The use of liver histopathology, lipid peroxidation and acetylcholinesterase assays as biomarkers of contaminant-induced stress in the Cape stumpnose, *Rhabdosargus holubi* (Teleostei: Sparidae), from selected South African estuaries

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Abstract

Three biomarkers of contaminant-induced stress (liver histopathology, the lipid peroxidation (LPx) assay and the acetyl-cholinesterase (AChE) assay) were adapted for application to the estuarine-dependent marine fish *Rhabdosargus holubi* (Steindachner, 1881). Specimens of *R. holubi* were collected using a seine net from 3 temporarily open/closed estuaries in the Eastern Cape, South Africa, each impacted by different anthropogenic activities. The East Kleinemonde estuary has a housing settlement on the banks in the lower reaches and some agriculture in its catchment. The Old Woman's estuary has a golf course adjacent to its lower and middle reaches and is crossed by a national road in its upper reaches. The Mtana estuary is virtually pristine, with limited cattle grazing occurring along the banks of the estuary and some subsistence agriculture in the catchment. According to the biomarker results from this study, *R. holubi* from the East Kleinemonde were in good health, as reflected by low LPx and high AChE levels. The liver histopathology did, however, suggest possible previous exposure to stress (increased melanomacrophage centres, increased perivascular connective tissue and severe vacuolation). Overall, liver histopathology results did not differ significantly between estuaries. Fish from the Old Womans recorded significantly inhibited AChE and increased LPx, while fish from the Mtana exhibited significantly increased LPx only, suggesting possible exposure to anticholinesterase contaminants in the former estuary and some form of oxidative stress in the latter. Although water samples were collected from each of the 3 estuaries and analysed for polychlorinated biphenyls, organochlorines, organophosphorous pesticides and pyrethroids, none of these chemicals were detected. As pesticide residues in water are highly variable, both temporally and spatially, future studies should focus on measuring tissue burdens of organisms in order to identify the contaminant stressor. This study has shown that while chemical analyses of water provide a 'snap-shot' of water quality at the time of sampling, biomonitoring can integrate past exposures to stress and is thus useful for identifying potential situations of concern that require further detailed investigation.

Keywords: biomonitoring, pollution, estuaries, AChE, LPx, histopathology

Introduction

Biomarkers can be sensitive indicators of sub-cellular stress in organisms exposed over short and longer periods to a range of pollutants (Adams et al., 2000). Several international studies have shown that biomarkers are useful tools in the monitoring of estuarine biotic 'health' and therefore give an indirect measure of possible environmental pollution or degradation (Cavanagh et al., 2000; Kirby et al., 2000; Fulton and Key, 2001; Lund Amado et al., 2006; Valavanidis et al., 2006; Sole et al., 2006). South African monitoring programmes in estuaries have focused on the use of chemical analyses of water and sediments to determine contaminant loadings (Schulz, 2001; Scharler and Baird, 2003; Bollmohr et al., 2007), and on fish community composition and structure to determine estuarine health (Harrison and Whitfield, 2006).

Chemical analyses of water and sediment cannot provide direct indications of the effect of contaminants on the biota of an estuary. This is because contaminant concentrations are not constant over time, toxicity is caused by the bioavailable fraction of the toxicant only (not the total measured concentration), and processes such as bioaccumulation can lead to an underestimation of toxic risk (Adams and Tremblay, 2003). Community indices have been criticised for only indicating severe stress that has already occurred (a change in the community structure), and do not provide any indication of the type of stressor that caused the change or early warnings of potential effects (Adams et al., 2005). Biomarkers have therefore been proposed as a method of detecting contaminant induced sub-organism level stress in the biota of an estuary before population- or community-level responses become apparent (Adams et al., 2005).

The aim of this pilot study was to determine the feasibility of using 3 biomarkers, at different sub-organismal levels, to detect pollutant-induced stress in the Cape stumpnose, *Rhabdosargus holubi*, in 3 temporary open/closed estuaries (TOCEs), each impacted by different anthropogenic activities (Richardson, 2008). TOCEs were selected for this biomarker study as, when

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closed, fish are not able to migrate in or out of the estuary, thereby ensuring that any measured effects in fish can be attributed to the circumstances within that particular estuary. In addition, the selected estuaries are in close proximity to each other, in the same biogeographical zone (warm temperate), and are similar in terms of hydrology and mouth morphology.

