INTEGRATING MULTIPLE BIOMARKERS OF FISH HEALTH: A CASE STUDY WITH FISH HEALTH IN PORTS

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Abstract

Biomarkers of fish health are recognised as valuable biomonitoring tools that inform on the impact of pollution on biota. The integration of a suite of biomarkers in a statistical analysis that better illustrates the effects of exposure to xenobiotics on living organisms is most informative however most published ecotoxicological studies base result interpretation on individual biomarkers rather than on the information they carry as a set. To contrast interpretation of results from individual biomarkers with an interpretation based on multivariate analysis, a case study was selected where fish health was examined in two species of fish captured in two ports located in Western Australia. The suite of variables selected included chemical analysis of the white muscle, body condition index, liver somatic index, hepatic ethoxyresorufin-*O*-deethylase (EROD) activity, serum sorbitol dehydrogenase (SDH) activity, biliary polycyclic aromatic hydrocarbons (PAH) metabolites, oxidative DNA damage measured by serum 8-oxo-dG, and stress protein HSP70 measured on gill tissue. Statistical analysis of individual biomarkers suggested little consistent evidence of the effects of contaminants on fish health. However, when biomarkers were integrated as a set by principal component analysis (PCA), there was evidence that the health status of fish in Fremantle port was compromised, mainly due to increased liver-somatic index and higher oxidative DNA damage in fish captured within the port area, relative to fish captured at a remote site. The conclusions achieved using the integrated set of biomarkers demonstrate the importance of viewing biomarkers of fish health as a set of variables rather than isolated biomarkers of fish health.

Keywords: EROD activity, SDH activity, PAH bile metabolites, DNA damage, 8-oxo-dG, heat shock proteins, HSP70, fish biomarkers, ports, PCA analysis

Introduction

Fish exposed to chemical stressors may exhibit outward signs of detrimental effects such as lesions, imbalance or death. Under laboratory conditions, organisms might be exposed to a single or to a few chemicals while under field conditions, exposure to multiple contaminants is simultaneous and occurs at constantly variable levels.

Biomarkers of exposure and effects can inform on induced metabolic perturbations caused by low-level exposure to contaminants. Biomarkers of effects are those that provide information on deviations related to the health status of an organism (van der Oost et al. 2003) while biomarkers of exposure can confirm exposure to contaminants without being directly associated to a known adverse health effect. In an environment with a potentially large range of chemical stressors, it is relevant to assess fish health using a battery of biomarkers of exposure and effect. Fish and other vertebrates are complex organisms with interconnected metabolic functions. It is therefore important to consider biomarkers of fish health as a set of data depicting an overall health status in exposed organisms rather than considering isolated biomarkers that can only provide fragmented information on the effects of exposure on biota.

The chronic and constantly variable exposure levels experienced by fish under field conditions ensues a higher biomarker variability. This implies that a larger number of organisms are required in field studies to demonstrate inter-site differences, if one exists (Gagnon and Hodson 2012). Despite apparent differences between sites, the higher biomarker variability often results in non-statistically significant outcomes when biomarkers of fish health are considered individually (e.g. Danion et al. 2014; Scarcia et al. 2014), with consequences that can result in the lack of managerial actions and further environmental deterioration.

This paper uses a case study to illustrate how interpretation might differ, should a suite of biomarkers of fish health be interpreted individually or should these same biomarkers be

analysed as a single set of data using multivariate methods. To illustrate the differing interpretation, the study investigated the health of fish inhabiting two typical ports, being the ports of Fremantle and the port of Albany, located in Western Australia and examining a suite of biomarkers of effect and exposure combined with chemical analysis of contaminant body burdens. It was hypothesised that the health of fish captured in the ports would be compromised by some of the stressors associated with the xenobiotic inputs released in the port environments. Interpretation of fish health status derived from individual biomarker is compared to an interpretation based on a multivariate analysis which integrates multiple biomarkers in a set of variables.

Case-Study: Fish Health in Ports

Coastal cities rely on ports as import and export pathways for produced goods. Bulk marine transport can be a highly polluting industry with ships representing a significant source of petroleum products, antifoulants, xenobiotics in ballast water, and other contaminants associated with their cargo to the waters of the ports they frequent (Hallegraeff and Bloch 1991, Bailey and Soloman 2004, Corbett, Winebrake et al. 2007, Cassi, Tolosa et al. 2008). Ports are often located in protected harbours and/or estuaries, which receive waters from agricultural areas often contributing organochlorine and organophosphate pesticides. Ports are usually closely associated with residential settlements and with growing public acknowledgement of the importance of the health of aquatic ecosystems, the potential impact of ports and port activities have been increasingly acknowledged as a social, political and environmental concern.

