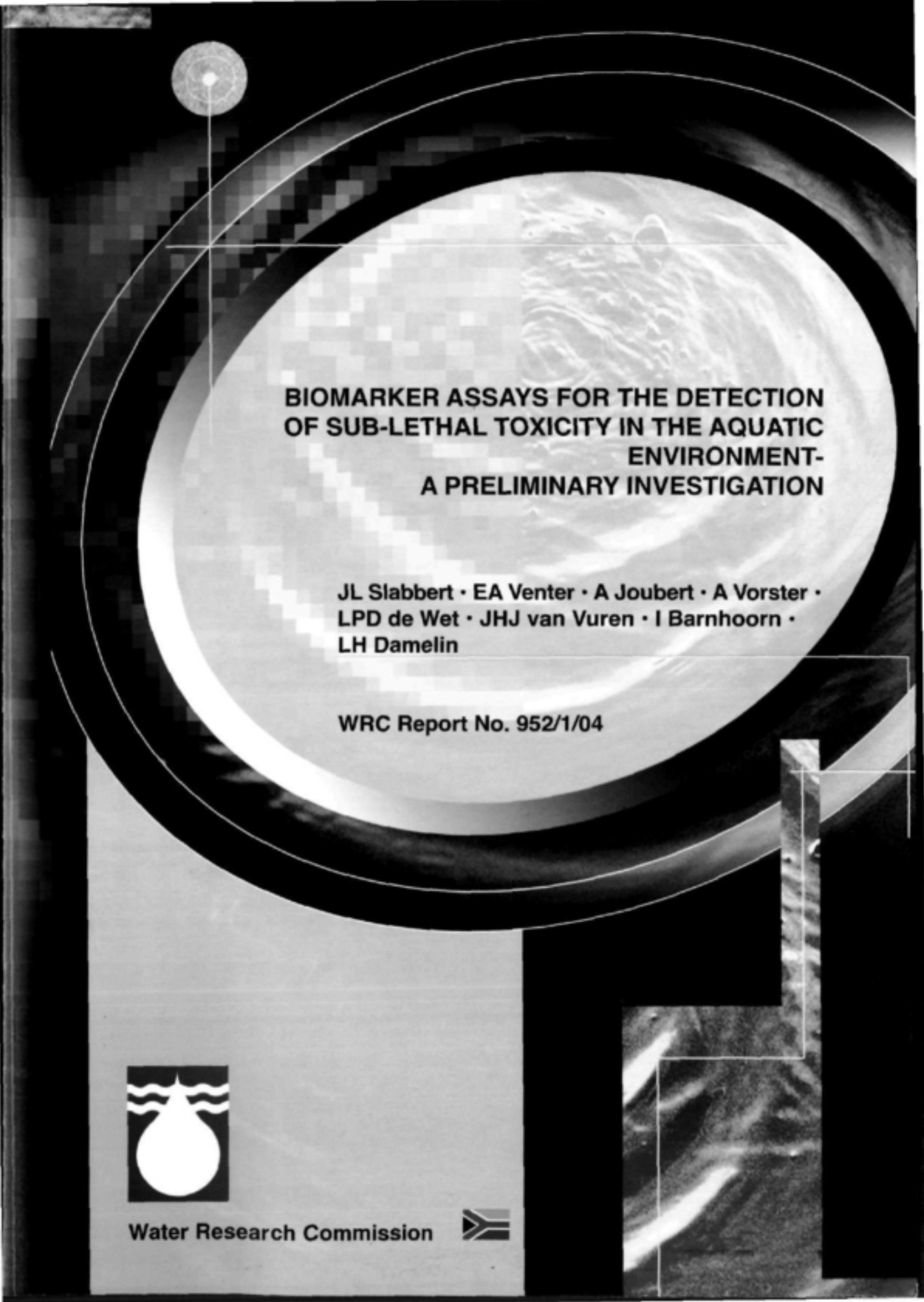

BIOMARKER ASSAYS FOR THE DETECTION OF SUB-LETHAL TOXICITY IN THE AQUATIC ENVIRONMENT- A PRELIMINARY INVESTIGATION

JL Slabbert · EA Venter · A Joubert · A Vorster · LPD de Wet · JHJ van Vuren · I Barnhoorn · LH Damelin

WRC Report No. 952/1/04

Water Research Commission

BIOMARKER ASSAYS FOR THE DETECTION OF SUB-LETHAL TOXICITY IN THE AQUATIC ENVIRONMENT-
A PRELIMINARY INVESTIGATION

Report to

the Water Research Commission

by

**JL Slabbert, EA Venter, A Joubert, A Vorster,
LPD de Wet, JHJ Van Vuren, I Barnhoorn and LH Damelin**

Environmentek, CSIR

Final report on the Project entitled :
'Biomarker Assays for the Detection of Chronic Toxicity
in the Aquatic Environment'

WRC Report No 952/1/04
ISBN No 1-77005-112-0

MARCH 2004

Printed by Silowa Printers: 012 804 1164

EXECUTIVE SUMMARY

1. Background and motivation

Many countries, including South Africa, have adopted an integrated management approach, that includes chemical and biological methods, to address toxic and non-conventional pollutants in the aquatic environment (Slabbert *et al.*, 1998a,b). Over the past 20 years, a range of biological tests measuring the effects of toxicants under controlled laboratory conditions have been established for South African use (Slabbert *et al.*, 1998a). Most of the tests are rapid/short-term tests, aimed at the detection of acute toxicity. Very little attention has so far been given to the use of sub-lethal toxicity tests that can be used alongside acute tests to obtain information on long-term effects of low levels of chemicals on aquatic organisms. Sub-lethal toxicity tests are important management tools when the toxicity of effluents is low and are indispensable to manage ambient water quality.

Traditionally, full life-cycle tests or shorter tests of about 30 days, known as early life-stage tests, are used to establish sub-lethal toxicity in the aquatic environment. Because such tests are labour intensive and expensive, most countries have shortened the duration of the tests to 7 to 10 days, focusing on the most sensitive life-cycle stages. These tests are known as short-term chronic tests (Slabbert *et al.*, 1998a; US EPA, 1991), and the primary endpoints are growth and reproduction. Examples of short-term chronic tests applied in the USA (Slabbert *et al.*,1998a) for fresh water testing include: a 7-day fish (*Pimephales promelas*) larval growth/survival test; a 7-day invertebrate (*Ceriodaphnia dubia*) reproduction/survival test; and a 96-h algal (*Selenastrum capricornutum* Printz) growth test.

In recent years, considerable attention has been given to the establishment of even more rapid measures of sub-lethal toxicity. A fascinating new approach is the use of biomarker assays that provides measures of the biochemical/molecular mechanisms underlying toxicity. These assays have been derived from mammalian medicine and allow for the rapid assessment of organism health (usually hours to a few days).

A biomarker may be biological material ranging from bio-molecules such as nucleic acids and proteins (e.g. enzymes) to organelles, cells, tissues, organs and whole organisms (Appendix A). Biomarkers indicating damage caused at the biochemical/molecular level in a cell have rapid response times and are highly sensitive. These biomarkers are currently the most applied in aquatic biomarker studies. Biomarkers of growth and reproductive responses are of high biological and ecological significance, but exhibit relatively low sensitivity to stress. Manifestations of stress at the tissue level represent an intermediate effect between the biochemical and reproductive levels. Histological changes occur earlier than reproductive changes and have been shown to be more sensitive than growth or reproductive measures, and as an integrative parameter, provides a better evaluation of organism health than a single biochemical marker.

Biomarkers can be used as indicators of exposure and effect, and can be categorised as non-specific and specific (Mayer *et al.*, 1992). Some of the non-specific biomarkers, e.g. ribonucleic acid/deoxyribonucleic acid, radiolabelled amino acid or nucleotide incorporation, and adenylate energy charge, give direct information on the growth rate or potential of an organism. These biomarkers cannot be used to identify the specific toxicant causing the effect. There are two types of specific indicators of stress, namely organ- and toxicant specific. Organ-specific biomarkers include organ specific enzymes and iso-enzymes, and often depend on the detection of enzymes at increased concentrations in organs. These enzymes appear in blood when organs are damaged and indicate the presence and extent of damage. Examples include lactate dehydrogenase (LDH), transaminases, creatine phosphokinase, lysosomal enzymes, alkaline phosphatases and mixed function oxidase (Mayer *et al.*, 1992; Gagné and Blaise, 1993). Toxicant-specific biomarkers indicate the exposure and effects due to a single chemical or group of chemicals, e.g. inhibition of acetylcholinesterase (AChE) by organophosphates and inhibition of cytochrome P monooxygenase, methallothionein and metal binding proteins by metals.

Since biomarkers measure events along the entire metabolic pathway during exposure to a chemical, and since the toxic effect on organisms is secondary to change inside cells, biomarkers hold promise for higher sensitivity and earlier detection of toxicants than the traditional assays measuring acute toxicity. Biomarkers can act as early warning signals of imminent, irreversible, permanent damage to an organism (Depledge, 1993).

2. Objectives

This project was carried out to establish biomarker assays, as rapid alternative measures to the more tedious growth and reproduction measures used in chronic toxicity tests, to detect sub-lethal toxicity in the South African aquatic environment.

The objectives of the project were:

1) To establish biological techniques to detect sub-lethal toxicity in the aquatic environment, and

2) To produce an operational manual on the established methodologies.

The focus of the project was on using biomarkers as sub-lethal endpoints in a laboratory fish test employing tilapia (*Oreochromis mossambicus*), to complement the acute (lethality) tilapia test developed for local use (Slabbert *et al.*, 1999). Since juveniles are much more sensitive than adult organisms, the aim was to use the youngest possible juvenile fish in the laboratory test. Once the biomarker assays were established for laboratory use, it was planned to apply the assays to tilapia and other organisms collected from selected field sites to evaluate their applicability for field use. The study was not aimed at extrapolating the laboratory results to the field data, but rather to establish two options (laboratory and field) for biomarker application.

3. Methodology

Assays for the following biomarkers were established and optimised: protein; AChE; ethoxyresorufin-O-deethylase (EROD); glucose; glycogen; delta-aminovulinic acid dehydratase (ALA-D); LDH; glucose-6-phosphate-dehydrogenase (G-6-P-DH); pyruvate kinase (PK); heat shock protein (Hsp 70) and osmotic ion analyses. Published protocols were followed. Where possible, techniques were miniaturised (microplate assays) or test kits were used.

The biomarker assays were applied in laboratory and field studies. The indigenous fish species *O. mossambicus* was selected as test organism. A preliminary evaluation using unexposed (control) fish indicated that 1 to 1.5 cm (2 to 4 week old) fish were the most suitable for use in laboratory tests with reference to detectability of biomarker activity, smaller variation, ease of use, small sample volumes and expected higher sensitivity. Laboratory fish were maintained and bred according to well-established protocols. Laboratory studies were carried out following static acute fish test protocols. Biomarker activity was evaluated in the laboratory by exposing 30 fish per container (single exposures) or 60 fish divided over 3 containers (triplicate exposures) to various concentrations of selected chemicals [cadmium, zinc, pentachlorophenol (PCP) and cyanide] using exposure periods of 8, 24 and 96 h. Selected biomarker assays were carried out on the supernatant of the homogenised fish. Assays were carried out in triplicate, which allowed the calculation of means and coefficients of variation (CVs). All the biomarker results, except Hsp 70, were expressed in terms of fish weight. Hsp 70 was expressed in terms of protein. The percentage fish lethality and biomarker induction/inhibition was calculated by comparing test and control results. The Rust de Winter Dam was selected as reference site, and the Loskop and Hartbeespoort Dams as test sites for field studies. Two field surveys were carried out, one in summer and the other in winter. Where possible, 20 fish (including males and females) were caught. Biomarker assays (single) were carried out on the blood (whole blood, plasma and erythrocytes), brain and liver of the fish. Biomarker results were expressed in terms of protein.

An analysis of variance (Student's t-test) was applied to laboratory biomarker data to establish whether or not results differed significantly at the $P<0.05$ level. This allowed the derivation of the toxicity endpoint referred to as a LOEC (lowest of consecutive test concentrations at which biomarker activity was significantly different from that of the control). Where possible, regression analysis (linear regression) was performed on exposure data (concentration-response curves) to establish EC_{20}s (the concentration causing a 20% induction or inhibition in biomarker activity). A two-way analysis of variance (ANOVA) and Dunnett's test were applied to field data to determine significant differences between localities and seasons. The significance level was set at $P<0.05$. Student' t-test was applied ($p<0.05$) to compare fish weights and lengths.

4. Summary of major results

4.1 Laboratory studies

The control results showed that, although values were low, all the biomarkers were present at measurable levels in the fish homogenate. The results obtained during single exposures were as follows: protein: 0.0166 to 0.0613 mg/mg fish; AChE (microplate assay): 0.0003 to 0.0041 abs/min/mg fish; AChE (test kit) (0.0003 to 0.0140 U/ℓ/mg fish; glucose: 0.0017 to 0.0044 mg/mg fish; EROD: 0.0699 to 0.3605 nM/min/mg fish; PK: 0.0201 to 0.6691 mU/mℓ/mg fish: LDH: 0.0011 to 0.0120 U/ℓ/mg fish; G-6-P-DH: 0.0562 to 4.6738 mU/ℓ/mg fish: Hsp 70 (WITS): 7.80 to 122.01 mm^2/20 µg protein; and Hsp 70 (RAU): 2 137 to 10 252 relative intensity/15 µg protein. The control values obtained during triplicate exposures were within the above ranges, except in the case of Hsp 70 (RAU), where values were considerably higher (6 238 to 15 685 relative intensity/15 µg protein.

A considerable fluctuation was noticed in the control biomarker values (mean of triplicate assays) of different single exposures. This was possibly because offspring of different weights and sizes (18 to 28 days old) were used in experiments while a fixed volume of buffer was used for homogenization. Glucose, protein, Hsp70 and EROD results exhibited the smallest fluctuation, and PK, AChE (test kit) and G-6-P-DH the largest fluctuation. When triplicate exposures were carried out, the fluctuation between the results of triplicate assays were much smaller, probably because the age of the fish used in these studies ranged between 21 and 24 days, reducing differences in weight and size.

Large inter-assay variations (triplicate assays) were noticed in the control results of single exposures in the case of: G-6-P-DH (14 to 72%), protein (0 to 92%), PK (5 to 98%), AChE (test kit) (2 to 109%) and LDH (2 to 110%). The best repeatability was obtained with the Hsp 70 (RAU) (CV: 5 to 28%) and glucose (CV: 0 to 32%) assays. With the exception of EROD, with CVs ranging from 8 to 102%, the inter-assay variation of triplicate exposures was much smaller (CVs: <50%) than the inter-assay variation of single exposures. The inter-experimental variation of triplicate exposures (triplicate data sets) of EROD and PK was >50% in some instances. With a few exceptions (mostly test kit data), most of the control CVs were in agreement with published values for fish homogenates.

In general, control lethalities were below 10% (detection limit for acute toxicity). A large lethality occurred in the control fish of the 96-h zinc exposure study (53%), which could have influenced biomarker activity. Lethality also occurred at some of the upper test concentrations during cadmium and zinc exposure studies. Oxygen concentrations in test and control containers during cyanide exposure studies were below the limit required for the sustenance of aquatic life (2.4 to 2.7 mg/ℓ), which could have influenced biomarker results.

The exposure studies showed that biomarkers were affected by the test chemicals. Biomarkers were induced or inhibited. Although large effects were observed in some instances, effects were not always significant because of the large variation between replicate results. The results of replicate experiments (PCP and cyanide) were often inconsistent. Test concentrations showing significant biomarker induction in one experiment did not affect the biomarkers in the other experiment or *vice versa*. This could be attributed to variability in the sensitivity of test fish or to large variations between replicate results, rendering test results not significantly different from control results.

Protein was significantly affected by PCP (inhibition) and cyanide (induction) during 24 h exposure, and by cadmium (inhibition) during 8 h exposure. AChE was significantly affected (induction or inhibition) by all the chemicals during the shorter exposure periods (8 and 24 h), while only PCP affected (induction) AChE during 96 h exposure. Glucose was significantly affected (induction and inhibition) by cadmium during 8 h exposure, by cyanide (induction) during 24 h exposure, and by zinc (inhibition) and PCP (induction) during 96 h exposure. EROD was significantly affected by cadmium (induction) during 8 and 96 h exposure, by zinc (induction and inhibition) and cyanide (induction) during 24 h exposure and by PCP (induction) during 96 h exposure. LDH was significantly inhibited by zinc during 96 h exposure and significantly induced by PCP during 24 and 96 h exposure. Hsp 70 displayed significant responses to cadmium (induction and inhibition) during 96 h exposure, to zinc (inhibition) during 96 h exposure, and to cyanide (induction) during 24 h exposure. PK was significantly induced by cyanide during 24 h exposure, while G-6-P-DH did not exhibit significant responses. In general, the effects exhibited during single and triplicate cadmium exposures were similar.

A comparison with data in literature, showed that some results were in agreement with published data, while other results differed from published data.

Results showed that concentrations of cadmium and zinc that exhibited significant biomarker responses during 8 and 24 h exposure, caused lethality during the 96 h exposure. The biomarkers thus acted as early warning signals for acute toxicity. The significant responses detected during 96 h exposure, generally occurred at lethal concentrations. PCP caused significant biomarker responses at sub-lethal concentrations during 24 and 96 h exposure, resulting in a 10 to 60 times enhancement of sensitivity upon longer exposure in some instances. As in the case of metals and PCP, cyanide exhibited significant biomarker responses at sub-lethal concentrations during 24 h exposure.

In general, no significant concentration-response functions were observed after 24 and 96 h exposure. Curves either showed an upward-downward or a downward-upward trend at increasing concentrations, or reached plateaus parallel to the x-axis. Significant linear fits were, however, obtained for all the biomarkers during 8 h exposure to cadmium. AChE, glucose, EROD and Hsp 70 showed elevated activity at higher concentrations, while protein levels decreased.

The following toxicity endpoints were derived for the different chemicals:

Chemical	Endpoint	Biomarker
Cadmium	8-h LOEC	Protein: 0.4 mg/ℓ; AChE: 0.2 mg/ℓ; glucose: ≤0.05 mg/ℓ; EROD: 0.2 mg/ℓ
	8-h EC_{20}	Protein: 0.0886 mg/ℓ; AChE: 0.0596 mg/ℓ; glucose: 0.2128 mg/ℓ; EROD: 0.0825 mg/ℓ; Hsp 70: 0.2818 mg/ℓ
	24-h LOEC	AChE: 0.0220 mg/ℓ
	96-h LOEC	EROD: 0.0125 mg/ℓ; Hsp 70: 0.0125 mg/ℓ
Zinc	24-h LOEC	AChE: 0.5 mg/ℓ; EROD: 1.0 mg/ℓ
	96-h LOEC	Glucose: ≤0.125 mg/ℓ; LDH: ≤0.125 mg/ℓ; Hsp 70: 0.5 mg/ℓ
PCP	24-h LOEC	AChE: 0.08 mg/ℓ; LDH: 0.08 mg/ℓ
	96-h LOEC	AChE: 0.005 mg/ℓ; glucose: 0.01 mg/ℓ; EROD: ≤0.00125 mg/ℓ; LDH: ≤0.00125 mg/ℓ
Cyanide	24-h LOEC	Protein: ≤0.0625 mg/ℓ; AChE: ≤0.0625 mg/ℓ; glucose: 0.25 mg/ℓ; EROD: ≤0.25 mg/ℓ; PK: ≤0.0625 mg/ℓ; Hsp 70: ≤0.0625 mg/ℓ

The 8-h LOECs of protein, AChE, EROD and Hsp 70 were between two to four times higher than the calculated EC_{20}s, while the LOEC of glucose was approximately four times lower than the EC_{20}. The endpoints different because a fixed variation of 20% was used for the EC_{20} while the actual variations of test and control data used for the analysis of variation (LOECs) were higher or lower. In some instances shorter exposure periods resulted in lower LOECs while in other instances LOECs were lower during longer exposure.

4.2 Field studies

In general, the lengths and weights of fish collected from the different dams differed significantly. Fish collected from the Hartbeespoort Dam in summer were small (mean weight: 0.36 kg; mean length: 24.9 cm) compared to the fish collected in winter, and from the other dams (mean weight: 0.90 to 1.71; mean length: 35.8 to 41.4 cm). Most of the fish collected in summer were female, while numbers were more equally distributed during winter sampling.

Protein levels were the highest in erythrocytes, followed in order of magnitude by whole blood, blood plasma and liver. The erythrocyte protein in replicate fish showed the least variation (CVs: 15 to 30%). Large variations occurred between the results obtained for whole blood, blood plasma and liver protein (CV: whole blood - 18 to 52%; erythrocytes - 5 to 85%; liver - 25 to 52%). Protein in fish homogenates were between 10 to 50 times lower than the protein levels in whole blood, between 5 to 25 times lower than the protein levels in blood plasma and between 3 to 20 times lower than the protein in liver.

Large variations occurred between some of the replicate biomarker results of fish collected at the reference (control) site (Rust de Winter Dam). The CVs for the different biomarkers were as follows: ALA-D: 47 to 52%; AChE (erythrocytes): 54 to 121%; AChE (brain): 22 to 51%; glucose: 34 to 54%; LDH (plasma): 97 to 119%; LDH (liver): 95 to 98%; calcium: 14 to 27%; potassium: 12 to 25%; sodium: 4 to 5%; EROD: 33 to 54%; G-6-P-DH: 170 to 219%; glycogen: 32 to 112% and PK: 138 to 164%. A considerable number of fish collected from the dams showed an absence of LDH, G-6-P-DH and PK activity.

All the biomarkers, except the AChE in erythrocytes, showed positive results on one or more of the sampling occasions. The effects of test sites on G-6-P-DH could not be determined because of the large variability in results. Induction as well as inhibition occurred. Results were generally in agreement with those found in literature. Since chemical analyses were not carried out, effects could not be attributed to specific toxicants. Seasonal changes (temperature, pH, oxygen concentration) and other stressors, e.g. availability of food and handling, could also have affected biomarker activity.

5. Conclusions

- In broad terms, the objectives of the project, namely 1) to establish biological techniques to detect sub-lethal toxicity in the aquatic environment, and 2) to produce an operational manual on the established methodologies, were met. However, certain issues, discussed below, were not adequately addressed.

- Some of the biomarker assays were successfully applied to the homogenates of 2 to 4 week old tilapia (*O. mossambicus*) used in laboratory toxicity tests, as well as to the organs of tilapia collected from the field.

- Although whole fish homogenates contained low levels of the studied biomarkers as a result of the dilution of enzyme levels in organs, the levels were sufficiently high to detect induction and inhibition in exposed laboratory fish.

- The biomarker assay protocols were relatively easily established in the participating laboratories, because well-established, published methodologies were used. The miniaturisation of some of the methods improved assays in terms of rapidity (small sample volumes and large numbers of samples), simplicity and cost. However, some of the tests, especially the test kits, appeared to be incompatible with whole fish homogenates, showing large variation in some instances.

- The inter-assay and inter-experimental variation in laboratory and field studies were very high for some of the biomarkers. Operational manipulation (sample preparation and assaying) and test method limitation (tissue compatibility) appeared to be the most likely causes of the variation. Sample storage played a minor role.

- Apart from operational manipulation and test method limitation, factors such as handling stress and the development stage of the fish could have contributed to the variation in biomarker values of field studies. It is therefore important to minimise stress and select fish of similar size/age when conducting field surveys.

- Although there was some degree of inconsistency in the results of laboratory exposure studies, sufficient evidence was obtained that biomarkers could be applied as sub-lethal measures of toxic activity in laboratory toxicity tests.

- The limited number of offspring obtained for tilapia maintained in the laboratory and the inconsistency in reproduction was a major constraint. As a result

    - ✓ elaborate experimental designs were not possible;
    - ✓ experiments were not repeated a sufficient number of times;
    - ✓ experiments were not carried out in replicate;
    - ✓ wide concentration ranges were not examined; and
    - ✓ a range of chemicals were not tested.

  Consequently, the sub-lethal test using biomarkers as endpoints was not sufficiently validated to enable informed decisions on its potential as a routine laboratory test.

- At this stage the sub-lethal test protocols, outlined in the Operational Manual (Venter *et al.*, 2003) is only applicable for qualitative studies. Protocols were optimised, but extensive testing/evaluation is required before the methods can be standardised for specific applications.

- Although the biomarkers were not applied to other test organisms in the field (e.g. worms, snails) as was initially envisaged, the established biomarker assays should be applicable to other organisms, including other fish, with little or no method modification.

- The concept of using a multi-organisational team seemed like a good idea to build capacity and transfer knowledge. However, this resulted in large delays in the execution of experiments. The use of students that came and went, instead of well-trained technicians, resulted in large inter-assay and inter-experimental variation which could not be remedied.

6. Recommendations

- The use of replicate sets of fish and replicate assays in laboratory exposure studies is recommended, to reduce experimental and assay variability. Replicate assays should also be carried out on fish of field studies to improve variability.

- It is also recommended to use well-trained operators to conduct biomarker assays and to follow quality assurance protocols and practices to ensure low variability.

- Because of the large differences in the control variation of the different biomarkers studied, and because no clear concentration-response functions were identified for most of the laboratory results, the use of a fixed endpoint such as an EC_{10} or an EC_{20} is not recommended. The LOEC, derived by means of an analysis of variance, proved to be a more appropriate sub-lethal toxicity endpoint.

- Based on the data obtained for different exposure periods, it is recommended that the sub-lethal fish toxicity test is carried out over 96 h. Any effects on biomarkers due to handling and stress manifested during the shorter exposure periods should have subsided after 96 h.

- The results of 8 and 24 h tests indicated that certain biomarkers can serve as early warning signals for acute toxicity. It is recommended that further studies are carried out (organism/cellular/sub-cellular level) to exploit the potential of biomarkers as rapid screening tests.

- Based on a combination of features such as sensitivity, selectivity, simplicity, repeatability and rapidity, the following biomarker assays are recommended for the detection of sub-lethal responses in the 96 h laboratory toxicity test: Protein (micro Bradford method), AChE (microplate assay), glucose, EROD, PK, LDH and Hsp 70. ALA-D (whole blood), AChE (microplate assay) (erythrocytes and brain), glucose (plasma), glycogen (liver), LDH (plasma and liver), EROD (liver), osmotic ions (plasma) and Hsp 70 are recommended for field use. The Hsp 70 assay is the most complex and time consuming of the assays. All the assays require relatively expensive equipment (e.g. spectrophotometers). The biomarkers should be used in battery form to allow for optimal detection of harmful pollutants. The test protocols are outlined in the Operational Manual (Venter *et al.*, 2003).

- The AChE microplate assay is preferred to the AChE test kit because of higher reproducibility, less fluctuation and more reliable data calculation. Likewise, the micro Bradford method (micro method) is preferred to the macro method because of rapidity and less variability.

- The G-6-P-DH assay is not recommended for use because it requires large sample volumes and produces unreliable and highly variable results. PK is not recommended for field use because of high variability and because its potential as an *in situ* marker is questioned by other researchers.

- It is recommended that fish weight is used to express biomarker activity in the laboratory toxicity test, instead of protein, because of smaller variation and fluctuation in results, and because protein acted as biomarker.

- A specific volume of buffer was added to a certain number of fish for homogenation. Because weight increases with age, this approach resulted in considerable fluctuation in laboratory control (unexposed fish) values. It is, therefore, recommended to base the volume of buffer on weight rather than on the number of fish.

- The established biomarkers should be used to complement fish lethality data and not to substitute chronic measurements such as growth and reproduction. Likewise, the biomarker approach is not a replacement for conventional field assessments, but a supplementary tool with great ecological relevance.

- Limited fish numbers is a major concern regarding the success of this fish as test organism in acute and sub-lethal toxicity tests. National breeding facilities should be established to ensure a consistent and healthy supply of the fish. In addition, other fish species, with the potential to regularly provide large numbers of offspring, should be investigated as test organisms.

