors, particularly those that denature proteins (Basu et al., 2002). In fish, a correlation between increased levels of heat shock proteins and exposure to stressors within an ecologically relevant range has been demonstrated, suggesting that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish (Basu et al., 2002). The increased HSP 70 expression at Site 1 decreasing downstream at Site 3 shows that the contaminants are more concentrated at Site 1 and decrease down the pollution gradient. Site 1 receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants that settle in Marais Dam, which acts as a sludge dam. Elevated levels of HSP 70 have been measured in tissues of fish exposed to environmental contaminants such as heavy metals (Boone et al., 2002), industrial effluents (Vijayan et al., 1998), pesticides and polycyclic aromatic hydrocarbons (Sanders, 1993). Elevated HSP 70 levels have been observed in hepatic, head and kidney tissues of coho salmon (*Oncorhnchus kisutch*) infected with *Reibaterium salmoninarium* the causative agent of a chronic disease of salmonids (Basu et al., 2002) thus providing evidence that a relationship exits between heat shock proteins and diseases in fish. In this study only disease free fish were used hence elevated HSP 70 levels can only be attributed to contaminants in the system. Studies in fish have shown that the appearance and decay of HSPs share a close temporal relationship with the induction and disappearance of thermotolerance (Mooser et al., 1987). Elevated HSP 70 levels in the Rietvlei System are similar to findings by Williams et al. (1996) where HSP 70 levels were significantly higher in juvenile rainbow trout exposed to metals in water and diet. This study indicates that *O. mossambicus* employ HSP 70 in their normal course of physiological adaptation to change in environmental conditions. Fader et al. (1994) also found that fish (*Ictalurus natalis*) respond to normal environmental variations in their native streams by producing HSP 70. HSP 70 from tissues of a number of fish species share common epitopes (Abukhalaf et al., 1994) hence increased HSP 70 expression in stressed fish enables the

detection of stress proteins in ecologically and commercially important fish species and consequently to establish the conditions that may prove hazardous to fish and other organisms. Although HSP 70 is a non-specific indicator of stress the results clearly show in this study that it is a useful and applicable biomarker in the active biomonitoring of the Rietvlei System using *O. mossambicus* as test organism.

Enzyme inhibition is a common mechanism of toxicity (Westlake et al., 1983). In this study LDH activity in *M. tuberculata* did not differ at all from the three sites and from the controls but in *O. mossambicuss* the activity of LDH was significantly reduced at Site 1 when compared to the other sites and the controls. This is similar to findings by Gill et al. (1990) where the activity of LDH was inhibited by mercury in the rosy barb. The lack of difference in activity in *M. tuberculata* indicates that LDH is not a sensitive biomarker in the Rietvlei system using *M. tuberculata*. The increase in LDH activity down the pollution gradient indicates that there are contaminants that inhibit LDH which are more concentrated at Site 1 and are less concentrated downstream. Escher et al. (1999) however found higher LDH activity in blood of brown trout (*Salmo trutta*) exposed to river and sewage plant effluent.

The biomarker analyses showed that the bioindicators responded to neurotoxic chemicals (i.e. decreased AChE activity) in the Rietvlei System at all three sites, with the degree of toxicity decreasing from Site 1 to Site 3. Low ACHE activity indicates a large degree of pollution by organophosphates, carbamates or similar neurotoxic compounds at the site of interest (Van der Oost et al., 2003). Based on the assumption that samples from the controls exhibited normal levels of activity, it was evident that exposure of test organisms to instream conditions generally resulted in decreased AChE indicating exposure to neurotoxins. When ranking sites with respect to levels of potential neurotoxicity (as observed in *O. mossambicus* and *M. tuberculata*) Site 3 is expected to have the least toxicity followed by Site 2 and Site 1 as the most toxic. The decrease in AChE activity could also be indication of metal pollution (Devi and Fingerman, 1995). According to Bouquene and Galgani (1996) AChE activity may be inhibited by phytotoxins released into the water column during phytoplankton blooms.

