Ecotoxicology, 13, 667–681, 2004

An *In Situ* Bioassay Integrating Individual and Biochemical Responses Using Small Fish Species

BRUNO BRANCO CASTRO,[1,2] OLÍMPIA SOBRAL,[1,2] LÚCIA GUILHERMINO[1,3] AND RUI RIBEIRO[1,*]
[1]*Departamento de Zoologia da Universidade de Coimbra, Instituto do Ambiente e Vida, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal*
[2]*Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123 Porto, Portugal*
[3]*ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Departamento de Estudos de Populações, Largo Abel Salazar 2, 4099-003 Porto, Portugal*

Accepted 3 December 2003

Abstract. The interest in the ecological relevance of risk assessments and, thus, in *in situ* bioassays has been increasing in the last years. The present study developed a time- and cost-effective *in situ* bioassay, aiming at obtaining, in a short period of time and with a minimum of resources, a set of ecologically relevant toxicological information in a site-specific approach. *Poecilia reticulata* and *Gambusia holbrooki* were chosen as test species. Post-exposure feeding inhibition and the biomarkers acetylcholinesterase, lactate dehydrogenase and glutathione *S*-transferases were the endpoints tested. The battery of biomarkers as a whole was sensitive to the *in situ* exposure in an acid mine drainage impacted effluent, although responses varied between test species. Post-exposure feeding inhibition was the most sensitive endpoint, and its association with biomarker responses was discussed. The linkage between individual responses, such as feeding, and biomarkers suggested that, at least in this case, biomarkers can be relevant at higher levels of biological organization. Altogether, the proposed short-term *in situ* bioassay seems to be a promising tool, since it represents a reasonable compromise between sensitivity, time/cost-effectiveness and ecological relevance.

Keywords: in situ bioassay; feeding inhibition; enzymatic biomarkers; *Poecilia reticulata*; *Gambusia holbrooki*

Introduction

In situ bioassays are useful tools in ecotoxicology, mainly because they integrate ecological relevance in toxicity testing, by incorporating field fluctuations (within semi-controlled conditions) in a cost-effective way. Fish have broadly been employed in *in situ* testing, and test chambers and general procedures have long been described (e.g., Wilde and Parrot, 1984; Jones and Sloan, 1989). Ideally, researchers should work with toxicity estimates derived from reproductive and demographic endpoints, especially in risk assessment.

*To whom correspondence may be addressed:
E-mail: rui.ribeiro@zoo.uc.pt.

However, prolonged field exposures are required to estimate sublethal/chronic toxicity. Such an *in situ* bioassay is neither cost- nor time-effective, and difficulties arise from a practical viewpoint. Therefore, a preferable alternative is to perform short-term field exposures and, afterwards, to quantify relevant sublethal endpoints capable of responding in such a short period of time. This has been accomplished by using biochemical or other subcellular responses (i.e., biomarkers) as test endpoints. Comprehensive field work has been developed in this area, namely in field caging experiments (Lindström-Seppä and Oikari, 1990; Soimasuo et al., 1995, 1998; Goksøyr et al., 1996; Fenet et al., 1998; Stien et al., 1998; Pacheco and Santos, 1999), as well as in biomonitoring surveys (e.g., Goksøyr et al., 1996; Van der Oost et al., 1996; Kosmala et al., 1998; Wall et al., 1998; Stephensen et al., 2000; Sturm et al., 2000), both measuring biochemical markers in several fish species. In general, these studies were performed with large species, such as salmonids (Lindström-Seppä and Oikari, 1990; Fenet et al., 1998), flatfish (Goksøyr et al., 1996; Wall et al., 1998) or eels (Van der Oost et al., 1996; Fenet et al., 1998; Pacheco and Santos, 1999). Although small fish do not provide such large amounts of tissue for biomarker quantification, they are preferred for logistic reasons (e.g., less complex test-chambers), as well as for usually having shorter life cycles.

In spite of their rapid responsiveness and sensitivity to contaminant exposure, biomarkers have questionable ecological relevance, as a result of being endpoints at a low level of biological organization (Peakall, 1992). Therefore, *in situ* bioassays using biochemical markers should combine them with more comprehensive sublethal variables, such as growth or behavioral responses (swimming activity, feeding, etc.), as test endpoints. Behavioral endpoints are commonly recognized as individually based responses resulting from the integration of a multiplicity of biochemical and/or physiological processes (Peakall, 1992; Heath, 1995). Feeding responses are usually regarded as behavioral endpoints, although effects on such variables may extend beyond their behavioral outcome. Any eventual depression in feeding rate will have implications in the energetic input, and thus in the growth, reproduction, or survival of the individual, and may, subsequently, have adverse effects at the population/community level (Kumar and Chapman, 1998; Taylor et al., 1998).

The aim of this study was to develop a sensitive and ecologically relevant short-term *in situ* bioassay with small fish species, which could work as an early warning tool, combining organismal (post-exposure feeding) and biochemical (biomarkers) responses. The biomarkers chosen were acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and glutathione *S*-transferases (GST). An acid mine drainage impacted aquatic reservoir was the location selected for deploying the *in situ* bioassays. It was also the objective of this work to establish a relationship between biochemical and organismal responses, in an effort to clarify the significance of the set of biomarkers at higher levels of organization.

Mosquitofish (*Gambusia holbrooki*) and guppy (*Poecilia reticulata*), both belonging to Poeciliidae, were used as test species in the *in situ* bioassay. *G. holbrooki* is an abundant fish species in European freshwaters, ever since its introduction in mosquito-control programs (Holcík, 1991; Almaça, 1995; García-Berthou, 1999). It occupies a key intermediate position in the trophic chain of lakes, since it preys on insects and zooplankton, while being eaten by piscivorous fish (some of commercial value) (Cabral et al., 1998; García-Berthou, 1999). *P. reticulata* is a standard test-organism, recommended by EEC (1992) and OECD (1982) guidelines for toxicity testing. Additionally, poeciliids are indigenous inhabitants of tropical inland waters, and there are substantial possibilities for using these methodologies in tropical countries, where rapid and ecologically relevant toxicological information is most needed.

Materials and methods

Study sites

The aquatic system surrounding São Domingos mine – an abandoned copper mine – in the SE of Portugal, was the location chosen for the deployment of the *in situ* bioassays. The mine effluent is strongly acidic due to the continuous oxidation of abandoned mine tailings, and contains large amounts of heavy metals (in decreasing order: Fe, Al, Zn, Cu, Mn, Co, Ni, Cd, Pb, Cr, As) (Lopes

et al., 1999; Pereira et al., 1999, 2000). After some dilution due to a small watercourse, the untreated effluent enters the Chança River reservoir (part of the Guadiana River basin) near an ancient dam. The source of contamination (pH and heavy metals) is isolated and well identified, since no other significant contamination sources are known (e.g., pesticides, industrial discharges or urban runoffs). Furthermore, there is extensive literature on the biology, ecology and water chemistry of the system (Pereira et al., 1995, 1999, 2000; Ribeiro et al., 1995; Lopes et al., 1999). Four sites were chosen to perform the *in situ* assays: three in the mine effluent, along the contamination gradient (S3, S2 and S1, from most to least contaminated), and a reference site (REF), located in a good water quality upstream lake (Fig. 1). This lake has no significant sources of contamination and it supports substantial communities of fish, amphibians, reptiles, birds, and mammals (Ribeiro et al., 1995). Apart from pH and metal concentrations, water chemical characteristics of REF were similar to those of contaminated sites. Furthermore, it has been successfully used as a reference site in previous *in situ* bioassays (Pereira et al., 1999, 2000).