- Tilapia were present in large numbers in the dams studied. This might not be the case in other environments. Before studying biomarkers in organisms in the field, their abundance should be established to ensure sufficient replicates.

- Since biomarkers have been proven to be useful measures of sub-lethal stresses, it is recommended that additional biomarkers, including metallothioneins, are investigated for application in laboratory and field studies.

- The present laboratory study was only conducted on individual chemicals. It is recommended that the study be extended to environmental samples (drinking water, effluents, sediments, etc) to evaluate the applicability of the biomarkers with reference to complex chemical mixtures.

- As the biomarker techniques are becoming more established for aquatic studies, more countries are applying such measures in continuous monitoring programmes. This approach should also be valuable for our National Toxicants Monitoring Programme that is currently being developed. Molluscs have been found excellent test organisms in such studies. It is, therefore, recommended that funds are invested in the establishment of an *in situ* biomarker test using a local mollusc.

7. Technology transfer

Training in fish culturing and biomarker application was provided at some stage during the project to a number of students: Ms F Buthane; Mr J Senyane; Ms N Ngwegwane; Ms A Joubert; Ms A Vorster; Mr L Damelin; and Ms I Barnhoorn.

The results were used in two M.Sc. studies:

Joubert, A. (2000) Evaluation of biomarkers for pollution in *Oreochromis mossambicus*: A laboratory and field study. M.Sc. Thesis, RAU, 116 pp.

Vorster, A. (2000) Evaluation of selected enzymes and osmotic ions as biomarkers for detecting pollution in the aquatic environment. M.Sc. Thesis, RAU, 112 pp.

The `results obtained during the project were presented at several conferences/symposia:

Joubert, A., Venter, E.A., Slabbert, J.L., Vorster, A., Van Vuren, J.H.J. and De Wet, L. (1999) Preliminary evaluation of biomarkers for the detection of chronic toxicity in *Oreochromis mossambicus*. Poster presented at the 9th International Symposium on Toxicity Assessment, held in Pretoria from 26 September to 1 October.

Joubert, A., Venter, E.A. and Slabbert, J.L. (1999) Evaluasie van biomerkers vir die bepaling van chroniese toksisiteit in die vis, *Oreochromis mossambicus*. Poster presented at the South African Academy of Science and Art - Biology Division Annual Congress, 15 to 16 September. The abstract was published in Die Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie, **19,** June 2000.

Vorster, A. and van Vuren, J.H.J. (1999) Biomarker assays for the detection of chronic toxicity in the aquatic environment. Poster presented at the 9th International Symposium on Toxicity Assessment, held in Pretoria from 26 September to 1 October.

Vorster, A. and van Vuren, J.H.J. (1999) Biomerkers en die bepaling van chroniese toksisiteit in die akwatiese omgewing. Poster presented at the South African Academy of Science and Art - Biology Division Annual Congress, 15 to 16 September. The abstract was published in Die Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie, **19,** June 2000.

Slabbert, J.L., Venter, E.A., Joubert, A., Vorster, A., van Vuren, J.H.J., De Wet, L., Barnhoorn, I. and Damelin, I. (2001) Evaluation of biomarker assays for the detection of sub-lethal toxicity in fish. Oral paper presented at the 10th International Symposium on Toxicity Assessment, held in Quebec City, Canada from 26 to 31 August.

The biomarker assays and results were also addressed in different courses:

Venter, E.A. and Slabbert, J.L. (1999; 2000; 2001) Standard laboratory organisms in aquatic toxicology. Presentation at Rhodes University, Grahamstown.

Slabbert, J.L. (1999; 2000; 2001) Aquatic toxicology and toxicity testing. Presentations to B.Sc. Hon. students at RAU.

Practical training courses in the different biomarker assays are planned by CSIR. It is envisaged that training will start in 2002.

ACKNOWLEDGEMENTS

The research in this report entitled 'Biomarker assays for the detection of sub-lethal toxicity in the aquatic environment - A preliminary investigation' emanated from a project funded by the Water Research Commission.

The Steering Committee responsible for this project consisted of the following members:

Mr HM Du Plessis	Water Research Commission (Chairman)
Dr NP Mjoli	Water Research Commission
Ms M du Preez	Environmentek, CSIR
Ms E Bofilatos	DWA&F
Dr S Jooste	IWQS, DWA&F
Dr AL Kühn	IWQS, DWA&F
Dr H du Preez	Rand Water
Mr M Graham	Umgeni Water
Dr EM Bey	Highveld Biological
Dr LPD de Wet	Waterlab Research

The financing of the project by the Water Research Commission and the contribution of the members of the Steering Committee is greatly acknowledged.

This project was only possible with the cooperation of many individuals and institutions. The authors therefore wish to record their sincere thanks to the following:

The Information Centres of Environmentek, Rand Afrikaans University (RAU), and the University of the Witwatersrand for assisting with literature searches;

Statcon (RAU) for conducting statistical analyses;

Mr W Fourie of Environmentek for assisting with data analysis;

Ms O Webb of Environmentek for typing of documents;

Mr J Morota of Environmentek for fish maintenance and general laboratory assistance;

Mr D Erlank and coworkers at RAU for fish breeding; and

The team of lecturers and scientists at RAU who assisted with the field sampling.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY

Acute	A stimulus severe enough to rapidly induce an effect in a short period of time. In aquatic toxicity tests an effect observed within 96 h or less is usually considered acute - an acute effect can be lethal or sub-lethal
Antibody	A specialised protein capable of combining chemically with the specific antigen which stimulated its production
Biomarker	A biomarker is an indicator in a living organism that reflects sub-lethal molecular and/or cellular changes (biochemical, physiological and histological) occurring along a metabolic pathway because of exposure to chemical or physiological changes
Buffer	A solution containing a mixture of a weak acid and its salt (conjugate base) or a weak base and its salt (conjugate acid). The pH changes only slightly, when a relatively large quantity of strong acid or strong base is added to a buffer solution
Chemical	Any element, compound, formulation or mixture of a chemical substance that might be mixed with, deposited in or found in association with water/effluent
Chronic	A stimulus that continues for a relatively long period of time (long–term effects of small doses and their cumulative effects over time). Chronic toxicity is measured in terms of sub-lethal effects. Endpoints include reproduction and growth. Most of the chronic aquatic tests in use today use a short exposure time of approximately 7 days and are called short-term chronic tests
Control	Duplicates all the conditions of the exposure treatment, but contains no toxicants. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g. salinity, temperature, health of test organisms, or effects due to handling of test organisms)
Coefficient of variation	A standard statistical measure of relative variation of a distribution of a set of data, defined as the standard deviation divided by the mean
Co-enzyme	An organic molecule required by some enzymes for activity
Deionised water	Fresh water from which the soluble ions has been removed
Diatoms	Microscopic unicellular algae having cell walls impregnated with silica
Erythrocytes	Red blood cells

Haemolysis	Breaking of erythrocytes resulting in the release of haemoglobin in the plasma
Heat–shock proteins	A subset of stress proteins induced by heat and other environmental stressors (e.g. anoxia, carcinogens, mutagens, teratogens, and toxicants such as cadmium) to protect the organism against these stressors
Homogenisation	The preparation of a homogeneous suspension of smaller pieces of a solid
Inter-assay variation	Variability detected when replicate assays were conducted
Inter-experimental variation	Variability detected between fish in replicate containers
Juvenile	Young organism, still sexually immature
Lysate	Lysed cells
Milli-Q water	Fresh water from which soluble ions and organic molecules have been removed
Monoclonal	A monoclonal antibody is derived from a single antibody producing cell and is specific to a single antigen determinant of the immunogen (monospecific)
Polyclonal	Polyconal antibodies contain many different antibody specificities to various antigen determinants of a structurally complex immunogen
Photoperiod	The duration of illumination and darkness within a 24-h period
Plasma	Fluid portion of blood
Replicate	Copy of the same thing under identical conditions
Regression analysis	The measure of the association between two variables, the one the dependent variable and the other the independent variable
Repeatability	Refers to the variation that occurs when replicate tests are carried out by one operator using the same experimental and environmental conditions (intra-laboratory variation)
Reproducibility	Refers to the variation that occurs when tests are carried out by different operators, in different laboratories (inter-laboratory variation)

S9 liver fraction	Liver preparation enriched with cytochrome P450-containing monooxygenases, e.g. ethoxy resorufin-O-deethylase (EROD)
Species	The largest unit of a population within which effective gene flow occurs or could occur
Standard curve	The graph of the absorbance values of increasing concentrations of the test chemical versus the concentrations read at a fixed wavelength. The linear plot is also known as a Beer's Law Plot
Standard protocol	A protocol that has been extensively evaluated and validated and is used in exactly the same way by numerous laboratories
Substrate	A substance acted on by an enzyme
Stock solution	A concentrated water solution of a substance to be tested. Test solutions are prepared by adding measured volumes of the stock solution to dilution water
Static test	A toxicity test in which test solutions are not renewed during the test
Sub-lethal	Detrimental but not causing death
Test kit	Commercially available test
Toxicity	The characteristic/inherent potential/capacity of a chemical or a group of chemicals to cause adverse effects on living organisms - adverse effects include lethality or those effects limiting an organism's ability to survive in nature - such effects could be acute or chronic
Western blotting	The use of transverse electrophoresis to move molecules separated by electrophoresis in a polyacrylamide gel onto a nitrocellulose membrane to which they bind in identical pattern

1. INTRODUCTION

Biological and ecological responses to toxic chemicals may range from changes at the molecular level, where genetic integrity and sub cellular processes are affected, to changes at population and community levels, where dynamics and structures of entire food chains are influenced.

Many countries, including South Africa, have adopted an integrated management approach, which includes chemical and biological methods, to address toxic and non-conventional pollutants in the aquatic environment (Slabbert *et al.*, 1998a,b). Over the past 20 years, a range of biological tests measuring the effects of toxicants under controlled laboratory conditions have been established for South African use (Slabbert *et al.*, 1998a). Most of the tests are rapid/short-term tests, aimed at the detection of acute toxicity. Not much attention has thus far been given to the establishment of sub-lethal aquatic toxicity tests that can provide information on long-term effects of low chemical concentrations. Sub-lethal toxicity tests are important management tools when the toxicity of effluents is low, and are indispensable to manage ambient water quality.

Traditionally, long-term effects in the aquatic environment is either measured in full life-cycle tests or in shorter tests of about 30 days, known as early life-stage tests. Because life-cycle tests are laborious and expensive, most countries have shortened the duration of the tests to 7 to 10 days, focussing on the most sensitive life-cycle stages. Such tests are known as short-term chronic tests (Slabbert *et al.*, 1998a; US EPA, 1991) and the primary endpoints are growth and reproduction. Examples of standardised short-term chronic tests applied in the USA (Slabbert *et al.*, 1998a) for fresh water testing include: a 7-day fish (*Pimephales promelas*) larval growth/survival test; a 7-day invertebrate (*Ceriodaphnia dubia*) reproduction/survival test; and a 96-h algal (*Selenastrum capricornutum* Printz) growth test.

Biomarker studies on a wide range of aquatic organisms have shown that biochemical and molecular responses provide rapid and sensitive measures of sub-lethal toxicity. The biomarker assays used in aquatic studies have been derived from mammalian medicine, and allow for the rapid assessment of organism health. The same biomarkers used in field applications can also be employed to monitor chemical effects under controlled laboratory conditions. More information on biomarkers and their applications can be obtained in Chapter 2 of this report and in the extensive literature study in Appendix A. Since biomarkers measure events along the entire metabolic pathway during exposure to a chemical, and the toxic effect on organisms is secondary to change inside cells, biomarkers hold promise for the earlier detection of toxicants, and at lower levels, than the traditional assays measuring acute toxicity. Biomarkers can potentially be used to determine to what degree an organism is affected (i.e. is recovery possible or will organism die?) and can act as an early warning signal of imminent, irreversible and permanent damage to the organism (Depledge, 1993).

The need for toxicity tests that reflect the effects of sub-lethal concentrations of chemicals on aquatic organisms in the South African water environment, and promising attributes of biomarkers as sub-lethal endpoints, prompted this investigation into the potential of biomarkers as rapid alternative measures to the more tedious growth and reproduction measures used for the detection of sub-lethal toxicity. The objectives of the project were:

1) To establish biological techniques to detect sub-lethal toxicity in the aquatic environment, and

2) To produce an operational manual on the established methodologies.

The emphasis of the project was on using biomarkers as sub-lethal endpoints in a laboratory fish test employing the indigenous fish species *Oreochromis mossambicus* (tilapia), to complement the acute (96-h lethality) tilapia toxicity test developed for local use (Slabbert *et al.*, 1999). Tilapia has been successfully cultured and maintained in captivity and is easy to work with. The fish is of academic importance as it has been used by various local laboratories to study the acute effect of chemical pollutants. In addition, the fish has already been used successfully in biomarker studies (Chapter 2). Since young organisms are often more sensitive to toxicants than adults (Slabbert *et al.*, 1998a,b), it was decided to use young juvenile fish in the sub-lethal fish test. It was also planned to apply the biomarker assays established for the laboratory fish test to tilapia and other organisms collected from selected field sites to evaluate their potential for *in situ* testing. The study was not aimed at extrapolating the laboratory results to the field data, but rather to establish two options (laboratory and field) for biomarker application.

Four organisations, namely Environmentek, CSIR, Pretoria; Waterlab Research, Pretoria; the Department of Zoology, Rand Afrikaans University (RAU); and the Department of Molecular and Cell Biology, University of the Witwatersrand (WITS, participated in the project. The various institutions were involved in the project to utilise the existing knowledge in fish culturing, fish sampling, toxicity testing and biomarker application, and to facilitate the rapid establishment of the technologies. Since the project required close collaboration, it provided an opportunity to establish how well different groups could cooperate. Senior staff members were involved in an advisory and mentoring capacity, while students developed/established and applied the various laboratory and field techniques. It was the intention to involve students and staff from previously disadvantaged institutions in the project. Recruitment of suitable students was, however, difficult.

An extensive literature study on existing biomarker assays, compiled by Environmentek, CSIR (Appendix A), was used to select suitable techniques to establish in the different laboratories. Each laboratory was required to select at least two biomarkers. The emphasis was placed on rapid and sensitive biochemical biomarker assays. The different biomarkers selected for the study and their attributes are discussed in the literature review in Chapter 2 and in Appendix A. The literature on biomarkers was continuously updated during the course of the study using reputable environmental toxicological journals and web-based literature searches. The use of *O. mossambicus* in biomarker studies and attributes as test organism are highlighted in Chapter 2 and in Appendix A. Fish were cultured at RAU and CSIR. Depending on fish availability, laboratory studies were carried out at either RAU or CSIR. Upon completion of an experiment, fish samples were shipped to each laboratory for biomarker assaying. Fish maintenance and test protocols are summarised in Chapter 3 of the report.

Laboratory exposure studies were preceded by biomarker assay and tissue preparation technique verification and optimisation. Juveniles of different ages were used to select a suitable fish size for the exposure studies. The effect of variables such as operator manipulation, sample storage time and sample containers on biomarker results were also investigated. The findings of the evaluation are presented in Chapter 4. Optimised protocols are summarised in Chapter 3. A number of toxicants were used during laboratory exposure studies to evaluate the sensitivity and applicability of the established biomarker assays. Various exposure periods were used to establish the effect of exposure time on biomarker sensitivity. The toxicants selected for exposure studies and potential effects of these toxicants on biomarkers are discussed in Chapter 2 and Appendix A. The experimental design and data analysis of the exposure studies and the results are, respectively, addressed in Chapters 3 and 4 of the report.

Individuals at the Institute for Water Quality Studies (IWQS), Department of Water Affairs and Forestry (DWA&F) were consulted to identify suitable areas for field studies. It was envisaged to select a control area (unpolluted) and polluted areas. The polluted areas could be single areas in different aquatic environments or a river system with different sources of pollution. Due to a number of factors (drought, fluids, availability of equipment), dams routinely monitored by RAU, were ultimately selected for the field study (Chapter 3). The application of the assays to other organisms was omitted from the study because of time and manpower constraints, and because molluscs/worms were not present in appropriate numbers at the selected sites. Students from RAU and CSIR conducted the field study. Two surveys, one in summer and another in winter, were carried out. The different organs were sampled and prepared on site and shipped on ice to the participating laboratories. Sample preparation protocols and data analysis are outlined in Chapter 3 of the report and the results are discussed in Chapter 4.

The conclusions and recommendations of the study are presented in Chapter 5 and the references are listed in Chapter 6.

The established protocols are compiled in a separate document, the Operational Manual (Venter *et al.*, 2003). It is anticipated that the manual will be used by individuals and laboratories involved in aquatic toxicology. Only those biomarker assays that proved to be applicable during laboratory and field studies are included in the manual. Biomarker assay protocols are presented in detail to allow easy replication and include the: test principle, reagents, materials and apparatus, procedures, expression of results, personal remarks and references. The introduction contains background information on biomarkers, information on the biomarkers addressed in the manual, and summaries of requirements and costs. The manual also includes detailed protocols for the sub-lethal fish toxicity test and sample preparation. Sampling and sample preparation of field samples are also outlined in detail.

2. LITERATURE REVIEW

2.1 Biomarkers

A biomarker is an indicator in a living organism that reflects sub-lethal molecular and/or cellular changes (biochemical, physiological and histological) occurring along a metabolic pathway because of exposure to a toxicant or physiological changes (Decaprio, 1997; Depledge and Fossi, 1994; Mayer *et al.*, 1992 and NRC, 1989). Biomarkers may be biological material ranging from bio-molecules such as nucleic acids and proteins (e.g. enzymes) to organelles, cells, tissues, organs and whole organisms. These biomarkers represent different levels of integration (complexity) of the effect of a toxicant on a living organism. Biomarkers indicating damage caused at the biochemical/molecular level in a cell have a rapid response time and are sensitive. Biomarkers of growth and reproductive responses have the advantage of high biological and ecological significance, but relatively low sensitivity to stress factors. Manifestations of stress at the tissue level represent an intermediate effect between the biological and reproductive levels. Histological changes occur earlier than reproductive changes and are more sensitive than growth or reproductive parameters and, as an integrative parameter, provide a better evaluation of organism health than a single biochemical parameter (Teh *et al.,* 1997).

Biomarkers can be used as indicators of general stress, organ specific stress, or toxicant specific stress. The specificity of the biomarker is determined by its localisation in the organism, biochemical structure, functional role and level in the sub-organismal structure.

Non-specific biomarkers (general stress) are normally present in all cells of the organism; e.g. deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), or play a role in the regulation of metabolic processes in the cell; e.g. hormones, or is involved with energy supply to the cells; e.g. adenalyte energy charge (AEC).

Organ specific biomarkers, on the other hand, are closely associated with the organ in question. At the highest level of the sub-organismal biomarker hierarchy, the histological structure or the functional integrity of the organ can be used to determine its health status. At a lower level of the biomarker hierarchy, the difference in concentration level of organ enzymes from the base-level concentration of these enzymes, or the appearance of organ specific isozymes in blood, can be used as indicators of organ damage.

The specificity of toxicant specific biomarkers depends on the biochemical structure of the biomolecules (Table 1). In the case of delta-aminolevulinc acid (ALA-D) and acetylcholinesterase (AChE), the toxicants in question interfere with the normal function of these enzymes; i.e. inhibiting their activation. A decrease in the breakdown of the enzyme substrate serves as a measure of the presence of the toxicants. In the other two examples given; i.e. metallothioneins and P450 enzymes, the synthesis of the biomolecules are induced in the presence of certain toxicants, and their normal function of detoxifying or activating these toxicants enhanced, because of the increased enzyme concentrations (Mayer *et al.*, 1992).

When selecting biomarkers for studies, knowledge about the cellular mechanisms involved with metabolism, distribution, and detoxification of toxicants is essential. It is also important that the analytical technology is available to identify the responses (Shugart, 1996).

TABLE 1: Examples of toxicant specific biomarkers

Biomarker	Toxicant	Reference
ALA-D	Lead	Gill *et al.*(1991); Ho and Ho (1997)
AChE	Organophosphates and carbamates	Murphy (1980)
Metallothioneins	Metals	Depledge and Fossi (1994)
Cytochrome P4501A	Chemicals with relatively flat molecular structures, e.g. aromatic hydrocarbons, dioxins, and co-planar polychlorinated biphenyls (PCBs)	Hansen and Addison (1990)

2.2 Use of Aquatic Organisms in Biomarker Studies

The criteria in Table 2 should be taken into account when selecting a test organism for chemical exposure studies (Nussey, 1994; Rand and Petrocelli, 1985; Wepener, 1997).

TABLE 2: Criteria to consider when selecting a test organism for chemical exposure studies

Criterium	Prerequisites
Sensitivity	Should have a broad sensitivity range; must accumulate pollutants rapidly
Availability	Indigenous; widely available throughout the year; abundant
Genetic composition and taxonomy	Low variability; easily identifiable
Information	Ecological, physiological, genetic and behavioural information should be available
Importance	Recreational; commercial; ecological (part of a food chain)
Laboratory use	Susceptible to routine laboratory culturing; sufficient number of organisms of same size and age; degree of repeatability should exist between individuals; simple affordable analyses; fish not too expensive

Aquatic organisms integrate all the stresses placed on aquatic ecosystems and they reflect the combined effects over periods of time (Heath and Claassen, 1999). They are good measures of environmental health, because activities taking place in the vicinity of a river or stream also have an effect on the quality of the river water due to run-off.

Fish are one of the best understood groups of organisms in the aquatic environment (Roux, 1994) and are excellent indicators of water quality. They produce large numbers of eggs, which means that fish are readily available as test organisms (Peakall, 1992). Because of the popularity of fish, a large number of biomarker studies reported in literature applied this test organism.

Biomarkers have also been studied in other aquatic organisms, e.g. protozoa, sponges, molluscs, water fleas, gammarids, crabs, amphibians and turtles (Tables 2 and 3 in Appendix A) (De Coen *et al.*, 2001; Gendron *et al.*, 1997; Müller, *et al.*, 1995; Yawetz *et al.*, 1997). AChE, pyruvate kinase (PK) and glucose appeared to be the most universally used biomarkers in these organisms. No information could be found in the literature on a comparison of the sensitivities of biomarkers in different organisms under similar exposure conditions.

In the case of laboratory studies, control is exercised over chemicals used in the study and the laboratory conditions. However, in natural systems a conglomerate of environmental factors can influence biomarker levels, leading to variability and complicating the interpretation of results. It is, however, necessary to apply biomarkers to organisms in natural systems to test their applicability for *in situ* testing. Current methods need additional attention, especially in the area of field validation, before they can be used extensively in hazard/risk assessment and regulatory toxicology (Mayer *et al.*, 1992). Shugart (1996) recommends that laboratory studies should be carried out in conjunction with field studies to assess whether pollutant responses in the laboratory are comparable to field data. The biomarker approach is not viewed as a replacement for conventional assessment techniques, but a supplementary tool with considerable ecological relevance (Depledge and Fossi, 1994). Evaluation of the general state of a population (birth rate, mortality, fertility index, relationship between ages) should be linked to biochemical changes to interpret the adverse effects on communities (Depledge and Fossi, 1994).

Sensitive species and life stages should be selected to reduce the cost and time involved in biomarker testing and to obtain a highly sensitive indicator of biological health. By using a suite of biomarkers with different degrees of specificity, ecosystem health can be more accurately reflected (Melancon, 1995; Peakall, 1992).

2.3 Fish as Test Organism in Biomarker Studies

There is an abundance of literature on the use of fish in laboratory and field studies (Table 3; Tables 2 and 3 in Appendix A; Joubert, 2000; Vorster, 2000). Most of the research have been carried out with adult fish in order to use the organs in biomarker studies. However, a number of scientists have investigated the use of embryos/larvae and young juvenile fish.

Fish embryos and larvae have poorly developed gills and kidneys (Lee *et al.*, 1996; Hwang and Yang, 1997). The skin is therefore the functional site of ionoregulation. This enlarged surface area enhances uptake of pollutants, and thus make embryos and larvae more sensitive than adult fish. A range of microsomal and cytosolic biomarkers were measured in the pooled whole homogenate (150 larvae) of 24- to 48-h old trout larvae after a 7-day exposure period (Viganò *et al.*, 1995). Several biotransformation enzyme activities were present and measurable in these larvae, although mostly at lower levels than in adult fish. Heath (1995) listed a number of practical limitations when working with larvae:

- Difficult to work with;
- Often not cultured in the laboratory;

- Biomarker measurements are difficult or impossible to do because of the small size of the organisms; and

- Pooling to obtain enough tissue eliminates individual variability.

Juvenile fish have also been found to be more sensitive than adult fish. For example, the rate of metal uptake correlates with the weight-specific metabolic rate, therefore young fish accumulate metals more rapidly than adult fish (Anderson and Spear, 1980; Heath, 1987). As in the case of larvae, the tissues/organs of young juvenile fish, containing specific biomarkers, cannot be removed. The biomarkers are determined in the pooled whole fish homogenate of several individuals, which results in the dilution of the biomarkers and lower biomarker levels. In spite of the lower levels of biomarkers in whole organism homogenates, biomarkers have been successfully applied to whole organism homogenates of a range of other organisms; e.g. molluscs and crustaceans (Tables 2 and 3 in Appendix A).

Table 3 shows some of the fish species, their sizes and life stages, as well as the experimental conditions, used in laboratory exposure studies. Some studies were carried out under static conditions, while others used static renewal or flow through conditions. Exposure times ranged from a few hours to several weeks. Fish were usually fed during longer exposure periods. Different laboratories used different numbers of fish in exposure studies. The number of fish used per exposure depended on organism availability and the number of biomarkers conducted. Fish larvae and juveniles were usually used in large numbers.

Control values (unexposed fish) obtained for selected biomarkers during laboratory fish exposure studies and the effect of a number of toxicants/complex samples on the biomarkers are presented in Table 4. The table shows that shorter exposure times were generally used with higher concentrations of a toxicant (e.g. cadmium) and that the exposure times increased to several weeks when the toxicant was present at sub-lethal levels. Biomarker assays were carried out on several individual fish from each exposure (4 to 11) or on replicates of pooled fish [3x150 fish (Viganò *et al.*, 1995) and 7x2 fish (Soengas *et al.*, 1996)]. The number of fish used for biomarker analysis were often not fixed due to mortalities in some of the test solutions. Different laboratories used different biomarker measurement units. Biomarkers are normally expressed in terms of volume when measured in blood or plasma, or in terms of wet weight or protein when measured in tissue. When the biomarker assay is based on a reaction rate, the time factor is also included. The effects reported in literature (Table 4) ranged from severe inhibition (glycogen: -72% after exposure to cadmium) to high inductions [heat stress protein (Hsp 70): +862% after exposure to paper mill effluent]. Coefficients of variation (CVs) ranged from low [0 to 1%: glycogen, lactate dehydrogenase (LDH) and Hsp 70] to high [52%: ethoxyresorufin-O-deethylase (EROD]). [Most of the results of exposure studies were reported as a mean biomarker value and standard deviation (SD) (Appendix A), and % effects and CVs had to be calculated to include in Table 4].