The main settlements in Western Australia are located in the south west of the state and are isolated by hundreds of kilometres from the rest of the country making it necessary to

transport large quantities of cargo by sea from, and to, domestic and international markets via ports with large capacity.

Ports are generally located on estuaries and are, or have been, areas of high ecological value. Estuaries provide habitat for a range of endemic aquatic flora and fauna, and can be nursery grounds for commercially and recreationally important fish species (Beck, Heck et al. 2001). Despite this, ports tend to be subject to a high degree of disturbance from shipping and related activities, and the ecosystem services they provide have been lessened. Most importantly, however, is the continuous input of contaminants that has the potential to affect the surrounding environments. While it is accepted that industrial port areas are highly disturbed environments, it is important that the potential impacts of port activities on the health of fish residing in the ports or surrounding areas and those with a transient residency are understood.

Fish Sampling Locations

Fremantle Harbour, located west of Perth at the mouth of the Swan-Canning estuary is the landing point for the majority of imports to Western Australia (Fig. 1). The second study site is the port of Albany. The city of Albany is located on the south coast of Western Australia, with its port located on Princess Royal Harbour which opens onto the Southern Ocean (Fig. 1). In 1983 the western side of Princess Royal Harbour was temporarily closed to fishing following the discovery of sediment and biota contaminated by mercury and lead from industrial discharges. This closure has been lifted in 1992 as a result of steadily declining metal concentrations in most fish species (Water and Rivers Commission, 1999). For comparative purposes, the remote site of Muir Rock, a small rocky outcrop 20 km offshore from Perth, away from any industrial outfalls, was selected. Two fish species were selected for this study based on availability from both port and remote sites. Yellowtail scad (*Trachurus novaezelandiae*) was collected from Fremantle port and the offshore remote site,

while skipjack trevally (*Pseudocaranx wrighti*) were collected from Albany port and the offshore remote site. Within the ports the fish were captured by handline while at the remote site the fish were captured in commercial nets.

Morphological Measurements and Biopsy Collection

Blood samples were collected from each fish immediately after capture using vacutainers (Becton Dickinson), allowed to clot on ice, centrifuged (1600*g* for 10 mins at 10°C) and the serum collected and stored in liquid nitrogen. The fish were sacrificed by *iki jime* (Robb et al. 2000) immediately following blood collection, weighed and the standard length measured. Fish were dissected along the ventral line using sharp scissors and the internal organs removed. The liver was weighed and sub-sampled to two cryovials before freezing in liquid nitrogen. Fresh bile was removed from the gall bladder using a syringe and immediately frozen in liquid nitrogen. Viscera and gonads were removed and the carcass weight (body weight less viscera and gonad weights) was recorded. A sample of white muscle (10-20 g) was collected and placed in aluminium foil pre-rinsed with HPLC-grade methanol for chemical analysis. Fish were handled according to the Curtin University of Technology Animal Ethics approval No. N39/08 . Using the standard length (SL), carcass weight (CW) and liver weight (LW), the body condition index ($BCI = 10^n \times CW / SL^3$) and the liver-somatic index ($LSI = 100 \times LW / CW$) were calculated.

Tissue Chemical Analysis

The white muscle samples were analysed for 8 metallic, 19 organochlorine and 15 organophosphate compounds. These compounds and the detection limits in the analyses are shown in Table 1. For comparative purposes with past studies, elemental mercury was measured rather than methylmercury. Metals were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES), and organochlorines and organophosphates were determined by gas chromatography – mass spectrometry (GC-MS).

Biomarkers

A suite of biomarkers of exposure and effect were quantified for each of the fish captured at the port and remote sites. The biomarkers studied and the related stressors and effects are summarised in Table 2.

Serum Sorbitol Dehydrogenase (S-SDH)

S-SDH levels were determined as described in Webb and Gagnon (2007). Briefly, frozen serum samples were thawed on ice and 50 µl of sample was mixed with 450 µl of 1.28 µM NADPH (in 0.1M Tris buffer - pH 7.5). After 10 min incubation at room temperature 100 ul of 4 M fructose solution was added to start the reaction. SDH levels were determined using a Pharmacia LKB-Biochrom 4060 spectrophotometer. This assay determines the reduction in fructose concentration using the co-enzyme NADPH by the spectrophotometric changes during the reaction time. Results are shown in milli-International Units (mIU), calculated by

$$mIU = \frac{\Delta A / min \times Vol\ Reaction}{Extin.\ Coeff. \times Vol\ Sample}$$

where ΔA is the change in absorbance (340 nm) over 1 minute. The total volume of the reaction is 0.6 ml, the micromolar extinction coefficient of NADPH at 340 nm is 0.0062 and the volume of serum used in the assay is 0.05 ml.