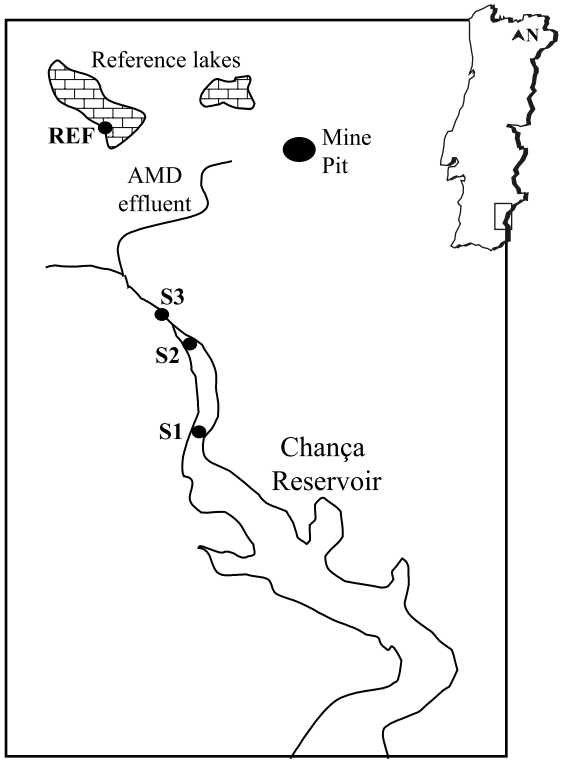

Figure 1. Schematic representation of the study area (São Domingos mine), showing the location of reference and contaminated sites.

Test organisms

Male adult guppies (*P. reticulata*), 2.0–2.5 cm standard length, were obtained from a local supplier and acclimated in the laboratory, for almost 2 weeks, to a temperature of 25 ± 2 °C and a photoperiod of 16 h L:8 h D. Fish were all from the same strain with the purpose of reducing genetic variability, and thus increasing experimental precision. Concern on this matter, especially when dealing with biochemical responses, such as enzymatic biomarkers, was already expressed in other studies (Garcia et al., 2000). Guppies were kept in glass aquaria with ASTM hardwater and were fed a commercial flake food diet (TetraMenu-Tetra-Werke, Germany) *ad libitum*, two times a day (the amount of food added was consumed 2–3 min subsequently to its addition). In the improvised field laboratory, guppies were subjected to a 3-day acclimation period, prior to testing. Initially, they were acclimated to field temperature fluctuations (25–30 °C), followed by gradual acclimation to filtered (150 μm) reference water. Only male guppies were used in the *in situ* bioassay, thus eradicating the chance of accidentally introducing an exotic species into Portuguese waters.

G. holbrooki were caught in the reference lake (REF) using hand nets (mesh size 1.0 mm). Adult males of appropriate size (2.0–2.5 cm long) were separated from the rest and kept in aerated containers with filtered (150 μm) reference water, until the deployment of the *in situ* bioassays (less than 24 h).

In situ *bioassay chamber design and general protocol*

In situ bioassay chambers were made from 1.5-l polyethylene terephthalate bottles (transparent), which are easily available and can be obtained at almost no cost. Although the sorption of organic contaminants was not a matter of concern in the present study, it may possibly occur in future studies elsewhere. An *in situ* pre-exposure of the test-chambers in local water can surpass this

shortcoming. Two lateral rectangular openings and a circular opening at the bottom were made on the bottles and covered with 1.0-mm nylon mesh (Fig. 2), allowing water flow inside the test-chambers. Ideally, the mesh-size used in *in situ* bioassay chambers should be small enough to prevent test-organisms from escaping, and large enough to allow a proper water flow/renewal and aeration (Sibley et al., 1999). A 1.0-mm mesh, as the one used in the present study, satisfied both these demands, although it did not retain autochthonous food sources (e.g., local zooplankton). The mesh was sealed to the chamber with white thermal glue (supplied by Elis-Taiwan, Taiwan, ref. TN122/WS, with a chemical composition of 50% ethylene–vinyl–acetate copolymer, 45% synthetic hydrocarbon, and 5% polyethylene wax), which has been shown to be non-toxic to cladocerans (Pereira et al., 1999).

Following the placement of the chambers *in situ*, organisms were introduced through the top opening of the bottle using a funnel-like apparatus. A polyethylene terephthalate screw cap was then placed on the top opening, and chambers were anchored to the shore with nylon rope. Two sets of two air-filled 1.5-ml microtubes attached to the outside of the chamber with transparent adhesive tape acted as floaters (Fig. 2), guaranteeing the buoyancy of the bioassay chamber in an appropriate position. The top opening is potentially useful if, in future studies, feeding the fish becomes necessary (e.g., growth bioassays). After unscrewing the cap, chambers were held in vertical position (cap up) and withdrawn from the water except for the last 5 cm (approximately). This enabled "trapping" the organisms in a confined volume of water, allowing a proper observation of the fish through the top opening, and therefore a clear assessment of their condition.

Short-term in situ bioassay with adult fish

The 4-d *in situ* bioassay was performed at S1, S2, S3 and REF with adult male *P. reticulata* and *G. holbrooki*. For both species, three fish per test chamber were used. Two sets of test chambers were placed in each site as described above: 2×3 chambers per site for the guppy bioassay; 2×4 chambers for the *Gambusia* bioassay. Organisms underwent a 24-h starvation period prior to the deployment of the *in situ* bioassay. No food was added to the chambers during the test. Physical–chemical parameters were measured and registered at the beginning and end of the *in situ* bioassay. Conductivity was measured with a WTW LF 330 conductivity meter (Reagente 5, Porto, Portugal), and pH measurements were performed using a WTW 340-A pH meter, with a SenTix electrode (Reagente 5). Dissolved oxygen concentrations were determined with a WTW OXI 320 oxygen meter (Reagente 5). A min/max thermometer was used to monitor water temperature fluctuations during the assay.

At the end of the test, test-chambers and fish were brought to the field laboratory, in local water, for further processing. Organisms from the first set of chambers were killed by decapitation for biomarker quantification (see below); gills, head, and dorsal muscles were immediately removed. This procedure was performed in ice-cold

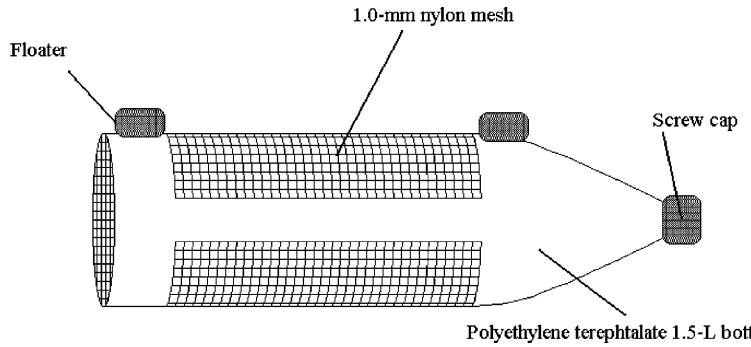

Figure 2. In situ bioassay chambers were made from transparent 1.5-l polyethylene terephthalate bottles. Three openings (2 lateral and 1 at the base), covered with 1-mm nylon mesh, allowed proper water flow. The *in situ* bioassay started with the introduction of the organisms (3 per test chamber) in the chambers, which were previously placed near the water surface.

phosphate buffer (0.1 M, pH = 7.2) and took, in average, about 3 min per fish. After removal of blood vestiges, each tissue was placed in the appropriate buffer for each biomarker: gills in phosphate buffer (0.1 M, pH = 6.5) for glutathione *S*-transferases determinations; head in phosphate buffer (0.1 M, pH = 7.2) for acetylcholinesterase analysis; dorsal muscle in Tris–NaCl buffer (0.1 M, pH = 7.2) for lactate dehydrogenase determinations. Samples were minced using a scissors and frozen at −20 °C (head and muscles) or in liquid nitrogen (gills). Fish from the second set of chambers were used in post-exposure feeding trials (see below), except for S3, where high mortalities resulted in an insufficient number of organisms to perform the test.