TABLE 3: Laboratory exposure studies: Summary of fish species and experimental conditions

Fish			Experimental condition					
Species	**Weight/ Length/ Age**	**Life stage**	**Type of exposure**	**Exposure time**	**Number of fish /container**	**Number of replicate containers**	**Feeding**	**Reference**
Channa punctatus	60±8 g 15±2 cm	NA	Static renewal: every 24 h	4 days	30	1	Yes	Sastry and Shukla (1994)
Rainbow trout (*Oncorhynchus mykiss*)	24 to 48 h	Larvae	Static: 2.8 ℓ	7 days	150	3	NA	Viganò *et al.* (1995)
Atlantic salmon (*Salmo salar*)	20.5±1.2 g 12.8±0.2 cm	Immature	Static	8 h	14	1	No	Soengas *et al.* (1996)
Flounder (*Pleuronectes flesus*)	54 to 304 g 18.1 to 29.6 cm	NA	Flow through	4 to 9 weeks	35	1	Yes	Johansson-Sjöbeck and Larsson (1978)
Tilapia (*O. mossambicus*)	10±1 g	Juvenile	Flow through	1 day	NA	1	Yes	Murthy *et al.* (1981)
Rainbow trout	3 months	Immature	Static	4 days	10	1	No	Vijayan *et al.* (1998)
Chinook salmon (*Oncorhynchus tshawytscha*)	8.8±0.28 g	Juvenile	Flow through	30 days	60	1	Yes	

NA Not available

TABLE 4: Laboratory exposure studies: Summary of control biomarker levels and effects of toxicants/complex samples on biomarkers

Experimental condition			Biomarker							Reference
Toxicant/ Complex sample	Concentration (mg/ℓ)	Exposure time	Name	Organ	Control level	CV (%)[1]	Replicates[2]	% Effect[3]	Significance (P<0.05)[4]	
Cadmium	11.2	96 h	Glucose	Blood	60.8 mg/100 mℓ	3	9	-14	S	Sastry and Shukla (1994)
			Total protein		3.89 mg/100 mℓ	3		-47	S	
			Glycogen	Liver	40.26 mg/g tissue (wet weight)	1		-3	NS	
			LDH		1.14 µg formazan/mg protein/h	1		-40	S	
			Total protein		112 mg/g tissue (wet weight)	2		-27	S	
			Glycogen	Muscle	1.98 mg/g tissue (wet weight)	1		-35	S	
			Total protein		168 mg/g tissue	1		-12	S	
			LDH		2.26 µg formazan/mg protein/h	6		+31	S	
	0.01	8 h	Glucose	Plasma	86.6 mg/100 mℓ	6	7	+10	NS	Soengas *et al.* (1996)
	0.1							+39	S	
	0.01		Glycogen	Liver	44.5 mg/g tissue (wet weight)	8		-35	S	
	0.1							-72	S	
	0.01		Protein	Liver	86.8 mg/g tissue (wet weight)	4		+8	NS	
	0.1							+19	NS	
	0.005	4 weeks	ALA-D	Blood	54.0 µg PBG/mℓ/h	15	9 to 11	+5	NS	Johansson-Sjöbeck and Larsson (1978)
	0.5							-18	NS	
	0.005	9 weeks			59.10 µg PBG/mℓ/h	13		-32	S	
	0.5							-17	NS	
	0.005	4 weeks	ALA-D	Spleen	106.8 µg PBG/g tissue (wet weight)/h	9		+2	NS	
	0.5							-25	S	
	0.005	9 weeks			140.0 µg PBG/g tissue (wet weight)/h	7		+17	NS	
	0.5							-3	NS	

[1] Calculated from standard deviation (SD) when not reported as CV

[2] Number of individual fish (or groups of pooled fish) on which biomarker assays were performed

[3] +: induction; -: inhibition

[4] S: significant; NS: not significant

TABLE 4: Laboratory exposure studies: Summary of control biomarker levels and effects of toxicants/complex samples on biomarkers (continued)

Experimental condition			Biomarker							Reference
Toxicant/ Complex sample	**Concentration**	**Exposure time**	**Name**	**Organ**	**Control level**	**CV (%)[1]**	**Replicates[2]**	**% Effect[3]**	**Significance (P<0.05)[4]**	
Acidic solution	pH < 4	24 h	Glycogen	White muscle	2.52 mg/g tissue (wet weight)	5	8	-21	S	Murthy *et al.* (1981)
			Glycogen	Red muscle	7.6 mg/g tissue (wet weight)	4		+40		
			G-6-P-DH[5]	White muscle	0.479 µmol formazan/mg protein/h	6		-9		
			G-6-P-DH	Red muscle	0.522 µmol formazan/mg protein/h	8		-22		
			LDH	White muscle	0.121 µmol formazan/mg protein/h	5		+76		
			LDH	Red muscle	0.183 µmol formazan/mg protein/h	6		+33		
Various sediment Extracts	250 g/ℓ	7 days	EROD	Whole organism homogenate	0.3 pmol resorufin/min/mg microsomal protein	52	3	-33 to +326	NS to S	Viganò *et al.* (1995)
			G-6-P-DH		4.1 nmol/min/mg cytosolic protein	22		+10 to +20	NS	
Sodium dodecyl sulphate (SDS)	10, 18 and 32 mg/ℓ	4 days	Hsp 70	Liver	45.8 arbitrary units/40 µg protein	16	4 to 6	+99, +140 and +81	S, S and NS	Vijayan *et al.* (1998)
Paper mill effluent	5.6, 10 and 18%	4 days			4.16 arbitrary units/40 µg protein	0	6	+92, +261 and +862	S, NS and S	
	2, 4, 8 and16%	30 days			17.14 arbitrary units/40 µg protein	8	6 to 8	+67, +52, +43 and +42	S, S, S and S	

[1] Calculated from standard deviation (SD) when not reported as CV

[2] Number of individual fish (or groups of pooled fish) on which biomarker assays were performed

[3] +: induction; -: inhibition

[4] S: significant; NS: not significant

[5] Glucose-6-phosphate-dehydrogenase

Information on fish and biomarkers used in field studies are summarised in Table 5. The fish were generally much larger than those used in laboratory studies and concomitantly the biomarkers were present at considerably higher levels. Adult fish collected from different field sites can display different sizes that can result in different levels of biomarkers. Chen *et al.* (1998) ascribed the lower enzyme activities in fish at two experimental sites compared to enzyme levels in fish at a reference site to less developed life stages of the fish (enzymatic system less developed), or to the presence of enzyme inhibitors (pollutants), or to other environmental factors such as food. The authors recommended that fish collected from different sites should be of similar size to enable optimal comparison of biomarker levels.

Some laboratories collected 3 to 5 fish per site (Table 5), while others used as many as 13 to 14 fish. The numbers collected depended on the abundance of fish at the sites. In the study by Schmitt *et al.* (1984), different species of suckers were used to overcome the problem with limited numbers of fish. Biomarker assays were carried out on between 5 and 12 replicate fish. Bainy *et al.* (1999) performed each biomarker assay in duplicate. Some of the biomarker units shown in Table 5 differed from the units used in laboratory studies (Table 4). Some biomarkers were inhibited (AChE: -49% during exposure to chlorinated hydrocarbons and heavy metals), while others were induced (EROD: +2 181% during exposure to industrial pollutants). The CVs ranged from 4% (AChE) to 49% (EROD).

2.4 Chemicals Used in Laboratory Exposure Studies and the Biomarkers Applied to Study their Effects

Various metals and organic chemicals have been used in laboratory exposure biomarker studies (Tables 2 and 3 in Appendix A). Metals entering waterways originate from a wide range of industrial effluents, old mines and agricultural activities. Acid precipitation can also lead to leaching of metals from surrounding soils. More than 300 million tons of anthropogenic organic chemicals (e.g. herbicides and insecticides) are manufactured world wide annually (Roux, 1994). These chemicals are freely applied in the environment and have been found in air, soil, water, human and animal tissue. Some of these chemicals, for example organochlorine insecticides, are highly fat-soluble and lengthy exposure to them results in a high accumulation in organisms and may produce adverse effects on ecosystems (Heath and Claassen, 1999).

Table 6 lists some of the chemicals and the biomarkers applied to study their effects. Some biomarkers, e.g. glucose, AChE and LDH are affected by metals as well as organic pollutants.

Table 7 summarises the effects of specific chemicals [(zinc, cadmium, cyanide and pentachlorophenol (PCP)] on selected biomarkers. It can be seen that the same chemical, e.g. zinc, can either inhibit or induce biomarkers [e.g. glucose (induction); glycogen (inhibition)]. Different metals (zinc and cadmium) can cause an induction or inhibition of a specific biomarker (e.g. blood glucose). Furthermore, pollutants from different chemical groups (e.g. zinc and PCP) can have the same effect on a biomarker (e.g. AChE).

TABLE 5: Field studies: Summary of fish species, control biomarker levels and effects of different environmental pollutants on biomarkers

Field exposure		*Fish*			*Biomarker*							Reference
Study area	**Pollution source**	**Species**	**Weight/ Length/ Age**	**Replicates[1]**	**Name**	**Organ**	**Control level**	**CV (%)[2]**	**Replicates[3]**	**% Effect[4] (range)**	**Significance (P<0.05)[5]**	
River	Mine tailings (zinc and lead)	Suckers (*Moxostoma duquesnei, M. erythrurum, Hypentelium nigrigans*)	21.3 to 28.4 cm 98 to 274.1 g	10	ALA-D	Blood	222.92 nmol/ℓ PBG/mℓ/h	9	10	-37 to -63	S	Schmitt *et al.* (1984)
Aquaculture ponds	Metal reclamation facility	Milkfish (*Chanos chanos*)	200 to 570 g 3 to 5 months	3 to 5	EROD	Liver	46.8 pmol/min/mg protein	22	5	-58 to +318	S	Chen *et al.* (1998)
River	Chlorinated hydrocarbons and heavy metals	Spotted garfish (*Lepisosteus oculatus*)	NA	5 to 8	AChE	Brain	569 nmol substrate hydrolysed/min/ mg protein	4	8	-49	S	Huang *et al.* (1997)
						Liver	153 nmol substrate hydrolised/ min/mg protein	4	8	-39	S	
Reservoir	Aryl hydrocarbons	Nile tilapia (*O. niloticus*)	15 to 20.6 cm 114.9 to 283.3 g	13 to 14	EROD[6]	Liver	8.9 nmol resorufin/ min/mg protein	49	12	+2 181	S	Bainy *et al.* (1999)

[1] Number of fish collected per field site

[2] Calculated from SD when not reported as CV

[3] Number of fish analysed

[4] +: induction; -: inhibition

[5] S: significant

[6] Assay performed in duplicate

NA Not available

TABLE 6: Chemicals used in laboratory exposure studies and the biomarkers applied to study their effects

Chemical group	Chemicals	Biomarkers
Metals	Cadmium Copper Lead Mercury Zinc	Glucose, glycogen, lactic acid, LDH, pyruvic acid, glycogen phosphorylase, glycogen synthetase, 6-phospho-fructokinase, lactate, AChE, cytochrome oxidase, hexokinase, PK, stress 70, cholesterol, cortisol, triodothryronine, thyroxine, insulin
Insecticides/ Herbicides	Aldicarb Azinphosmethyl Chlorpyrifos Cypermethrin Diazinon Delamethrin Endosulfan Fenitrothion Malathion Parathion-methyl Paraquat	Erythrocytes, free fatty acids, lipids, AChE, ammonia, alanine amino transferase, aspartate amino transferase, free amino acids, glutamine, protease, proteins (soluble, structural, total), glucose, lactate, EROD, lipids, cholesterol, glycerol, phospholipids, LDH
Phenols	Phenol P-chlorophenol P-cresol	Glutamate transaminases
PCBs	Arochlor 1254	Cytochrome P4501A
Organic acids	Hexachlorobenzene Hydrocyanic acid	Glutamate transaminases, dehydrogenases (glutamate, malate), kinases (hexo and pyruvate), ALA-D, estradiol, triiodothryronine, vitellogenin
Polyaliphatic hydrocarbons (PAHs)	Benzo[a]pyrene	EROD

TABLE 7: Effects of specific chemicals on selected biomarkers

Chemical	Biomarker	Effect	Reference
Zinc	Glycogen (muscle and liver)	Inhibition	Heath (1987); Wagner and McKeown (1982)
	AChE (gill)	Inhibition	Gill *et al.* (1990)
	LDH (blood)	Induction	Rojik *et al.* (1983)
	Glucose (blood)	Induction	Wagner and McKeown (1982)
Cadmium	Ions (calcium) (blood)	Inhibition	Rattner and Heath (1995)
	ALA-D (kidney)	Induction	Heath (1987)
	Glycogen (liver)	Inhibition	
	AChE		Spry and Weiner (1991)
	G-6-P-DH		
	LDH (liver)		Sastry and Shukla (1994)
	Glucose (blood)		
Cyanide	Glucose (blood)	Induction	Solomonson (1981)
	Liver	Degeneration	DWA&F (1996b)
PCP	PK	Inhibition	Murty (1986)
	LDH		
	G-6-P-DH	Induction	
	EROD		Fossi *et al.* (1995)
	AChE	Inhibition	

The bioavailability and toxicity of chemicals are determined by the physical-chemical properties of water, i.e. temperature, hardness, pH, dissolved oxygen (DO), salinity, and the presence of other chemicals. Great care should, therefore, be taken to control physical-chemical parameters during laboratory exposure studies (Slabbert *et al.*, 1998a).

2.5 Justification for the Selection of the Test Organism, Biomarkers and Test Chemicals for the Water Research Commission Biomarker Study

2.5.1 Test organism

Fish are good indicators of environmental health and are therefore very suitable to use in aquatic toxicity tests. One of the acute toxicity tests recommended for local use is based on fish lethality (Slabbert *et al.*, 1998a). It was decided to use an indigenous fish species, the Mozambique tilapia (*O. mossambicus*), as test organism in this study. *O. mossambicus* is suitable for toxicity testing because it meets most of the criteria outlined in Table 2. The fish is abundant and widely distributed throughout the year. It is also a recreational and ecologically important species, and adequate background information exists on the species (Skelton, 1993). The fish breeds in summer, grows fast and reaches sexual maturity within one year. *O. mossambicus* has been found relatively easy to culture and maintain in the laboratory (Slabbert and Pienaar, 1998) and laboratory culturing techniques exist (Slabbert and Pienaar, 1998). Different local laboratories have been using the fish to study aquatic pollutants and information on sensitivity is, therefore, available. An acute toxicity test employing 3 to 4 week old juveniles has also been developed for local use (Slabbert *et al.*, 1999). *O. mossambicus* has been successfully employed as test organism in a number of biomarker studies (Hwang and Yang, 1997; Lee *et al.*, 1996; Murthy *et al.*, 1981; Nussey, 1994; Pratap and Wendelaar, 1993; Swallow and Fleming, 1970).

In order to achieve a high level of sensitivity it was decided to use the youngest possible fish in the laboratory sub-lethal toxicity test. Literature has shown that larvae and young juveniles are the most sensitive life stages of fish because they accumulate toxic chemicals much faster than adult fish (section 2.3), and that biomarkers have been successfully measured (detectable levels) in pooled whole organism homogenates. The use of young fish also involves lower maintenance and operational costs (simple culturing system, smaller culture and experimental chambers, shorter periods of feeding and no dissection required).

Following the international approach, it was decided to use adult fish in field studies. This would allow an evaluation of the applicability of the selected biomarkers for field monitoring and a comparison with an abundance of published data. Although it was initially planned to also investigate the applicability of biomarkers to homogenates of other aquatic organisms collected from the field, e.g. molluscs or worms, such organisms were either absent or present at insignificant numbers at selected field sites.

2.5.2 Biomarkers

The universality of biomarkers (presence in range of organisms) played an important role in the selection of biomarkers for the study, as it was originally planned to apply the biomarkers to fish and other aquatic organisms. It was also important that the biomarkers would be applicable to whole organism homogenates. It was decided to investigate toxicant specific biomarkers (section 2.1), as well as biomarkers that are more general, in order to establish a suite of biomarkers with varying specificities. It was also important to select biomarkers that would elicit responses at sub-lethal levels, to allow a high level of sensitivity. The importance of the biomarker in major physiological and biochemical functions of the organism also played a role in the selection process.

As far as the selection choices of the individual laboratories are concerned, the availability of expertise and equipment played an important role as well as the need to establish the protocol in the laboratory for use in other research projects. The biomarkers selected by the participating laboratories and the justification for their selection are summarised in Table 8.

TABLE 8: Biomarkers selected by the different laboratories for use in the study and justification for their selection

Biomarker	Laboratory	Justification
Protein	CSIR, RAU, WITS	• A measure used to express biomarker activity • Widely applied in biomarker studies • Expertise available
AChE	CSIR, RAU	• Specific biomarker for organophosphate and carbamate pesticides stress, but also responds to heavy metals • Well studied in fish and other organisms (universal) • Expertise already available (used as acute toxicity assay at CSIR; RAU can obtain a test kit)
EROD	CSIR	• Specific for organic chemicals with flat molecular structures • Efficient early warning measure • CSIR had access to a fluorometer required for assay
Glucose	Waterlab[1]	• General/universal biomarker • Test kit available
Glycogen	Waterlab[1]	• General biomarker • Good indicator of stress
ALA-D	Waterlab[1]	• Specific biomarker for lead
LDH	RAU	• General biomarker • Test kit available
G-6-P-DH	RAU	• General biomarker • Test kit available
PK	RAU	• General/universal biomarker • Test kit available
Ions	RAU	• General biomarker • Already in use
Hsp	WITS[2]	• General biomarker • Expertise already available
Metallothionein[3]	WITS	• Specific for heavy metal pollution

[1] Conducted by the student appointed by CSIR because of the unavailability of a student for Waterlab

[2] WITS transferred the technology to RAU when they withdrew from the project

[3] Later omitted, because available assays were too complicated

The different test protocols available for conducting the biomarker assays listed in Table 8, the protocols selected for this study and the justification for selecting the specific protocols are outlined in Table 9. Rapidity, simplicity and cost efficiency (Table 6 in Appendix A), as well as availability of equipment, skilled manpower, prior knowledge of the method and a good track record of the method (AChE - Ellman *et al.*, 1961) were used as criteria during the selection process.

2.5.3 Test chemicals

Two metals, cadmium and zinc, a pesticide, PCP, and cyanide were selected for evaluation in the sub-lethal toxicity test. (It was envisaged to also use lead and an organophosphate based pesticide but due to time constraints these chemicals were omitted from the study). The particular chemicals were selected because:

- Literature (Appendix A) has shown that they affect the biomarkers selected for the study. (It was anticipated that metals would exhibit effects on AChE, glucose, glycogen, LDH, PK, Hsp 70 and possibly ALA-D; PCP would exhibit effects on EROD, glucose, glycogen, LDH, G-6-P-DH, PK and Hsp 70; and cyanide would exhibit effects on glucose, glycogen, LDH, G-6-P-DH and PK);

- They are highly toxic to fish (Heath, 1987);

- Acute toxicity data are available for the test fish *O. mossambicus* (Slabbert *et al.*, 1999); and

- They are widely distributed in the aquatic environment, have been well studied, and represent various types of pollution.

2.5.3.1 Cadmium

Cadmium is a non-essential trace metal with no biological function (Viarengo, 1985). It is widely distributed in natural waters due to industrial discharge (Pratap and Wendelaar, 1993). Other sources of cadmium in the environment are from mining, agriculture (fertilisers and pesticides) and burning of fossil fuels.

Cadmium also occurs naturally in the earth's crust and is released with the natural weathering of rocks (DWA&F, 1996b). Generally, fish are very sensitive to cadmium poisoning. However, some species; e.g. roach, perch and tilapia, can tolerate high concentrations of cadmium (Pelgrom *et al.*, 1997). The toxicity of cadmium is strictly dependent on chemical speciation and water composition (Carpene *et al.*, 1987). Cadmium toxicity increases with an increase in temperature. Hardness and high pH reduces toxicity (Rattner and Heath, 1995; DWA&F, 1996b). At lower salinities cadmium uptake increases because more cadmium is available as free ions (Weis and Weis, 1991; DWA&F, 1996b). Spry and Weiner (1991) found that cadmium toxicity and accumulation is reduced at lower pH, because hydrogen ions compete with cadmium ions for binding sites on the cell membranes of gill surfaces.

TABLE 9: Protocols available for conducting biomarker assays, the protocols selected for the study and the justification for selecting the specific protocols

Biomarker	Test protocols available	Protocols selected for study	Justification
Protein	Biuret Lowry Bradford	Biuret (Weichselbaum, 1946): test kit - RAU Bradford (Bradford, 1976): macro method - WITS; micro method - CSIR	RAU selected the Biuret method because it was available in kit form; WITS selected the macro Bradford method because it was already in use and the CSIR selected the micro Bradford method because of the lack of interference by sucrose and Tris as well as the small volumes required making it possible to perform the assay in a microplate.
AChE	Colorimetric Electrometric Titrimetric Manometric Radiometric Potentiometric	Colorimetric (Ellman *et al.*, 1961): test kit - RAU; microplate assay - CSIR	The majority of articles reviewed used Ellman's colorimetric method. The test kit was already in use at RAU and the CSIR had expertise in a version of the assay used for acute toxicity testing. A spectrophotometer was required for measurement, which is standard equipment in any analytical laboratory.
EROD	Spectrophotometric Fluorometric	Fluorometric (Burke and Mayer, 1974): microplate assay - CSIR	The fluorometric method was selected because a fluorometer was available. This method is much more rapid than the spectrophotometric method.
Glucose	Automated glucose analyzer Several enzymatic colorimetric test kits	Colorimetric (Trinder, 1969): test kit - CSIR	The colorimetric test kit was selected because limited experience in its use already existed. The method is rapid and simple, and results are available within 1 h.
Glycogen	Various Colorimetric	(Seifter *et al.*,1950): microplateassay - CSIR	The protocol is rapid and simple
ALA-D	Various Colorimetric	(Schaller and Berlin, 1984) microplate assay - CSIR	Simple and rapid colorimetric method. Standard spectrophotometer required
LDH	Various Colorimetric	(Z. klin. Chem. u klin. Biochem, 1970): test kit - RAU	Test kit already in use
G-6-P-DH	Various Colorimetric	(Kornberg and Horecker., 1955): test kit -RAU	Test kit already in use
PK	Various Colorimetric	(Beisenherz *et al.*, 1953): test kit - RAU	Test kit already in use
Ions	Standard chemical analytical techniques	Automated analyzer - RAU	Already carried out by RAU
Hsp 70	Involves various steps: separation on SDS gel, Western blotting, enhanced chemiluminescence, quantification by laser densitometry	(Sambrook, 1989): WITS and RAU	Standard laboratory protocols, already in use at WITS

Cadmium can disrupt the ionic balance by altering the permeability of cell membranes (Viarengo, 1985), or by structural injuries to the gills, i.e. ruptured epithelum cells (Lee *et al.*, 1996). Cadmium levels as low as 10 µg/ℓ cause hypocalcemia in *O. mossambicus* (Hwang and Yang, 1997).

2.5.3.2 Zinc

Zinc is an essential trace element in living organisms. It acts as co–factor for many enzymes and also binds to the structural components of cell membranes to help them maintain their structure and function (Viarengo, 1985). This metal concentrates mainly in skin, bone and the liver, and is excreted by the bile (Heath, 1987). In aquatic ecosystems, zinc occurs in two oxidation states namely, as Zn^{2+} and the metal (Zn) (DWA&F, 1996b). The zinc ion (Zn^{2+}) is toxic to fish and other aquatic organisms at relatively low concentrations (0.02 mg/ℓ) (Nussey, 1998). The effects of metals take place at the gills (damaged by zinc) before they are transported to other parts of the body (Hoogstrand *et al.*, 1994). The uptake of zinc by the gills are affected by the following physical properties of water: temperature (LC_{50} of zinc higher in warm water); hardness (increase in hardness reduces uptake); and DO (lower DO increases toxicity of zinc) (Rattner and Heath, 1995). Zinc has an antagonistic and protective action in the uptake and toxic action of other metals; e.g. cadmium, by inhibiting uptake of cadmium, accelerating cadmium transport to other organs, or inducing metallothionein which binds with cadmium and detoxifies it (Kargin and Cogun, 1991).

2.5.3.3 Pentachlorophenol (PCP)

PCP represents the substituted halogenated aromatic family of pesticides, many of which exhibit insecticide and fungicide activity (Davidson, 1995). It was once one of the most widely used chemicals. Before restrictions were placed on its use in 1987, it was used as insecticide, herbicide, algicide, fungicide, molluscicide, as wood preservative, and to a lesser extent as biocide in cooling towers and pulp and paper mills. Currently, PCP is used in commercial wood preservatives for power line poles, cross arms and fence posts. PCP does not occur naturally in the environment and is released into the environment by domestic and commercial pesticide usage (Murty, 1986), industrial waste discharge, hazardous waste sites and treated wood. PCP is highly toxic to most organisms (Kim *et al.*, 1996). PCP accumulates in the liver, which is the primary organ for metabolism of PCP (Heath, 1987).

2.5.3.4 Cyanide

"Free cyanide" (CN^- and HCN) occurs mostly as molecular hydrogen cyanide (Heath, 1987). Cyanide salts ionise in water to release these cyanide ions (CN^-), which hydrolyse to form hydrocyanic acid (HCN), the most toxic form of cyanide. Cyanide is commonly found in surface waters originating from industrial wastes (petrochemical, mining, metal electroplating, steel and photographic) or biological sources. Bacteria, fungi and algae produce hydrogen cyanide (HCN) as part of their nitrogen metabolism. Cyanogenic glucosides in plants release HCN when their cellular structure is disrupted during composition (Leduc, 1981). Some seeds; e.g. the pit of the wild cherry, contain cyanide, while vegetables, like sweet potatoes, maize, millet and lima beans, contain cyanogenic glucosides. Other sources of cyanide and thiocyanate are tobacco smoke, malfunctioning car pollution control devices, plastics burnt in home fires and organic thiocyanates, used as insecticides (Solomonson, 1981; Way, 1981).

The toxicity of cyanide is affected by the following:

- Temperature: At low cyanide concentrations, cyanide is more toxic when the temperature is reduced; at high cyanide concentrations, cyanide is more toxic when the temperature increases;

- DO: Low DO increases toxicity; and

- Other water constituents: Mixtures of cyanide and ammonia have been found to be more toxic than the chemicals alone (addition) (DWA&F, 1996a,b).

3. MATERIALS AND METHODS

3.1 Biomarker Protocols

This section of the report contains a short summary of each biomarker assay. The detailed protocols are outlined in the Operational Manual (Venter *et al.*, 2003)

3.1.1 Protein assays

3.1.1.1 Bradford method

This method (Bradford, 1976) involves the binding of Coomassie Brilliant Blue G250 to protein. The colour is stable for 1 h. The original method (macro method) and a miniaturised version (micro method) were applied. Both methods were carried out at room temperature. In the macro method 100 µℓ of the sample was added to 5 mℓ of the Coomassie Brillant Blue reagent and the absorbance measured after 5 min using a spectrophotometer (595 nm). In the micro method the sample and reagent volumes were reduced to 10 µℓ and 200 µℓ, respectively. Tests were carried out in 96-well microplates that allowed for the simultaneous analyses of multiple samples. Absorbance was measured after 2 min at 600 nm on a microplate reader.

Protein concentration (mg/mℓ) was determined by means of a standard protein curve.

3.1.1.2 Boehringer Mannheim test kit

The test kit is based on the Biuret method as described by Weichselbaum (1946). Protein present in a sample forms a coloured complex with cupric ions in an alkaline reagent. 20 µℓ of sample was added to 1 mℓ of the reagent. The mixture was mixed and incubated for 30 min at room temperature. The absorbance of a sample was measured against a blank using a spectrophotometer (546 nm).

Protein concentration is expressed as mg/mℓ.

3.1.2 Acetylcholinesterase (AChE) assays

3.1.2.1 Microplate assay

The protocol of Ellman *et al.* (1961) was followed. This is a colorimetric method that measures the enzyme activity in terms of acetylcholine breakdown. A reaction mixture, consisting of buffer (2.1 mℓ), substrate (s-acetylthiocholine iodide) (100 µℓ) and Ellman's reagent (2,2'-dinitro-5,5-dithio-benzoic acid) (100 µℓ), was incubated for 5 min at 37°C. 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 acid 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 a microplate reader.

The enzyme activity [absorbance (abs)/min] was determined using a standard curve.