EROD Activity

Liver samples were thawed on ice and homogenised for *ca.* 5 seconds in 0.02 M HEPES buffer using a Heidolph Diax homogeniser. The resulting homogenate was centrifuged (9000*g* for 20 mins at 4ºC) and supernatant (S9 fraction) collected. The protein content of each supernatant sample was determined using Folin phenol reagent as described by Lowry et al. (1951).

A standard curve of the fluorescence of known resorufin concentrations was generated prior conducting the assay. Samples were prepared in duplicate following the method described in

Webb and Gagnon (2002) and the rate of resorufin formation (Ex = 530 nm, Em = 585 nm) measured on a Perkin Elmer LS45 Luminescence spectrometer. The fluorescence of each sample was converted to a concentration of resorufin using the standard curve and results presented as the pmol resorufin produced / minute / mg protein.

PAH Bile Metabolites

PAH biliary metabolites of naphthalene, pyrene and benzo(a)pyrene were quantified using fixed wavelength fluorescence as described in Webb et al. (2005). This method measures several metabolites issued from a parent compound that are fluorescing at a specific wavelength. Each bile sample was run in duplicate and diluted in order for the fluorescence readings to fall within the range of the linear standard curve. Assay parameters are described in Table 3. Pyrene and benzo(a)pyrene metabolite concentrations are reported in ng / ml and naphthalene metabolite concentrations are reported in mg / ml.

HSP70

HSP70 concentration in gill tissues was measured following the method of Martin (1996) as adapted by Webb and Gagnon (2009). Gill tissues were mechanically homogenised in 10 mM Tris buffer (with 0.1 M phenylmethylsulfonylfluoride) and centrifuged (13700 *g* for 98 mins at 4^{o}C). Supernatant protein content was measured by the method described in Lowry et al. (1951) , diluted to a total concentration of 2 µg protein /µl in DI water, further diluted (1:2, v:v) in Laemmli sample buffer (Sigma-Aldrich) and heated in a 95^{o}C water bath for 4 minutes. Samples and standards were loaded into the outer wells of a 12% Tris-Glycine gel and electrophoresed in a Bio-Rad Mini Protean module (225 V, 120 mA, 40 mins). The proteins were transferred to 0.2 µm Whatman Protran nitrocellulose membrane (Interpath) in a Bio-Rad Mini Trans Blot Electrode Module (100 V, 250 mA, 60 mins) and probed with a mouse anti-HSP70 monoclonal antibody (IgG1). The membrane was rinsed and a secondary antibody (goat anti-mouse IgG peroxidise conjugate – Sigma) applied for 2 hours.

Membranes were developed in a dark room using a commercial chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate Kit – Quantum Scientific). The pixel density of visible bands in the 70 kDa range was quantified using Scion Image software for windows. The bands on different blots were calibrated to standardized heat shocked control samples to enable comparison between blots, as described in Webb and Gagnon (2009). HSP70 concentrations are expressed as pixel density per µg total protein.

Oxidative DNA damage

Oxidative DNA damage was quantified using a commercially available ELISA kit for the quantification of 8-oxo-2'-deoxyguanosine (8-oxo-dG), a kit especially designed for measurements of 8-oxo-dG in liquid samples such as serum (Trevigen). 8-oxo-dG is removed from DNA by the base excision repair pathway and subsequently transported into saliva, urine and plasma, with higher levels of 8-oxo-dG suggesting higher oxidative DNA damage (Trevigen 2015). Diluted serum samples and 8-oxo-dG standard were added to a 96-well microplate pre-coated with an 8-oxo-dG conjugate in the presence of a monoclonal anti-8-oxo-dG antibody and incubated for 1 hour at room temperature. The plate was washed and a secondary antibody (anti-mouse IgG: HRP conjugate) added to each well. The plate was again washed and developed with a stabilised tetramethylbenzidine (TMB) substrate and the reaction stopped with 2M H_2SO_4. The absorbance at 450 nm is inversely proportional to the amount of 8-oxo-dG in the original sample and the resulting value was standardised against the total protein in the original serum sample.