Induction of CYP450 enzyme systems has been attributed to PAH exposure although many other contaminants (PCB's, polybrominated biphenyls or dibenzodioxin) have an effect on these enzymatic systems. Biological responses to these organic compounds can be evaluated by measuring the induction of EROD activity. The activity of EROD is one of the best-known and widespread biomarkers and its utilization is successful in indicating organic pollution (Stegeman and Hahn, 1994). During this study the EROD assays showed no significant differences in EROD activity between the sites and between sites and the controls. However it is known that under highly stressful pollution conditions could alter the liver physiology of fish such that minimal CYP450 activity and organic xenobiotic metabolism may occur (Stegeman and Hahn, 1994). This can explain the low levels of EROD activity obtained in fish measured from polluted water during this study. However the low activity could also be attributed to non-induction due to the absence of sufficiently large amounts of organic pollutants. Additional biomarkers of exposure must therefore always be utilized in association with biomarkers of effect able to define health status of the animals (Cajaraville et al., 2000). Chemicals such as PCB's have been shown to induce heme oxygenase and CYP1A and inhibit EROD activity in various fish species (Hahn and Chandran, 1996). The levels of PCBs are higher in some species where direct correlations between EROD and CYP1A expression occur. Where concentrations are high they induce heme oxygenase, which has been shown to degrade CYP in other species (Hahn and Chandran, 1996). It has been proposed that PCBs are not only inhibiting catalytic activity but also leading to the degradation of CYP1A either through direct induction of heme oxygenase or through generation of oxidative stress brought about by the incomplete oxygenation of PCB's by CYP (Hahn and Chandran, 1996). It is therefore necessary to determine whether low EROD activity is due to degradation of CYP or due to low induction of EROD by PAH or PCB exposure, before conclusive remarks can be made. Stegeman and Hahn (1994) have also demonstrated that exposure to high concentrations of PCB's can inhibit CYP1A catalytic activity. This is a possible explanation for the low levels of EROD exhibited by the exposed organisms. No influences of other contributing factors such as age, temperature or feeding behavior were considered in this study.

No between site differences were observed with in the MT content. Metallothioneins do not have enzymatic functions and are thus used to indicate exposure to metal pollution. Evaluation of MT content has been widely used to verify the presence of heavy metal pollution in experimental conditions (Viarengo et al., 1997). Metallothioneins occur in the cytosol and have been detected in the nucleus and lysosomes following laboratory exposure or exposure in the field to essential and non-essential metal ions (Viarengo et al., 1997). Induction has been demonstrated in organisms from polluted sites following exposure to class B metals e.g. Ag, Cu, Cd, Hg and Zn. In this study both the fish and invertebrates from ABM assessment showed increases of MT following exposure to water from the Rietvlei Wetland System, indicating possible heavy metal contamination. It is important that the use of MT in biomonitoring should be supported by knowledge about the physiology of stress responses in bioindicator organisms. In fish, MT induction should be considered a general stress response and in mollusks, MTs represent a stress response to heavy metals as well (Viarengo et al., 1999). Usually the MT content is very low in uncontaminated organisms (Viarengo et al., 1997). Involvement of metal sequestration is more evident in gills, digestive glands and kidney reflecting the significance of these tissues in uptake of storage and excretion of metals (Bebianno et al., 1993).

Multivariate analysis techniques are a useful tool for interpreting biomarker data as they produce a two-dimensional pattern of the degree of similarity between different groups of data based on responses. The resultant MDS ordination identified that organisms at Sites 2 and 3 were reacting differently to the ambient environment than organisms at Site 1. Based on the information of individual biomarkers in the preceding paragraphs it is evident that Sites 1 was subjected to organic pollutants with distinct neurotoxic action (as seen from decreased AChE and increased EROD activities) and Sites 2 and 3 were subjected to a mixture of predominantly metal pollution (as seen from increased MT concentrations) and some organic pollutants.

The analyses of biomarker data using multivariate statistical techniques showed that it has the potential of being an effective ecotoxicological tool. In this particular instance the method proved to be more sensitive than using conventional toxicity tests as the mortality-based WET testing was unable to any toxicity. The results also indicated a pollution gradient, suggesting that animals from the uppermost site, where the water enters the system, were more stressed than the control animals and that there was a decrease in pollution stress gradient down the system. MDS is particularly useful for highlighting sites at which more detailed analysis of chemical contamination would be useful.

The use of transplanted organisms (fish and mollusks) is a sensitive and easily applicable active biomonitoring tool that can be used to assess water quality and pollution in field conditions. Evidence from the present study confirms that the combination of WET and *in situ* exposures may provide some insight into understanding and predicting effects of chemicals on natural communities under realistic exposure conditions and would thereby contribute towards improving the interpretation of laboratory to *in situ* extrapolation. If biomarkers and multivariate analysis are to become established as recognised monitoring tools it is important to find a suite of biomarkers that most compliment each other. The analyses of a large suite of biomarkers, as described in the study, preclude its applicability in routine *in situ* biomonitoring programmes. Recently Smolders et al. (2003, 2004c) demonstrated that energy budgets could successfully be used to link cellular effects with effects at higher levels of biological organisation. Indeed unpublished results from our research group showed that the spatial delineation in the Rietvlei System, based cellular energy allocation biomarker responses, were similar to the suite of biomarker responses utilized in this study (Moolman, 2004). It still remains to be verified whether this relationship is applicable to other systems with a less clear pollution gradient. This is a topic of ongoing research.

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