3.1.2.2 Boehringer Mannheim test kit

The test kit is based on the method of Ellman *et al.* (1961). 750 µℓ of the reaction mixture (consisting of buffer and Ellman's reagent), 5 µℓ of sample, and 25 µℓ of the substrate were pipetted into a test tube and mixed. The absorbance of the mixture was measured every 30 s for a period of 90 s, starting at time 0, using a spectrophometer (405 nm).

The enzyme activity (U/ℓ) was calculated as the abs/30s x 23 460 (specific factor).

3.1.3 Ethoxyresorufin-O-deethylase (EROD) assay

The direct fluorometric 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. 10 µℓ β-nicotinoamide adenine dinucleotide phosphate (NADPH) was mixed into the mixture to start the reaction. The progressive increase in fluorescence (formation of resorufin) was recorded at 1 s intervals for 2 min (room temperature) in a fluorometer at 510 and 586 nm, respectively.

EROD activity (nM/min) was determined using a standard curve.

3.1.4 Glucose assay

Glucose was determined with a glucose oxidase test kit of Boehringer Mannheim. The method is described by Trinder (1969). This is a colorimetric assay where glucose is oxidised by glucose oxidase to gluconate and hydrogen peroxide. Hydrogen peroxide is converted to a coloured product, hydrogen peroxidase, in the presence of the chromogen and phenol. 2 mℓ of the reagent solution containing buffer, enzymes, chromogen and phenol was added to 200 µℓ of sample and incubated for 35 to 60 min at room temperature. The absorbance was measured with a spectrophotometer at 546 nm.

The glucose concentration is expressed as mg/mℓ.

3.1.5 Glycogen assay

The method is described by Seifter *et al.* (1950). Fish tissue was digested with potassium hydroxide to liberate the glycogen (a polysaccharide made up of condensed glucose residues), which was precipitated with ethanol. 4 mℓ anthrone reagent was added to 2 mℓ diluted glycogen mixture (1:20 dilution) and incubated for 10 min at room temperature. The glucose residues condensed with anthrone to form a blue-coloured product (McDonald *et al.*, 1989). Absorbance was measured at 620 nm using a spectrophotometer.

The glycogen concentration is expressed as mg/100 g sample.

3.1.6 Delta-aminovulinic acid dehydratase (ALA-D) assay

ALA-D activity was determined using the rapid method of Schaller and Berlin (1984).

100 μℓ of the substrate (aminolevulinic acid), 1.3 mℓ water and 200 μℓ sample were mixed in a test tube and incubated for 1 h at 37°C. After the addition of a trichloroacetic acid-mercury chloride solution (1 mℓ) the mixture was centrifuged (room temperature). Ehrlich's reagent (1 mℓ) was added to the filtered supernatant (1 mℓ) and the mixture incubated for 5 min at room temperature. The absorbance of the mixture was read at 555 nm in a spectrophotometer.

ALA-D activity (U) was determined using a standard curve.

3.1.7 Lactate dehydrogenase (LDH) assay

The commercially available kit from Boehringer and Mannheim is based on a method described in *Z. klin. Chem. u klin. Biochem* (1970). LDH converts pyruvate to lactate with the aid of the co-enzyme NADH, which at the same time is oxidised to NAD^+. The reagent solution (1 500 μℓ) and the sample (50 μℓ) were incubated at room temperature for 30 s. The reaction (removal of NADH) was then recorded on a spectrophotometer (340 nm) at 1 min intervals for 3 min, starting at time 0.

The enzyme activity (U/ℓ) was calculated as the abs/min x 4 921(specific factor).

3.1.8 Glucose-6-phosphate-dehydrogenase (G-6-P-DH) assay

A commercially available kit from Boehringer and Mannheim was used. The test kit is based on a method by Kornberg and Horecker (1955). The enzyme is incubated in the presence of an excess of the substrate, G-6-P, and the co-enzyme $NADP^+$. The oxidised co-enzyme is reduced to NADPH with a maximum absorption at 340 nm. 500 μℓ of the sample is incubated for 10 min at room temperature in the presence of the prepared reagent solutions, where after the absorbance is measured on a spectrophotometer (340 nm) at 1 min intervals for 3 min, starting at time 0.

The enzyme activity (mU/mℓ) was calculated as the abs/min x 500 (specific factor).

3.1.9 Pyruvate kinase (PK) assay

A commercially available kit from Boehringer and Mannheim was applied. The test kit is based on the method of Beisenherz *et al.* (1953). In this assay $NADP^+$ is produced in a double-coupled enzyme reaction, involving PK and LDH. PK converts phosphoenolpyruvate to pyruvate, which serves as substrate for LDH. LDH then converts pyruvate to lactate with the aid of the co-enzyme NADPH, which at the same time is oxidised to $NADP^+$. The test sample (125 μℓ) is pre-incubated for 5 min at room temperature with three of the prepared reagent solutions. Absorbance is then measured at 340 nm with a spectrophotometer (A1). The measurement is repeated after 10 min (A2). The final (fourth) solution is then added and absorbance immediately measured (A3). The measurement is repeated after 10 min (A4).

The enzyme activity (mU/mℓ) was calculated as $[(A_3 - A_4) - (A_1 - A_2)]$ x 103.2 (specific factor).

3.1.10 Heat shock protein (Hsp 70) assay

Equivalent amounts of protein (15 to 30 µℓ sample containing 20 or 15 µg protein) from samples separated on 12% polyacrylamine gels are transferred by electroblotting onto a nitrocellulose membrane. The membrane is then incubated with an antiHsp 70 monoclonal antibody, which in turn is detected by a peroxidase coupled anti-mouse polyclonal antibody. The level of peroxidase is quantified by exposure to enhanced chemiluminescence reagents which release an amount of light proportional to the quantity of peroxidase coupled secondary antibody. An X-ray film is then exposed to the membrane for 5 min and developed. The band intensity on the X-ray film, which is proportional to the quantity of Hsp 70, is then quantified using laser densitometry. RAU expressed their results as relative density/15 µg protein and WITS as the area under the curve (mm^2/20 µg protein).

3.1.11 Osmotic ion analyses

Calcium, sodium and potassium were determined by means of the KONE microlyte 3+2 Ion Selective Analyser. Results are expressed in mmol/ℓ.

3.2 Laboratory Studies

3.2.1 Test fish

Laboratory studies were carried out with the Mozambique tilapia, *Oreochromis mossambicus*. Fish maintenance and breeding protocols are described in the Operational Manual (Venter *et al.*, 2003). Fish were supplied by the CSIR and RAU breeding facilities.

At CSIR, the breeding stock was kept in aerated tap water in a static system at a water temperature of 27±1°C. The young juveniles were moved from the breeding facility to the experimental room (water temperature: 23±2°C), 7 to 10 days after hatching and allowed to acclimatise for at least one week before use in experiments. RAU used an aerated flow-through system consisting of several tanks filled with borehole water, installed in an environmental room with an air temperature of 23±1°C. Juveniles of different ages were kept in separate tanks in the flow-through system.

Two days before experimentation, the aerated tap/borehole water in tanks holding the test fish was gradually replaced by moderately hard reconstituted water (Table 10). This allowed the fish to acclimatise to the synthetic water used for control testing and sample dilution.

3.2.2 Experimental design

3.2.2.1 Studies on unexposed (control) fish

Experiments were carried out with three sizes juvenile fish, namely 1 to 1.5 cm (2 to 4 weeks old), 5 cm and 10 cm. The 1 to 1.5 cm fish were placed in glass containers with moderately hard reconstituted water for 96 h (loading: 5 fish per 250 mℓ water). The experimental conditions outlined in section 3.2.2.2 were followed. The 5 and 10 cm fish were kept in borehole water in a flow-through system for the same period. Fish were starved for 24 h before experimentation and were not fed during experiments.

After 96 h, the fish were removed from the water, dried with paper towel, weighed (only 5 and 10 cm fish) and homogenised for biomarker analysis (section 3.2.3). The 1 to 1.5 cm fish were pooled in batches of 15 for homogenisation, while the larger fish were homogenised individually.

TABLE 10: Moderately hard reconstituted water[1]

Reagent added[2] (mg/ℓ)	$NaHCO_3$	96.0
	$CaSO_4.2H_2O$	60.0
	$MgSO_4$	60.0
	KCl	4.0
Nominal water quality range	pH	7.4 to 7.8
	Hardness[3]	80 to 100
	Alkalinity	60 to 70

[1] According to US EPA (1991)

[2] CSIR used Milli-Q water and RAU deionised water for preparation

[3] As mg/ℓ $CaCO_3$

3.2.2.2 Exposure studies

Tests were carried out under static conditions at a water temperature of 23±2°C, in ambient light (9 to 14 h daylight, depending on time of year), following standard acute toxicity test procedures (Slabbert *et al.*,1998a, 1999). Each test included a control (moderately hard reconstituted water) and a number of test solutions. The detailed protocol is outlined in the Operational Manual (Venter *et al*, 2003).

2 to 4 week old juveniles were used for toxicity testing. Fish were starved for 24 h prior to use and were not fed during tests. Each experiment was carried out with the offspring of a single female. The number of offspring per female ranged from 150 to 250. Fish were randomly transferred from a holding tank into the test containers (loading: 5 fish per 250 mℓ water):

Single exposures

30 fish were placed in 1.5 ℓ sample in 2 ℓ glass containers (no replicates). Fish were exposed to two to three toxicant concentrations and control water for periods of 8, 24 and 96 h.

Triplicate exposures

60 fish, divided over triplicate 2 ℓ flasks containing 1 ℓ sample (20 fish per container), were used per toxicant concentration and control. Fish were exposed to two test concentrations and the control water for 96 h. Three experiments (triplicate containers) were carried out with control water only.

The pH, DO and water temperature of each solution (including the control) were measured at the beginning and end of each exposure study. Chemical analyses were carried out on selected samples.

Fish lethality was recorded on a daily basis. Dead fish were removed at regular intervals to limit bacterial contamination. The % lethality was calculated as follows:

$(N_{t0} - N_{t96})/N_{t0} \times 100\%$, where

N_{t0}: Number of live fish at t = 0 h
N_{t96}: Number of live fish at t = 96 h

After exposure, the fish in each container were pooled for biomarker analysis. The fish were removed from the water, dried on a paper towel and placed in pre-weighed Eppendorf tubes. After weighing, the tubes were kept at -70°C until homogenisation (section 3.2.3)

3.2.3 Preparation of fish samples for biomarker analysis

Fish sample preparation is described in detail in the Operational Manual (Venter *et al.*, 2003). A 50 mM Tris (hydroxymethyl-aminomethane) buffer (pH: 7.4) containing 0.25 M sucrose was used for tissue preparation, because all the biomarker assays could be performed in this buffer, and at this pH.

After homogenisation the tissue suspension was centrifuged in an Eppendorf centrifuge at 11 000 rpm for 10 min at 4°C (Besselink *et al.*, 1997; Ibrahim *et al.*, 1998). The supernatants of the homogenates were divided into three aliquots and stored at -70°C until distribution to all participating laboratories for biomarker analyses.

3.2.3.1 Studies on unexposed (control) fish

2 mℓ buffer was used to homogenise the 15 pooled fish. The 5 and 10 cm fish were homogenised with 3 and 8 mℓ buffer, respectively.

3.2.3.2 Exposure studies

4 and 6 mℓ buffer was used for the homogenisation of the 20 and 30 fish, respectively. When fish lethality occurred, the volume of buffer used was adjusted accordingly.

3.2.4 Analysis of fish homogenates for biomarker activity

3.2.4.1 Studies on unexposed (control) fish

Protein was determined by means of all three methods described in section 3.1.1. Other assays included AChE (microplate assay and test kit), EROD, PK, LDH, G-6-P-DH and Hsp 70. The glucose assay was not applied because the protocol was not established. ALA-D and osmotic ion assays are only applicable to blood and glycogen to liver, and were, therefore, omitted from the evaluations.

Single assays were carried out (no replication), because homogenate volumes were limited.

3.2.4.2 Exposure studies

Single exposures

All the biomarker assays, except ALA-D, glycogen and osmotic ions (organ specific biomarkers), were applied. Protein was determined with the micro and macro Bradford methods, and AChE with both the microplate assay and test kit.

Triplicate exposures

The G-6-P-DH assay was omitted from the battery of assays because of unacceptable results and the large sample volumes required for assaying. Protein was determined with the micro Bradford method, and AChE with the microplate assay and test kit.

Each biomarker assay was carried out in triplicate (except where stated differently) and means and coefficients of variation (CVs) calculated [CV = mean/standard deviation (SD) x 100%].

The effect (% induction or inhibition) of chemicals on biomarker activity was established by means of the following formula:

$(B_t - B_c)/B_c \times 100\%$, where

B_t: Mean test biomarker value, and
B_c: Mean control biomarker value

3.2.5 Toxicants used in exposure studies

Toxicity tests were carried out with four chemicals, namely cadmium ($CdCl_2$), zinc ($ZnCl_2$), PCP (Cl_5C_6ONa) and cyanide (NaCN). Stock solutions of 100 mg/ℓ were prepared with Milli-Q water. Where required, the stock solution was diluted further with Milli-Q water to a suitable working solution (e.g. 1 or 10 mg/ℓ). Test solutions were prepared as 1.5 ℓ volumes in measuring cylinders with moderately hard reconstituted water (Table 10). (In the case of triplicate tests, 4.5 ℓ sample was prepared). The results of tilapia lethality tests (Slabbert *et al.*, 1999) were used to select a suitable sub-lethal concentration range for the exposure studies. The test concentration ranges were as follows (mg/ℓ):

Cadmium:	0.003 to 0.4
Zinc:	0.125 to 2.0
Cyanide:	0.0625 to 0.25
PCP:	0.00125 to 0.1

3.2.6 Statistical analysis

3.2.6.1 Analysis of variance

Data sets were compared by means of Student's t-test (Clarke, 1969) to establish whether or not results differed significantly at the $P<0.05$ level. This allowed the derivation of the toxicity endpoint referred to as a LOEC. The LOEC is the lowest of consecutive toxicant concentrations at which the biomarker activity was significantly different from that of the control.

3.2.6.2 Regression analysis

Where possible, linear regression was applied to the test data (% effect versus log concentration) to establish the EC_{20} (the concentration causing a 20% induction/inhibition in biomarker activity compared to the control activity).

3.3 Field Studies

The field studies were carried out at the sites where students of RAU have been doing work for several years. The Rust de Winter Dam was selected as reference site and the Loskop and Hartbeespoort Dams as test sites. Historical water quality data are presented in Table 11.

TABLE 11: Historical water quality data (mean±SD) of the selected dams (obtained from DWA&F)

Variable (mg/ℓ, except when indicated otherwise)	Rust de Winter Dam		Loskop Dam		Hartbeespoort Dam	
	1995	2000	1995	1996	1995	2000
pH	8.0	7.9	7.9	7.8	8.4	8.3
Conductivity (mS/m)	19.0±2.0	19.0±2.0	35.0±1.0	32.0±2.0	50.0±4.0	45.0±6.0
Alkalinity ($CaCO_3$)	64.0±6.0	56.0±4.0	43.0±3.0	44.0±2.0	99.0±10.0	98.0±14.0
Calcium (Ca)	10.0±0.8	9.0±1.0	21.0±1.0	20.0±0.6	29.0±4.0	26.0±6.0
Magnesium (Mg)	5.0±0.5	5.0±0.8	10.0±1.0	10.0±0.4	14.0±1.0	14.0±2.0
Sodium (Na)	16.0±2.0	16.0±1.0	26.0±2.0	26.0±0.8	44.0±4.0	33.0±9.0
Potassium (K)	6.0±0.5	4.0±0.2	6.0±0.4	5.0±0.7	10.0±0.6	7.0±1.0
Silica (Si)	3.0±0.9	5.0±0.5	2.0±0.3	2.0±0.2	2.1±1.0	4.0±2.0
Ammonia (N)	0.05±0.02	0.04	0.04	0.04±0.01	0.3±0.3	0.1±0.2
Nitrate-nitrite (N)	0.07±0.05	0.05±0.01	0.06±0.03	0.06±0.05	1.8±0.7	1.4±0.7
Phosphate (PO_4)	0.01±0.01	0.02	0.11±0.2	0.02	0.05±0.03	0.1
Sulphate (SO_4)	10.0±2.0	7.0±2.0	89.0±11.0	74.0±3.0	67.0±4.0	52.0±8.0
Fluoride (F)	0.4±0.07	0.32±0.1	0.2±0.03	0.3	0.5±0.1	0.3
Chloride (Cl)	10.0±0.6	14.0±1.0	15.0±1.0	15.0±2.0	46.0±3.0	38.0±8.0

3.3.1 Study sites

3.3.1.1 Rust de Winter Dam

The Rust de Winter Dam is the uppermost major dam in the Elands River and is surrounded by a nature reserve. The Elands River rises a few km south of Rayton near Kaztan, north of the N4-highway and drains northwards through hilly country for approximately 60 km to the dam. From this point it flows in a westerly direction to the south of the Springbok Flats to its confluence with the Olifants River, downstream of Marble Hall. The dam has a catchment area of 1 147 km^2 and a total storage capacity of 28.1 x 10^6 m^3. The dam was initially used for irrigation of vegetable farms below the dam, but has recently become a popular recreational area for fishing and boating (Butty *et al.*, 1980; Grimsehl and Pullen, 1991). There is no development that can have a major impact on the water. The largest portion of the catchment is undeveloped bushveld, which is used for cattle ranching.

3.3.1.2 Loskop Dam

The Upper Olifants River Catchment comprises the drainage areas of the Olifants River, Klein Olifants River and Wilge River with tributaries down to the Loskop Dam. The headwaters of these rivers are located along the Highveld Ridge in the Secunda-Bethal areas and rivers then flow in a northern direction towards the Loskop Dam. The major impoundments upstream of the dam include the Witbank Dam, Middelburg Dam, Bronkhorstspruit Dam and Premiere Mine Dams. The dam is 48 km north of Middelburg and has a catchment area of 12 261 km^2 and a storage capacity of 180 x 10^6 m^3 to 348.1 x 10^6 m^3 (SANCOLD, 1978). The total catchment incorporates the most industrialised region of the Olifants River basin. A large number of mines, predominantly coal mines, are located in the Loskop Dam Catchment. There is also a large number of agricultural activities in the area. The Loskop Dam has been described as a sink for heavy metals and other chemicals (e.g. pesticides). Presently Loskop Dam supplies water for domestic, industrial and irrigation purposes.

3.3.1.3 Hartbeespoort Dam

The Hartbeespoort Dam is situated on the Crocodile River, about 16 km southwest of the town of Brits and 37 km due east of Pretoria. The Hartbeespoort Dam was built downstream of the confluence of the Crocodile and Magalies Rivers and has a catchment area of 4 112 km^2 and a storage capacity of 185.49 x 10^6 m^3 (Rossouw, 1992). The catchment area includes the city of Johannesburg and its northern suburbs. Other towns include Midrand, Clayville, Olifantsfontein, Tembisa, Alexandra, Hartbeespoort, Magaliesburg and parts of Atteridgeville and Saulsville. The intense urbanisation in the area has the potential to decrease the water quality due to dumping of effluents and solid wastes, mines, industrial activities, etc. There are also a large number of sewage treatment plants and storm water run-off in the area

3.3.2 Fish sampling

Fish sampling is described in detail in the Operational Manual (Venter *et al.*, 2003). Two field surveys were carried out, namely in January 2000 (summer) and June 2000 (winter). Where possible, 20 adults (including males and females) were caught. Total length (cm), weight (kg) and sex of the fish were recorded.

Blood was drawn from the caudal vein and kept on ice in 5 mℓ Vacutainers. The brain and liver were removed, placed into Cryotubes, and kept in liquid nitrogen.

The water temperature, pH, conductivity, total dissolved solids (TDS) and DO were determined at each site.

3.3.3 Preparation of fish samples for biomarker analysis

210 µℓ of each blood sample was transferred to a separate Vacutainer and stored at -20°C for use in the ALA-D assay. The preparation of the whole blood for ALA-D and protein analyses is described in the Operational Manual (Venter *et al.*, 2003). The remaining blood was centrifuged for 10 min at 3 000 rpm. The red blood cells were placed at -20°C and the plasma was kept in liquid nitrogen until analysis. Brain and liver samples were weighed and homogenised in buffer at a ratio of 1:1 (weight/volume). The homogenate was centrifuged at 15 000 rpm for 20 min at 4°C and the supernatant kept at -20°C until analysis. 500 mg liver sample was kept for the glycogen assay and prepared as described in the Operational Manual (Venter *et al.*, 2003).

3.3.4 Analysis of fish samples for biomarker activity

The micro Bradford protein assay was applied to whole blood, erythrocytes, blood plasma, and liver homogenate. ALA-D activity was determined in whole blood and AChE activity in erythrocytes (microplate assay) and brain homogenate (test kit). Blood plasma was analysed for glucose and LDH activity, and osmotic ion concentrations. EROD, PK, LDH, G-6-P-DH and glycogen assays were carried out on liver samples.

3.3.5 Statistical analysis

Biomarker data was statistically analysed (Statcon, RAU) using the SPSS computer programme. Two-way analysis of variance (ANOVA) and Dunnett's test were employed to determine significant differences between localities and season. The significance level was set at $P<0.05$.

Fish weight and length were statistically analysed by means of Student's t-test.

4. RESULTS AND DISCUSSION

4.1 Laboratory Studies

4.1.1 Verification and optimisation of protocols

4.1.1.1 Biomarker levels in unexposed (control) fish

Biomarker analysis were carried out on different sizes unexposed (control) fish to:

- Establish, evaluate and optimise the different biomarker methodologies and sample preparation protocols;
- Establish the biomarker levels in the homogenates of different sizes fish to enable the selection of a suitable fish size for exposure studies; and
- Establish the variation between data.

The biomarkers were determined in a number of homogenates of pooled 1 to 1.5 cm fish and individual fish (5 and 10 cm). 15 of the 1 to 1.5 cm fish were pooled. 2.5 cm fish (6 weeks) were initially included in studies but the samples were destroyed when the freezer defrosted. Single assays were carried out because of limited homogenate volumes.

Protein levels

A micro and macro Bradford method, and a Boehringer Mannheim test kit were used to determine protein. The results (range, mean and CV) obtained for four pooled fish samples, and eight individual 5 and 10 cm fish samples are presented in Table 12.

The micro and macro Bradford results were fairly similar in some instances (e.g. 2.9 and 3.1 mg/ℓ protein), while in others they showed large differences (e.g. 4.1 and 10.7 mg/ℓ protein). The best agreement between results of the two methods was obtained in the case of the 1 to 1.5 cm fish, while the results of the larger fish differed considerably. In general, the protein values of the micro method were lower than the protein values of the macro method. The differences between the results of the two Bradford methods were possibly due to the fact that 10 μℓ is used in the micro method and 100 μℓ sample is used in the macro method. The smaller volume will amplify any pipetting error made by an inexperienced analyst.

The protein values of the Boehringer Mannheim test kit were between 10 and 25 times higher than the values of the Bradford methods. The higher values could be ascribed to possible interference of the Tris buffer with the Biuret reaction on which the assay is based.

The protein values (all three methods) of the 5 and 10 cm fish were fairly similar, which could have been due to incomplete homogenisation. The values of the 1 to 1.5 cm fish obtained with the Boehringer Mannheim test kit were in the value range of the larger fish, confirming the possible interference of the buffer with the Biuret reaction. The results obtained for the 1 to 1.5 cm fish using the Bradford methods were between two to three times lower than the results obtained for the larger fish.

In general, the variation (CV) between the results (all three protein methods) obtained for the eight individual 5 and 10 cm fish was larger than the variation between the results of the four sets of 1 to 1.5 cm fish. The individual fish displayed a range of weights (Table 12), while a constant volume of buffer was used for homogenisation, which could explain the variation in the protein values.

TABLE 12: Protein levels in the homogenates of different sizes fish, determined by means of a micro and macro Bradford method and a Boehringer Mannheim test kit

	Fish weight (g)	Micro Bradford method	Macro Bradford method	Boehringer Mannheim kit
		mg protein/mℓ homogenate		
15 pooled 1 to 1.5 cm fish (n=4)				
Range	ND	3.0 to 3.6	2.1 to 4.8	61.4 to 78.9
Mean	ND	3.2	3.5	71.8
CV (%)	ND	9	35	10
Individual 5 cm fish (n=8)				
Range	1.9 to 10.2	4.1 to 13.2	8.4 to 11.1	64.4 to 136.0
Mean	4.6	8.1	9.9	91.1
CV (%)	61	36	9	27
Individual 10 cm fish (n=8)				
Range	9.7 to 24.7	5.0 to 9.2	8.6 to 12.2	65.9 to 104.3
Mean	15.7	6.9	10.0	84.9
CV (%)	33	17	11	16

n Replicates
ND Not determined
CV Coefficient of variation

Values obtained for other biomarkers

The different fish homogenates were also analysed for AChE (microplate assay and test kit), PK, LDH, G-6-P-DH and Hsp 70. Biomarker activity is usually expressed in terms of protein. The results of the macro Bradford method was used to calculate the biomarker activities, because this was the most established method at the time. The results (range, mean and CV) are summarised in Table 13.

The AChE values obtained with the microplate assay were between two to four times higher than the test kit values. One of the four 1 to 1.5 cm fish homogenates showed an absence of AChE with the test kit. AChE values in the 5 cm fish were higher than the values in the 10 cm fish. In general, the variation between test kit results were larger than the variation between microplate assay results. The variation between results of both methods was particularly large in the case of the 1 to 1.5 cm fish (microplate assay: 101%; test kit: 113%). Table 14 shows that there was a good correlation between the AChE enzyme reaction rates (slope: abs versus time) of the different 10 cm fish homogenates in the microplate assay.

PK, LDH, G-6-P-DH and Hsp 70 were detected in all the 1 to 1.5 cm fish homogenates. A variation of 102% was noticed between the LDH results. The variation between the results of the other markers ranged from 21 to 49%. None of the 5 cm fish homogenates and only four of the 10 cm fish homogenates contained PK. The results of the 10 cm fish homogenates showed a very large variation (181%). On average, PK levels in the 1 to 1.5 cm fish were approximately 20 times higher than in the 10 cm fish. Two of the 5 cm fish and three of the 10 cm fish homogenates did not contain LDH. The variation between the results was high (5 cm fish: 221%; 10 cm fish: 93%). The 1 to 1.5 cm fish contained the highest LDH levels (0.0551 U/ℓ/mg protein) and the 10 cm fish the lowest (0.0008 U/ℓ/mg protein). G-6-P-DH was absent from four of the 5 cm fish and five of the 10 cm fish homogenates. The variation between the results of these homogenates was very high (5 cm fish: 247%; 10 cm fish: 158%). As in the case of PK and LDH, the larger fish contained lower G-6-P-DH levels. A high degree of variation was found to occur between the results of the 5 cm fish homogenates (132%), where three of the eight samples did not contain Hsp 70, and two yielded very low levels of Hsp 70. The 10 cm fish contained the highest Hsp 70 levels. The results showed a variation of 26%.