Data Analysis

The aims of the study were (1) to compare interpretation of biomarkers when statistically analysed one biomarker at a time, or analysed using multivariate tools, and (2) to investigate if differences in biomarker levels could be identified between the fish captured in ports relative to biomarker levels in fish collected outside port infrastructures. Although the design

could be considered to contain two analysable factors (location as either port or remote, and species) a fully factorial analysis across these factors was not considered appropriate as the *a priori* hypotheses were not concerned with differences between species but the influence of the location only. Similarly, given the fact that there are likely to be differences in the response of different species to similar contaminants (e.g., metabolism *vs.* accumulation, biomarker induction potential) pooling of data for the two different species collected within the port and remote locations was not appropriate. Therefore, data were firstly analysed using single factor ANOVA between populations of yellowtail scad from the Fremantle port (n=10) and the remote site (n=7), and skipjack trevally from the Albany port (n=12) and the remote site (n=6) using the SPSS statistical package (V. 17.0). As these were generally small sized fish it was not always possible to collect all biopsies (especially bile samples) resulting in a reduced sample size for some biomarkers. Since multiple measurements were recorded on each specimen, the use of a data reduction method (e.g., multivariate analyses) was also considered an appropriate method capable of providing further information on the overall health status of the studied fish. Between and among group differences across measured endpoints were examined using analyses of similarity (ANOSIM) on a normalised, Euclidean distance resemblance matrix. Therefore there were some missing data points which meant that is was not possible to consider all biomarkers in a single multivariate analysis as described above. Consequently the bile metabolite variables were analysed separately from the remainder of the biomarkers. The ANOSIM could consider only fish with a complete set of biomarker data (other than bile metabolites) and therefore, the number of fish included in the analysis might be substantially reduced at some sites. Where ANOSIM suggested a significant difference between groups at port and remote sites a principal components analysis (PCA) was run to investigate which biomarkers were important in describing the differences. All multivariate analyses were conducted using the PRIMER multivariate statistical package.

Results

Ten (10) yellowtail scad were captured at the port of Fremantle, while sixteen (16) skipjack trevally were collected in the port of Albany. At the remote site of Muir Rock, seven (7) yellowtail scad and six (6) skipjack trevally were captured. All fish were juvenile according to their weight and length (Fishbase), and had no signs of gonadal development.

Fish Morphology

The yellowtail scad captured at Fremantle was statistically similar in standard length, weight, liver-somatic index and body condition index as those captured at the remote site (Table 4). The skipjack trevally captured at Albany port had the same standard length, body weight and liver-somatic index as those captured from the remote site but had a significantly reduced body condition index (ANOVA, $F_{3, 29} = 21.37$, $p < 0.001$) (Table 4).

Tissue Chemistry

White muscle concentrations of the organochlorine and organophosphate compounds included in the analysis were all below the respective assay detection limits. As, Cu, Hg and Zn were detected in all samples. There was no significant difference between the tissue concentrations or As, Cu or Zn in fish captured in the remote site and either of the port sites in the respective species (Table 5). Cr, Ni and Pb were all detected in only one sample each, as flows: yellowtail scad from Muir Rock: 0.13 mg Cr/kg, yellowtail scad from Fremantle port: 0.07 mg Ni/kg, and skipjack trevally from Albany port; 0.2 mg Pb/kg. Cd was not detected in any of the fish tissue samples. While Hg concentrations were similar in tissues in yellowtail scad from Fremantle and the Muir Rock remote site they were higher in muscle tissue from skipjack trevally from Albany port compared to those from Muir Rock (Table 5).

S-SDH

Serum sorbitol dehydrogenase (s-SDH) activity measured in fish from Muir Rock was not significantly different to that measured in fish either from Fremantle port (ANOVA, $F_{1, 15}$ = 1.103, p = 0.330, Fig. 2) or from the Albany port (ANOVA, $F_{1,15}$= 0.926, p = 0.351, Fig. 2).

EROD

Basal EROD activity was estimated from levels measured in fish from the remote site of Muir Rock as slightly higher in skipjack trevally (22.8 pmol res/min/mg protein) than in yellowtail scad (12.35 pmol res/min/mg protein). In both species there was no statistically significant induction in fish captured from the port sites relative to fish from the remote site (Fremantle: ANOVA, $F_{1, 14}$ = 1.024, p = 0.329; Albany: ANOVA, $F_{1, 19}$ = 1.735, p = 0.203) (Fig. 2).

PAH Bile Metabolites

The background fluorescence levels of the biliary secretions for each fish species, at each PAH wavelength measured, were estimated from values measured from fish collected at the remote site of Muir Rock. There was no significant biliary metabolite accumulation above these background levels for naphthalene (Fremantle: ANOVA, $F_{1, 8}$ = 2.685, p = 0.140; Albany: ANOVA, $F_{1,9}$ = 2.240, p = 0.169), or benzo(a)pyrene (Fremantle: ANOVA, $F_{1,8}$ = 0.023, p = 0.883, Albany: ANOVA, $F_{1,9}$ = 3.736, p = 0.085 either for yellowtail scad from Fremantle port or for skipjack trevally from Albany port (Fig. 3). Similarly, levels of pyrene metabolites were not significantly elevated in fish collected at the Fremantle port (ANOVA, $F_{1,8}$ = 3.131, p = 0.115), however, pyrene metabolites levels were higher in fish collected within the Albany port relative to those from the remote site (ANOVA, $F_{1,9}$ = 7.687, p = 0.022) (Fig. 3).