Biomarker quantification

In the laboratory, frozen samples were thawed and homogenized using an Ystral GmbH Dottingen homogeniser (Reagente 5). Samples were always kept on ice, as well as throughout all subsequent processes, until the enzymatic assay. Gill homogenates were centrifuged at $9000 \times g$ for 30 min (at 4 °C), while muscle and head homogenates were centrifuged at $5000 \times g$ for 3 min. Supernatants were then normalized to approximate protein contents of 0.3 and 0.9 mg/ml for head and muscle samples, respectively. The activity of AChE was determined by the method of Ellman et al. (1961) adapted to microplate, as described in Guilhermino et al. (1996). GST activity was determined by the technique described by Habig et al. (1974), adapted to microplate. The activity of LDH was determined according to Diamantino et al. (2001), which is an adaptation to microplate of the spectrophotometric method described by Vassault (1983). All enzymatic assays were performed in triplicate, at 25 °C, and activity was expressed as Units (U) per mg of protein; a U is a nmol (for AChE and GST) or μmol (for LDH) of substrate hydrolyzed per minute. The concentration of protein was determined in triplicate by the Bradford (1976) method adapted to microplate, using bovine γ-globulin as standard. A Labsystem Multiskan EX microplate reader (supplied by Paralab, Porto, Portugal) was used.

Post-exposure feeding trials

Ideally, feeding impairment should be assessed during the test, but *in situ* exposures pose too many technical difficulties. Therefore, two types of feeding trials were performed following the *in situ* exposure: a 3-min continuous-observation assay, and a 90-min feeding trial. Subsequently to the *in situ* exposure, fish were kept in the field laboratory in aerated containers with respective local water until the beginning of the feeding trials (between 2 and 8 h later). Ideally, this time gap should be reduced to a minimum and all treatments should be given an identical waiting time. Fish were then individually transferred to polyethylene terephthalate vessels with 20 cm of diameter (1 organism per vessel) containing 330 ml of filtered (150 μm) reference water. After a 5-min period for the fish to become calm, the 3-min feeding trial was started with the addition of 12 particles of TetraMenu, between 500 and 1000 μm (obtained using a simple sieving procedure). Food flakes were evenly spread over the surface of the test water, and the individual fish feeding behavior was monitored. Small fish, like guppies or mosquitofish, would have difficulties ingesting particles larger than 1 mm, which could lead them to split the food flakes in two or more pieces in their feeding strikes, thus misleading the observer. Nevertheless, particles should be large enough to allow a proper observation, and thus the choice of a minimum flake size (500 μm). The time elapsed between the beginning of the trial (addition of food) and each feeding strike (i.e., feeding movement where ingestion actually occurred) was registered, through direct individual observation. The feeding trial was stopped when the 3-min period expired or when the fish completed 12 successful strikes. Preliminary experimentation had shown that healthy fish would eagerly eat the food particles, and that a small number of particles would be preferable for improved visualization. These experiments also showed that, under these conditions, healthy guppies would eat all the particles in less than 1 min, although *Gambusia* were not so voracious. Since some sort of feeding impairment was expected on the contaminated mine effluent, a 3-min test period seemed adequate to monitor changes in the fish feeding behavior between sites. A similar feeding trial was described by Little and DeLonay (1996) involving video recordings.

Subsequently to the continuous-observation assay, a pre-quantified amount – 12.06 ± 0.84 (average ± SD, $n = 5$) mg/organism – of 500–1000 μm food particles was added, thus setting off the long-term (90 min) feeding trial. Preliminary experimentation indicated that, during the 90-min period, healthy fish would consume a major percentage of this quantity of food. After removal of the fish, the test water was filtered through a 200-μm nylon mesh, thus gathering the uneaten food particles. Prior to this procedure, faecal pellets were removed with a plastic pipette, if present in the bottom of the test vessel. Food particles were then carefully collected from the mesh and stored in 70% ethanol (to avoid putrefaction) in 1.5-ml microtubes. In the laboratory, the surplus of ethanol was removed with a pipette, and food particles were transferred to pre-weighed foil cups and dried for 48 h at 60 °C. Dry weights of the uneaten food were then obtained on a METTLER UMT2 microbalance (Soquimica, Porto, Portugal) to the nearest microgram. Food intake was expressed as the proportion of food consumed relatively to the initial amount of food (12 mg, for calculation purposes). All feeding trials were performed in similar conditions, at a temperature of 25 ± 2 °C. As stated above, high mortalities in S3 resulted in an insufficient number of organisms to perform the feeding trials in fish from this particular site.

Statistical analysis

Mortality was tested for significance using the Fisher exact test. Biomarker data (AChE, LDH and GST) were separately analyzed for differences between sites using one-way analysis of variance (ANOVA). Food intake (after arcsine transformation) was also tested for significance with one-way ANOVA. In both cases, a Tukey multiple comparison test followed the ANOVA, when applicable. Data resulting from the continuous-observation feeding trial were analyzed using non-parametric techniques (Log-rank and Wilcoxon statistics), usually employed in survival-time analysis, which allow the analysis of censored data. Correlation analysis was used to analyze the relationships between food intake (after arcsine transformation) and biomarkers. These analyses were performed with the mean values of the biomarkers, since fish were from a different set of test-chambers.

Results

Short-term in situ *bioassay with adult fish*

The range of physical and chemical parameters registered during the *in situ* bioassays are presented in Table 1. High temperatures were registered in all sites, and a progressive acidification and increased conductivity were observed in the most contaminated sites (S2 and S3, see Table 1). Dissolved oxygen (DO) was more or less constant during the *in situ* exposures, except in S3, where it decreased to 3.7 mg/l. Some fishes escaped from the test chambers, through gaps formed in the mesh-bottle linkage (Table 2). The worst case happened in REF with *G. holbrooki*, where four organisms escaped.

A mortality increase (Table 2) of *P. reticulata* was observed along the contamination gradient. Nevertheless, mortality was significantly higher than REF only in S3 (the most contaminated site), where 70.6% of the organisms died. With *G. holbrooki*, mortality was only registered in S2 and S3, and in both cases it was significantly different from REF.

Biomarkers response was considerably different between *P. reticulata* (Fig. 3) and *G. holbrooki* (Fig. 4). In comparison to the REF station, *P. reticulata* AChE activity was inhibited (ANOVA: $F_{(3,26)} = 7.27$, $p = 0.001$) in the most contaminated sites (S2 and S3). LDH activity was significantly stimulated (ANOVA: $F_{(3,24)} = 3.95$, $p = 0.020$) in S1, and GST was not significantly affected (ANOVA: $F_{(3,26)} = 0.87$, $p = 0.471$). In *G. holbrooki* adults, only LDH activity was

Table 1. Range of physical and chemical parameters registered in the field during the *in situ* exposures

Site	Temperature (°C)	D.O. (mg/l)	pH	Cond. (μS/cm)
REF	18–34	8.2–8.4	7.2–7.8	185–185
S1	18–34	7.0–9.3	6.3–6.9	260–266
S2	18–34	7.4–9.5	4.5–5.2	350–458
S3	18–34	3.7–5.0	3.7–5.2	365–558

Table 2. Mortality and recovery of adult fish at the end of the short-term *in situ* bioassay

Species	Site	$N_{initial}$	N_{recov}	N_{dead}	Mortality (%)
P. reticulata	REF	18	18	1	5.6
	S1	18	18	2	11.1
	S2	18	16	4	25.0
	S3	18	17	12	70.6**
G. holbrooki	REF	24	20	0	0
	S1	24	24	0	0
	S2	24	23	7	30.4*
	S3	24	21	15	71.4***

Fisher exact test: significantly different from REF (*$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$).
$N_{initial}$ – number of fish at day 0; N_{recov} – number of fish retrieved at the end of the test (including dead ones); N_{dead} – number of dead fish at the end of the bioassay.

significantly affected (ANOVA: $F_{(3,35)} = 4.23$, $p = 0.012$), with a clear inhibitory effect being observed. Significant differences were not found between sites, either for AChE (ANOVA: $F_{(3,35)} = 2.31$, $p = 0.093$) or for GST (ANOVA: $F_{(3,32)} = 1.16$, $p = 0.339$).