Rhabdosargus holubi was identified as a potential bioindicator species for South African estuaries due to its abundance and wide distribution on both the south and east coasts. This species is dependent on estuaries as nursery areas during its juvenile phase (therefore only juvenile individuals can be used as bioindicators of estuarine health), with the subadults and adults restricted to the marine environment (Blaber, 1974a). The Cape stumpnose uses estuaries in a similar manner to a number of other important angling species in South African waters and therefore the management of estuaries using *R. holubi* as an indicator species could have positive outcomes for other estuary-associated marine taxa. Aspects of the biology and ecology of this easily identified species have been well studied (Blaber, 1973a; 1973b; 1974a; 1974b; 1974c; Whitfield, 1984; Cowley et al., 2001) and it has been successfully used in other pollution-related experiments (De Kock and Lord, 1988).

Knowledge of contaminant concentrations in most South African estuaries is limited. Orr et al. (2008) have reported the presence of heavy metals in the Kariega estuary sediments and to a lesser extent in the East Kleinemonde and Riet estuary sediments. Although no analyses of pesticide contamination have occurred in the Eastern Cape estuaries investigated during this study, Schulz (2001), Bollmohr et al. (2007) and Bollmohr et al. (2009) have shown that pesticides are periodically washed into estuaries along the Western Cape coast. Since one of the estuaries in this study is situated adjacent to a golf course that uses pesticides, the acetylcholinesterase activity (AChE) assay was chosen as a biomarker. Dipping of cattle belonging to rural subsistence farmers is undertaken by state veterinarians on a frequent basis in the Eastern Cape and these cattle often wade through estuaries. The potential impact of this on the ichthyofauna of estuaries was investigated using 2 broad biomarkers of exposure to contaminants, the lipid peroxidation (LPx) assay and liver histopathology.

Lipid peroxidation occurs as result of oxidative stress damage to the lipid components of cellular membranes (Kelly et al., 1998). This reaction occurs, to a limited degree, during natural immunity processes (e.g. phagocytosis) but is caused to a much greater extent by xenobiotics. The LPx assay measures malondialdehyde, a by-product of LPx, and can be used as a quantitative measurement of the extent of oxidative damage an organ has undergone. The liver was chosen for the LPx analyses in order to allow for comparison with the results from the histopathological investigations.

Histopathological analyses and LPx assay of the liver can provide a good indication of the overall health of an animal, including fish. The liver is a key organ of overall homeostasis, in terms of nutrition, defence against toxicants and reproductive development (Hinton and Lauren, 1990). Examples of redox cycling compounds that have been shown to affect LPx levels include quinones, certain dyes, bipyridyl herbicides, metals, aromatic nitro compounds and certain pesticides (Kelly et al., 1998; Dorval et al., 2005; Valavanidis et al., 2006).

Histological alterations in the liver have been shown to provide definite biological end points of exposure to a number of contaminants and have been successfully applied in several biomonitoring programs in Europe and North America (Malins et al., 1988; Kohler, 1989; Hinton and Lauren, 1990; Stentiford et al., 2003). Laboratory and field-based studies have established relationships between contaminant exposure and toxicopathic lesion formation (Naigaga, 2002; Stentiford et al., 2003; Van Dyk, 2003).

The AChE enzyme is found in the brain and muscle tissue of fish and controls a large proportion of physiological and behavioural responses in the animal; thus any changes to these regulatory abilities could be potentially detrimental to fish (Pan and Dutta, 1998; Kirby et al., 2000). This assay is regarded as a good biomarker and has been successfully used in fish to detect exposure to a range of neurotoxic compounds (Kirby et al., 2000; Roex et al., 2003; Eder et al., 2004; Lau et al., 2004). Until recently, the AChE assay was considered to be a specific biomarker of exposure to pesticides and insecticides; however recent studies have shown that AChE activity is also inhibited by metals, organochlorines, herbicides and surfactants (Guilhermino et al., 1998; Guilhermino et al., 2000; Corsi et al., 2003; Roy et al., 2006; Bervoets et al., 2009, Mochida et al., 2009; Modesto and Martinez, 2010).
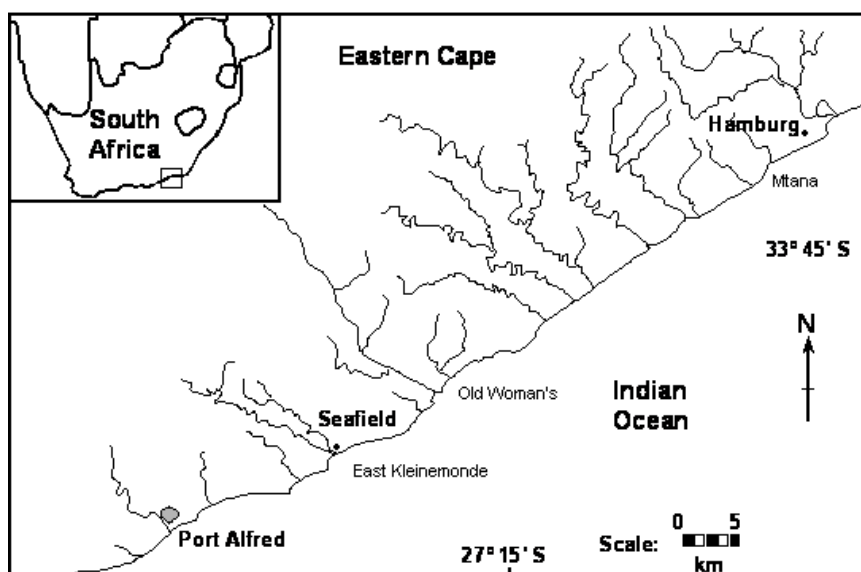