HSP70 protein levels

There was no significant difference in the gill HSP70 concentrations in fish collected in the Fremantle port compared to the levels in fish originating from the remote site (ANOVA, $F_{1, 13} = 0.032$, $p = 0.860$) or between the HSP70 concentration in fish captured at Albany port relative to the levels observed in the yellowtail scad collected at the remote site (ANOVA, $F_{1,20} = 0.006$, $p = 0.941$) (Fig. 4).

Oxidative DNA damage

Background levels of 8-oxo-dG were estimated from the concentrations measured at the Muir Rock remote site and these were similar for each of the two species included in the study (yellowtail scad = 27.9 ng/ml; skipjack trevally = 23.2 ng/ml). For both fish species, the mean 8-oxo-dG concentrations measured at Fremantle port or at Albany ports was not significantly different from the levels measured at the remote site (Fremantle: t-test, t = 1.225, $p = 0.244$: Albany: t-test, t = 1.931, $p = 0.098$) (Fig. 4).

Multivariate Analyses

When the pyrene, nathpthalene and benbzo(a)pyrene metabolite concentrations were considered together, there was no difference between the bile of fish captured at Fremantle port and at the remote site (ANOSIM, Global-R = 0.088, $p = 0.278$) and the same was the case for fish captured at Albany compared to the remote site (ANOSIM, Global-R = 0.148, $p = 0.238$) (Table 6). When the remainder of the biomarkers were analysed together there was no detectable difference between the fish captured at Albany Port and the fish captured at the remote site (ANOSIM, Global-R = -0.004, $p = 0.475$) (Table 6). It is important to note that because the ANOSIM could only consider fish that had a complete set of biomarker data, the number of fish at Albany was reduced to 3 individuals. A PCA of the Albany data supports this lack of separation based on biomarker profile and suggests that most of the within site

variation at the remote site is explained by oxidative DNA damage and condition index (Fig. 5).

There was an overall difference between the biomarker profile of fish captured at Fremantle and that in the fish captured in the remote site (ANOSIM, Global-R = 0.376, $p = 0.006$) (Table 6). A PCA of these data indicated that the fish from the remote site were more similar in biomarker profile than those at the port, in which there was greater variability (Fig. 5). Much of the variation between the sites and within the port site occurred along PC1 which was described most by body condition index, liver somatic index, hepatic EROD activity and oxidative DNA damage (Fig. 5). Differences in the biomarker profile of fish from the remote site were explained most by differences in SDH (Fig. 5).

Discussion

Accumulation

The fish captured at Fremantle and Albany ports and the remote site had not accumulated any of the tested organochlorine or organophosphate compounds analysed in muscle tissues. Many of the organochlorines measured are highly accumulative so a lack of any measureable quantity is interpreted as a lack of exposure. Zn and Cu are important micronutrients that are metabolically regulated in vertebrates (Papagiannis et al. 2004) and consequently, low concentrations in the muscle tissue would be expected. For both species of fish, the Zn and Cu values recorded at all sites are well below the generally expected level (GEL) 90th percentile trigger levels for these metals derived for human consumption purposes (Australia New Zealand Food Standards 2015).

The finding that Hg concentrations were increased in fish captured at Albany port suggests the bioavailability of this contaminant in this environment. The interpretation of the Hg

concentration in Albany requires special consideration of historical contamination. Areas adjacent to the site of capture of these fish were closed to fishing in the 1980s due to excessive levels of these metals in both sediments and biota. Francesconi and Lenanton (1992) reported Hg levels in fish from Albany port of up to 3.6 mg / kg, much higher than those measured in the current study but lower than those which caused the fishing ban. Comparatively, Hg levels of 0.2 mg/Kg wet weight are still higher in Albany port relative to levels reported in fish flesh collected in other port environment. For example, Bagherpour and Soltanialvar (2014) evaluated Hg levels in black sole (*Brachirus orientalis*) from Abadan port as 0.043 mg/Kg wet weight. Results from the Albany port Hg levels in fish flesh suggest the influence of legacy contamination, which requires ongoing monitoring, rather than current activities within the port of Albany.