TABLE 13: Biomarker levels in the homogenates of different sizes fish

	AChE		PK (mU/mℓ/mg protein)	LDH (U/ℓ/mg protein)	G-6-P-DH (mU/mℓ/mg protein)	Hsp 70 (mm^2/20 μg protein)
	Microplate assay (abs/min/mg protein)	Test kit (U/ℓ/mg protein)				
15 pooled 1 to 1.5 cm fish (n=4)						
Range	0.0458 to 0.3810	0 to 0.1490	4.4290 to 18.9200	0.0055 to 0.1323	6.9840 to 15.3175	640 to 1 020
Mean	0.1531	0.0572	12.2091	0.0551	10.2350	845
CV (%)	101	113	49	102	30	21
Individual 5 cm fish (n=8)						
Range	0.3410 to 1.0500	0.1113 to 0.2690	0	0 to 0.0187	0 to 5.0340	0 to 1 664
Mean	0.5623	0.1820	0	0.0029	0.7078	381
CV (%)	41	30	0	221	247	132
Individual 10 cm fish (n=8)						
Range	0.3307 to 0.4652	0.0561 to 0.2513	0 to 2.9011	0 to 0.0019	0 to 2.8816	640 to 1 872
Mean	0.3907	0.1384	0.5753	0.0008	0.6800	1 434
CV (%)	10	49	181	93	158	26

n	Replicates
CV	Coefficient of variation
abs	Absorbance

TABLE 14: Slopes obtained with the AChE microplate assay for individual 10 cm fish (n=13)

Slope range (abs versus time)	Correlation range (R)
0.0013 to 0.0036	0.9992 to 1.0

n	Replicates
abs	Absorbance

EROD activity was only determined in the 1 to 1.5 cm fish homogenates. All the samples showed an absence of this biomarker activity. The assay was verified on the S9 liver fraction of rats injected with Arochlor 1254 (a mutagen inducing EROD synthesis), which showed high activity. The absence of EROD activity in the fish homogenates can probably be ascribed to the fact that in the absence of toxicants inducing EROD synthesis, only basal levels of this enzyme, which is primarily found in the liver, are present. In this case the enzyme was even further diluted in the whole fish homogenate.

Conclusions and recommendations

- The studies succeeded in establishing the majority of biomarker assays and in obtaining satisfactory values for the different biomarkers on unexposed (control) fish.

- Although some of the biomarker levels were low or undetectable in the unexposed fish, it was decided not to omit any of the assays from the exposure studies because the possibility existed that biomarkers would be induced by certain toxicants and therefore would be higher in exposed fish.

- Because of the possible interference of the Tris buffer with the Biuret reaction in the Boehringer Mannheim test kit, it was decided to omit this method from further studies, but to continue with the two Bradford methods to verify which of the two methods is the most suitable.

- Although the AChE microplate assay performed better than the AChE test kit in terms of lower variability and higher enzyme levels, a final decision could not be made on whether or not to omit the test kit method. It was decided to include both methods in the battery of biomarker assays for exposure studies to enable a more justified recommendation.

- The variation between data sets raised serious concern. Most of the CVs for data sets were >10%, in fact, a large number of CVs were >100%. The highest CV reported in literature (section 2.3) for fish homogenates is 52%. In order to reduce variability of exposure study results, it was decided to conduct each biomarker assay in triplicate and to do replicate fish exposures wherever possible. It was also decided to ensure that the biochemical analytical skills of the participating students meet minimum requirements to reduce variability.

- Since the biomarker levels in the 1 to 1.5 cm fish were not markedly lower than those obtained with the 5 and 10 cm fish (in some instances they were higher) and the variation between data sets were generally smaller, it was decided to use 2 to 4 week old juveniles in exposure studies. The homogenates of the 1 to 1.5 cm fish were much more easy to prepare (the 5 and 10 cm fish had scales and were hard). Furthermore, the 1 to 1.5 cm fish were easier to handle and smaller sample volumes were required.

- The studies were hampered by limited numbers of fish. A large effort would be required to ensure large enough numbers of fish for replicate biomarker analyses and replicate experiments during exposure studies.

4.1.1.2 Effect of variables on biomarker results

The repeatability and reproducibility of test results can be affected by one or more variables, e.g. test method, sample (preparation, handing, storage), operator manipulation, equipment and the experimental environment. Since large variations were noticed between data sets in section 4.1.1.1, the effect of some variables that could affect test data was investigated to optimise protocols. These included:

- Operator manipulation;
- Sample storage time; and
- Sample containers.

Operator manipulation

Two students with training in biomarker assays, determined protein in three fish homogenates using the Biuret method. The assays were carried out with the same equipment and in the same laboratory. Table 15 shows that the protein values of Operator 1 were higher than the protein values of Operator 2. A larger variation was also noticed between the replicate assays of Operator 1. An analysis of variance (Student's test) on the data sets showed that the results were, however, not significantly different at $P<0.05$.

TABLE 15: Protein levels (mg/mℓ) established by two trained operators in three fish homogenates using the Biuret method

Sample	Operator 1		Operator 2		Significant difference at P<0.05
	Mean (n=3)	CV (%)	Mean (n=3)	CV (%)	
1	85.98	23	75.29	6	No
2	91.68	9	75.34	6	No
3	99.09	13	81.83	7	No

n Replicates
CV Coefficient of variation

In a second experiment one of the trained students (Operator 1) and a researcher not regularly involved in biomarker assays (Operator 2) applied the micro Bradford protein assay to four fish homogenates. The assays were carried out in the same laboratory using the same equipment. Operator 1 performed triplicate assays, and Operator 2 single assays. The result of Operator 2 for sample 1 was very high compared to the result of Operator 1. Likewise, the result of Operator 2 for sample 2 was very low. An analysis of variance showed that these two data sets were significantly different at $P<0.05$. The values of Operator 2 for the other two samples were also lower than the values of Operator 1, but were not significantly different. In general, the variation between replicate results was large ranging from 9 to 51 %.

Sample storage time

Fish homogenates were often frozen for extended periods of time before biomarker analysis. Table 17 shows the protein results of three samples analysed by the micro Bradford method after 2 weeks and 11 months storage at -70°C. The assays were carried out by one operator, using the same equipment and experimental environment.

The variation between replicate assays was small (0 to17%), and the results obtained after 11 months storage were similar to the results obtained after 2 weeks storage. An analysis of variance indicated that results did not differ significantly at $P<0.05$.

TABLE 16: Protein levels (mg/mℓ) established by one trained and one inexperienced operator in four fish homogenates using the micro Bradford method

Sample	Operator 1		Operator 2		Significant difference at P<0.05?
	Mean (n=3)	CV (%)	Mean (n=1)	CV (%)	
1	5.12	51	12.12	ND	Yes
2	7.90	9	2.57	ND	Yes
3	9.11	28	5.00	ND	No
4	6.87	31	5.00	ND	No

n Replicates
CV Coefficient of variation
ND Not determined – single value

TABLE 17: Protein levels (mg/mℓ) established with the micro Bradford method in three fish homogenates after different storage periods at -70°C

Sample	Storage: 2 weeks		Storage: 11 months		Significant difference at P<0.05
	Mean (n=3)	CV (%)	Mean (n=3)	CV (%)	
1	2.48	3	2.63	5	No
2	2.83	0	2.70	15	No
3	2.75	6	3.04	17	No

n Replicates
CV Coefficient of variation

Sample containers

Fish homogenates were dispensed into different Eppendorf tubes for shipment to the different laboratories. The effect of sample containers on test data was examined by conducting a micro Bradford assay on the contents of two sets of four Eppendorf tubes. The one set of tubes contained a protein standard (0.5 mg/mℓ bovine serum albumin) and the other set contained a fish homogenate. The assays were carried out by one operator, using the same equipment and experimental environment. The results of replicate assays were close to the standard value of 0.5 mg/mℓ (Table 18) and showed little variation (1 to 6%). An analysis of variance indicated that the results of replicate containers did not differ significantly at $P<0.05$. The variation between replicate assays on the fish homogenates ranged between 1 and 16%. A significant difference was established between the results of tubes 3 and 4.

Conclusions and recommendations

- The findings confirmed that individual variables such as operator manipulation and sample containers contribute to significant differences between data.
- Sample storage did not significantly affect data.
- Because of the lower biomarker levels in young fish, the variation between the results is expected to be amplified. Extreme care should, therefore, be taken to limit the effect of variables, to ensure that small changes in biomarker activity as a result of toxicity are detected.

TABLE 18: Protein levels (mg/mℓ) established with the micro Bradford method in a protein standard (0.5 mg/mℓ bovine serum albumin) and fish homogenate dispensed in different Eppendorf tubes

Sample	0.5 mg/mℓ bovine serum albumin		Fish homogenate	
	Mean (n=3)	CV (%)	Mean (n=3)	CV (%)
1	0.494	3	2.37	16
2	0.502	4	2.47	12
3	0.481	6	2.79*	1
4	0.480	1	2.31*	2

n	Replicates
CV	Coefficient of variation
*	Data sets differed significantly at $P<0.05$

4.1.1.3 Selection of an appropriate protein method

During studies on unexposed fish (section 4.1.1.1) the results of the two Bradford methods showed considerable variation. It was decided to use both methods in further studies, to allow for the selection of the most appropriate method.

Table 19 presents the protein results obtained in a number of experiments (mean and CV of replicate assays and the mean and CV of replicate data sets). The Bradford method was conducted by two laboratories (WITS and RAU). WITS did not provide individual data, which rendered the calculation of CVs impossible.

Large differences were noticed between the results of the two Bradford methods. 58% of the micro and macro (RAU) Bradford data sets were significantly different ($P<0.05$). In general, the results of the micro method was lower than the results of the macro method. The results of the two laboratories conducting the macro Bradford method also differed considerably. The results of RAU were generally higher than the results of WITS. Differences were noticed between the data within an experiment and between the data of different experiments. Triplicate protein values ranged between 3.54 and 9.11 mg/mℓ in the case of the micro Bradford method, between 1.5 and 14.9 mg/mℓ in the case of the macro Bradford method used by WITS, and between 3.51 and 14.47 in the case of the macro Bradford method used by RAU. In general, the results of the micro Bradford method showed less fluctuation than the results of the macro Bradford method. The CVs of replicate data sets ranged from 4 to 28% for the micro Bradford method, from 12 to 38% for the macro Bradford method used by WITS, and from 17 to 21% for the macro Bradford method used by RAU. The variation between the triplicate assays of both Bradford methods was generally small, indicating good experimental conduct. Some of the triplicate assays in experiments 3 and 4 showed high CVs for the micro Bradford method.

TABLE 19: Protein levels (mg/mℓ) established by means of the micro and macro Bradford methods

Experiment	**Data set**	**Micro method**		**Macro method**		
				WITS[1]	**RAU**	
		Mean (n=3)	**CV (%)**	**Mean (n=3)**	**Mean (n=3)**	**CV (%)**
1	1	4.51	11	7.4	13.65	SV
	2	5.38*	9	6.7	**10.01***	3
	3	3.54	3	6.0	8.84	SV
	4	4.03*	7	6.7	**9.19***	5
	5	3.92*	10	4.8	**10.49***	1
	Mean (n=15)	4.28 (17%)		6.32 (16%)	10.44 (18%)	
2	1	5.83	6	4.1	6.07	3
	2	6.22*	5	3.4	**5.11***	4
	3	5.96*	6	3.7	**3.51***	6
	4	6.37*	5	1.9	**5.11***	3
	5	6.26*	2	1.5	**7.11***	6
	Mean (n=15)	6.13 (4%)		2.92 (39%)	4.95 (21%)	
3	1	3.85*	41	5.7	**9.32***	10
	2	4.17	53	6.0	7.58	4
	3	5.80	10	5.2	5.85	4
	4	5.46	18	3.4	ND	ND
	5	7.61	8	6.3	7.37	4
	Mean (n=15)	5.38 (28%)		5.32 (22%)	7.53 (19%)	
4	1	5.12*	51	11.6	**12.96***	9
	2	7.90*	9	12.7	**9.45***	3
	3	9.11	28	11.9	13.09	8
	4	6.87*	31	14.9	**14.47***	13
	Mean (n=12)	7.25 (23%)		12.78 (12%)	12.49 (17%)	

1	Calculation of CVs not possible
n	Replicates
ND	Not determined
SV	Single value
CV	Coefficient of variation
*	Data sets differed significantly at P<0.05
	CV of replicate data sets in brackets

Conclusions and recommendations

- The micro Bradford method proved to be the most appropriate assay for protein determination, because the results were more consistent and more repeatable than the results of the macro Bradford method.

- Since protein analysis is an integral part of stress protein analysis and should be carried out on site, it was decided that WITS and RAU would continue to use the macro Bradford method to establish protein for Hsp 70 analysis.

4.1.1.4 Protein *versus* fish weight for the expression of biomarker activity

Biomarker activity in tissue can be expressed in terms of protein or wet weight. The large variation occurring in protein values raised the question if fish weight instead of protein should not be used to express biomarker activity in fish homogenates.

Table 20 compares the protein values and fish weights obtained for a number of data sets. Generally, the weight data sets showed less variation than the protein data sets. 56% of the weight CVs were below the CVs obtained for protein, and 63% of the weight CVs were <10%, while only 38% of the protein CVs were <10%.

TABLE 20: Comparison between protein values and fish weight

Data set	Protein			Fish weight		
	Mean (mg/mℓ)	Replicates (n)	CV (%)	Mean (mg/mℓ)	Replicates (n)	CV (%)
1	4.27	15	17	155.61	5	6
2	3.28	15	10	98.73	5	8
3	6.15	15	5	111.67	5	5
4	6.13	15	4	106.67	5	4
5	5.38	15	28	177.12	5	9
6	7.25	15	23	273.56	5	7
7	4.27	15	47	178.90	5	17
8	2.57	12	13	147.09	4	7
9	2.69	9	7	92.26	3	23
10	2.37	12	10	113.89	4	18
11	2.25	12	9	110.92	4	17
12	4.38	9	18	115.36	3	4
13	7.35	9	2	141.01	3	18
14	10.08	9	31	170.43	3	9
15	4.73	9	35	148.85	3	4
16	2.54	9	16	81.8	3	18

CV Coefficient of variation

Conclusions and recommendations

- Based on the small variability between fish weight data, it was decided to use weight rather than protein to express biomarker data in exposure studies.

- Because of the large differences obtained in some experiments between the control and test protein data, it was decided to include protein as biomarker and express protein data in terms of fish weight.

- Hsp 70 will be expressed in terms of protein (section 4.1.1.3)

4.1.2 Exposure studies

Each experiment (toxicity test) was carried out with the offspring of a single female to ensure homogeneity (limit variability due to age and genetic differences). The number of offspring per female ranged from 150 to 200. In order to accommodate all the selected biomarkers, 30 fish were pooled. This meant that between five and seven batches of 30 fish were available for a toxicity test.

To obtain sufficient information on sensitivity, the effect of exposure time, and possible concentration-response relationships, it was necessary to conduct tests on a range of test concentrations (definitive test). A number of experiments were, therefore, carried out on single batches of 30 fish (single exposures). Each assay was carried out in triplicate to establish inter-assay variation. A few experiments were carried out with triplicate batches of 20 fish (triplicate exposures) to establish inter-experimental variation. Some biomarkers were omitted from the latter experiments and, therefore, fewer fish were pooled. Only a control and two test concentrations, or three controls, could be accommodated during triplicate exposures.

4.1.2.1 Control biomarker results

Each experiment included a control that duplicated all the conditions of the exposure treatment, but contained no toxicants. A control is used to determine the absence of measurable toxicity due to basic test conditions (e.g. salinity, temperature, health of organisms, or effects due to handling) (Slabbert *et al.*, 1998a).

All the biomarker results, except Hsp 70, are expressed in terms fish weight. Hsp 70 is expressed as protein (macro Bradford method). Protein (micro Bradford method) was included as biomarker.

Single exposures

The control results (means and CVs) of single exposures (1x 30 fish) are summarised in Table 21. G-6-P-DH assays were discontinued during later experiments because of insufficient sample volumes (this test required 1.5 mℓ sample). Hsp assays were initially carried out by WITS, and later by RAU.

Because young juveniles were used in the exposure studies, it was expected that the biomarker values would be lower than the values presented in literature for adult fish. A comparison with the data reported in literature (Table 4) was difficult, because different units were used to express biomarker activities. The protein values (mg/mg fish) presented in Table 21 are between two and six times lower than the values presented for tissue and liver in Table 4. Similar ratios are expected for the other biomarker values. The biomarker values obtained during the verification and optimisation of protocols (Table 13) were higher that the values in Table 21, because protein was used for the expression of results (protein levels considerably lower than fish weight - Table 20).

The AChE values of the test kit (Table 21) were generally higher than the values of the microplate test. Likewise, the Hsp 70 values obtained by RAU were much higher than the values obtained by WITS. The differences between the results could be attributed to the use of different units (AChE: abs/min/mg fish and U/ℓ/mg fish; Hsp 70: mm^2/20 µg protein and relative density/15 µg protein).

The biomarker values of the different experiments fluctuated considerably (Table 21). This could be attributed to differences in the weight and size of the offspring (a fixed volume of buffer was used in all experiments, irrespective of the weight and size of the fish). *The fish weight ranged from 72.5 to 251 mg/mℓ/Tto* 0.0613 mg/mg fish), glucose (0.0017 to 0.0044 mg/mg fish), Hsp 70 (RAU) (2137 to 10 252 relative intensity/15 µg protein) and EROD (0.0699 to 0.3605 nM/min/mg fish) exhibited the smallest fluctuation and PK (0.0201 to 0.6691 mU/mℓ/mg fish), AChE (test kit) (0.0003 to 0.0140 U/ℓ/mg fish) and G-6-P-DH (0.0562 to 4.6738 mU/ℓ/mg fish) the largest fluctuation.

Large inter-assay variations were noticed in the case of G-6-P-DH (CV: 14 to 72%), protein (CV: 0 to 92%), PK (CV: 5 to 98%), AChE (test kit) (CV: 2 to 109%) and LDH (CV: 2 to 110%). These variations could be attributed to experimental error during sample manipulation (e.g. the handling of the micropipette) or in the case of the test kits to test method limitation (incompatibility with the fish homogenate). The best repeatability was obtained with the Hsp 70 (RAU) (CV: 5 to 28%) and glucose (CV: 0 to 32%) assays. An evaluation of microsomal and cytosolic biomarkers in a 7-day laboratory larval trout sediment toxicity test (Viganò *et al.*, 1995) showed control CVs of between 8 and 52%. With a few exceptions (mostly test kit data) the control CVs in Table 21 were in agreement with the published values. Table 4 in Chapter 2 shows that variability decreased (lower CVs) when replicates were increased.

Triplicate exposures

The control results (assay means and CV ranges, experimental means and CVs) of triplicate exposures (3x20 fish) are summarised in Table 22. G-6-P-DH was omitted from all experiments because of the large sample volume required. AChE (test kit) and Hsp 70 were only applied to three of the experiments.

The biomarker values in Table 22 were within the ranges reported in Table 21, except in the case of Hsp 70, where some of the results were considerably higher. The fluctuation between assay means were much smaller than previously reported (Table 21), probably because the age of the fish used in these studies all ranged between 21 and 24 days. PK exhibited the largest fluctuation, with values ranging from 0.0537 to 0.3886 mU/mℓ/mg fish. With the exception of EROD, with CVs ranging from 8 to102%, the inter-assay variation in Table 22 was much smaller (CVs: <50%) than the inter-assay variation displayed in Table 21, indicating an improvement in sample handing and the execution of triplicate assays. The inter-experimental variation (triplicate data sets) of EROD and PK was >50% in some instances.

An analysis of variance on the triplicate data sets of each experiment (Table 22) showed that there were significant differences (P<0.05) between some of the biomarker values. In a few instances all three data sets differed significantly. Some degree of variation is expected between triplicate exposures, as a result of natural variation.

TABLE 21: Control biomarker values established in juvenile *O. mossambicus* during single exposures

Experiment	Weight	Protein		AChE				Glucose		EROD		PK		LDH		G-6-P-DH		Hsp 70		
				Microplate assay		Test kit												WITS	RAU	
	mg/mℓ	mg/mg fish^1	CV (%)	Abs/min/ mg fish^1	CV (%)	U/ℓ/mg fish^1	CV (%)	mg/mg fish^1	CV (%)	nM/min/mg fish^1	CV (%)	mU/mℓ/ mg fish^1	CV (%)	U/ℓ/mg fish^1	CV (%)	mU/mℓ/ mg fish^1	CV (%)	mm^2/20 µg protein^1	Relative density/15 µg protein^1	CV (%)
2	106.90	0.0287	7	0.0009	25	0.0134	12	0.0023	8	0.1338	sv	0.4471	7	0.0085	2	3.20	25	53.12	ND	ND
3	105.00	0.0613	5	0.0012	29	0.0108	27	0.0044	5	0.1808	13	0.3097	8	0.0092	9	4.16	14	16.76	ND	ND
4	105.00	0.0555	6	0.0012	18	0.0125	28	0.0043	6	0.0822	5	0.3215	31	0.0034	55	4.67	72	74.86	ND	ND
5	160.17	0.0240	41	0.0033	32	0.0061	30	0.0019	5	0.0944	2	0.2154	27	0.0065	6	0.06	15	45.50	ND	ND
6	251.00	0.0204	51	0.0041	7	0.0099	16	0.0017	2	0.1064	4	0.0320	98	0.0013	48	ND	ND	44.40	ND	ND
7	139.64	0.0193	92	0.0033	40	0.0081	10	0.0024	5	0.1241	3	0.1150	15	0.0071	9	ND	ND	122.01	10 252; 5 095	7; 13
8	142.58	0.0169	0	0.0006	43	0.0003	76	0.0018	3	0.2231	8	0.2952	11	0.0067	10	ND	ND	ND	2 715	16
9	72.50	0.0342	3	0.0014	35	0.0008	66	0.0043	3	0.3605	15	0.6691	6	0.0120	13	ND	ND	ND	3 589	28
10	143.57	0.0166	2	0.0004	33	0.0003	73		2	0.1387	27	0.3939	5	0.0053	9	ND	ND	ND	4 033; 4 806	14; 5
11	123.97	0.0181	1	0.0003	34	0.0011	109	0.0026	8	0.1612	25	0.4443	12	0.0011	110	ND	ND	ND	7423; 6152	15; 8
Range	72.50 to 251.00	0.0166 to 0.0613	0 to 92	0.0003 to 0.0041	0 to 46	0.0003 to 0.0140	2 to 109	0.0017 to 0.0044	0 to 32	0.0699 to 0.3605	2 to 45	0.0201 to 0.6691	5 to 98	0.0011 to 0.0120	2 to 110	0.06 to 4.67	14 to 72	7.80 to 122.01	2 137 to 10 252	5 to 28

1	Mean of triplicate assays
CV	Coefficient of variation
ND	Not determined
SV	Single value
abs	Absorbance

TABLE 22: Control biomarker values established in juvevenile *O. mossambicus* during triplicate exposures

Experiment		Weight	Protein	AChE		Glucose	EROD	PK	LDH	Hsp 70
				Microplate assay	*Test kit*					
		mg/mℓ	mg/mg fish	Abs/min/ mg fish	U/ℓ/mg fish	mg/mg fish	nM/min/ mg fish	mU/mℓ/ mg fish	U/ℓ/mg fish	Relative density/15 µg protein
1	Assay mean range (n=3)	112.00 to 121.05	0.0034 to 0.0435 (0)	0.0017 to 0.0032 (1)	0.0037 to 0.0077 (1)	0.0015 to 0.0017 (0)	0.0827 to 0.2241 (0)	0.3391 to 0.3886 (0)	0.0079 to 0.0099 (2)	12 520 to 13 746 (0)
	Assay CV (%) range	ND	6 to 31	10 to 25	5 to 30	6 to 14	46 to 102	9 to 25	2 to 11	6 to 13
	Experimental mean (n=9)	115.36	0.0378	0.0024	0.0054	0.0015	0.1671	0.3684	0.0087	13 043
	Experimental CV (%)	4	19	35	41	10	87	18	12	9
2	Assay mean range (n=3)	113.79 to 164.58	0.0440 to 0.0639 (2)	0.0015 to 0.0028 (2)	0.0030 to 0.0069 (1)	0.0011 to 0.0017 (1)	0.0642 to 0.0950 (1)	0.0720 to 0.2721 (2)	0.0083 to 0.0095 (0)	14 339 to 15 685 (0)
	Assay CV (%) range	ND	5 to 13	13 to 23	12 to 41	8 to 16	8 to 34	5 to 33	6 to 29	3 to 6
	Experimental mean (n=9)	141.01	0.0534	0.0020	0.0047	0.0014	0.0832	0.1852	0.0088	14 834
	Experimental CV (%)	18	18	30	43	22	27	54	17	6
3	Assay mean range (n=3)	153.70 to 180.00	0.0448 to 0.0758 (1)	0.0012 to 0.0015 (0)	0.0049 to 0.0055 (5)	0.0011 to 0.0013 (0)	0.1069 to 0.1723 (0)	0.1226 to 0.2592 (0)	0.0059 to 0.0082 (1)	6 238 to 7 287 (1)
	Assay CV (%) range	ND	3 to 39	7 to 17	7 to 14	7 to 10	50 to 57	2 to 13	1 to 9	3 to 4
	Experimental mean (n=9)	170.43	0.0589	0.0014	0.0052	0.0012	0.1386	0.1909	0.0071	6 763
	Experimental CV (%)	9	30	16	12	10	52	40	19	9
4	Assay mean range (n=3)	65.00 to 91.00	0.0257 to 0.0358 (1)	0.0012 to 0.0021 (2)	ND	0.0021 to 0.0033 (3)	0.1037 to 0.1819 (0)	0.0537 to 0.1111 (0)	0.0029 to 0.0051 (3)	ND
	Assay CV (%) range	ND	3 to 18	11 to 19	ND	4 to 11	16 to 50	17 to 46	3 to 10	ND
	Experimental mean (n=9)	81.80	0.0315	0.0016	ND	0.0026	0.1443	0.0710	0.0040	ND
	Experimental CV (%)	18	17	33	ND	22	42	40	24	ND
5	Assay mean range (n=3)	144.12 to 155.56	0.0199 to 0.0380 (2)	0.0013 to 0.0016 (0)	ND	0.0012 to 0.0015 (1)	0.0701 to 0.1424 (0)	0.0958 to 0.2922 (2)	0.0069 to 0.0082	ND
	Assay CV (%) range	ND	4 to 15	13 to 22	ND	6 to 17	9 to 61	8 to 38	1 to 12	ND
	Experimental mean (n=9)	148.85	0.0316	0.0015	ND	0.0013	0.1009	0.1749	0.0075	ND
	Experimental CV (%)	4	29	18	ND	15	44	58	10	ND

n	Replicates
CV	Coefficient of variation
ND	Not determined
Abs	Absorbance
	Number of data sets significantly different (P<0.05) in brackets

Large variations were most probably due to the non-random distribution of the test fish or fish handling.

4.1.2.2 Lethal effect of toxicants on juvenile *O. mossambicus*

Single exposures

Experiments were carried out with cadmium, zinc, PCP and cyanide. Exposure periods of 8, 24 and 96 h were used in cadmium studies, 24 and 96 h in zinc and PCP studies, and 24 h in cyanide studies. Between three and four test concentrations were included in each experiment. The age of the fish used in experiments ranged from 18 to 28 days.

In general, control lethality was within the limit required for acute toxicity tests, namely ≤10% during 96 h exposure (Table 23). A large lethality (53%) occurred in the control fish during the 96 h exposure studies on zinc. The test concentrations in this experiment exhibited lethalities ranging from 10 to 43%. The offspring used in this experiment were probably sick or stressed. The 0.025 mg/ℓ cadmium concentration exhibited 33% lethality after 96 h exposure.

Control and test pHs were within the limits required for aquatic life (7.0 to 8.5), and remained fairly stabile during exposure. The oxygen concentrations of the control and test solutions were >3.0 mg/ℓ (required minimum limit for aquatic tests) during cadmium, zinc and PCP experiments. However, oxygen concentrations in test and control containers were low (between 2.4 and 2.7 mg/ℓ) during cyanide experiments.

Metals can interact with the chemicals used in moderately hard water at high pHs to form precipitates. The pH of metal concentrations ranged between 7.7 and 8.4. Although low metal concentrations were tested, and precipitation was not expected, it was important to verify that chemical interaction did not occur. The measured concentrations of cadmium and zinc in some of the test solutions are shown in Table 23. In most instances the measured values were slightly lower than the prepared concentrations. It is expected that the prepared concentrations were more accurate than the measured values, as large discrepancies were noticed between the analysed results of two analytical facilities. Chemical analyses were not carried out on PCP and cyanide because interference with the chemicals in the moderately hard water was not expected.