Post-exposure feeding trials

Post-exposure feeding trials were successfully conducted with *P. reticulata* but not with *G. holbrooki*, since the latter did not eat any TetraMenu particles during the feeding trials. The 3-min continuous observation assay monitored the feeding activity of the fish, as illustrated by Fig. 5a. Fish exposed in REF ate all the food particles quickly (<80 s), while fish from S1 did not eat all the particles within the 3-min interval. Organisms from S1 occasionally refused food particles by spitting them out (not accounted as successful feeding strikes). Almost no feeding response was recorded in fish from S2, with only one fish demonstrating interest in the food particles, eating one at 41 s and refusing two others. All other fish from S2 were hypoactive, and showed no curiosity towards the food. Significant differences (log-rank and Wilcoxon statistics: $p \leq 0.05$) between REF, S1 and S2 feeding curves were registered (Fig. 5a).

The 90-min post-exposure feeding trial was also successful only in *P. reticulata*. Food intake (the proportion of food consumed per mg of initial food supplied) was impaired in the contaminated sites (S1 and S2), relatively to the reference (REF) site (Fig. 5b), as revealed by the significant differences registered between sites (ANOVA: $F_{(2,15)} = 16.9$, $p = 1.45 \times 10^{-4}$). No feeding trials were performed with fish from S3, because of high mortalities in that site.

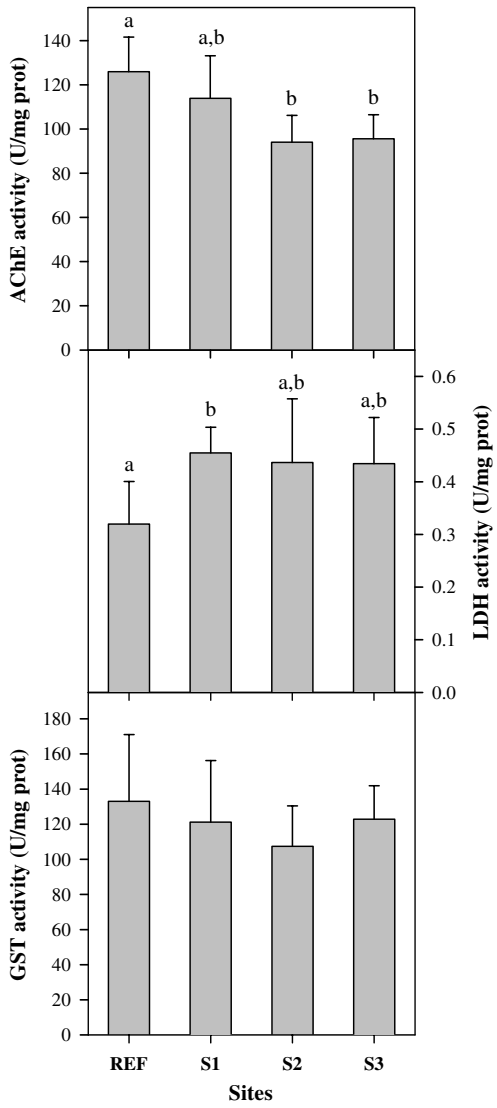

Figure 3. Enzymatic activities of AChE, LDH, and GST from *P. reticulata* after the 96-h *in situ* exposure. Error bars represent standard deviation. Different letters (a, b) represent statistically significant differences between sites, for each biomarker ($p \leqslant 0.05$). REF – reference site; S1, S2, and S3 – sites along the contamination gradient.

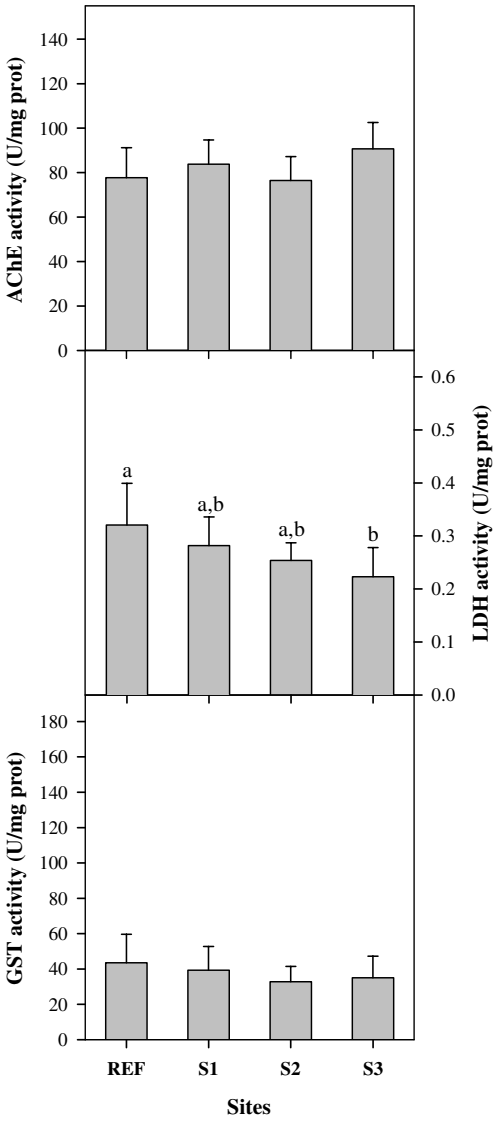

Figure 4. Enzymatic activities of AChE, LDH, and GST from *G. holbrooki* after the 96-h *in situ* exposure. Error bars represent standard deviation. Different letters (a, b) represent statistically significant differences between sites, for each biomarker ($p \leqslant 0.05$). REF – reference site; S1, S2, and S3 – sites along the contamination gradient.

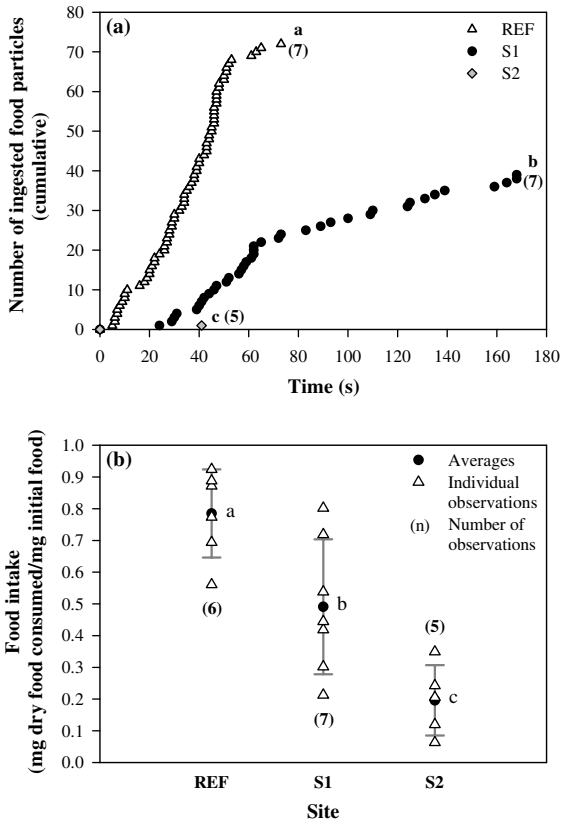

Figure 5. (a) Feeding activity of *P. reticulata* after *in situ* exposure in REF, S1 and S2. Each set of points represents the cumulative number of food particles ingested by the organisms during the 3-min feeding trial, for each treatment. Different letters (a, b, c) represent statistically significant differences between feeding curves (Log-rank and Wilcoxon statistics: $p \leqslant 0.05$). Numbers in brackets stand for the number of organisms used in each of the treatments. REF – reference site; S1 and S2 – sites along the contamination gradient. (b) Food intake (mg dry food consumed/mg of initial food) in *P. reticulata* quantified through a post-exposure feeding trial. Individual observations (triangles) and means (circles) are represented for each site. Error bars represent standard deviations. Different letters (a, b, c) represent statistically significant differences in food intake, between sites ($p \leqslant 0.05$).