While the concentrations of Hg measured in fish captured in the port of Albany were higher than those collected at the remote site they were at the lower end of concentrations reported in the literature. For example Goldstein et al. (1996) reported concentrations up to 0.38 mg/kg in carp and cited studies which measured up to 0.77 mg/kg in muscle tissues of other freshwater species. Estuarine fish in Florida had even higher total mercury concentrations up to 4.98 mg/kg (Kannan, Smith et al. 1998). In a review of the impacts of Hg to wildlife Eisler (1987) indicated that laboratory induced body burdens of 20 to 30 mg/kg led to sublethal changes on appetite and maintenance of equilibrium in rainbow trout but there appears little evidence in the literature of environmentally measured body burdens of Hg (generally an order of magnitude lower than these laboratory induced concentrations) impacting on fish health. It is, therefore, unlikely that the concentrations of Hg measured in skipjack trevally captured in the port of Albany would cause detrimental effects on the health of the fish.

The levels of As were not increased in either of the ports above the background levels measured in fish from the remote site. However the concentration of As in the muscle of

skipjack trevally exceeded the Australian New Zealand Food Standard trigger value of 2 mg/kg in 7 of the 10 yellowtail scad and 1 of the 10 skipjack trevally captured for this study. While this does not provide information on the health status of the fish in the ports it is worth noting that As levels in skipjack trevally collected at Fremantle port or at Muir Rock may be above background levels.

Exposure

There was little evidence to suggest that the fish captured within the two port zones of Fremantle and Albany were exposed to high levels of organochlorines or organophosphates. EROD activity is a sensitive biomarker for exposure to compounds which induce the production of CYP1A in response to binding to the aryl hydrocarbon receptor (particularly the co-planar chlorinated aromatics; PCBs, dioxins and furans; reviewed by Whyte et al. 2000). While these compounds are highly hydrophobic and generally in much higher concentration in sediments, exposure through bioaccumulation and magnification in predatory species can be high (Gobas 1990, Stow and Carpenter 1994, Jones, Kannan et al. 2001, Otchere 2005, Magnusson, Ekelund et al. 2006). A few published studies report EROD induction in fish captured in ports as significantly higher than that measured in remote fish (Richardson, Davies et al. 2001, Fuentes-Rios, Orrego et al. 2005). In the current study there was no evidence of exposure to CYP1A inducing compound based on chemical analysis of the flesh, or on EROD activity.

Fish are able to quickly metabolise PAHs, with biliary metabolite levels having been shown to return to background levels within 6 days following the end of exposure (Gagnon and Holdway 2000). Since this high metabolism of PAHs precludes the direct measurement in body tissues, PAH biliary metabolites have been shown to be an effective measure of this metabolism and consequently, represent an indirect measure of exposure to PAHs (Ariese, Kok et al. 1993, da Silva, Buzitis et al. 2006). While, there was also no consistent,

significantly higher levels of PAH biliary metabolites in fish captured in the ports above the levels measured in fish captured in the remote site a few points of interest should be considered. The significant increase in pyrene metabolites above background levels (i.e. levels measured in fish collected at the remote site) in the skipjack trevally captured from Albany port is indicative of the trend across all PAH metabolites (though there was no statistical relationship for benzo(a)pyrene and naphthalene). This is an indication that the fish at this port had indeed been exposed to PAHs. Conversely there is no consistent evidence or trends to suggest that the fish captured in the Fremantle port had been exposed to PAHs at all. It could be reasonably expected that the most common pollutants in ports would be of petrochemical origin. While, the majority of these pollutants are aliphatic hydrocarbons, the more bioactive are aromatic PAHs. Exposure to PAHs has been related to a multitude of detrimental effects in aquatic biota including DNA damage (Dévier et al. 2013). Given the high metabolism of these compounds by fish, the biliary metabolite levels observed in the skipjack trevally from Albany port (a very popular recreational fishing area) suggest a continuous exposure of fish to these compounds.

Effects

8-oxo-dG is a common marker for oxidative DNA damage occurring as a result of oxy-radical formation which can be induced by exposure to a range of contaminants commonly found in port environments including metals and PAHs (Valavanidis, Vlachoogianni et al. 2009). In the present study there was a trend toward increased oxidative DNA damage at the port sites, however when tested with a regular ANOVA the lack of statistical significance makes the drawing of clear conclusions challenging. It is likely that the fish captured within in the ports are subject to greater oxidative stress than those at the remote site.

There was an indication that some individual fish may have compromised liver integrity (as measured by increased SDH activity), at both ports but, again, this was not sufficient to drive

a statistical difference. A decrease in liver integrity could result in a reduction in the ability of the animal to effectively metabolise potentially damaging compounds such as PAHs.

In general, there was little morphological indication that the health of the fish collected from within the ports was any different to those at the remote site. The fish were slightly (but not significantly) shorter in length at the remote site but were of similar weight. There was, however, a decrease in the condition of fish at Albany compared to the remote fish. Decrease in condition can be the result of a number of factors, but particularly can relate to food availability. In the present study fish are compared across a wide geographic range and it is not possible to rule out differences in food resources as a potential driver for a difference in body condition.