Figure 1
Map indicating the position of the East Kleinemonde, Old Woman's and Mtana estuaries on the Eastern Cape coast.

Table 1
Main physical and catchment characteristics of each estuary

Estuary	Size (ha)	Mean Annual Runoff	Catchment activities
East Kleinemonde	35.0	$2.00 \times 10^6 \, m^3$	Cattle grazing and pineapple farming in catchment, residential development around estuary
Old Woman's	21.5	$1.16 \times 10^6 \, m^3$	Golf course adjacent to estuary, low intensity cattle grazing in upper catchment
Mtana	15.7	$2.29 \times 10^6 \, m^3$	Cattle grazing around estuary and in catchment, small scale subsistence farming in catchment

Table 2
Grading system for the determination of histological alterations observed in *R. holubi* liver sections from the East Kleinemonde, Old Woman's and Mtana estuaries (adapted from Van Dyk et al., 2007)

Grade	Histological characteristic
0	Typical hepatic cord structure and hepatopancreatic structure.
1	Typical hepatic cord structure and hepatopancreatic structure. Mild lipid accumulation (vacuolation) within hepatocytes.
2	Typical hepatic cord structure and hepatopancreatic structure. Moderate lipid accumulation (vacuolation) within hepatocytes.
3	Typical hepatic cord structure and hepatopancreatic structure with 2 to 5 of the following characteristics: • Severe lipid accumulation (vacuolation) within hepatocytes. • Congestion of hepatic blood vessels. • Increase in perivascular connective tissue. • Hyalinization within hepatocytes. • Cellular swelling due to hydropic change. • Increased melanomacrophage centres. • Lymphocyte infiltration.
4	Loss of hepatic cord structure with 2 to 5 of the histological changes listed under Category 3.

Study sites

The 3 TOCEs chosen for this study, the East Kleinemonde (EK), Old Woman's (OW) and Mtana (MTN), are of similar morphology and are located within the same biogeographical zone in the Eastern Cape Province (Fig. 1). The estuaries differ according to the types of anthropogenic impacts occurring within their catchments (Table 1). The EK is characterised by housing developments along the banks of the lower estuary reaches and pineapple and cattle farms in the catchment area. The OW catchment has a golf course along the banks of the lower and middle reaches and is traversed by a national road in the upper reaches. Some low intensity cattle grazing occurs in the catchment of this estuary. The MTN catchment is virtually undeveloped although limited subsistence farming is recorded in certain areas. Low intensity cattle grazing occurs throughout the catchment and along the banks of the estuary.

Materials and methods

Sampling methods

At the time of sampling, all 3 estuaries were closed and had been for 4 months, thereby ensuring that the fish in a particular estuary had been exposed to the conditions in that particular estuary for at least that period. One hundred juvenile *R. holubi* of similar size (9.3 ± 1.6 cm standard length) were collected during the course of a morning between 18 and 25 April 2005 from the lower reaches of each estuary. Fish were caught using a 30 m x 1.7 m x 15 mm bar mesh seine net fitted with a 5 mm bar mesh cod end and then held in the estuary in a submerged, covered container that allowed free exchange of water. All fish were processed within 3 h of capture. For each individual, the

spinal cord was severed before the brain and liver were excised. Dissecting tools were rinsed in 70% ethanol, washed in de-ionised water and dried with paper towel between each dissection. The brain and half of the liver were immediately frozen in liquid nitrogen and stored at -70 ºC until further processing for AChE and LPx assays could be conducted. The remaining half of the liver was placed in 10% buffered formalin for 72 h and then transferred to 70% alcohol for later histopathological analysis (Richardson, 2008).