Feeding versus biomarkers

Since no data on feeding was available for *G. holbrooki*, only *P. reticulata* results were used in this analysis. A significant correlation was found between AChE and food intake ($r = 0.823$, $p < 0.001$), LDH and food intake ($r = -0.654$, $p = 0.003$), and GST and food intake ($r = 0.831$, $p < 0.001$).

Discussion

Short-term in situ *bioassay with adult fish*

The *in situ* bioassay chambers allowed successful exposure and retrieval of the fish, although a small

minority escaped. Low pH and heavy metals in the mine effluent caused significant mortality in the most contaminated stations, in both guppy and mosquitofish. Low mortalities were to be expected but the strong evaporation led to a progressive acidification, and consequent increase in the number of deaths. In S3, low oxygen levels might have worked as a confounding factor in this variable. Nevertheless, a clear increase in mortality was observed along the contamination gradient in both species. Both metals and low pH are known to affect ion regulation (Lacroix and Townsend, 1987; Heath, 1995). Lacroix and Townsend (1987) have shown that pH alone can be responsible for severe ionic imbalance, leading to mortality of the fish, and that ionic depletion was further enhanced by Al (in combination with low pH).

Besides mortality, metals and acidity also produce numerous sublethal effects, such as feeding impairment and other behavioral alterations (Lacroix and Townsend, 1987; Blaxter and Hallers-Tjabbes, 1992; Peakall, 1992; Heath, 1995; Gerhardt, 1998; Beaumont et al., 2000). Biomarker alterations are also among the sublethal outcomes caused by metals. Although several types of contaminants can affect AChE (Guilhermino et al., 1998; Garcia et al., 2000), it is still successfully employed as a specific biomarker of exposure to organophosphorous and carbamate pesticides (Beyers and Sikoski, 1994; Stien et al., 1998; McLoughlin et al., 2000; Sturm et al., 2000). Metals usually inhibit AChE *in vitro* (Gill et al., 1991, 1992; Garcia et al., 2000), but *in vivo* responses of AChE seem to vary. Gill et al. (1991, 1992) have shown that *in vivo* AChE activity can be unaffected, stimulated or inhibited in different organs of rosy barb, by copper and cadmium, respectively. The underlying physiological mechanism for *in vivo* AChE stimulation or inhibition by metals is still unclear. The results from the *in situ* bioassay suggested that mosquitofish AChE was insensitive to metals and to low pH, while guppy AChE was inhibited in the most contaminated sites. This result suggests an adverse effect for guppy on this key enzyme, with harmful consequences in the central nervous system and neuromuscular function.

LDH is a glycolytic enzyme, and its activity can be used to assess the metabolic state of the organisms. It is considered a nonspecific indicator of cell integrity when quantified in plasma samples, which is not the present case. LDH has been shown to be related with growth (Pelletier et al., 1995), although its response to metals has revealed to vary with the tissue (Gill et al., 1991, 1992). Nevertheless, both copper (Gill et al., 1992) and cadmium (Gill et al., 1991) did not significantly affect LDH activity from skeletal muscle of rosy barb. In the present study, LDH muscle activity responded differently in tested species. This subject should be further exploited in future studies, since both inhibition (as seen in the mosquitofish) and stimulation (as seen in the guppy) of LDH activity can be explained by the existing literature. The inhibition of LDH activity could be due to the direct effect of metals in the enzymatic activity, as shown by Diamantino et al. (2001) and Gill et al. (1991). LDH stimulation, on the other hand, may be a consequence of a local hypoxia "syndrome", at the muscular level, resulting from an ammonia-mediated toxicity mechanism (may be a general stress response). Beaumont et al. (2000) described this mechanism in brown trout exposed to copper at low pH, after observing high lactate concentration and glycogen depletion in the red and white muscle, which was consistent with tissue hypoxia (Heath, 1995). These authors also suggested that this phenomenon was not due to the limitation of the oxygen supply to the muscles, but resulted instead from a general stress response in combination with an electrophysiological disruption. Hyperammonaemia appeared to be related with all of these metabolic changes, although the exact role of ammonia was not fully understood. Whether it is a general stress response or a more complex mechanism, it is apparent that low pH and copper (this may be true for other metals) induce a metabolic shift at the muscular level, promoting anaerobic metabolism. The increase observed in the muscular LDH activity of *P. reticulata* may result from this metabolic shift, as reviewed by Heath (1995). The two opposite response patterns of LDH activity between the two species tested remain, however, unanswered.

GST are commonly used biomarkers for hydrophobic organic chemicals, including PCBs and PAHs, which are detoxified via glutathione conjugation (Phase II detoxification mechanism) (Habig et al., 1974; Van der Oost et al., 1996; Fenet et al., 1998; McLoughlin et al., 2000).

Although metals are not natural substrata for these enzymes, recent studies have shown *in vivo* GST induction in fish (Paris-Palacios et al., 2000) and mussels (Canesi et al., 1999) exposed to heavy metals. GST are among the antioxidant defenses of vertebrates and invertebrates, and metals are known promoters of oxidative stress (Heath, 1995; Paris-Palacios et al., 2000). It is therefore possible that GST metabolize oxidative stress-originated byproducts, which are responsible for the induction of these enzymes (Canesi et al., 1999; Paris-Palacios et al., 2000). Nevertheless, Serafini and Romeu (1991), for example, have shown that metals can also inhibit GST, namely *in vivo*. In the present study, however, no significant effects were recognizable in GST activity, although a decrease in their activity was apparent. Other authors have also found a lack of response of fish GST to contaminants (Jensen et al., 1991; Fenet et al., 1998), or a less sensitive response than other related biomarkers (Huuskonen et al., 1996; Van der Oost et al., 1996). Although their practical application may thus be questionable, GST have successfully been included in biomonitoring studies (e.g., Stien et al., 1998).

In general terms, the battery of biomarkers chosen revealed to be sensitive to the effluent exposure, and, as a whole, it was more sensitive than mortality. McLoughlin et al. (2000) have used ChE and GST for evaluating sublethal effects of several toxic chemicals (zinc, LAS, lindane, pirimiphos-methyl, and permethrin). Their results showed that these biomarkers were more sensitive than mortality and may work in a complementary way, especially if further complemented with other measures (e.g., feeding inhibition). Other authors have shown that a more or less diversified battery of biomarkers can be successfully used, and in a time-effective manner, in *in situ* monitoring of effluents, whether it is in biomonitoring (Van der Oost et al., 1996; Stephensen et al., 2000) or in field-caging studies (Soimasuo et al., 1995; Stien et al., 1998).

The set of biomarkers used in the present study were chosen as indicators of key physiological functions: AChE for neurotoxicity, GST for detoxification/oxidative stress, and LDH as an indicator of the metabolic condition of the organisms. Peakall (1992) first suggested this approach, recognizing the utility of biochemical markers apart from their more popular use as contaminant-specific indicators. The battery here proposed did not intend to be specific for acid mine drainage contamination and there is evidence, from our laboratories research (Guilhermino et al., 1994, 1996, 1998, 2000; Ribeiro et al., 1999; Diamantino et al., 2000, 2001; Garcia et al., 2000) and from the work of McLoughlin (2000), that these biomarkers will respond to several other types of contaminants. Ideally, a large set of biochemical endpoints should be used to properly check-up the normal physiological condition and, at the same time, to identify the causal nature of the physiological disturbance due to the comprehensive contaminant specificity of the selected biomarkers. Thus, both general and highly specific biomarkers can be integrated in the bioassay, although, as seen here, a simple battery can also provide a sensitive response.

Based solely on the results, it would be sound to recommend guppy, not mosquitofish, as a preferable test-species. However, it is fundamental that the use of native species is encouraged, although this might require additional optimization of methodologies (the use of live local food is recommended – see below). Here, mosquitofish and guppy were used due to their availability, but virtually any small fish species has potential to be included in the bioassay.