Multivariate analysis of biomarker data

When considered individually, the suite of biomarkers of effect studied presented no consistent evidence that the health of the fish captured with in the ports was impaired. Given the non-significant trends described above it was expected that the multivariate analyses would provide a clearer picture as to the overall relative health, as measured by biomarkers, of individual fish from each location. Despite the geographical separation between the fish captured in Albany port and those at the remote site there was no difference in the overall health status between the fish living in these locations which could be related to either this spatial distance or the presence of port activities.

The liver somatic index was 0.21x higher in the Fremantle yellowtail scad relative to those from the remote site, suggesting a metabolic change in fish chronically exposed to pollutants in this location (van der Oost et al. 2003). Biochemical markers of fish health e.g. PAH metabolites, combined with altered LSI suggest that the fish collected in the Fremantle port had an impaired health status.

While the individual analysis of biomarker results suggests that there was little difference between the fish collected in the port and Fremantle and from the remote site, combining the data indicates that there are some notable differences. The most significant of these is the fact that the inter-individual variation in biomarker levels is much greater in fish captured within the port. This is likely due to the differential sensitivities of individual fish to the range of stressors experienced within their habitat. Most of this inter-individual variation was explained by the body condition index, liver-somatic index and DNA damage. The individual stressors causing the biomarker variability between the fish are unclear, as are the potential long-term impacts on these fish. However, despite the lack of statistical difference when biomarkers are considered individually, the multivariate analyses made it clear that differences do exist between the health status of the two fish populations sampled at the Fremantle port and at the remote site. A limitation of this multivariate analysis is that only fish with complete set of biomarker data can be included in the analysis, meaning that a much reduced number of fish can be used in the multivariate process. Nevertheless, the results from the multivariate analysis demonstrate the importance of viewing biomarkers of fish health as a set of indicators rather than individual variables. Other studies using multivariate analysis were also able to establish links between variables, links that were not evident when considering biomarkers individually (Patino et al. 2015). While statistical differences between the skipjack trevally collected at Fremantle and at the remote site suggests differences in health status, further large scale studies would be require to confirm if the observed differences translate into effects at population levels.

Conclusion

Biomarkers of exposure and effects have become recognised monitoring tools used by decision-makers to evaluate the potential impacts of pollution on biota. While it is important to select a suite of biomarkers that are relevant to specific situations, individual biomarkers taken in isolation can only contribute limited information on the overall health status of

460 animals. The case study presented describes such a scenario where the suite of biomarkers demonstrated no consistent evidence that the health of the fish captured with in the ports was altered. However, the integration of the same suite of biomarkers in a multivariate analysis clearly evidenced the effects of exposure to xenobiotics on living organisms, and demonstrated the advantage of multivariate analysis as an effective tool to evaluate the status 465 of impacted systems.

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Tables

575 **Table 1. Analytes in fish white tissue analysis. Figures in brackets are the chemical detection limits for the analyte in mg/kg. Detection limits for all organochlorine and organophosphate compounds were 10 µg/kg except where indicated.**

Metals	Organochlorines	Organophosphates
Arsenic (0.1)	Aldrin	Dichlorvos[b]
Cadmium (0.02)	α-BHC	Demeton-S-methyl
Chromium (0.1)	β-BHC	Diazinon
Copper (0.1)	γ-BHC (Lindane)	Chlorpyrifos-methyl
Mercury (0.01)	δ-BHC	Parathion-methyl
Nickel (0.05)	*cis*-Chlordane	Pirimiphos-methyl
Lead (0.15)	*trans*-Chlordane	Fenitrothion
Zinc (0.06)	*p,p'*-DDD	Chlorpyrifos
	p,p'-DDE	Fenthion
	p,p'-DDT	Parathion
	Dieldrin[a]	Chlorfenvinphos[b]
	alpha-Endosulfan[a]	Bromophos-ethyl
	beta-Endosulfan[a]	Methidathion[a]
	Endosulfan sulphate[a]	Prothiofos
	Endrin ketone	Ethion
	Heptachlor	
	Heptachlor epoxide	
	Hexachlorobenzene	
	Methoxychlor	

[a] limit of detection = 100 µg/kg

[b] limit of detection = 50 µg/kg

580 **Table 2. Suite of biomarkers used in the study with the exposure or effect indicated by each described.**

Type of biomarker		Suggest exposure to:	An indicator of:	
Exposure	PAH bile metabolites	PAHs		Ariese et al. 1993
	EROD activity	PCBs, dioxins, furans		Whyte et al. 2000
Effect	Serum SDH		Liver damage	Dixon et al. 1987
	Body Condition Index		General chronic effect	van der Oost et al. 2003
	Liver-somatic Index		Liver metabolic activity, energy reserves	van der Oost et al. 2003
	HSP70 proteins		General stress response	Webb and Gagnon 2009
	DNA damage 8-oxo-dG		DNA oxidative stress	Tomasello et al. 2012

Table 3 Assay parameters for quantification of PAH biliary metabolites.