Triplicate exposures

Fish were exposed to cadmium for 24 and 96 h. Two cadmium concentrations, namely 0.0025 and 0.025 mg/ℓ, were used in exposure studies. The age of the test fish ranged from 21 to 24 days.

Control lethality was ≤10% (Table 24). Cadmium did not cause lethality .P atlkoqna qne c 52 d atlkoqna(pda , ,,.1 ic ℓ concentration exhibited 17 % lethality and the 0.025 mg/ℓ concentration exhibited 25% lethality.

TABLE 23: Lethal effect of cadmium, zinc, PCP and cyanide on juvenile *O. mossambicus* during single exposures

Toxicant	Exposure period (h)	Age of fish (days)	Test concentration (mg/ℓ)	pH	Oxygen concentration (mg/ℓ)		Lethality (%)
					Start	Completion	
Cadmium	8	28	Control	8.3	5.1	4.0	0
			0.05 (0.029)	ND	ND	ND	0
			0.1 (0.054)	ND	N	ND	0
			0.2 (0.115)	ND	ND	ND	0
			0.4 (0.25)	7.8	4.5	4.3	0
	24	21	Control	8.5	5.4	5.4	0
			0.011	ND	ND	ND	0
			0.022 (0.015)	ND	ND	ND	0
			0.05	ND	ND	ND	0
			0.1 (0.081)	7.7	4.0	3.0	0
	96	28	Control	8.3	5.1	3.5	**13**
			0.0031	ND	ND	ND	**10**
			0.0063	ND	ND	ND	**13**
			0.0125 (0.017)	ND	ND	ND	**13**
			0.025 (0.024)	8.1	4.6	3.9	**33**
Zinc	24	28	Control	8.5	3.5	ND	0
			0.25	8.5	3.7	ND	0
			0.5	8.5	3.7	ND	0
			1.0 (0.059)	8.4	3.6	ND	0
			2.0 (0.115)	8.4	3.5	ND	0
	96	18	Control	8.1	3.7	3.4	**53**
			0.125	ND	ND	ND	**10**
			0.25	ND	ND	ND	**43**
			0.5 (0.305)	ND	ND	ND	**47**
			1.0 (0.606)	8.0	3.6	3.5	**30**
PCP	24	21	Control	7.0	4.3	4.0	**10**
			0.02	7.1	4.3	4.0	0
			0.04	7.2	4.3	4.0	3
			0.08	7.3	4.3	4.0	7
			0.1	7.3	4.3	4.0	3
	96	18	Control	7.2	4.7	5.2	0
			0.00125	ND	ND	ND	0
			0.0025	ND	ND	ND	**10**
			0.005	ND	ND	ND	0
			0.01	7.1	4.8	5.3	0
	96	18	Control	7.3	4.4	4.3	0
			0.00125	7.3	4.5	4.4	0
			0.0025	7.2	4.6	4.3	0
			0.005	7.2	4.5	4.4	0
			0.01	7.3	4.6	4.4	0
Cyanide	24	21	Control	7.9	2.5	ND	7
			0.0625	8.0	2.7	ND	0
			0.125	7.9	2.4	ND	0
			0.25	7.9	2.4	ND	7
	24	21	Control	7.9	2.5	ND	3
			0.0625	8.0	2.7	ND	0
			0.125	7.9	2.4	ND	0
			0.25	7.9	2.4	ND	3

ND Not determined
Results in bold indicate acute toxicity
Measured concentration in brackets

TABLE 24: Lethal effect of cadmium on juvenile *O. mossambicus* during triplicate exposures

Exposure period (h)	Age of fish (days)	Test concentration (mg/ℓ)	Lethality (%)
24	21	Control	0
		0.0025	0
		0.025	0
96	24	Control	0
		0.0025	**17**
		0.025	**25**

Results in bold indicate acute toxicity

4.1.2.3 Effect of toxicants on biomarker activity

Single exposures

The effect of cadmium, zinc, PCP and cyanide on biomarker activity is shown in Table 25. Results are expressed as the percentage induction or inhibition, calculated in terms of the control data presented in Table 21.

Analysis of variance

An analysis of variance (Student's t-test) was carried out on test and control data to establish if effects were significant at the $P<0.05$ level.

In general, protein was not significantly affected by cadmium (Table 25). Only one concentration, namely 0.4 mg/ℓ, exhibited a significant inhibition (76%) during the 8 h exposure period. AChE activity was significantly induced by the upper test concentrations during the 8 and 24 h exposures. Glucose showed a significant induction during 8 h exposure to 0.4 mg/ℓ cadmium. The lower concentrations (0.05 to 2.0 mg/ℓ) caused significant inhibitions. EROD activity was significantly induced by 0.2 and 0.4 mg/ℓ cadmium during the 8 h exposure period. Likewise, the 0.0125 and 0.025 mg/ℓ concentrations induced EROD activity during the 96 h exposure period. In general, Hsp 70 activity was not affected during 8 and 24 h exposure. During the 96 h exposure period, Hsp 70 was significantly inhibited by the 0.025 mg/ℓ concentration, and significantly induced by the 0.0125 mg/ℓ concentration. Fish homogenates were not analysed for AChE (test kit), PK, LDH and G-6-P-DH, because samples were lost due to a power failure.

Zinc caused large effects on protein (Table 25), but because of the large variations between triplicate test and control data, effects were not significant. AChE activity was significantly inhibited by the upper test concentrations.during 24 h exposure when using the microplate assay. The same concentrations caused significant inductions when AChE activity was measured with the test kit. EROD was significantly induced by the 1.0 and 2.0 mg/ℓ zinc concentrations during 24 h exposure. Effects were not significant during 96 h exposure. G-6-P-DH activity was not significantly affected by zinc during the 24 h exposure period. The assay could not be applied to the homogenates of the 96 h experiment, because sample volumes were limited as a result of the lethalities

TABLE 25: Effect of cadmium, zinc, PCP and cyanide on the biomarker activity of test fish during single exposures

Toxicant	Exposure period (h)	Test concentration (mg/ℓ)	Protein	AChE		Glucose	EROD	PK	LDH	G-6-P-DH	Hsp 70
				Microplate assay	Test kit						
			Induction (+) or inhibition (-) (%)								
Cadmium	8	0.05	-20	+29	ND 20*		+24	ND	ND	ND	+1
		0.1	-7	+44	ND	**-31***	-3	ND	ND	ND	+1
		0.2	-28	**+159***	ND	**-25***	**+34***	ND	ND	ND	**+16***
		0.4	**-76***	**+277***	ND	**+82***	**+214***	ND	ND	ND	+28
	24	0.011	+8	+28	ND	0	-33	ND	ND	ND	**-40***
		0.022	+2	**+188***	ND	+9	-17	ND	ND	ND	-9
		0.05	+4	**+12***	ND	+9	-9	ND	ND	ND	-1
		0.1	+4	**+64***	ND	+13	+4	ND	ND	ND	ND
	96	0.0031	-12	-42	ND	-29	+7	ND	ND	ND	+5
		0.0063	-29	-40	ND	+1	+6	ND	ND	ND	ND
		0.0125	+1	-28	ND	+8	**+41***	ND	ND	ND	**+41***
		0.025	+14	-39	ND	-11	**+26***	ND	ND	ND	**-39***
Zinc	24	0.25	+34	-55	+22	**-34***	**-37***	**-92***	**-44***	-80	-92
		0.5	+46	**-73***	**+109***	0	+13	+12	+2	ND	-42
		1.0	+47	**-76***	**+86***	0	**+41***	-17	-6	-30	ND
		2.0	+3	**-52***	**+132***	-2	**+22***	-29	-11	-81	-90
	96	0.125	+85	+30	+13	**-18***	+10	-35	**-33***	ND	-12
		0.25	+31	+24	-26	**-30***	8	-9	**-40***	ND	-13
		0.5	+51	-39	+14	**-31***	0	-42	**-37***	ND	**-66***
		1.0	-53	+64	+15	**-17***	+12	+9	-16	ND	**-61***
PCP	24	0.02	**-11***	-17	**-47***	+5	0	-43	**+62***	+60*	-72
		0.04	**-17***	+83	-30	-12	+8	-71	+8	+66	-58
		0.08	**-28***	0	**-45***	0	-32	-75	**+149***	-23	+126
		0.1		-33*	-22*	-1	+32	-89	**+144***	+19	-57
	96	0.00125	-36	-23	+33	+8	+62	-3	-19	**+152***	-5
		0.0025	-37	+368	+132	+30	+40	+45	-3	+33	-32
		0.005	-32	+627	**+121***	+8	+34	+41	+44	+56	-7
		0.01	-37	**+381***	**+97***	**+50***	+68	+69	**+44***	-36	-1
	96	0.00125	+7	+8	-47	-4	**+66***	+13	**+126***	-38	+190
		0.0025	+1	+50	-16	-8	**+79***	+10	**+122***	+39	+22
		0.005	-1	+25	+8	-6	**+72***	+17	**+131***	-26	-80
		0.01	+10	+50	+38	+2	**+77***	-20	**+149***	+3	-19
Cyanide	24	0.0625	**+27***	+50	+59	+18	+39	**+49***	+9	ND	**+26***
		0.125	**+62***	+25	+50	+28	+31	**+60***	+7	ND	-18
		0.25	**+25***	+75	+102	**+27***	+26	**+66***	+30	ND	+19
	24	0.0625	**+36***	**+133***	-86	+24	+4	**+31***	-86	ND	**+30***
		0.125	**+28***	**+100***	+17	+11	+20	**+67***	+17	ND	**+72***
		0.25	**+30***	**+67***	-11	+8	**+120***	+15	-11	ND	**+116***

ND **Not determined** **Results marked with an asterisk and in bold are significantly different from controls at P<0.05**

occurring during the experiment (Table 23). In general, glucose, PK and LDH were not significantly affected during the 24 h exposure studies. During 96 h exposure, glucose and LDH were significantly inhibited, while effects on PK were not significant. The upper test concentrations caused significant inhibitions in Hsp 70 activity during 96 h exposure. Some of the inhibiting effects observed during 24 h exposure were very high, but results could not be analysed for significance because triplicate data were not available. Large lethalities occurred in test and control fish during the 96 h exposure study (Table 23). This could have caused or contributed to the inhibitions observed in glucose, LDH and Hsp 70.

Protein was significantly inhibited during 24 h exposure to PCP (Table 25). Effects were not significant during longer exposure. AChE was significantly inhibited by some test concentrations during 24 h exposure, and significantly induced during 96 h exposure. Effects on glucose and EROD were not significant during 24 h exposure. Some of the upper test concentrations induced glucose and EROD during 96 h exposure. In general, PK and G-6-P-DH were not significantly affected by PCP. Significant inductions occurred in LDH activity during 24 and 96 h exposure. The Hsp 70 results were erratic, showing inhibition in some instances and induction in others. Since triplicate results were not available, an analysis of variance could not be carried out.

Protein was significantly induced by cyanide after 24 h exposure (Table 25). AChE showed significant induction in the microplate assay, while no significant effects were displayed with the test kit. Glucose, EROD, PK and Hsp 70 were significantly induced by some of the test concentrations, while effects on LDH were not significant. G-6-P-DH assays were not carried out because of limited sample volumes. The low oxygen concentrations detected in test and control containers during cyanide exposures (Table 23) could have stressed the fish, contributing to the increased protein, Hsp 70 and PK levels.

Total protein has been shown to be a useful biomarker in several fish exposure studies. Sastry and Shukla (1994) found that cadmium (11.2 mg/ℓ) inhibited the muscle protein of the fish *Channa punctatus* during 24 day exposure studies (-27%). The brain, gill, liver, muscle and blood protein of carp, killifish and catfish was also affected by mercury, cypermethrin and aldrin (Appendix 1). Reddy and Bashamohideen (1995) reported inhibitions of between 21 and 30% during 24 h exposure of fish to 20 µg/ℓ cypermethrin. No information could be found on the effect of the other chemicals on protein. The result obtained with 0.4 mg/ℓ cadmium during 8 h exposure (-76%) (Table 25) was in agreement with the above findings. However, no significant inhibition in protein was noticed at lower cadmium concentrations or during exposure to zinc. PCP significantly inhibited protein during 24 h exposure, while cyanide increased protein levels. The major variations observed in protein values could be an indication of protein degradation.

AChE activity is not only affected by organophosphate and carbamate pesticides. Adverse effects by several metals have been reported (Heath, 1995). The binding of metallic cations to AChE can alter enzyme activity by inhibiting or stimulating the catalytic function of the enzyme. Najimi *et al.* (1997) found AChE inhibition during cadmium and zinc exposure studies. The neurotoxin, PCP, has also been found to

inhibit AChE activity (Fossi *et al.*, 1995; Parks and LeBlanc, 1996). During our exposure studies, some of the zinc concentrations caused a significant inhibition in AChE activity when using the microplate inhibited by PCP during
24 h exposure, but induced during 96 h exposure. The enzyme was also induced by cadmium and cyanide during the shorter exposure periods.

Glucose levels can be increased or reduced. An increase in blood glucose levels (hyperglycaemia) is a general response to stress. During exposure to chemicals such as cadmium and zinc, the metal accumulates in the pancreatic isles of the fish and causes damage to the insulin-producing cells in the pancreas. The decease in insulin-secretion rate causes hyperglycaemia (Heath, 1995; Larsson *et al.*, 1976). When a reduction in glucose occurs during exposure to metals, it could be due to the depletion of liver glycogen or the excretion of glucose by the kidneys. Fish exposed to PCP tend to mobilise liver glycogen into blood glucose (glucose induction). PCP also enhances tissue metabolism and there is a possibility that PCP causes damage to the insulin producing beta cells in the pancreas (Heath, 1995). Sub-lethal concentrations of cyanide alter the carbohydrate metabolism by causing an increase in glycogen breakdown, elevation in blood glucose levels and a shift from aerobic to anaerobic metabolism in fish (Solomonson, 1981). In general, glucose was inhibited by cadmium and zinc during our exposure studies. Some PCP and cyanide concentrations caused *an increase in glucose.*

Organic chemicals cause the induction of EROD synthesis, but exposure to metals can lower the mixed function monooxygenase activity (Heath, 1995). Inhibition of cytochrome P450 1A mediated catalytic activity occurs when fish are exposed to high levels of cadmium (Jašić *et al.*, 1998; George, 1989; Livingstone, 1993). Fossi *et al.* (1995) found that the EROD enzyme activity was highly induced in fish exposed to PCP. The sensitivity of the cytochrome P450 system to cyanide is so low that this chemical has very little biological significance to the cytochrome P450 system. However, in a study by Solomonson (1981), P450 was inhibited by sub-acute levels of cyanide. In our studies (Table 25) EROD was induced by cadmium, instead of inhibited. The conflicting results could be due to the low cadmium concentrations used in our studies. EROD was also significantly induced by some of the zinc, PCP and cyanide concentrations.

Metals such as zinc were found to inhibit PK activity in fish (Isani *et al.*, 1994). PCP exposure causes significant alterations in metabolic enzyme activities. PCP enhances aerobic activity, which decreases glycolysis. PK is therefore reduced by PCP (Böstrom and Johansson, 1972). Cyanide inhibits aerobic respiration (Rattner and Heath, 1995; Lanno and Dixon, 1996). Glycolysis is therefore reduced with a concomitant reduction in PK. PK was not significantly inhibited during our cadmium, zinc and PCP exposure studies (Tables 25). PK was induced by cyanide, instead of reduced.

Metal exposure causes damage to gill tissue (Hoogstrand *et al.*, 1994). The resulting cell necrosis causes the release of LDH in the blood (Roijk *et al.*, 1983; Nemcsók and Benedeczky, 1995). Exposure to zinc should therefore induce LDH. PCP and cyanide have been found to inhibit LDH activity (Böstrom and Johansson, 1972; Rattner and Heath, 1995; Lanno and Dixon, 1996). Our results showed LDH inhibition by zinc instead of induction. PCP caused an induction in LDH instead of an inhibition as reported in literature.

G-6-P-DH is a biomarker for metabolic stress (Viganò *et al.*, 1995) and is not affected by metal contaminants. PCP enhances aerobic activity, which decreases glycolysis. As a result G-6-P-DH activity is increased due to higher substrate availability (Böstrom and Johansson, 1972). The activity of the pentose phosphate shunt enzyme, G-6-P-DH is significantly increased when fish are exposed to cyanide (Solomonson, 1981). Zinc exposure studies showed that G-6-P-DH was not affected by zinc, which was in agreement with the findings of Viganò *et al.* (1995). Likewise, the enzyme activity was generally not affected by PCP.

Information on the effect of chemicals on Hsp 70 activity in aquatic systems was limited. Vedel and Depledge (1995) found an induction of 47% in Hsp 70 in the gills of crab using 100 µg/ℓ copper and a two week exposure period. The significant inductions in Hsp 70 during some of the cadmium and the cyanide exposures are in agreement with the findings of Vedel and Depledge (1995). However, Hsp 70 showed inhibition during zinc exposure.

Comparison between lethal and sub-lethal effects

The results in Tables 23 and 25 show that the concentrations of cadmium and zinc that exhibited significant biomarker responses during 8 and 24 h exposure, caused lethality during the 96 h exposure. The biomarkers thus acted as early warning signals for acute toxicity. The significant responses detected during 96 h exposure, occurred at lethal concentrations. This suggested that the biomarkers were not sufficiently sensitive to detect the metals, cadmium and zinc, at sub-lethal concentrations. PCP caused significant biomarker responses at sub-lethal concentrations during shorter (24 h) and longer exposure (96 h) periods. The sensitivity of some biomarkers was enhanced by 10 to 60 times upon longer exposure. As in the case of metals and PCP, cyanide exhibited significant biomarker responses at sub-lethal concentrations during 24 h exposure. Unfortunately, no comparisons could be made with longer exposure periods, as only 24 h studies were carried out.

Regression analysis

The concentration-response curves obtained for some of the biomarker data sets in Table 25 are presented in Figures 1 to 4. In general, no significant concentration-response functions were observed after 24 and 96 h exposure. Curves either showed an upward-downward or downward-upward trend at increasing concentrations, or reached plateaus parallel to the x-axis (Figs. 2, 3 and 4). Similar concentration-response relationships were observed in a study by De Coen *et al.* (2001) on G-6-P-DH, PK and LDH activity in the crustacean *Daphnia magna* after 48 and 96-h exposure to mercury and lindane. Significant fits (linear regression) were obtained for all the

biomakers after 8 h exposure to cadmium (Figure 1), which allowed the calculation of toxicity endpoints. AChE, glucose, EROD and Hsp 70 showed elevated activity at higher test concentrations, while protein levels decreased.

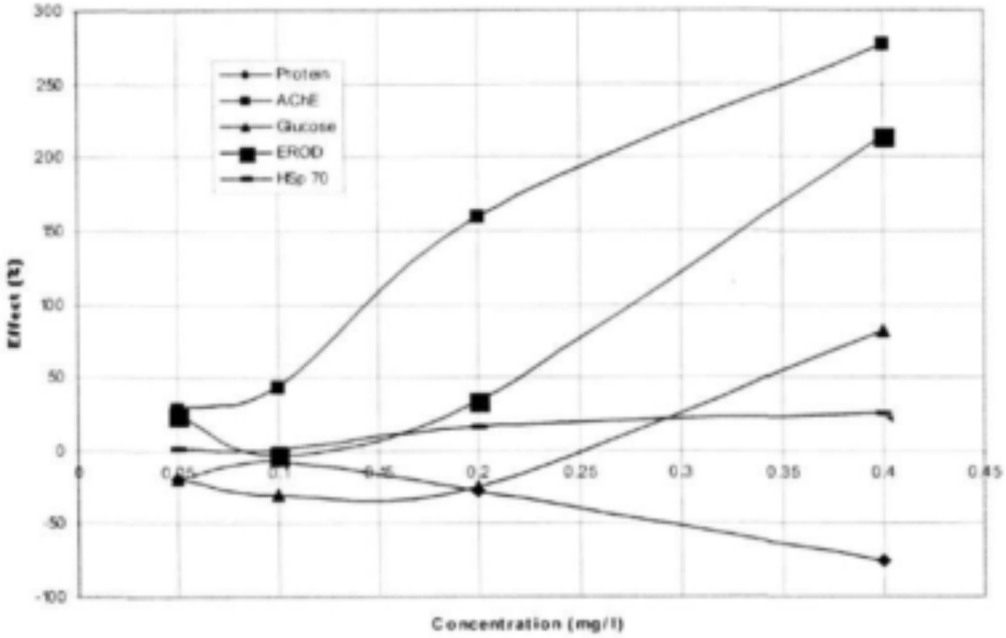

FIGURE 1: Concentration-response curves obtained for protein, AChE, glucose, EROD and Hsp 70 after 8 h exposure to cadmium

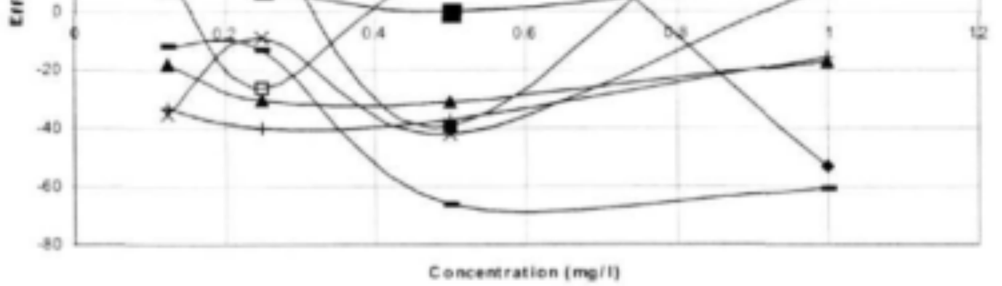

FIGURE 2: Concentration-response curves obtained for protein, AChE (M – microplate assay; T - test kit) glucose, EROD, PK, LDH and Hsp 70

after 96 h exposure to zinc

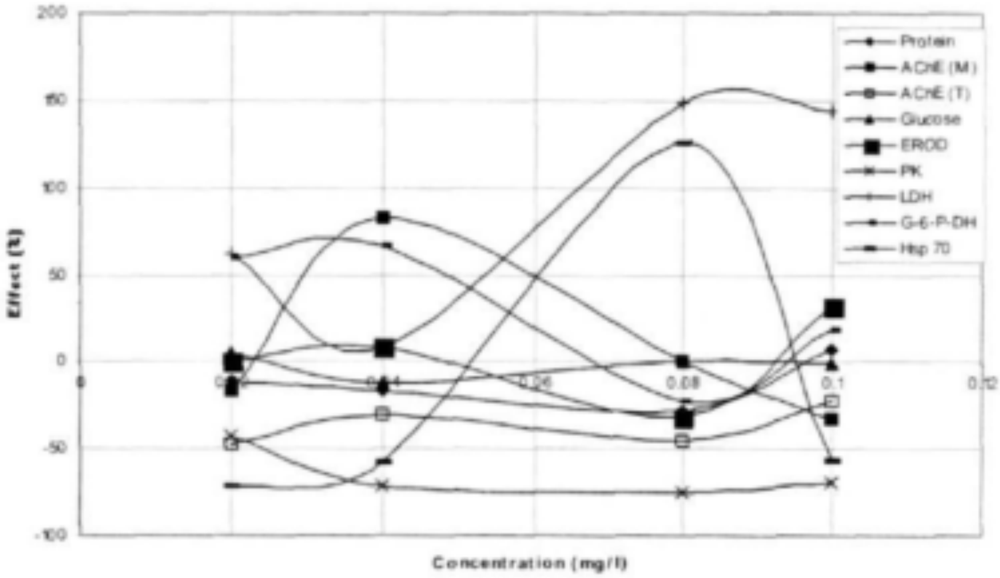

FIGURE 3: Concentration-response curves obtained for protein, AChE (M – microplate assay; T - test kit) glucose, EROD, PK, LDH, G-6-P-DH and Hsp 70 after 24 h exposure to PCP

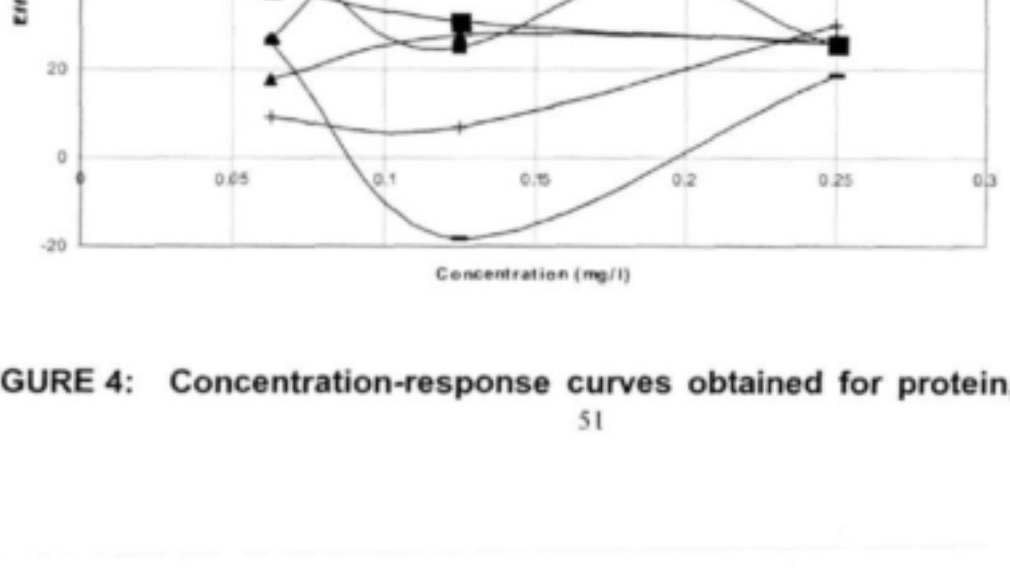

FIGURE 4: Concentration-response curves obtained for protein, AChE (M –

microplate assay; T - test kit) glucose, EROD, PK, LDH and Hsp 70 after 24 h exposure to cyanide (first of two experiments)

Toxicity endpoints

The toxicity endpoints (effect threshold values) derived by means of analysis of variance (LOEC - lowest of consecutive concentrations at which biomarker activity was significantly different from the control) (Table 25) and regression analysis (EC_{20} - *concentration causing a 20% induction or inhibition in biomarker activity*) (Figure 1) are summarised in Table 26.

Minimum effect concentrations of sub-lethal tests are usually expressed as an EC_{10} or EC_{20}. These toxicity endpoints are based on the variability of control results (Slabbert *et al.*, 1998a). Since large variations occurred in control biomarker activities (Tables 21 and 22), it was decided to use an EC_{20} rather than an EC_{10} as endpoint for regression analysis.

The 8-h LOECs of protein, AChE, EROD and Hsp 70 (Table 26) were between two to four times higher than the calculated EC_{20}s, while the LOEC of glucose was approximately four times lower than the EC_{20}. The endpoints differed because a fixed variation of 20% was used for the EC_{20} while the actual variations of test and control data used for the analysis of variance (LOECs) were higher (protein, AChE, EROD and Hsp 70) or lower (glucose). Although control biomarker variation in the study of De Coen *et al.* (2001) was generally <10%, the EC_{10}s (used as endpoint for regression analysis) were also between two to six times lower than the LOECs. Similar differences were noticed between the EC_{10}s and LOECs of 21-day *Daphnia* growth and reproduction tests (De Coen *et al.*, 2001). Because of the large differences in the control variation of the different biomarkers studied (section 4.1.2.1 and Table 21), and because regression analysis was not possible for most of the results, the use of a fixed endpoint such as an EC_{20} does not appear to be appropriate.