Histopathological analyses

Liver tissues obtained from 24 individuals (10 from the EK, 5 from the OW and 9 from the MTN) were embedded in paraffin wax, sectioned into 5 μm slices and stained using haematoxylin and eosin. Each slide was graded between 0 and 4 (Table 2) according to methods adapted from Van Dyk et al. (2007). Grading involved examining the liver slides under the microscope in order to identify a range of lesions such as melanomacrophage centres, increases in perivascular tissue, hydropic change and vacuolation in the hepatocytes. Each type of change recorded was assigned a specific score (Table 2).

LPx determination

Liver samples were assayed according to the methods by Ringwood et al. (2003). Each section of liver was weighed, added to cold 50 mM potassium phosphate buffer solution (pH 7.0) based on 4 times the gram weight of the sample, homogenised using Teflon Eppendorf micropestles, and then centrifuged for 5 min at 13 000 *g* at 4ºC. Sample supernatant (50 μℓ) was then added to 700 μℓ of 10 mM 0.375% (w/v) thiobarbituric acid and 7 μℓ of 2% (w/v) butylated hydroxytoluene and

Table 3			
List of chemicals tested for in each estuary using OACA International (1995) method			
	Chemical type		
	Polychlorinated biphenyls (PCBs):	**Organochlorines**	**Organophosphorous pesticides**	**Pyrethroids**
Chemical name	PCB-52	Alpha-BHC	Chlorpyrifos-Me	Cyhalothrin
	PCB-87	Lindane	Fenitrothion	Cyfluthrin
	PCB-153	Heptachlor	Malathion	Cypermethrin
	PCB-138	Aldrin	Chlorpyrifos	Deltamethrin
	PCB-180	Heptachlor epoxide	Chlorfenvinphos	
		pp'-DDE	Profenophos	
		Dieldrin	Dichlorvos	
		Endrin	Sulfotepp	
		β-Endosulfan	Diazinon	
		Methoxychlor	Parathion,	
		p-p'-DDT	Mevinphos	
		o-p'-DDT	Pirlmiphos-methyl	
		p-p'DDE	Parathion-methyl	

incubated in a heating block at 100ºC for 15 min. The samples were centrifuged at 13 000 g for 5 min at 22 ± 2ºC. A 100 $\mu\ell$ sample of supernatant was placed in the well of a 96-well microtiter plate and read at 532 nm using a microtiter plate reader (PowerWave$_x$, Bio-Tek Instruments Inc, USA). A standard curve was prepared using serial dilutions of malondialdehyde tetraethylacetal (MDA) in a concentration range of 6.25 to 3 200 μM MDA. Each sample and standard was analysed in quadruplicate and the results were expressed as the median ± 95% confidence intervals (Richardson, 2008).

AChE activity determination

Methods for the determination of AChE activity in the brain tissue were optimised for *R. holubi* based on methods described by McLoughlin et al. (2000). In brief, each brain sample was homogenised on ice in 30 $\mu\ell$ cold phosphate buffer saline (PBS) (pH 8.0) containing 1% (v/v) Triton X-100, using Teflon Eppendorf micropestles. The homogenate was diluted with 270 $\mu\ell$ PBS and centrifuged for 20 min at 13 000 g at 4ºC. A 50 $\mu\ell$ sample of this supernatant was diluted with 950 $\mu\ell$ cold PBS containing 0.1% (v/v) Triton X-100 in preparation for the assay. Enzyme activity was determined by adding 100 $\mu\ell$ of 8 mM 5,5'-dithio-*bis* (2-nitrobenzoic acid) and 50 $\mu\ell$ of each sample's supernatant to a well in a 96-well microtiter plate kept on ice. The microtiter plate was incubated at 30˚C in a microtiter plate reader (PowerWave$_x$, Bio-Tek Instruments Inc, USA) for 3 min. An aliquot of 50 $\mu\ell$ of 16 mM acetylthiocholine iodide was added to each well, after which a reading was taken at 405 nm every 30 s for 10 min (McLoughlin et al., 2000). Enzyme activity was calculated using the following equation:

$$\text{Activity (}\mu\text{mol}\ \text{m}\ell^{-1}\ \text{min}^{-1}) = \left(\frac{\Delta abs / \min}{\varepsilon \times l}\right) \times (df) \times 10^3$$

where an extinction coefficient (ε) value of 1.36 x 10^4 ℓ·mol^{-1}·cm^{-1} was used, and where l is the pathlength in cm, Δabs is the change in absorbance per minute and df is the dilution factor (Ellman et al., 1961).