Post-exposure feeding trials

The post-exposure feeding trials were not successful with *G. holbrooki*, maybe because fish caught in the field may require a pre-acclimation to the food used. Little and DeLonay (1996) recommend such a procedure when it is intended to use a novel (i.e., strange) food source in the feeding trial. However, post-exposure feeding trials with *P. reticulata* were successfully conducted using TetraMenu, which were fed with this same type of food during laboratory rearing. For locally collected fish, it could be preferable to use a different food source, such as indigenous zooplankton.

The 3-min feeding trial aimed at analyzing the feeding behavior of the fish, after the *in situ* exposure. Similar behavioral assays were proposed by other authors, which analyzed the number of feeding strikes (Buckler et al., 1995; Little and

DeLonay, 1996). Guppy feeding behavior was impaired in the contaminated stations, and was subjectively qualified as active feeding, in REF, sluggish feeding, in S1, and no feeding, in S2. Fish were increasingly hypoactive along the effluent sites, revealing decreasing interest in the food particles. The underlying mechanism of this decrease in the fish interest to feed is unclear. Other authors that have observed feeding inhibition provoked by metals, also hypothesized on the mechanism leading to feeding impairment. Some suggested that damages to sensory organs and receptors induced by metals and low pH cause loss or impairment of sensorial perception of the fish (namely olfaction) (Waiwood and Beamish, 1978; Blaxter and Haller-Tjabbes, 1992). Taylor et al. (1998) suggested gut poisoning as the toxicity mechanism of cadmium in feeding-impaired *Daphnia*. The inhibition of AChE is responsible for an impairment of neuronal and neuromuscular function, and could also affect the feeding behavior of the fish (Dell'Omo et al., 1997; Jensen et al., 1997; Beauvais et al., 2000; Brewer et al., 2001). However, in the present study, feeding impairment was observed where no AChE inhibition occurred (site S1). The most probable cause of feeding inhibition is, however, a combination of several or all of these factors, and more work is required to clarify this issue.

The observed reduction in the feeding activity had consequences in the amount of food consumed, as seen by the results of the 90-min feeding trial. Similar feeding trials were successfully conducted by Waiwood and Beamish (1978) and Kumar and Chapman (1998), for copper and profenofos, respectively. In both cases, feeding was significantly impaired. Feeding inhibition revealed to be, in this study, the most sensitive parameter measured, in all of the feeding trials. Feeding inhibition of *Gammarus pulex* was also the most sensitive endpoint tested in a study by McLoughlin et al. (2000), where mortality, AChE activity, GST activity, and feeding rate were compared in terms of their sensitivity to five different compounds (zinc, LAS, lindane, pirimiphos-methyl, and permethrin). Feeding inhibition was almost always the most sensitive endpoint and it was affected by all of the contaminants tested.

Several contaminants, including metals and low pH, affect the feeding of fish. Lacroix and Townsend (1987) have shown that pH alone can depress feeding responses in juvenile Atlantic salmon, in an *in situ* experiment. Feeding activity in this species has also shown to be an extremely sensitive response to pH alone and to aluminium (Buckler et al., 1995). In rainbow trout, food consumption and gross conversion efficiency were significantly impaired by copper and low pH (Waiwood and Beamish, 1978). Thus, not only the feeding rate of the fish can be affected by heavy metals, but also a reduction in the metabolic efficiency can occur (Waiwood and Beamish, 1978; Kumar and Chapman, 1998).

Ultimately, food intake can be considered to be a rough measure of energy input, and a reduction in energy input will affect growth and reproduction. Copper- and pH-induced growth reduction has been related to feeding inhibition in rainbow trout (Waiwood and Beamish, 1978). Kumar and Chapman (1998) have shown evidences of growth impairment and reduction in lipid content associated with reduced feeding rate in rainbow fish exposed to an organophosphate (profenofos). The energy status of the organisms is also a main factor when analyzing reproductive endpoints. Maltby and Naylor (1990) showed a correspondence between feeding rate depression and long-term reproductive impairment in *Gammarus pulex*, after zinc exposure. Impairment of feeding rate can also have serious consequences at the community level, and several authors have focused their attention on this issue. Taylor et al. (1998) questioned the possible consequences in the fish and phytoplankton communities of feeding inhibition observed in populations of a primary consumer (*Daphnia*). Maltby and Naylor (1990) and Forrow and Maltby (2000) addressed the problem of reduced efficiency of detritus processing and nutrient cycling, as a plausible consequence of feeding inhibition observed in key benthic detritivores, such as *Gammarus*.

Feeding versus biomarkers

The approach proposed in the present study was to attempt linking subcellular responses (AChE, GST and LDH) with ecologically relevant individual endpoints (in this case, feeding inhibition), as the first step to assess the ecological relevance of biomarkers. This approach is not new, although

previous studies have only exploited individual biomarkers, based on some sort of underlying physiological mechanism, such as AChE inhibition and behavioral changes (Dell'Omo et al., 1997; Jensen et al., 1997; Beauvais et al., 2000; Brewer et al., 2001). Pelletier et al. (1995) have shown a strong positive relationship between glycolytic enzymes (including LDH) and growth rate, but did not study the response of this association to toxicants.

It is difficult to understand the significance of biomarkers at higher levels of biological organization level, although their role as early warning tools is recognized. Toxicant-induced changes at the individual level are an outcome of the integrated molecular, subcellular, cellular, and physiological alterations (Heath, 1995). Plus, ecologically based changes result from the integrated alterations in growth, feeding and reproduction of the organisms. Amidst all of these integrative phenomena, it is difficult to comprehend what is the ecological significance behind subcellular or molecular alterations.

In the present study, significant correlations between biomarkers and food intake were observed, especially for AChE and GST, but this was feeble for LDH. Since biomarkers will respond differently to different contaminants, a battery of biomarkers is strongly preferred, when linking biomarkers with endpoints at higher levels of biological organization. As previously stated, the set of biomarkers used can be broadened, but it is clear that these three biomarkers alone were able to detect physiological alterations due to low pH and high heavy metals concentration and were relevant at higher levels of organization. At least for AChE, there could be an underlying mechanistic linkage with feeding behavior.

The connection between early warning signals (subindividual responses) and ecologically relevant parameters (population and community effects) is vital in order to enable the use of biomarkers in environmental effects assessment. Recently, De Coen and Janssen (2003b) have proposed a successful quantitative approach for predicting population level effects from short-term biomarker endpoints in *Daphnia*. In a previous study by the same authors (De Coen and Janssen, 2003a), they were able to establish a relevant connection between cellular energy allocation (CEA) and population level responses in *Daphnia*. This parameter is most likely related to feeding, which, due to its behavioral nature, also requires an extrapolation basis to higher-level parameters in order to be regularly used in environmental effects assessment. Future research is therefore required to further establish a reliable connection between early warning responses, such as biomarkers and other individual or subindividual parameters (including feeding), and population-level responses. The present study presented a first step in that direction, although future comparisons with population-level responses are needed. Nevertheless, we emphasize the role of this assay as a short-term and cost-effective early warning tool, which is especially useful to scientists in developing countries, where this type of information is most needed.

Conclusion

The aim of this work was to develop an *in situ* bioassay with small fish integrating feeding and biomarkers. There is novelty in this approach, namely because: (i) *in situ* exposures reduce the uncertainty related with sample manipulation, while, at the same time, allowing a much more realistic scenario of exposure to contaminants; (ii) caging (or *in situ*) experiments are usually performed with large species, such as salmonids; (iii) endpoints at two levels of organization are included in the assay. The proposed assay should be used as an early warning tool, especially while biochemical endpoints are not fully linked with alterations at ecologically relevant levels of biological organization, such as population or community. Many other parameters can be assessed at the end of the assay, depending on the purpose of the study: behavioral changes and general physiological biomarkers can be introduced in the assay in order to understand the mode of action of the pollutant(s); contaminant-specific biomarkers can elucidate which classes of contaminants are present, as well as which pose more drastic consequences to the test organisms. Laboratory research must also continue, especially to build a robust database on the effects of contaminants (metals, pesticides, hydrocarbons, etc.) and water chemistry (pH, alkalinity, etc.) on the selected endpoints.