Table 26 shows that in some instances shorter exposure periods resulted in lower LOECs (e.g. cadmium 8-h LOEC for glucose; zinc 24-h LOECs for AChE and EROD), while in other instances LOECs were lower during longer exposure (e.g. cadmium 96-h LOECs for EROD and Hsp 70; zinc 96-h LOECs for glucose, LDH and Hsp 70; PCP 96-h LOECs for EROD and LDH).

The differences in sensitivity of duplicate experiments could be attributed to larger variations between replicate results, and therefore test results were not significantly different from control results, or to variability in the sensitivity of the test fish.

Triplicate exposures

The results of cadmium exposure studies (percentage induction or inhibition) carried out on triplicate sets of fish are shown in Table 27. In general, responses were similar to those observed during single exposure studies (Table 25). Cadmium was more toxic during 24 h exposure than during 96 h exposure, causing significant inductions at the 0.0025 and 0.025 mg/ℓ concentrations on AChE and EROD. As was found during single exposures, the concentrations of cadmium that exhibited significant biomarker responses during 24 h exposure, caused lethality during 96 h exposure (Tables 24 and 26).

TABLE 26: LOEC and EC$_{20}$ values obtained for the different biomarkers after 8, 24 and 96 h exposure to cadmium, zinc, PCP and cyanide

Toxicant	Exposure period (h)	Upper test concentration (mg/ℓ)	Toxicity endpoint (mg/ℓ)	Biomarker									
				Protein	AChE		Glucose	EROD	PK	LDH	G-6-P-DH	Hsp 70
					Micro-plate assay	Test kit						
Cadmium	8	0.4	LOEC	0.4	0.2	ND	≤0.05	0.2	ND	ND	ND	NT
			EC$_{20}$ (ind)	NA	0.0596	ND	0.2128	0.0825	ND	ND	ND	0.2818
			EC$_{20}$ (inh)	0.0886	NA	ND	NA	NA	ND	ND	ND	NA
	24	0.1	LOEC	NT	0.022	ND	NT	NT	ND	ND	ND	NT
	96	0.025		NT	NT	ND	NT	0.0125	ND	ND	ND	0.0125
Zinc	24	2.0		NT	0.5	0.5	NT	1.0	NT	NT	NT	NT
	96	1.0		NT	NT	NT	≤0.125	NT	NT	≤0.125	ND	0.5
PCP	24	1.0		NT	0.1	0.08	NT	NT	NT	0.08	NT	NT
	96	0.01		NT	0.01	0.005	0.01	NT	NT	0.01	NT	NT
	96	0.01		NT	NT	NT	NT	≤0.00125	NT	≤0.00125	NT	NT
Cyanide	24	0.25		≤0.0625	NT	NT	0.25	NT	≤0.0625	NT	ND	NT
	24	0.25		≤0.0625	≤0.0625	NT	NT	0.25	NT	NT	ND	≤0.0625

NA	Not applicable
ND	Not determined
NT	Not toxic
ind	20% induction in biomarker activity
inh	20

TABLE 27: Effect of cadmium on the biomarker activity of test fish during triplicate exposures

Exposure period (h)	Test concentration (mg/ℓ)	Protein	AChE	Glucose	EROD	PK	LDH
		Induction (+) or inhibition (-) (%)					
24	0.0025	+7	**+675***	**+42***	**+66***	+3	**-42***
	0.025	-11	**+269***	+2	**+123***	-39	-13
96	0.0025	-5	-13	+9	**-40***	-24	0
	0.025	-8	+33	+28	-7	-18	+27

Results marked with an asterisk and in bold are significantly different from controls at $P<0.05$

4.2 Field Studies

Field studies were carried out on the Rust de Winter Dam (reference site), Loskop Dam and Hartbeespoort Dam. Fish were sampled in January (summer) and June 2000 (winter). Where possible 20 adult fish (including males and females) were caught.

4.2.1 Physical-chemical quality of dam water

The water quality results of the different dams are presented in Table 28. The pHs of the samples were within the required limits for aquatic life. With the exception of the Rust de Winter Dam sample taken in winter, having a low pH of 6.3, the pHs were generally higher than those reported in Table 11 (historical data). The conductivity of the Rust de Winter Dam sample taken in winter was lower than the historical data (Table 11), while the conductivity obtained for the Loskop Dam summer sample was higher. DOs were low in the Rust de Winter and Hartbeespoort Dam samples taken in winter (3.0 and 3.2 mg/ℓ, respectively).

TABLE 28: Measured quality of dam water

Determinand	Rust de Winter Dam		Loskop Dam		Hartbeespoort Dam	
	Summer	Winter	Summer	Winter	Summer	Winter
Date	14/01/00	05/06/00	15/01/00	06/06/00	08/02/00	08/06/00
Temperature (°C)	25.7	15.5	26.3	18.1	23.1	16.8
pH	8.3	6.3	9.2	8.6	9.3	8.5
DO (mg/ℓ)	11.1	3.0	12.3	5.2	7.6	3.2
Conductivity (mS/m)	17.8	13.6	40.9	30.9	51.0	48.0
TDS (mg/ℓ)	88.9	ND	203	308	233	ND

ND Not determined

4.2.2 Fish weight, length and gender

Table 29 shows the weight, length and gender of the fish collected in the various dams. The lengths and weights of the fish collected during summer in the different dams were all significantly different ($P<0.05$) from each other (fish in Loskop Dam were the largest, followed in order of magnitude by fish in Rust de Winter Dam and fish in Hartbeespoort Dam). The length of the fish collected in winter in the Loskop Dam were significantly different from that of the fish in Rust de Winter Dam, while there was no significant difference between the length of the fish in the Rust de Winter Dam and the Hartbeespoort Dam and between the length of the fish in the Loskop Dam and Hartbeespoort Dam. The weight of the fish collected in winter were all significantly different from each other (fish in Loskop Dam were the largest, followed in order of magnitude by fish in Hartbeespoort Dam and fish in Rust de Winter Dam). There was no significant difference between the sizes (lengths and weights) of the fish collected in summer and winter in the Rust de Winter Dam. However, the fish collected in winter in the Loskop and Hartbeespoort Dams were significantly larger in winter than in summer. Differences in fish size can result in different biomarker levels (Chen *et al.*,1998). For the best comparison it is important to collect the same size fish at reference and polluted sites.

TABLE 29: Weight, length and gender of fish present in dams

Parameter		Rust de Winter Dam		Loskop Dam		Hartbeespoort Dam	
		Summer	Winter	Summer	Winter	Summer	Winter
Number of fish		18	20	20	20	15	15
Length (cm)	Range	28.5 to42.0	25.0 to43.0	35.0 to47.0	36.0 to49.0	23.0 to34.0	28.5 to47.0
	Mean	35.8	37.9	39.0	41.4	24.9	40.3
	CV (%)	11	11	9	8	11	12
Weight (kg)	Range	0.5 to1.7	0.23 to1.6	0.75 to2.1	1.15 to2.5	0.3 to 0.55	1.1 to 1.9
	Mean	0.90	1.07	1.21	1.71	0.36	1.36
	CV (%)	36	28	34	22	18	28
Gender (number)	Male	5	11	2	11	3	6
	Female	13	9	18	9	12	8

During the summer sampling period, most of the fish were female, while in winter the distribution was more equal.

4.2.3 Biomarker activity in fish present in different dams

The biomarker activity in fish collected from dams are expressed in terms of protein. All the organs except the brain was analysed for protein. Protein was determined with the micro Bradford assay. The protein levels detected in whole blood, erythrocytes, blood plasma, and liver homogenate are summarised in Table 30.

Protein levels were the highest in erythrocytes, followed in order of magnitude by whole blood, blood plasma and liver. The whole blood protein levels were higher in Rust de Winter and Loskop Dam fish sampled in winter, while the levels were similar in

Hartbeespoort Dam fish. Erythrocyte levels were lower in Rust de Winter and Loskop Dam fish sampled in winter, but higher in the Hartbeespoort Dam fish sampled in winter. Blood plasma protein were slightly lower in fish sampled in winter. Liver protein was higher in winter in Rust de Winter and Hartbeespoort Dam fish, but similar in Loskop Dam fish.

The erythrocyte protein in replicate fish showed the least variation (CVs: 15 to 30%). Large variations occurred between the results obtained for whole blood, blood plasma and liver protein (CV: whole blood - 18 to 52%; erythrocytes - 5 to 85%; liver - 25 to 52%).

Protein in fish homogenates (Table 20) were between 10 and 50 times lower than the protein levels in whole blood, between 5 and 25 times lower than the protein levels in blood plasma and between 3 and 20 times lower than the protein in liver.

TABLE 30: Protein levels found in whole blood, erythrocytes, plasma and liver of fish collected from dams

Dam	Season	Measure	Whole blood	Erythro-cytes	Blood plasma	Liver
Rust de Winter	Summer	Number of samples	18	17	18	18
		Mean (mg/mℓ)	63.60	326.94	53.39	29.09
		CV (%)	36	86	26	39
	Winter	Number of samples	19	20	17	20
		Mean (mg/mℓ)	130.27	225.12	47.15	43.07
		CV (%)	27	12	20	27
Loskop	Summer	Number of samples	19	19	19	20
		Mean (mg/mℓ)	58.36	509.77	57.10	34.57
		CV (%)	30	55	27	37
	Winter	Number of samples	20	20	20	20
		Mean (mg/mℓ)	123.60	259.37	46.27	34.83
		CV (%)	52	19	15	26
Hartbees-poort	Summer	Number of samples	14	15	14	15
		Mean (mg/mℓ)	123.35	184.11	53.27	18.78
		CV (%)	18	5	30	52
	Winter	Number of samples	14	14	14	14
		Mean (mg/mℓ)	127.75	302.11	42.883	39.90
		CV (%)	35	52	19	25

CV Coefficient of variation

Biomarker levels in fish sampled from different dams are shown in Tables 31 and 32. All the biomarkers, except ions (mmol/ℓ) and AChE in brain (U/ℓ), are expressed as mg protein. Single assays were carried out on all the samples because of limited sample volumes. ALA-D activity was determined in whole blood and AChE in erythrocytes

Table 31: Biomarker levels found in fish present in different dams during summer

Dam	Measure	Whole blood	Erythrocytes	Plasma					Liver					Brain
		ALA-D activity (U/mg protein)	AChE activity (abs/min/ mg protein)	Glucose level (mg/mg protein)	LDH activity (U/ℓ/mg protein)	Osmotic ions (mmol/ℓ)			EROD activity (nM/min/ mg protein)	G-6-P-DH activity (mU/mℓ/ mg protein)	Glycogen level (mg/100 mg liver)	LDH activity (U/ℓ/mg protein)	PK activity (mU/mℓ/ mg protein	AChE activity (U/ℓ)
						Calcium	Potassium	Sodium						
Rust de Winter	Number of samples	18	15	18	18	18	15	18	18	17	18	16	17	18
	Range	4.51 to 32.03	0.0001 to 0.003	0.013 to 0.064	0 to 0.0246	0.88 to 1.86	6.6 to 9.8	146 to 167	0.345 to 1.169	0 to 33.84	0.050 to 23.74	0 to 0.0314	0 to 0.6241	0.1017 to 1.8221
	Mean	16.09	0.0006	0.024	0.0080	1.34	8.15	155	0.813	4.67	567.0	0.0090	0.1692	0.9884
	CV (%)	47	121	54	97	27	12	5	33	219	112	95	138	51
Loskop Dam	Number of samples	19	14	17	19	19	12	19	20	20	20	19	20	20
	Range	0.82 to 21.14	0.00003 to 0.0006	0.012 to 0.038	0 to 0.0136	0.87 to 1.98	8.0 to 10.0	146 to 197	0.36 to 1.79	0 to 31.98	31.24 to 1 228.76	0.0049 to 0.0160	0 to 0.0553	0 to 1.517
	Mean	5.60	0.0002	0.022	0.0035	1.55	8.9	168	1.011	6.79	515.39	0.0110	0.2290	0.5928
	CV (%)	78	73	36	102	22	7	8	33	142	65	32	93	59
	Significant effect?	Yes (inh)	No	No	No	No	No	Yes (inc)	No	ND	No	No	No	No
Hartbeespoort Dam	Number of samples	14	15	14	13	13	7	13	15	15	15	15	15	13
	Range	2.60 to 15.63	0.00006 to 0.001	0.001 to 0.047	0.0059 to 0.0274	0.54 to 1.40	6.0 to 9.7	138 to 163	0.539 to 1.538	0-93 to 53	4.8 to 576.0	0.0084 to 0.0583	0 to 1.0623	0.2268 to 1.1808
	Mean	7.73	0.0006	0.017	0.0122	0.87	8.3	150	1.059	25.57	86.0	0.0249	0.1241	0.7267
	CV (%)	14	47	76	53	33	16	5	27	125	172	67	229	37
	Significant effect ?	Yes (inh)	No	No	Yes (ind)	Yes (red)	No	Yes (inc)	No	ND	No	Yes (ind)	No	No

CV	Coefficient of variation
ND	Not determined
Ind	Induction
Inh	Inhibition
Red	Reduction
Inc	Increase

Table 32: Biomarker levels found in fish present in different dams during winter

Dam	Measure	Whole blood	Erythrocytes	Plasma					Liver					Brain
		ALA-D activity (U/mg protein)	AChE activity (abs/min/mg protein)	Glucose level (mg/mg protein)	LDH activity (U/ℓ/mg protein)	Osmotic ions (mmol/ℓ)			EROD activity (nM/min/mg protein)	G-6-P-DH activity (mU/mℓ/mg protein)	Glycogen level (mg/100 mg liver)	LDH activity (U/ℓ/mg protein)	PK activity (mU/m/mg protein)	AChE activity (U/ℓ/mg protein)
						Calcium	Potassium	Sodium						
Rust de Winter	Number of samples	19	17	17	16	17	15	17	20	20	20	20	20	20
	Range	0.094 to 2.535	0.0002 to 0.001	0.005 to 0.023	0 to 0.0189	0.95 to 1.44	4.4 to 9.9	154 to 182	0.453 to 2.063	0 to 23.02	766.86 to 2 554.38	0 to 0.0067	0 to 0.3208	0.5318 to 1.3138
	Mean	1.22	0.0005	0.016	0.0046	1.10	5.8	170	0.874	4.33	1 349.86	0.0020	0.0579	0.7609
	CV (%)	52	54	34	119	14	25	4	54	170	32	98	164	22
Loskop Dam	Number of samples	17	18	20	19	20	20	12	19	19	20	19	19	19
	Range	0.152 to 5.095	0.0002 to 0.001	0.006 to 0.033	0 to 0.0139	1.89 to 2.68	2.9 to 7.2	182 td 196	0.070 to 1.356	0 to 36.04	684.162 to 3 428.12	0 to 0.0174	0 to 1.6798	0.5396 to 1.2903
	Mean	0.91	0.0005	0.021	0.0043	2.16	4.46	189	0.493	6.47	1 450.89	0.0044	0.2200	0.9170
	CV (%)	126	51	35	104	11	22	3	77	154	41	111	198	20
	Significant effect?	No	No	Yes (ind)	No	Yes (inc)	Yes (red)	Yes (inc)	Yes (inh)	ND	No	No	No	Yes (ind)
Hartbeespoort Dam	Number of samples	11	11	14	12	14	14	7	14	14	14	14	14	14
	Range	0.181 to 3.415	0.0001 to 0.001	0.012 to 0.057	0.005 to 0.0302	1.74 to 2.44	3.9 to 6.3	186 to 200	0.849 to 2.350	0 to 40.71	208.354 to 2 571.01	0 to 0.0111	0 to 0.8099	0.43 to 2.0879
	Mean	1.12	0.0005	0.024	0.0083	2.06	4.6	192	1.295	9.00	1 569.39	0.0034	0.2207	1.0697
	CV (%)	90	57	47	102	11	14	3	29	150	39	105	124	39
	Significant effect ?	No	No	Yes (ind)	No	Yes (inc)	No	No	Yes (ind)	ND	No	No	Yes (ind)	No

CV	Coefficient of variation
ND	Not determined
Ind	Induction
Inh	Inhibition
Red	Reduction
Inc	Increase

(miain homogenate (test kit). Blood plasma was analysed for glucose and LDH activity, and osmotic ion concentrations. EROD, PK, LDH, G-6-P-DH and glycogen assays were carried out on liver samples. Hsp 70 was not determined. The tables indicate where significant differences ($P<0.05$) between the Rust de Winter samples (reference) and the other dam samples (polluted) occurred.

ALA-D activity was not significantly affected in winter, while the summer samples showed inhibition. AChE in blood showed no significant induction or inhibition at any of the sampling occasions. AChE in the brain of fish of Loskop Dam was induced in winter. The Loskop Dam and Hartbeespoort Dam fish showed increased glucose levels in blood plasma in winter, but not in summer. The AChE activity in brain was considerably higher than the AChE activity in blood. LDH activity in blood plasma was not affected in winter, but the fish collected from the Hartbeespoort Dam in summer showed induced activity. The sodium levels were significantly higher in the blood of Loskop Dam and Hartbeespoort Dam fish in summer, while the calcium in the blood of Hartbeespoort Dam fish was significantly lower. Potassium levels were unaffected. An increase in blood calcium was noticed in Loskop Dam and Hartbeespoort Dam fish in winter. The Loskop Dam fish collected in winter also showed a significant reduction in potassium but an increase in sodium. EROD activity was induced in the liver of fish of Loskop Dam and Hartbeespoort Dam in winter, while no significant effects occurred in summer. Liver glycogen was not affected at any of the sampling occasions. LDH liver activity was induced in Hartbeespoort Dam fish during summer. No significant changes were noticed in the fish in winter. The LDH activity in Loskop Dam fish was not affected. In general, the LDH levels in the liver and blood were similar. The liver PK activity was not significantly affected in summer, but in winter the Hartbeespoort Dam fish showed an induction. PK results showed large variation (CV: 93 to 229%). G-6-P-DH results could not be calculated because of extreme variations (CV: 125 to 219%) and because a large percentage of the fish samples showed an absence of the biomarker activity.

The inhibition of ALA-D activity is specific for lead (Wepener, 1990; Johansson-Sjöbeck and Larsson, 1979) and a reduction in the enzyme activity occurs rapidly and can be detected at exposure concentrations near the 'no effect' level (Schmitt *et al.*, 1984). Fish exposed to other heavy metals such as cadmium, copper, zinc and mercury showed no erythrocyte ALA-D inhibition. The effects exhibited on ALA-D in fish collected from Loskop Dam and Hartbeespoort Dam suggest the possible presence of lead in the water.

AChE activity in fish is often referred to as one of the most successful examples of a clinical test in aquatic toxicology. AChE activity is not only affected by organophosphate and carbamate compounds but also by heavy metals such as zinc, mercury, cadmium and copper (Heath, 1995). Sharp reductions in the enzyme activity may also be caused by non-toxic stressors. The inhibition of blood AChE activity is usually short lived and more definitive results are obtained with brain AChE (Melancon, 1995). This is possibly the reason why brain AChE in Loskop Dam fish was affected and not the blood AChE.

For continuous exposure studies, blood sampling has the advantage that the organism does not have to be sacrificed and samples can be taken from the same organism over an extended period of time.

Blood glucose levels are elevated in fish during exposure to various pollutants, including pesticides. These stressful stimuli elicit the rapid secretion of both hormones, glucocorticoids and catecholamines from the adrenal tissue of fish, producing rapid hyperglycemia (Cerón *et al.*, 1996). Short-term changes in glucose levels can be induced by handling stress, changes in temperature, pH, water velocity, hypoxia, or other seasonal variations (Folmar, 1993). Differences in blood glucose levels can also be attributed to differences in rate and degree of digestion, absorption and utilisation of glucose, i.e. impaired carbohydrate metabolism (Hilmy and Shabana, 1980). The effects on glucose collected from Loskop Dam and Hartbeespoort Dam could have been due to chemical pollutants in the water or to the seasonal change.

A stress response in fish is generally characterised by an increase in adrenalin causing mobilisation of liver glycogen into blood glucose (Swallow and Flemming, 1970). This means lower glycogen levels and higher glucose levels. However, the blood glucose levels do not necessarily reflect the level of glycogen (Love, 1980), as was seen in our study (no significant change in liver glycogen).

Muscle tissue damage, organ damage and cell necrosis leads to the release of specific enzymes, such as LDH, in the bloodstream. Increased liver LDH levels is thus accompanied by similar increases in plasma enzyme activity. Plasma LDH reveals the degree of tissue damage, but does not indicate the specific sites of damage (Nemcsók and Benedeczky, 1995). Fish handling can also increase LDH levels. When fish struggle in nets, there is a strain on the muscles. The demand for energy as ATP is increased, which depletes glucose levels. This leads to lactate production and an increase in LDH activity (Everse and Kaplan, 1973). Seasonal changes (e.g. increased temperature) can also induce this enzyme activity (Sauer and Haider, 1977). General stress due to pollutants may cause induced LDH activity. The LDH containing tissue is however not damaged by metal pollutants (Mayer *et al.*, 1992). Loskop Dam, that is mainly polluted by heavy metals, showed no significant changes in LDH activity. The increased LDH levels in the blood and liver of the Hartbeespoort Dam fish collected during summer could be attributed to pollutants other than metals or to seasonal effects. Food shortages have been reported to inhibit the enzyme activity (Blier and Guderley, 1986).

Osmoregulation is influenced by various biotic and abiotic factors, which complicate monitoring (Mayer *et al.*, 1992). Chronic pollution does not always cause changes in ion levels, due to acclimatisation of animals by means of adaptive mechanisms. Plasma ion concentrations react differently to different types of pollutants (Heath, 1987). Some chemicals cause increased ion levels and others decrease ions levels, depending on the structure of the chemical. Osmotic ions can, therefore, be used as non-specific biomarkers to indicate adverse conditions in natural systems. Organic pollutants cause fluctuations in plasma sodium and chloride levels (Mayer *et al.*, 1992), while metals influence osmotic balance by competing for calcium binding sites on the gill surface, or by inhibiting sodium/potassium-ATPase (Heath, 1987). Metals such as cadmium, copper, zinc and manganese have the largest effect on calcium (McDonald *et al.*,1989), but pesticide exposure could also damage the bronchial epithelium and inhibit calcium uptake (Singh *et al.*, 1996).

Organic pollutants like PAHs, PCBs and dioxins as well as complex chemical mixtures including municipal and industrial effluents cause induction in EROD activity (Jimenez and Stegeman, 1990). EROD activity can also be inhibited. Heavy metals have been found to inhibit cytochrome P4501A in fish hepatoma cells (Chen *et al.*, 1998; Gagné and Blaise, 1993). Copper inhibits enzyme activity by binding to SH-residues of the enzymatic proteins of the MFO system, or by enhancing lipid peroxidation of the membranes. Environmental variables such as seasonal changes, which are associated with temperature and sexual factors, age and nutritional status of the fish, are the most important influences on the MFO activity of fish (Jimenez and Stegeman, 1990). The induction in EROD activity by Loskop Dam and Hartbeespoort Dam water could have been due to chemical pollutants such as those mentioned, or to the seasonal change.

influenced by environmental factors. The efficiency of PK as an *in situ* biomarker is thus questioned by researchers (Mayer *et al.*, 1992) and more research is recommended to prove its value. Generally, metal or organic pollution causes a decrease in PK activity in fish (Mayer *et al.*, 1992). Metals inhibit this enzyme by displacing the cofactor magnesium and thus reducing phosphoryl transfer (Hansen *et al.*, 1992). PK activity is also affected by exercise and nutritional status (Johnston and Moon, 1980).

5. CONCLUSIONS AND RECOMMENDATIONS

- In broad terms, the objectives of the project, namely 1) to establish biological techniques to detect sub-lethal toxicity in the aquatic environment, and 2) to produce an operational manual on the established methodologies, were met. However, certain important issues, discussed below, were not adequately addressed.

- Certain biomarker assays were successfully applied to the homogenates of 2 to 4 week old tilapia (*O. mossambicus*) used in laboratory toxicity tests, as well as to the organs of tilapia collected from the field.

- Although whole fish homogenates contained low levels of the studied biomarkers as a result of the dilution of enzyme levels in organs, the levels were sufficiently high to detect induction and inhibition in exposed laboratory fish.

- The biomarker assay protocols were relatively easily established in the participating laboratories, because well-established, published methodologies were used. The miniaturisation of some of the methods improved assays in terms of rapidity (small sample volumes and large numbers of samples), simplicity and cost. However, some of the tests, especially the test kits, appeared to be incompatible with whole fish homogenates, showing large variation in ome instances.

- The inter-assay and inter-experimental variation in laboratory and field studies were very high for some of the biomarkers. Operational manipulation (sample preparation and assaying) and test method limitation (tissue compatibility) appeared to be the most likely causes of the variation. Sample storage played a minor role.

- Apart from operational manipulation and test method limitation, factors such as handling stress and the development stage of the fish could have contributed to the variation in biomarker values of field studies. It is therefore important to minimise stress and select fish of similar size/age when conducting field surveys.

- The use of replicate sets of fish (e.g. fish divided over triplicate containers) and replicate assays in laboratory exposure studies is recommended, to reduce variability. Replicate assays should also be carried out on fish of field studies to improve variability.

- It is also expected that variability will be significantly reduced if the biomarker assays are carried out by a well-trained operator in a laboratory with well-established quality assurance protocols and practices.

- Because of the large differences in the control variation of the different biomarkers studied, and because no clear concentration-response functions were identified for most of the laboratory results, the use of a fixed endpoint such as an EC_{10} or an EC_{20} is not recommended. The LOEC, derived by means of an analysis of variance, proved to be a more appropriate sub-lethal toxicity endpoint.

- Although there was some degree of inconsistency in the results of laboratory exposure studies, sufficient evidence was obtained that biomarkers could be applied as sub-lethal measures of toxic activity in laboratory toxicity tests.

- Based on the data obtained for different exposure periods, it is recommended that the sub-lethal fish toxicity test is carried out over 96 h. Any effects on biomarkers due to handling and stress manifested during the shorter exposure periods should have subsided after 96 h. A longer exposure period might result in higher sensitivity but fish will have to be fed, which will include other factors that could affect biomarker activity. Because growth and reproduction are very sensitive measures of chronic (sub-lethal) toxicity in fish and are used in a 7-day test, such a test should rather be performed than a longer test employing biomarkers.

- The results of 8 and 24 h tests indicated that certain biomarkers can serve as early warning signals for acute toxicity. It is recommended that further studies are carried out (organism/cellular/sub-cellular level) to exploit the potential of biomarkers as rapid screening tests.

- Based on a combination of features such as sensitivity, selectivity, simplicity, repeatability and rapidity, the following biomarker assays are recommended for the detection of sub-lethal responses in the 96 h laboratory toxicity test: Protein (micro Bradford method), AChE (microplate assay), glucose, EROD, PK, LDH and Hsp 70. ALA-D (whole blood), AChE (microplate assay) (erythrocytes and brain), glucose (plasma), glycogen (liver), LDH (plasma and liver), EROD (liver), osmotic ions (plasma) and Hsp 70 are recommended for field use. Hsp 70 was not carried out on field samples, but because of its high acclaim in literature and promising results in laboratory studies this marker is recommended for field use. The Hsp 70 assay is the most complex and time consuming of the assays. All the assays require relatively expensive equipment (e.g. spectrophotometers). The biomarkers should be used in battery form to allow for optimal detection of harmful pollutants. The test protocols are outlined in the Operational Manual (Venter *et al.,* 2003).

- The AChE microplate assay is preferred to the AChE test kit because of higher reproducibility, less fluctuation and more reliable data calculation. Likewise, the micro Bradford method (microplate) is preferred to the macro method because of rapidity and less variability.

- The G-6-P-DH assay is not recommended for use because it requires large sample volumes and produces unreliable and highly variable results. PK is not recommended for field use because of high variability and because its potential as an *in situ* marker is questioned by other researchers.