Fresh standards were prepared daily using commercial AChE (1 mg·mℓ^{-1}) at a concentration range between 0 mg·mℓ^{-1} and 0.125 mg·mℓ^{-1} to produce a standard curve. Specific standard concentrations were included in subsequent plates to ensure consistently accurate readings. Each sample and standard was analysed in quadruplicate (Richardson, 2008).

Protein content determination

Liver and brain sample protein content was determined according to the method described in Bradford (1976). For the liver samples, a dilution was made using 2 $\mu\ell$ of the original liver homogenate (containing 4 times the gram weight of the liver in millilitres of 50 mM potassium phosphate buffer, pH 7.0) and 198 $\mu\ell$ of 50 mM potassium phosphate buffer (pH 7.0). A portion of this diluted sample (20 $\mu\ell$) was placed in the well of a 96-well microtiter plate and 230 $\mu\ell$ of Bradford reagent added. The plate was allowed to stand for 5 min at 22 ± 2ºC whereafter a reading was taken at 595 nm.

For the brain tissue, 20 $\mu\ell$ of brain sample homogenate (containing 50 $\mu\ell$ of the original homogenate diluted with 950 $\mu\ell$ cold PBS containing 0.1% (v/v) Triton X-100) was placed in a 96-well microtiter plate well. Bradford reagent (230 $\mu\ell$) was added and the plate was allowed to stand for 5 min at 22 ± 2ºC, whereafter a reading was taken at 595 nm.

Standards were prepared using commercial Bovine Serum Albumin (BSA) and ranged between 0 and 0.4 mg·mℓ^{-1}. This standard curve was used to calculate the protein content in each liver and brain sample. Final malondialdehyde concentrations were expressed as nmol MDA produced·h^{-1}·mg protein^{-1}. For the brain tissue, sample AChE activities (μmol·mℓ^{-1}·min^{-1}) were divided by the sample's protein content (mg·mℓ^{-1}) and multiplied by 1 000 to present AChE activity in nmol·min^{-1}·mg protein^{-1} (Richardson, 2008).

Water analyses

Subsurface (<1 m) water samples were collected from the lower, middle and upper reaches of each estuary during the course of 1 morning and were immediately sent, on ice, to the South African Bureau of Standards (SABS) for analysis. Single determinations were carried out by employing the method as described in the *Official Methods of Analysis of AOAC International* manual (AOAC, 1995). Quantitative analyses were conducted by gas chromatography-electron capture detector for organochlorine pesticides (OCs), pyrethroids and polychlorinated biphenyls (PCBs). Organophosphorous

Table 4
Most commonly recorded lesions in the livers of *Rhabdosargus holubi* from the East Kleinemonde, the Old Woman's and Mtana estuaries

Estuary	Mean histological alteration grade	Lesion			
		Increase in melanomacrophage centres	Increase in perivascular connective tissue	Hydropic change in the hepatocytes	Vacuolation of the hepatocytes
East Kleinemonde	2.9	5/10	4/10	1/10	5/10
Old Woman's	2.6	5/5	1/5	2/5	2/5
Mtana	3.0	7/9	4/9	1/9	0/9

Table 5
Biomarker results from *Rhabdosargus holubi*, and sample size for each biomarker (n) measured in individuals from the East Kleinemonde, Old Woman's and Mtana estuaries

Biomarker	East Kleinemonde	n	Old Woman's	n	Mtana	n
AChE (nmol·min^{-1}·mg protein^{-1})	55.06 (26.31-97.41)	96	36.54 [a] (23.60-80.28)	89	55.94 (36.56-164.50)	94
LPx (nmol MDA produced·h^{-1}·mg protein^{-1})	4.51[a] (2.031-8.83)	96	6.88 (2.48-48.17)	89	5.06 (3.20-13.13)	94
Liver histopathology	9/10	10	4/5	5	7/9	9

Notes: 95% confidence intervals are given in brackets for AChE and LPx assays.
The incidence of pathological change in the liver of each fish is presented as a ratio of all fish sampled in the particular estuary.
[a] significantly different (P < 0.01).

pesticide (OPs) concentrations were determined using gas chromatography – flame photometric detection methods. The presence of pesticide residues were confirmed using gas chromatography – mass spectrometry. This multi-residue analysis was undertaken to test for a range of pesticides and pyrethroids (listed in Table 3) as these are the most commonly detected contaminants in South African estuaries (Schulz, 2001; Bollmohr et al., 2007; Bollmohr et al., 2009). Under the conditions of the analyses employed by the SABS, the lowest limit of detection was 0.5 µg·ℓ$^{-1}$.