The results here presented have shown that the 96-h *in situ* bioassay is sensitive and cost/time-effective, since it identified a contamination gradient at an impacted reservoir. Furthermore, the *in situ* approach makes this a site-specific tool for ecotoxicological studies, allowing the rapid attainment of ecologically relevant information. In this study, it was also shown that biomarker responses were related to feeding inhibition. This is a first step to support the idea that a battery of biomarkers may be an indicator of biological effects at higher levels of organization. Future work should further attempt to elucidate the link between biomarkers and effects at the population or community levels.

Acknowledgements

B.B. Castro was a recipient of a M.Sc. grant from Fundação para a Ciência e a Tecnologia (Portugal) – PRAXIS XXI. This work was partially funded by Fundação para a Ciência e a Tecnologia (project SENSOR, ref. PRAXIS/PCNA/C/BIA/0157/96) and by the European Commission (project TROCA-WET, ref. ERBIC18-CT98-0264). Authors wish to thank S. Moreira for assistance during fieldwork and two anonymous reviewers for significantly improving the relevance of the manuscript.

References

Almaça, C. (1995). *Fish Species and Varieties Introduced into Portuguese Inland Waters*. Lisboa: Museu Nacional de História Natural.

Beaumont, M.W., Butler, P.J. and Taylor, E.W. (2000). Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. *Aquat. Toxicol.* **51**, 259–72.

Beauvais, S.L., Jones, S.B., Brewer, S.K. and Little, E.E. (2000). Physiological measures of neurotoxicity of diazon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. *Environ. Toxicol. Chem.* **19**, 1875–1880.

Beyers, D.W. and Sikoski, P.J. (1994). Acetylcholinesterase inhibition in federally endangered Colorado squawfish exposed to carbaryl and malathion. *Environ. Toxicol. Chem.* **13**, 935–939.

Blaxter, J.H.S. and Hallers-Tjabbes, C.C.T. (1992). The effect of pollutants on sensory systems and behavior of aquatic animals. *Netherlands J. Aquat. Ecol.* **26,** 43–58.

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

Brewer, S.K., Little, E.E., DeLonay, A.J., Beauvais, S.L., Jones, S.B. and Ellersieck, M.R. (2001). Behavioral dysfunctions correlate to altered physiology in rainbow trout (*Oncorhynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. *Arch. Environ. Contam. Toxicol.* **40**, 70–76.

Buckler, D.R., Cleveland, L., Little, E.E. and Brumbaugh, W.G. (1995). Survival, sublethal responses, and tissue residues of Atlantic salmon exposed to acidic pH and aluminium. *Aquat. Toxicol.* **31**, 203-216.

Cabral, J.A., Mieiro, C.L. and Marques, J.C. (1998). Environmental and biological factors influence the relationship between a predator fish, *Gambusia holbrooki*, and its main prey in rice fields of the lower Mondego River valley (Portugal). *Hydrobiologia* **382**, 41–51.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G. (1999). Heavy metals and glutathione metabolism in mussel tissues. *Aquat. Toxicol.* **46**, 67–76.

De Coen, W.M., Janssen, C.R. (2003a). The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environ. Toxicol. Chem.* **22**, 1632–1641.

De Coen, W.M., Janssen, C.R. (2003b). A multivariate biomarker-based model predicting population-level responses of *Daphnia magna*. *Environ. Toxicol. Chem.* **22**, 2195–2201.

Dell'Omo, G., Bryenton, R. and Shore, R.F. (1997). Effects of exposure to an organophosphate pesticide on behavior and acetylcholinesterase activity in the common shrew *Sorex araneus*. *Environ. Toxicol. Chem.* **16**, 272–276.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M. and Guilhermino, L. (2001). Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. *Chemosphere* **45**, 553–560.

Diamantino, T.C., Guilhermino, L., Almeida, E. and Soares, A.M.V.M. (2000). Toxicity of sodium molybdate and sodium dichromate to *Daphnia magna* Straus. *Ecotoxicol. Environ. Saf.* **45**, 253–259.

EEC (1992). Acute toxicity to fish. Seventh amendment of directive 67/548/EEC, Annex V – Part C: Methods for the determination of ecotoxicity, Directive 92/32/EEC. EEC.

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.

Fenet, H., Casellas, C. and Bontoux, J. (1998). Laboratory and field–caging studies on hepatic enzymatic activities in European eel and rainbow trout. *Ecotoxicol. Environ. Saf.* **40**, 137–143.

Forrow, D.M. and Maltby, L. (2000). Toward a mechanistic understanding of contaminant-induced changes in detritus processing in streams: direct and indirect effects on detritivore feeding. *Environ. Toxicol. Chem.* **19**, 2100–2106.

Garcia, L.M., Castro, B., Ribeiro, R. and Guilhermino, L. (2000). Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its *in vitro* inhibition by environmental contaminants. *Biomarkers* **5**, 274–284.

García-Berthou, E. (1999). Food of introduced mosquitofish: ontogenic diet shift and prey selection. *J. Fish Biol.* **55**, 135–147.

Gerhardt, A. (1998). Whole effluent toxicity testing with *Oncorhynchus mykiss* (Walbaum 1792): survival and behavioral responses to a dilution series of a mining effluent in South Africa. *Arch. Environ. Contam. Toxicol.* **35**, 309–316.

Gill, T.S., Tewari, H. and Pande, J. (1991). *In vivo* and *in vitro* effects of cadmium on selected enzymes in different organs of the fish *Barbus conchonius* Ham. (rosy barb). *Comp. Biochem. Physiol.* **100C**, 501–505.

Gill, T.S., Tewari, H. and Pande, J. (1992). Short- and long-term effects of copper on the rosy barb (*Puntius conchonius* Ham.). *Ecotoxicol. Environ. Saf.* **23**, 294–306.

Goksøyr, A., Beyer, J., Egaas, E., Grøsvik, B.E., Hylland, K., Sandvik, M. and Skaare, J.U. (1996). Biomarker responses in flounder (*Platichthys flesus*) and their use in pollution monitoring. *Mar. Pollut. Bull.* **33**, 36–45.

Guilhermino, L., Lopes, M.C., Donato, A., Silveira, L., Soares, A.M.V.M. and Carvalho, A.P. (1994). Comparative study between the toxicity of 3,4-dichloroaniline and sodium bromide with the 21-day chronic test and using dehydrogenase activity of *Daphnia magna* Straus. *Chemosphere* **28**, 2021–2027.

Guilhermino, L., Lopes, M.C., Carvalho, A.P. and Soares, A.M.V.M. (1996). Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. *Chemosphere* **32**, 727-738.

Guilhermino, L., Barros, P., Silva, M.C. and Soares, A.M.V.M. (1998). Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Biomarkers* **3**, 157–163.

Guilhermino, L., Lacerda, M.N., Nogueira, A. and Soares, A.M.V.M. (2000). *In vitro* and *in vivo* inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contaminant biomonitoring. *Sci. Total Environ.* **247**, 137–141.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130–7139.

Heath, A.G. (1995). *Water Pollution and Fish Physiology*, 2nd edn. USA: CRC Press, Inc.

Holcík, J. (1991). Fish introductions in Europe with particular reference to its central and eastern part. *Can. J. Fish. Aquat. Sci.* **48**, 13–23.

Huuskonen, S., Lindström-Seppä, P., Koponen, K. and Roy, S. (1996). Effects of non-ortho-substituted polychlorinated biphenyls (congeners 77 and 126) on cytochrome P4501A and conjugation activities in rainbow trout (*Onchorhynchus mykiss*). *Comp. Biochem. Physiol.* **113C**, 205–213.

Jensen, C.S., Garsdal, L. and Baatrup, E. (1997). Acetylcholinesterase inhibition and altered locomotor behavior in the carabid beetle *Pterostichus cupreus*. A linkage between biomarkers at two levels of biological complexity. *Environ. Toxicol. Chem.* **16**, 1727–1732.