Parent Compound	Excitation nm	Emission nm	Slit nm	Standard
Naphthalene	290	335	10	1-naphthol
Pyrene	290	335	10	1-hydroxypyrene
Benzo(a)pyrene	380	430	10	1-hydroxypyrene

585 **Table 1. Mean (95CI) of morphological measures on fish captured at Fremantle and Albany ports and a remote site. * = significantly different to the corresponding measure in fish from the remote site (ANOVA, $\alpha = 0.05$).**

		Std Length (mm)		Body Weight (g)		LSI		CF	
Fremantle (yellowtail scad)	Port	158	(8.1)	62	(10.3)	0.91	(0.41)	1.41	(0.13)
	Ref	151	(12.9)	52	(12.5)	0.59	(0.23)	1.36	(0.11)
Albany (skipjack trevally)	Port	164	(8.5)	85	(13.7)	0.74	(0.08)	1.83	(0.11)*
	Ref	147	(47.0)	83	(68.5)	1.06	(0.93)	2.21	(0.35)

Std length: standard length; LSI: liver somatic index; BCI: body condition index

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Table 5. Mean (± SEM) in mg/kg wet weight for the metals measured in all white muscle tissues at all sites (n=5 per site). *p* is the value from a single factor (location) ANOVA (α = 0.05).

	Fremantle (yellowtail scad)			Albany (skipjack trevally)		
	Remote	Port	*p*	Remote	Port	*p*
As	2.14 (0.22)	2.02 (0.35)	0.782	1.06 (0.18)	1.44 (0.18)	0.537
Cu	0.42 (0.13)	0.41 (0.09)	0.932	0.47 (0.06)	0.39 (0.05)	0.343
Hg	0.07 (0.02)	0.12 (0.02)	0.108	0.07 (0.02)	0.20 (0.01)	<0.001
Zn	3.80 (0.33)	3.44 (0.37)	0.493	4.02 (0.15)	3.42 (0.34)	0.147

595 **Table 6. Results of multivariate analyses of similarity on normalised biomarker data. Variables included in the "biomarkers" analyses were body condition index, liver-somatic index, serum sorbitol dehydrogenase (SDH), ethoxyresorufin-O-deethylase (EROD) activity, gill heat shock proteins (HSP70) and oxidative DNA damage. ** represents a significant difference between groups of fish captured at the port and remote sites.**

		Variables	Global R	Sig.	
Fremantle	Biomarkers	6	0.376	0.006	**
(yellowtail scad)	Bile Metabolites	3	0.088	0.278	
Albany	Biomarkers	6	-0.004	0.475	
(skipjack trevally)	Bile Metabolites	3	0.148	0.238	

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LIST OF FIGURES

Fig. 1 Location of port sampling sites and the remote site of Muir Rock in Western Australia

Fig. 2 Serum sorbitol dehydrogenase (mIU) and EROD activity (pmol resorufin/min/mg protein) in fish from Albany and Fremantle ports. Boxes represent the interquartile range (IQR). Lines within the boxes denote the median value. Whiskers extend to the last datum within 1.5 x IQR. Open circles are data points outside ± 1.5 x IQR.

Fig. 3 Concentrations of PAH metabolites in the bile of fish captured in Albany port, Fremantle port and a remote site. * = significantly different from the measured value at the remote site (ANOVA, α = 0.05). Boxes represent the interquartile range (IQR). Lines within the boxes denote the median value. Whiskers extend to the last datum within 1.5 x IQR. Open circles are data points outside ± 1.5 x IQR.

Fig. 4 Relative gill HSP70 content (top) and oxidative DNA damage (8-oxo-dG concentration in serum, bottom) in fish collected from Fremantle and Albany ports. Boxes represent the interquartile range (IQR). Lines within the boxes denote the median value. Whiskers extend to the last datum within 1.5 x IQR. Open circles are data points outside ± 1.5 x IQR.

Fig. 5 Biplot of PCA for fish collected from (top) Fremantle port (closed circles) and the remote site (open circles), and (bottom) Albany port (closed circle) and the remote site (open circles). Vectors have been scaled (x2). Inset shows the cumulative variance explained by PCs. SDH = sorbitol dehydrogenase. Condition = Body Condition Index. LSI = liver somatic index.