Statistical analyses

Normality of the data from each biomarker analysis was tested using the Kolmogorov-Smirnov test ($P \leq 0.05$). All the data were non-parametric and therefore the Kruskal-Wallis test was performed, and multiple comparisons of the data sets between estuaries were undertaken using the Mann Whitney U-test. Comparisons were conducted between each pair of estuaries to determine for significant differences in biomarker responses in fish from the 3 estuaries. A Bonferroni adjusted level of significance of $P<0.02$ was used when determining these differences (Zar, 1999). All analyses were conducted using STATISTICA™ Version 7.0.

Results

None of the data were normally distributed; consequently the results are presented as medians with 95% confidence intervals. The 3 estuaries did not differ significantly in terms of the results from the histological analyses performed (Kruskal-Wallis, H=0.6378, df=2, 24, $P=0.727$). The predominant lesions found in fish from the EK were increased melanomacrophage centres, increased perivascular connective tissue and severe vacuolation (Table 4). Nine out of ten fish from the EK showed pathological changes (Table 5). Four out of five fish from the OW (Table 5) showed pathological changes with the most common lesions being increased melanomacrophage centres,

cellular swelling due to hydropic change and severe vacuolation (Table 4). Seven out of nine fish from the MTN showed pathological changes (Table 5), with the predominant lesions being increased melanomacrophage centres and perivascular connective tissue (Table 4). The mean scores for histological alterations as determined from the grading system were 2.9, 2.6 and 3.0 for the EK, OW and MTN estuaries, respectively (Table 4).

The LPx levels in liver samples from the 3 estuaries were found to be significantly different (Kruskal-Wallis, H=23.76, df=287, P<0.01). Levels in fish from the MTN and OW were significantly different (higher) to those in the EK (EK/MTN: Mann Whitney U test, z=-3.557, $P<0.001$. EK/OW: Mann Whitney U test, z=-4.329, $P<0.001$), while the OW and MTN were not significantly different from one another (Mann Whitney U test, z=2.206, $P=0.03$) (Table 5).

Significant differences in brain AChE activity were measured from the 3 estuaries (Kruskal-Wallis, H=44.09, df=293, $P<0.001$). While fish from the EK and MTN did not have significantly different AChE activities (Mann Whitney U test, z=-1.476, $P=0.14$), fish from the OW had significantly lower AChE activities than fish from both the EK (Mann Whitney U test, z=4.781, $P<0.001$) and the MTN (Mann Whitney U test, z=-6.409, $P<0.001$) (Table 5).

Water samples collected from the upper, middle and lower reaches in all 3 estuaries showed pesticide concentrations below the detection limit of the tests employed. Unfortunately, no historical water chemistry data exists for these 3 estuaries either.

Discussion

The estuaries investigated during this study were all temporarily open/closed systems. The last mouth-opening event was 4 months prior to sampling, consequently migration of *R. holubi* into or out of the estuaries during this time was prevented. Thus, the fish investigated during this study had all inhabited their respective estuaries for at least 4 months prior to sampling and were exposed to any pollution events during

this period. Although toxicological analysis of water samples from the lower, middle and upper reaches of the study estuaries failed to reveal the presence of polychlorinated biphenyls, organochlorines, organophosphorous pesticides or pyrethroids (Richardson, 2008), this does not mean that these contaminants were absent from these systems in the prior 4 months. Since pesticide residues are highly variable, both temporally and spatially (Bollmohr et al., 2009), future studies should focus on analysing regular water samples from the study estuaries, as well as measuring tissue burdens of organisms over an extended period in order to identify contaminant stress.

Liver histopathology of fish from the 3 estuaries did not conclusively distinguish between the anthropogenic activities (and associated potential pollutant stressors) present within each catchment. An increase in melanomacrophage centres can be indicative of previous exposure to toxicants but may also be a result of parasitic infection (Hinton and Lauren, 1990; Dezfuli et al., 2007). A macroscopic inspection of each fish was conducted prior to removal of the liver and only 1 sample from the OW was found to have parasites in the mesenteries surrounding the stomach and this specimen was not used for analysis. Parasites can, however, also be present on the gills and in the muscle tissue of fish and these areas were not investigated due to time constraints in the field. A study conducted using flounder (*Platichthys flesus*) from 4 estuaries in England found that melanomacrophage centres were more prevalent in samples collected from contaminated estuaries compared to uncontaminated estuaries (Stentiford et al., 2003). Although the increase in melanomacrophage centres recorded in the present study could possibly suggest exposure to pollutants, further intensive contaminant analysis of the 3 estuaries is required to confirm this.