Jensen, E.G., Skaare, J.U., Egaas, E. and Goksøyr, A. (1991). Response of xenobiotic metabolizing enzymes in rainbow trout (*Onchorhynchus mykiss*) to endosulfan, detected by enzyme activities and immunochemical methods. *Aquat. Toxicol.* **21**, 81–92.

Jones, P.A. and Sloan, R.J. (1989). An *in situ* river exposure vessel for bioaccumulation studies with juvenile fish. *Environ. Toxicol. Chem.* **8**, 151–155.

Kosmala, A., Migeon, B., Flammarion, P. and Garric, J. (1998). Impact assessment of a wastewater treatment plant effluent using the fish biomarker ethoxyresorufin-*O*-deethylase: field and on-site experiments. *Ecotoxicol. Environ. Saf.* **41**, 19–28.

Kumar, A. and Chapman, J.C. (1998). Profenofos toxicity to the eastern rainbow fish (*Melanotaenia duboulayi*). *Environ. Toxicol. Chem.* **17**, 1799–1806.

Lacroix, G.L. and Townsend, D.R. (1987). Responses of juvenile Atlantic salmon (*Salmo salar*) to episodic increases in acidity of Nova Scotia rivers. *Can. J. Fish. Aquat. Sci.* **44**, 1475–1484.

Lindström-Seppä, P. and Oikari, A. (1990). Biotransformation and other toxicological and physiological responses in rainbow trout (*Salmo gairdneri* Richardson) caged in a lake receiving effluents of pulp and paper industry. *Aquat. Toxicol.* **16**, 187–204.

Little, E.E. and DeLonay, A. (1996). Measures of fish behavior as indicators of sublethal toxicosis during standard toxicity tests, Vol. 4, ASTM STP 1262. In T.W. La Point, F.T. Price, E.E. Little (eds). *Environmental Toxicology and Risk Assessment*. USA: American Society for Testing and Materials, pp. 216–233.

Lopes, I., Gonçalves, F., Soares, A.M.V.M. and Ribeiro, R. (1999). Discriminating the ecotoxicity due to metals and to low pH in acid mine drainage. *Ecotoxicol. Environ. Saf.* **44**, 207–214.

Maltby, L. and Naylor, C. (1990). Preliminary observations on the ecological relevance of the *Gammarus* 'scope for growth' assay: effect of zinc on reproduction. *Funct. Ecol.* **4**, 393–397.

McLoughlin, N., Yin, D., Maltby, L., Wood, R.M. and Yu, H. (2000). Evaluation of sensitivity and specificity of two crustacean biochemical biomarkers. *Environ. Toxicol. Chem.* **19**, 2085–2092.

OECD (1982). Fish, acute toxicity test. OECD Guideline for Testing of Chemicals 203. Paris: OECD, pp. 1–15.

Pacheco, M. and Santos, M.A. (1999). Biochemical and genotoxic responses of adult eel (*Anguilla anguilla* L.) to resin acids and pulp mill effluent: laboratory and field experiments. *Ecotoxicol. Environ. Saf.* **42**, 81–93.

Paris-Palacios, S., Biagianti-Risbourg, S., Vernet, G. (2000). Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulphate. *Aquat. Toxicol.* **50**, 109–124.

Peakall, D. (1992). *Animal Biomarkers as Pollution Indicators*. London, UK: Chapman & Hall.

Pelletier, D., Blier, P.U., Dutil, J.D. and Guderley, H. (1995). How should enzyme activities be used in fish growth studies? *J. Exp. Biol.* **198**, 1493–1497.

Pereira, E.G., Moura, I., Costa, J.R., Mahony, J.D., Thomann, R.V. (1995). The S. Domingos Mine: a study of heavy metals contamination in the water column and sediments of the Chança river basin by discharge from an ancient cupriferous pyrite mine (Portugal). *Mar. Freshwater Res.* **46**, 145–151.

Pereira, A.M.M., Soares, A.M.V.M., Gonçalves, F. and Ribeiro, R. (1999). Test chambers and test procedures for *in situ* toxicity testing with zooplankton. *Environ. Toxicol. Chem.* **18**, 1956–1964.

Pereira, A.M.M., Ribeiro, R., Gonçalves, F. and Soares, A.M.V.M. (2000). Water-column, sediment and *in situ* chronic bioassays with cladocerans. *Ecotoxicol. Environ. Saf.* **47**, 27–38.

Ribeiro, R., Martins, A.M.A., Correia, J.C.A., Lopes, I., Pereira, A., Canteiro, M.H.S.F., Gonçalves, F. and Soares, A.M.V.M. (1995). Vertebrados da zona da Mina de São Domingos (Baixo Alentejo). *Ciência Biológica – Ecol. Systemat.* **15**, 33–47.

Ribeiro, S., Guilhermino, L., Sousa, J.P. and Soares, A.M.V.M (1999). Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods. *Ecotoxicol. Environ. Saf.* **44**, 287–293.

Serafini, M.T. and Romeu, A. (1991). Cu(II) and Cd(II) inhibition of rat–liver glutathione-*S*-transferase – a steady-state kinetic study. *Biochem. Int.* **24**, 497–505.

Sibley, P.K., Benoit, D.A., Balcer, M.D., Phipps, G.L., West, C.W., Hoke, R.A. and Ankley, G.T. (1999). *In situ* bioassay chamber for assessment of sediment toxicity and bioaccumulation using benthic invertebrates. *Environ. Toxicol. Chem.* **18**, 2325–2336.

Soimasuo, M.R., Jokinen, I., Kukkonen, J., Oikari, A. and Ristola, T. (1995). Biomarker responses along a pollution gradient: effects of pulp and paper mill effluent on caged whitefish. *Aquat. Toxicol.* **31**, 329–345.

Soimasuo, M.R., Karels, A.E., Oikari, O.J., Santti, R. and Oikari, A.O.J. (1998). Biomarker responses in whitefish (*Coregonus lavaretus* L. s.l.) experimentally exposed in a large lake receiving effluents from pulp and paper industry. *Arch. Environ. Contam. Toxicol.* **34**, 69–80.

Stephensen, E., Svavarsson, J., Sturve, J., Ericson, G., Adolfsson-Erici, M. and Förlin, L. (2000). Biochemical indicators of pollution exposure in shorthorn sculpin (*Myoxocephalus scorpius*), caught in four harbours on the southwest coast of Iceland. *Aquat. Toxicol.* **48**, 431–442.

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M. and Lafaurie, M. (1998). Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean Sea. *Environ. Pollut.* **99**, 339–345.

Sturm, A., Wogram, J., Segner, H. and Liess, M. (2000). Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback (*Gasterosteus aculeatus*): application in biomonitoring. *Environ. Toxicol. Chem.* 19(6) 1607–1615.

Taylor, G., Baird, D.J. and Soares, A.M.V.M. (1998). Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. *Environ. Toxicol. Chem.* **17**, 412–419.

Van der Oost, R., Goksøyr, A., Celander, M., Heida, H. and Vermeulen, N.P.E. (1996). Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*) – II. Biomarkers: pollution–induced biochemical responses. *Aquat. Toxicol.* **36**, 189–222.

Vassault, A. (1983). Lactate dehydrogenase: UV-method with pyruvate and NADH. In H.O. Bergmeyer (ed.). Methods of Enzymatic Analysis, Vol. III Enzymes: Oxireductases, Transferases, 3rd edn, Vol. III. pp. 118–126. New York: Academic Press.

Waiwood, K.G. and Beamish, W.H. (1978). The effect of copper, hardness and pH on the growth of rainbow trout, *Salmo gairdneri*. *J. Fish Biol.* **13**, 591–598.

Wall, K.L., Jessen-Eller, K. and Crivello, J.F. (1998). Assessment of various biomarkers in winter flounder from coastal Massachusetts, USA. *Environ. Toxicol. Chem.* **17**, 2504–2511.

Wilde, E.W. and Parrot, A.B. (1984). A simple, inexpensive *in situ* method for assessing acute toxicity of effluents to fish. *Water Res.* **18**, 783–785.