- It is recommended that fish weight is used to express biomarker activity in the laboratory toxicity test, instead of protein, because of smaller variation and fluctuation in results, and because protein acted as biomarker.

- A specific volume of buffer was added to a certain number of fish for homogenation. Because weight increases with age, this approach resulted in considerable fluctuation in laboratory control (unexposed fish) values. It is, therefore, recommended to base the volume of buffer on weight rather than on the number of fish.

- The limited number of offspring obtained for tilapia maintained in the laboratory and the inconsistency in reproduction was a major constraint. As a result

  - ✓ elaborate experimental designs were not possible;
  - ✓ experiments were not repeated a sufficient number of times;
  - ✓ experiments were not carried out in replicate;
  - ✓ wide concentration ranges were not examined; and
  - ✓ a range of chemicals were not tested.

  Consequently, the sub-lethal test using biomarkers as endpoints was not sufficiently validated to enable informed decisions on its potential as a routine laboratory test.

- At this stage the sub-lethal test protocols, outlined in the Operational Manual (Venter *et al.*, 2003) is only applicable for qualitative studies. Protocols were optimised, but extensive testing/evaluation is required before the methods can be standardised for specific applications.

- The established biomarkers should be used to complement fish lethality data and not to substitute chronic measurements such as growth and reproduction. Likewise, the biomarker approach is not a replacement for conventional field assessments, but a supplementary tool with great ecological relevance.

- Limited fish numbers is a major concern regarding the success of this fish as test organism in acute and sub-lethal toxicity tests. National breeding facilities should be established to ensure a consistent and healthy supply of the fish. In addition, other fish species, with the potential to regularly provide large numbers of offspring, should be investigated as test organisms.

- Tilapia were present in large numbers in the dams studied. This might not be the case in other environments. Before studying biomarkers in organisms in the field, their abundance should be established to ensure sufficient replicates.

- Although the biomarkers were not applied to other test organisms in the field (e.g. worms, snails) as was initially envisaged, the established biomarker assays should be applicable to other organisms, including other fish, with little or no method modification.

- Since biomarkers have been proven to be useful measures of sub-lethal stresses, it is recommended that additional biomarkers, including metallothioneins, are investigated for application in laboratory and field studies.

- The present laboratory study was only conducted on individual chemicals. It is recommended that the study be extended to environmental samples (drinking water, effluents, sediments, etc) to evaluate the applicability of the biomarkers with reference to complex chemical mixtures.

- As the biomarker techniques have become more established for aquatic studies, more countries are applying such measures in continuous monitoring programmes. This approach should also be valuable for our National Toxicants Monitoring Programme that is currently being developed. Molluscs have been found excellent test organisms in such studies. It is, therefore, recommended that funds are invested in the establishment of an *in situ* biomarker test using a local mollusc.

- The concept of using a multi-organisational team seemed like a good idea to build capacity and transfer knowledge. However, this resulted in large delays in the execution of experiments. The use of students that came and went, instead of well-trained technicians, resulted in large inter-assay and inter-experimental variation which could not be remedied. It is recommended that any future bioassay technique development is carried out in one laboratory, and that the use of students in complex studies is avoided.

6. REFERENCES

Anderson, P.D. and Spear, P.A. (1980) Copper pharmakinetics in fish gills. I. Kinetics in pumpkinseed sunfish, *Lepomis gibossus*, of different body sizes. *Wat. Res.* **14**, 1101 - 1110.

Bainy, A.C.D., Woodin, B.R. and Stegeman, J.J. (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir-São Paulo, Brazil. *Aquat. Toxicol.* **44**, 289 - 305.

Beisenherz, Von G., Boltze, H.J., Bücher, Th., Czok, R., Garbade, K.H., Meyer-Arendt, E. und Pfleiderer, G. (1953) Diphosphofructose-aldolase, Phosphoglyceraldehyd-dehydrogenase, Milchsäure-dehydrogenase, Glycerophosphat-dehydrogenase und Pyruvat-kinase aus Kaninchenmuskulature in einem Arbeitsgang. *Z. Naturforschg.* **8b**, 555 - 577.

Besselink, H.T., Van Santen, E., Vorstman, W., Vethaak, A.D., Koeman, J.H. and Brouwer, A. (1997) High induction of cytochrome P4501A activity without changes in retinoid and thyroid hormone levels in flounder (*Platichthys flesus*) exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Environ. Toxicol. Chem.* **16**, 816 - 823.

Blier, P. and Guderley, H. (1986) The enzymatic and metabolic effects of extended food deprivation in *Rana pipiens. Physiol. Zool.* **59**, 230 - 239.

Böstrom, S.L. and Johansson, R.G. (1972) Effects of pentachlorophenol on enzymes involved in energy metabolism in the liver of eel. *Comp. Biochem. Physiol.* **41B**, 359 - 369.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248 - 254.

Burke, M.D. and Mayer, R.T. (1974) Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Disp.* **2**, 583 - 588.

Butty, M., Walmsley, R.D. and Alexander, C.J. (1980) The limnology of some selected South African impoundments. Water Research Commission Report, Pretoria, 71 - 80.

Carpene, E., Cortesi, P., Tacconi, S., Cattani, O., Isani, G. and Serrazanetti, G.P. (1987) Cd-Metallothionein and metal-enzymes interactions in the goldfish, *Carassius auratus. Comp. Biochem. Physiol.* **86C**, 267 - 272.

Cerón, J.J., Sancho, E., Ferrando, M.D., Gutierrez, C. and Andreu, E. (1996) Metabolic effects of diazinon on the European eel *Anguilla anguilla. J. Environ. Sci. Health* **B31**, 1029 - 1040.

Chen, C.-M., Ueng, T.-H, Wang, H.-W, Lee, S.Z. and Wang, J.S. (1998) Microsomal monooxygenase activity in milkfish (*Chanos chanos*) from aquaculture ponds near metal reclamation facilities. *Bull. Environ. Contam. Toxicol.* **61**, 378 - 383.

Clarke, G.M. (1969) *Statistics and Experimental Design.* Arnold, London, 148 pp.

Davidson, M.W. (1995) Molecular expressions: The pesticide collection - pentachlorophenol. World Wide Web, accessed on 08/01/03.
http://www.micro.magnet.fsu.edu/pesticides/pages/pentachlo.html.

Decaprio, A.P. (1997) Biomarkers: Coming of age for environmental health and risk assessment. *Environ. Sci. Tech.* **31**, 1837 - 1848.

De Coen, W.M., Janssen, C.R. and Segner, H. (2001) The use of biomarkers in *Daphnia magna* toxicity testing. V. *In vivo* alterations in the carbohydrate metabolism of *Daphnia magna* exposed to sublethal concentrations of mercury and lindane. *Ecotoxicol. Environ. Safety* **48**, 223 - 234.

Depledge, M.H. (1993) The rationale basis for the use of biomarkers as ecotoxicological tools. In: *Non-destructive Biomarkers in Vertebrates,* edited by M.C. Fossi and C. Leonzio, Lewis Publishers, Boca Raton, Florida, 271 - 295.

Depledge, M.H. and Fossi, M.C. (1994) The role of biomarkers in environmental assessment. 2. Invertebrates. *Ecotoxicol.* **3**, 161 - 172.

DWA&F (1996a) *South African Water Quality Guidelines - Agricultural Use: Aquaculture.* 2nd Ed., Vol. 6, edited by S. Holmes, Department of Water Affairs and Forestry, Pretoria, 194 pp.

DWA&F (1996b) *South African Water Quality Guidelines - Aquatic Ecosystems.* 2nd Ed., Vol. 7, edited by S. Holmes, Department of Water Affairs and Forestry, Pretoria, 155 pp.

Ellman, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88 - 92.

Everse, J. and Kaplan, N.O. (1973) Lactate dehydrogenases: Structure and function. In: *Advances in Enzymology.* Vol. 37, edited by A. Meister, John Wiley & Sons, New York.

Folmar, L.C. (1993) Effects of chemical contaminants on blood chemistry of teleost fish: A bibliography and synopsis of selected effects. *Environ. Toxicol. Chem.* **12**, 337 - 375.

Fossi, M.C., Focardi, S., Leonzio, C., Gavilán, J.F. and Parra, O. (1995) Use of biomarkers to evaluate the effects of xenobiotic compounds in the Biobio basin (central Chile). *Bull. Environ. Contam. Toxicol.* **55**, 36 - 42.

Gagné, F. and Blaise, C. (1993) Hepatic metallothionein level and mixed function oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. *Wat. Res.* **27**, 1669 - 1682.

George, S.G. (1989) Cadmium effects on plaice liver xenobiotic and metal detoxification systems: dose-response. *Aquat. Toxicol.* **15**, 303 - 310.

Gendron, A.D., Bishop, C.A., Fortin, R. and Hontela, A. (1997) In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (amphibia) exposed to chlorinated hydrocarbons in the wild. *Environ. Toxicol. Chem.* **16**, 1694 - 1706.

Gill, T.S., Tewari, J. and Pande, J. (1990) Use of the fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. *Comp. Biochem. Physiol.* **97C**, 287 - 292.

Gill, T.S., Tewari, J., Pande, J. and Las, S. (1991) *In vivo* tissue activities in the rosy barb *(Barbus conchonius* Hamilton) experimentally exposed to lead. *Bull. Environ. Contam. Toxicol.* **47**, 939 - 946.

Grimsehl, T.P. and Pullen, R.A. (1991) Water resource planning of the Olifants River Basin. Report No. PB V100/00/0591, Vol. 3, Part 3, Department of Water Affairs and Forestry, Pretoria.

Hansen, P.D. and Addison, R.F. (1990) The use of mixed function oxygenase (MFO) to support biological effects monitoring in the sea. Int. Council Explor, Sea E:33, Marine Environmental Quality Committee.

Hansen, J.I., Mustafa, T. and Depledge, M. (1992) Mechanisms of copper toxicity in the shore crab, *Carcinus maenas*. II. Effects on key metabolic enzymes, metabolites and energy charge potential. *Mar. Biol.* **114**, 259 - 264.

Heath, A.G. (1987) Some introductory concepts. In: *Water Pollution and Fish Physiology*. CRC Press Inc., Boca Raton, Florida, 245 pp.

Heath, A.G. (1995) Approaches to understanding sublethal effects of contaminants to larval fishes. In: *Contaminant Effects on Fish*, Proceedings of the International Congress on the Biology of Fishes, edited by B. Barton and D. MacKinley.

Heath, R.G.M. and Claassen, M. (1999) An overview of the pesticide and metal levels present in populations of the larger indigenous fish species of selected South African rivers. Report No. 428/1/99, Water Research Commission, Pretoria, 318 pp.

Hilmy, A.M. and Shabana, M.B. (1980) Blood chemistry levels after acute and chronic exposure to $HgCl_2$ in the killifish *Aphanius dispar* (rupp). *Wat. Air Soil Pollut.* **14**, 409 - 417.

Ho, J.W. and Ho, A.W. (1997) Environmental lead exposure induces changes in the heme biosynthetic pathway. *Environ. Toxicol. Wat. Qual.* **12**, 245 - 248.

Hoogstrand, C., Wilson, R.W., Polgar, D. and Wood, C.M. (1994) Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. *J. Exp. Biol.* **168**, 55 - 73.

Huang, T.L., Obih, P.O., Jaiswal, R., Hartley, W.R. and Thiyagarajah, A. (1997) Evaluation of liver and brain esterases in the spotted gar fish (*Lepisosteus oculatus*) as biomarkers of effect in the lower Mississipi River Basin. *Bull. Environ. Contam. Toxicol.* **58**, 688 - 695.

Hwang, P.P. and Yang, C.H. (1997) Modulation of cadmium pretreated tilapia *(Oreochromis mossambicus)* larvae. *Fish Physiol. Biochem.* **16**, 403 - 410.

Ibrahim, H., Kheir, R., Helmi, S., Lewis, J. and Crane, M. (1998) Effects of organophosphorus, carbamate, pyrethroid and organochlorine pesticides, and a heavy metal on survival and cholinesterase activity of *Chironomus riparius* Meigen. *Bull. Environ. Contam. Toxicol.* **60**, 448 - 455.

Isani, G., Cattani, O., Carpene, E. and Cortesi, P. (1994) Kinetic properties of liver and muscle pyruvate kinase of a marine teolost, sea bass (*Dicentrarchus labrax* L.). *Comp. Biochem. Physiol.* **107B**, 617 - 624.

Jašić, Z., Bihari, N., Müller, W.E.G., Zahn, R.K. and Batel, R. (1998) Modulation of cytochrome P4501A in sea bass liver by model substances and seawater extracts. *Aquat. Toxicol.* **40**, 265 - 273.

Jimenez, B.D. and Stegeman, J.J. (1990) Detoxification enzymes as indicators of environmental stress on fish. In: *Biological Indicators of Stress in Fish*, Proceedings of the American Fisheries Symposium 8, edited by S.M. Adams, Bethesda, Maryland, 67 - 79.

Johansson-Sjöbeck, M.-L. and Larsson, Á. (1978) The effect of cadmium on the haematology and on the activity of δ–aminolevulinic acid dehydratase (ALA-D) in blood and hematopoietic tissues of the flounder, *Pleuronectes flesus* L. *Environ. Res.* **17**, 191 - 204.

Johansson-Sjöbeck, M.-L. and Larsson, Á. (1979) Effect of inorganic lead on delta-aminolevulinic acid dehydratase activity and hematological variables in the rainbow trout, *Salmo gairdneri. Arch. Environ. Contam. Toxicol.* **8**, 419 - 431.

Johnston, I.A. and Moon, T.W. (1980) Exercise training in skeletal muscle of brook trout (*Salvelinus fontinalis*). *J. Exp. Biol.* **87**, 177 - 194.

Joubert, A. (2000) Evaluation of biomarkers for pollution in *Oreochromis mossambicus*: A laboratory and field study. M.Sc. Thesis, RAU, 116 pp.

Kargin, F. and Cogun, H.Y. (1991) Metal interactions during accumulation and elimination of zinc and cadmium in tissues of the freshwater fish, *Tilapia nilotica. Bull. Environ. Contam. Toxicol.* **63**, 511 - 519.

Kim, W.S. Jeon, J.K., Lee, S.H. and Huh, H.T. (1996) Effects of pentachlorophenol (PCP) on the oxygen consumption rate of the river puffer fish *Takifugu obscurus*. *Mar. Ecol. Progr. Ser.* **143**, 9 - 14.

Kornberg, A. and Horecker, B.L. (1955) Glucose-6-phosphate dehydrogenase. *Meths. Enzymol.* **1**, 323 - 327.

Lanno, R.P. and D.G. Dixon. (1996) The comparative chronic toxicity of thiocyanate an *Aquat. Toxicol.* **36**, 177 - 187.

Larsson, Á., Bengtsson, B.-E. and Svanberg, O. (1976) Some haematological and biochemical effects of cadmium on fish. In: *Effects of Pollutants on Aquatic Organisms*, edited by A.P.M. Lockwood, Cambridge University Press, Cambridge, 35 - 45.

Leduc, G. (1981) Ecotoxicology of cyanides in freshwater. In: *Cyanide in Biology*, edited by B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley and F. Wissing, Academic Press Inc., London, 548 pp.

Lee, H.S., Hwang, P.P. and Lin, H.C. (1996) Morphological changes of integument chloride cells to ambient cadmium during the early development of the teleost, *Oreochromis mossambicus*. *Environ. Biol. Fishes* **45**, 95 - 02.

Livingstone, D.R. (1993) Biotechnology and pollution monitoring: Use of molecular biomarkers in the aquatic environment. *J. Chem. Tech. Biotech.* **57**, 195 - 211.

Love, R.M. (1980) *The Chemical Biology of Fishes. Advances 1968-1977*, Vol. 2, Academic Press Inc., London, 943 pp.

Mayer, F.L., Versteeg, D.J., McKee, M.J., Folmar, L.C., Graney, R.L., McCume, D.C. and Rattner, B.A. (1992). Physiological and non-specific biomarkers. In: *Biomarkers: Biological, Physiological and Histological Markers of Anthropogenic Stress*, edited by R.J. Huggett, R.A. Kimerke, P.M. Mehrle, Jr. and H.L. Bergman, Lewis Publishers, Boca Raton, Florida, 235 - 335.

McDonald, D.G., Reader, J.P. and Dalziel, T.R. (1989) The combined effects of pH and trace metals on fish ionoregulation. In: *Acid Toxicity and Aquatic Animals*, edited by R. Morris, E.W. Taylor, D.J.A. Brown and J.A. Brown, Cambridge University Press, Cambridge.

Melancon, M.J. (1995) Bioindicators used in aquatic and terrestrial monitoring. In: *Handbook of Ecotoxicology*, edited by D.J. Hofmann, B.A. Rattner, G.A. Burton, Jr. and J. Cairns, Jr., Lewis Publishers, London.

Müller, W.E.G., Koziol, C., Kurelec, B., Dapper, J., Batel, R. and Rinkevich, B. (1995) Combinatory effects of temperature stress and non-ionic organic pollutants on stress protein (Hsp70) gene expression in the freshwater sponge *Ephydatia fluviatilis*. *Environ. Toxicol. Chem.* **14**, 1203 -1208.

Murphy, S.D. (1980) Pesticides. In: *Toxicology: The Basic Science of Poisons.* 2nd Ed., edited by J. Doull, C.D. Klaassen and M.O. Amdur, MacMillan Publishing Company, New York, 357 - 407.

Murthy, V.K., Reddanna, P., Bhaskar, M. and Govindappa, S. (1981) Muscle metabolism of freshwater fish, *Tilapia mossambica* (Peters), during acute exposure and acclimation to sublethal acidic water. *Can. J. Zool.* **59**, 1909 - 1915.

Murty, A.S. (1986) *Toxicity of Pesticides to Fish.* Vol. 2, CRT Press, Florida.

Najimi, S., Bouhaimi, A., Daubèze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F. and Moukrim, A. (1997) Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir Marine Bay (South of Morocco). *Bull. Environ. Contam. Toxicol.* **58**, 901 - 908.

Nemcsók, J. and Benedeczky, I. (1995) Pesticide metabolism and the adverse effects of metabolites on fishes. In: *Environmental and Ecological Biochemistry*, edited by P.W. Hochachka and T.P. Mommsen, Elsevier Science, Amsterdam.

NRC. (1989) *Biological Markers in Reproductive Toxicology.* National Academy Press-1X, Washington DC.

Nussey, G. (1994) The effect of copper on the blood coagulation and general haematology of *Oreochromis mossambicus* (Cichlidae). M.Sc. Thesis, RAU.

Nussey, G. (1998) Metal ecotoxicology of the upper Olifants River at selected localities and the effect of copper and zinc on fish blood physiology. Ph.D. Thesis, RAU.

Parks, L.G. and LeBlanc, G.A. (1996) Reductions in steroid hormone biotransformation/elimination as a biomarker of pentachlorophenol chronic toxicity. *Aquat. Toxicol.* **34**, 291 - 303.

Peakall, D. (1992) *Animal Biomarkers as Pollution Indicators.* Ecotoxicological Series I, Chapman and Hall, London and New York, 290 pp.

Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M. and Wendelaar B.S.E. (1997) Calcium fluxes in juvenile tilapia, *Oreochromis mossambicus,* exposed to sublethal waterborne Cd, Cu or mixtures of these metals. *Environ. Toxicol. Chem.* **16**, 770 - 774.

Pratap, H.B. and Wendelaar, B.S.E. (1993) Effect of ambient and dietary cadmium on pavement cells, chloride cells and Na^+/K^+ATPase activity in the gills of the freshwater teleost *Oreochromis mossambicus* at normal and high calcium levels in the ambient water. *Aquat. Toxicol.* **26**, 133 - 150.

Rand, G.M. and Petrocelli, S.R. (Eds.) (1985) Chapter 1: Introduction. In: *Fundamentals of Aquatic Toxicology.* Hemisphere Publishing Corporation, USA, 1 - 8.

Rattner, B.A. and Heath, A.G. (1995) Environmental factors affecting contaminant toxicity in aquatic and terrestrial vertebrates. In: *Handbook of Ecotoxicology*, edited by

D.J. Hoffman, B.A. Rattner, G.A. Burton, Jr. and J. Cairns, Jr., Lewis Publishers, Boca Raton, Florida.

Reddy, P.M. and Bashamohideen, Md. (1995) Alterations in protein metabolism in selected tissues of fish, *Cyprinus carpio*, during sublethal concentration of cypermethrin. *Environ. Monit. Assess.* **36**, 183 - 190.

Rojik, I., Nemcsók, J. and Boross, L. (1983) Morphological and biochemical studies on liver, kidney and gill of fishes affected by pesticides. *Acta Biol. Hungar.* **34**, 81 - 92.

Rossouw, J.D. (1992) Krokodilrivier (Wes-Transvaal) opvangsgebiedstudie. Beskrywing van die fisiese waterstelstel, Verslag Nr. PA200/00/1292, Departement van Waterwese en Bosbou, Pretoria.

Roux, D.J. (1994) Role of biological monitoring in water quality assessment and a case study on the Crocodile River, Eastern Transvaal. M.Sc. Thesis, RAU,130 pp.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual.* 2nd Ed., Cold Spring Harbour Laboratory Press, New York.

SANCOLD (South African National Committee on Large Dams). (1978) *Typical Large Dams in South Africa*, CIGB ICOLD.

Sastry, K.V. and Shukla, V. (1994) Acute and chronic toxic effects of cadmium on some haematological, biochemical, and enzymological parameters in the fresh water teleost fish *Channa punctatus. Acta hydrochim. hydrobiol.* **22**, 171 - 176.

Sauer, D.M. and Haider, G. (1977) Enzyme activities in the serum of rainbow trout, *Salmo gairdneri* Richardson; the effects of water temperature. *J. Fish Biol.* **11**, 605 - 612.

Schaller, K.-H. and Berlin, A. (1984) δ-Aminolaevulinate dehydratase. In: *Methods of Enzymatic Analysis. Enzymes 2: Esterases, Glycosidases, Lyases, Ligases.* 3rd Ed., Vol. IV, edited by H.U. Bergmeyer, J. Bergmeyer and M. Grass, Verlag Chemie, Weinheim, 363 - 368.

Schmitt, C.J., Dwyer, F.J. and Finger, S.E. (1984) Bioavailability of Pb and Zn from mine tailings as indicated by erythrocyte δ-aminolevulinic acid dehydratase (ALA-D) activity in suckers (Pisces: Catostomidae). *Can. J. Fish. Aquat. Sci.* **41**, 1030 - 1040.

Seifter, S., Dayton, S., Novic, B. and Muntwyler, E. (1950). The estimation of glycogen with anthrone reagent. *Arch. Biochem.* **25**, 191 - 200.

Shugart, L.R. (1996) Biological Markers. In: *Ecotoxicology, a hierachial treatment*, edited by M.C. Newman and C.H. Jagoe, Lewis Publishers, Boca Raton, Florida.

Singh, N.N., Das, V.K. and Singh, S. (1996) Effect of aldrin on carbohydrate, protein and ionic metabolism of freshwater catfish, *Heteropneustes fossilis. Bull. Environ. Contam. Toxicol.* **57**, 204 - 210.

Skelton, P.H. (1993) *'n Volledige gids tot die varswatervisse van Suider-Afrika.* Southern Publishers, Halfway House, 387 pp.

Slabbert, J.L. and Pienaar, E. (1998) Fish culturing methods for local use. Report No. ENV-P-I 98022, Division of Water, Environment and Forestry Technology, CSIR, Pretoria.

Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. (1998a) Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa. Report No. 358/1/98, Water Research Commission, Pretoria.

Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. (1998b) Development of procedures to assess whole effluent toxicity. Report No. 453/1/98, Water Research Commission, Pretoria.

Slabbert, J.L., Venter, E.A., Pienaar, E. and Bouwer, L. (1999) Water resource assessment tools - Biotoxicology. Report No. ENV-P-I 98202, Division of Water, Environment and Forestry Technology, CSIR, Pretoria.

Soengas, J.L., Agra-Lago M.J., Carballo, B., Andrés, M.D. and Veira, J.A.R. (1996) Effect of an acute exposure to sublethal concentrations of cadmium on liver carbohydrate metabolism of Atlantic salmon (*Salmo salar*). *Bull. Environ. Contam. Toxicol.* **57**, 625 - 631.

Solomonson, L.P. (1981) Cyanide as a metabolic inhibitor. In: *Cyanide in Biology*, edited by B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley and F. Wissing, Academic Press Inc., London, 548 pp.

Spry, D. and Weiner, J.G. (1991) Metal bioavailability and toxicity to fish in low-alkalinity lakes: A critical review. *Environ. Pollut.* **71**, 243 - 304.

Swallow, R.L. and Fleming, W.R. (1970) The effect of oxaloacetate, ACTH and cortisol on the liver glycogen levels of *Tilapia mossambica. Comp. Biochem. Physiol.* **36**, 93 - 98.

Teh, S.J., Adams, S.M. and Hinton, D.E. (1997) Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. *Aquat. Toxicol.* **37**, 51 - 70.

Trinder, P. (1969) *Ann. Clin. Biochem.* **6**, 24.

US EPA. (1991) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 4th Ed., EPA-600/4-90/027, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

Vedel, G.R. and Depledge, M.H. (1995) Stress-70 levels in the gills of *Carcinus maenus* exposed to copper. *Mar. Pollut. Bull.* **31**, 84 - 86.

Venter, E.A., Slabbert, J.L., Joubert, A., Vorster, A. and I. Barnhoorn. (2003) Biomarker assays for the detection of sub-lethal toxicity in fish - Operational Manual. Report No. 952/2/03, Water Research Commission, Pretoria.

Viarengo, A. (1985) Biochemical effects of trace metals. *Mar. Pollut. Bull.* **16**, 153 - 158.

Viganò, L., Arillo, A., De Flora, S. and Lazorchak, J. (1995) Evaluation of microsomal and cytosolic biomarkers in a seven-day larval trout sediment toxicity test. *Aquat. Toxicol.* **31**, 189 - 202.

Vijayan, M.M., Pereira, C., Kruzynski, G. and Iwama, G.K. (1998) Sublethal concentrations of contaminant induce the expression of hepatic shock protein 70 in two salmonids. *Aquat. Toxicol.* **40**, 101 - 108.

Vorster, A. (2000) Evaluation of selected enzymes and osmotic ions as biomarkers for detecting pollution in the aquatic environment. M.Sc. Thesis, RAU, 112 pp.

Wagner, G.F. and McKeown, B.A. (1982) Changes in plasma insulin and carbohydrate metabolism of zinc-stressed rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **60**, 2079 - 2084.

Way, J.L. (1981) Pharmacology aspects of cyanide and its antagonism. In: *Cyanide in Biology*, edited by B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley and F. Wissing, Academic Press Inc., London, 548 pp.

Weichselbaum, T.E. (1946) An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Amer. J. Clin. Path.* **16**, 40 - 49.

Weis, P. and Weis, J.S. (1991) The developmental toxicity of metals and metalloids in fish. In: *Metal Ecotoxicology Concepts and Applications*, edited by M.C. Newman and A.W. McIntosh, Lewis Publishers, Michigan.

Wepener, V. (1990) Die effek van swaarmetale by variërende pH op die bloedfisiologie en metaboliese ensieme van *Tilapia sparrmanii* (Cichlidae). M.Sc. Thesis, RAU.

Wepener, V. (1997) Metal Ecotoxicology of the Olifants River in the Kruger National Park and the effect thereof on fish haematology. Ph.D. Thesis, RAU.

Yawetz, A., Benedek-Segal, M. and Woodin, B. (1997) Cytochrome P4501A immunoassay in freshwater turtles and exposure to PCBs and environmental pollutants. *Environ. Toxicol. Chem.* **16**, 1802 - 1806.

Z. klin. Chem. u klin. Biochem. (1970) **8**, 658; (1972) **10**, 182.