An increase in connective tissue in the parenchyma was found in fish from all 3 estuaries (Table 4) and, according to Hibiya (1982), this lesion is a result of phagocyte infiltration to a focal area of necrosis. Van Dyk (2003) conducted a histopathological study on *Clarias gariepinus* collected from 3 sites: 2 were considered polluted and the 3rd was a site that was considered to be in pristine condition. The researcher found an increase in connective tissue in the parenchyma in 60% of the fish caught in one of the polluted sites and 35% of the fish caught from the 2nd polluted site. None of the fish collected from the control site had an increase in perivascular tissue. The above results, coupled with findings from the present study, suggest that this type of lesion may be a potentially useful indicator of exposure to xenobiotics in *R. holubi*.

Hydropic change in a marine flatfish (*Pleuronectes vetulus*) and in 3 estuary-associated species (*Platichthys flesus, Pomatoschistus minutus* and *Zoarces viviparous*) was described in 2 northern hemisphere studies and in both cases was shown to be correlated with exposure to hydrocarbons (Stehr et al., 1998; Stentiford et al., 2003). In addition, reduced occurrences of hydropic change in the flounder, *Pleuronectes americanus*, occurred when improved sewage management practices in Massachusetts resulted in decreases in heavy metal and organic compounds within the sewage effluent entering receiving waters (Moore et al., 1996). A few *R. holubi* from each estuary in this study showed evidence of hydropic change in their hepatocytes. However, quantification of the natural level of occurrence of hydropic change in *R. holubi* is required in order to ascertain the extent of exposure to contaminants.

Vacuolation was only found in fish from the EK and OW (Table 4). Vacuolation has been shown to occur as a result of exposure to xenobiotics. For example, Wester et al. (1990)

exposed *Oryzias latipes* to various concentrations of bis(tri-*n*-butyltin)oxide and di-*n*-butyltindichloride, and found a dose-dependent increase in glycogen-related vacuolation in the liver in response to exposure to both of these chemicals. In a similar study, Crestani et al. (2007) investigated the effects of short-term exposure to herbicide clomazone on liver histology and vacuole formation in silver catfish, *Rhamdia quelen*. These authors reported vacuolation of hepatocytes for various exposure durations and both the above studies suggest that vacuolation may be indicative of short-term toxicant exposure.

The fish from the OW and MTN had significantly higher LPx levels compared to the EK (Table 5), suggesting that these fish experienced oxidative stress. Dorval et al. (2005) investigated LPx levels using the white sucker (*Catostomus commersoni*) and found that the fish from 3 polluted sites showed significantly higher LPx levels in the liver tissue than those taken from the control site. These authors also found that plasma AChE levels were depressed when compared to a control site, probably as a consequence of pesticides in the water at the polluted sites. Similar results were reported by Sole et al. (2006), who found significant increases in LPx in *Pomatoschistus minutus* following the release of pesticide-containing water from an upstream dam. *Pomatoschistus minutus* was sampled throughout the year, and increased LPx levels were only reported during periods of high rainfall or when water was released from the upstream dam, coinciding with a significant decrease in AChE activity (Sole et al., 2006). Interestingly, Sole et al. (2006) noted that LPx levels were relatively uniform (low standard errors) during low levels of LPx activity, a pattern that was mirrored in the samples from the EK, with high standard errors during periods of high LPx levels; this was also the pattern noted in the data from the OW (Table 5) in this study.

The brain AChE levels in fish from the OW were 35% lower than the values obtained for fish from the EK and MTN (Table 4). Varo et al. (2003) consider inhibitions greater than 20% to be indicative of exposure to acetylcholinesterase inhibiting chemicals. The per cent inhibitions recorded during the present study are less than those found in related studies which used similar techniques (Coppage and Braidech, 1976; Minier et al., 2000; Kirby et al., 2000; De la Torre et al., 2002; Sole et al., 2006). However, all of those studies were undertaken in heavily industrialised rivers, possibly explaining the high inhibition levels recorded. In contrast, the OW is not heavily influenced by industrial land-use, but could possibly be impacted by pesticides during spray-drift and rainfall runoff from the nearby golf course.

When considered in combination, the biomarker results from the EK suggest that the *R. holubi* in this estuary were in good health at the time of sampling. The overall score for the EK in terms of the histology was less than 3, despite a high (9 out of the 10 samples) occurrence of pathological changes to the liver. The AChE levels were high and the LPx levels were low, suggesting that the brain and liver tissues were not affected by anticholinesterase compounds or oxidative stressors respectively. The predominant lesions that were recorded using the histopathology were an increase in melanomacrophage centres and an increase in perivascular tissue. Both of these pathologies suggest previous exposure to stressors as they are indicative of cellular repairing mechanisms. The stressors may have been of anthropogenic (e.g. chemical) or natural origin (e.g. parasites). The low LPx and absence of other histopathological signs of stress, such as a degeneration of the parenchyma, suggests that the liver was no longer undergoing stress at the time of sampling (Richardson, 2008).

The biomarker results suggest fish from the OW were stressed. Four of the five histological samples of fish from the OW showed signs of pathological changes to the liver. In addition, there was large AChE inhibition and high LPx levels in the fish sampled from this estuary. Although the golf course appears to be the principal potential pollution source (since there are no other anthropogenic activities in the catchment), analyses of the water samples did not detect the presence of pesticides. Future studies should investigate both the water quality of precipitation run-off from the golf course into the estuary and monitor the accumulation of contaminants in the body tissues of fish within the system.

The fish from the MTN estuary exhibited increased LPx levels, but AChE activities remained high. In addition, 2 fish from this estuary scored a grade of 4 for the histological analyses. The grade of 4 indicates a degeneration of the liver parenchyma and is indicative of fish that have been strongly affected by hepatic disease. This, combined with the LPx data, suggested that some fish from the MTN were exposed to xenobiotics. The causes of these biomarker responses are unknown since there are limited land-use activities (primarily small-scale subsistence crop farming and cattle grazing) within this catchment. The fact that the AChE levels were unaffected implies that the pollutant is probably not an anticholinesterase agent. However, the only possible source of contaminants to the estuary was from biocide-dipped cattle belonging to local inhabitants. These cattle were regularly observed wading through the estuary as well as feeding on vegetation adjacent to the estuary. Cattle are routinely immersed in a biocide solution to prevent parasites (especially ticks) by the state veterinary services. Dipping occurs every 2 to 3 weeks in winter and every 1 to 2 weeks in summer. Plunge dips are used and the dip solution is Amitraz (chemical name N,N'-[(methylimino) dimethyllidyne] di-2,4-xylidine) (Pretorius, *YEAR*). Further water chemistry and sediment sampling of the MTN estuary, as well as toxicological examinations of the particular pesticide used on the cattle in the area, would be required in order to determine the causes of the biomarker responses measured.

The biomarker methods used during this study were successfully implemented to provide a preliminary assessment of whether anthropogenic activities in the 3 systems were having an effect on the health of resident *R. holubi* or not. The histology methodology applied to *R. holubi* in this study provided a quantitative assessment of the lesions observed, although further investigation is required to fully understand the relationship between the histological responses in *R. holubi* and chemical exposure. The AChE and LPx assays were relatively straightforward to perform and many (~ 35 samples/day) samples could be analysed simultaneously. Other assays related to the liver tissue such as the glutathione assay, the superoxide dimutases assay or measuring metallothionein levels could further assist in determining more precisely which group of chemicals were responsible for the lesions seen in the fish livers, particularly in the EK.

The fact that the chemicals tested for in water from each of the estuaries were below the test detection limits may be due to the time of sampling, as there had been no recent rainfall event to wash any land-based chemicals into the estuaries. Other factors to consider are the short half-life of some pesticides and the influence of salinity on the bioavailabilty of pesticides. Furthermore, the chemicals may have come out of solution and bound to the sediments (De la Torre et al., 2002; Bollmohr et al., 2009), or were present in concentrations below the detection limit of the analyses. These possibilities highlight the shortcomings of using water sample chemical analyses to detect pollution, as the results provide limited insight into the longer-term effects of harmful chemicals on the biota of an ecosystem. Chemical analyses provide a 'snap shot' of the water quality at the time of sampling (Bollmohr et al., 2007) and do not indicate medium or long-term ecosystem health. Biomonitoring has the advantage of integrating past pollution events and can be used to determine the effect of longer-term pollution problems on the aquatic biota (McCarthy and Shugart, 1990). However, most biomarkers lack ecological relevance as they cannot be linked to effects at population or community levels, and consequently should not replace community-level indicators, but rather complement such investigations.

Acknowledgements

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