*Biomarkers*, 2009; 14(7): 452–464

informa
healthcare

RESEARCH ARTICLE

# Two novel non-destructive biomarkers to assess PAH-induced oxidative stress and porphyrinogenic effects in crabs

Samuel Koenig[1], Candida Savage[1], and Jonathan P. Kim[2]

[1]*Departments of Marine Science, and* [2]*Chemistry, University of Otago, Dunedin, New Zealand*



**Abstract**

Two novel, non-destructive assays were developed to evaluate contaminant-induced lipid peroxidation (thiobarbituric acid-reacting substances, TBARS, levels) and haem biosynthesis disruption (porphyrin excretion) in decapod crabs. A laboratory experiment was conducted whereby pie-crust crabs (*Cancer novaezelandiae*) were fed cockles (*Austrovenus stutchburyi*) collected from a contaminated and reference site and TBARS levels and porphyrin excretion determined using fluorometric analysis in urine samples. Pyrene metabolite levels were also measured in the same urine samples to assess polycyclic aromatic hydrocarbon (PAH) exposure. Contaminant-exposed crabs exhibited elevated urinary TBARS and porphyrin levels and a strong correlation was found between these two assays and the urinary pyrene metabolite concentrations. However, there was large within-treatment variability, which precluded a clear separation between the control and the impacted group. Nevertheless, consistency in the direction of the response shows that the biomarkers reflect pollutant levels and validates the use of these simple techniques from human medicine for environmental assessments.

**Keywords:** *Non-destructive biomarker; PAH; crab urine; fluorescence spectrometry; oxidative stress; porphyrin*



## Introduction

Many of the biomarkers applied in environmental assessments have been adopted from human medical tests. In clinical examination, a suite of laboratory tests, rather than an individual test, is used to diagnose a particular disease state. Similarly, it has been suggested that multiple biomarkers be used in pollution-monitoring studies to allow a weight of evidence approach in environmental risk assessment (Galloway et al. 2006). When developing novel biomarkers, it is important to consider the generic nature of the endpoint (i.e. biomarker response), which facilitates the interpretation of responses when applied to organisms across different taxa.

A consequence of normal oxygen metabolism is the production of potentially harmful reactive oxygen species (ROS), also referred to as oxyradicals. Oxidative stress is caused by an imbalance between the production of ROS (i.e. pro-oxidant forces) and an organism's ability to detoxify readily the reactive intermediates (i.e. antioxidant defences) and can lead to enzyme inactivation, lipid peroxidation, DNA damage and, ultimately, cell death (Winston & Di Giulio 1991). Despite the numerous endogenous sources of ROS production, several environmental contaminants (e.g. polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), heavy metals) enhance the intracellular generation of oxyradicals through different mechanisms including the redox cycle, the cytochrome P450-dependent oxidative metabolism of PAHs, and the Fenton reaction in the presence of some transition metals (Winston & Di Giulio 1991, Winston

*Address for Correspondence*: Candida Savage, Department of Marine Science, University of Otago, PO Box 56, Dunedin 9016, New Zealand. Tel.: +64 3 4798324. Fax: +64 3 4798336. E-mail: candida.savage@stonebow.otago.ac.nz

*(Received 09 June 2008; revised 06 January 2009; accepted 16 January 2009)*

ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.3109/13547500903123026

et al. 1996, Livingstone 2001, Regoli et al. 2002, van der Oost et al. 2003).

Lipid peroxidation (i.e. the oxidation of polyunsaturated fatty acids) is one of the most important biochemical perturbations resulting from oxidative stress as it may lead to a free radical chain reaction and result in degenerative propagation reactions. These reactions are usually accompanied by the formation of various products, some of which are toxic by themselves and serve as second messengers for radical damage (De Zwart et al. 1999). Products resulting from the peroxidation of lipids are thus attractive parameters to monitor the adverse effects of oxidative stress and are particularly promising with regard to the development of biomarkers. The most widely used technique for the determination of lipid peroxidation is the measurement of secondary degradation products such as ketones and aldehydes, including malondialdehyde (MDA) (De Zwart et al. 1999). MDA forms a 1:2 adduct with thiobarbituric acid (TBA) to produce a compound that can be measured by fluorometry or spectrophotometry (Uchiyama & Mihara 1978, Camejo et al. 1998).

The measurement of TBA-reacting substances (TBARS), such as MDA, is a well-established method for the evaluation of oxyradical-mediated lipid peroxidation used in the diagnosis of numerous diseases and disorders in human medicine (e.g. diabetes, Alzheimer's disease, Down's Syndrome, asthma) (Liu et al. 1997, De Zwart et al. 1999) where increased MDA levels have been recorded in different human tissues (e.g. plasma, urine, hair) (De Zwart et al. 1999, Sheu et al. 2003). Furthermore, the TBARS assay has been used as a biomarker in a number of environmental studies on aquatic organisms, including bivalve mussels (whole body homogenates, digestive gland) (Viarengo et al. 1989, 1991, Wilhelm Filho et al. 2001, Aloisio Torres et al. 2002, Downs et al. 2002, Romeo et al. 2003, Damiens et al. 2007, Gorbi et al. 2008), decapod crabs (hepatopancreas, haemolymph, gonads) (Vijayavel et al. 2004, 2005, Chaufan et al. 2006) and fish (liver, gonad, plasma) (Ando & Yanagida 1999, Oakes & Van Der Kraak 2003, Oakes et al. 2004, Almroth et al. 2005).

Porphyrins are cyclic tetrapyrrole compounds formed as intermediate metabolites of haem biosynthesis (e.g. protoporphyrin) or their oxidative byproducts (coproporphyrin and uroporphyrin). They are produced and accumulate in erythropoietic tissues (i.e. relating to the formation of red blood cells), liver and kidney and are excreted via urine and faeces (Lim 1991).

Various xenobiotics, including organic contaminants, such as PCBs, organophosphorus pesticides and PAHs, and heavy metals (e.g. mercury, arsenic, lead) interfere with the haem biosynthesis by increasing the rate of oxidation of intermediate porphyrinogens or by direct interference with enzyme activities of the haem biosynthetic pathway, causing modifications in the profile of porphyrin accumulation and excretion (Lamola et al. 1975, Marks 1985, Marks et al. 1987, 1988, Woods 1996, Casini et al. 2003, Ng et al. 2005, dos Santos et al. 2007). Several studies have investigated the porphyrinogenic effects of different classes of pollutants on a range of aquatic organisms (Table 1) and the disruption of the haem biosynthesis has been proposed as a biomarker

**Table 1.** Studies that have investigated the porphyrinogenic effects of one or more classes of pollutants on aquatic organisms.

| | Species | Tissue | Pollutant | References |
|---|---|---|---|---|
| Bivalve molluscs | Common cockle, *Cerastoderma edule*; lagoon cockle, *Cerastoderma lamarcki* | Hepatopancreas | Mercury | (Brock 1992) |
| Crabs | Burrowing crab, *Chasmagnathus granulatus* | Hepatopancreas | Hexachlorobenzene | (Chaufan et al. 2006) |
| | Mediterraneancrab, *Carcinus aestuarii* | Midgut gland, hepatopancreas, faeces | Benzo[a]pyrene, methylmercury, polychlorinated biphenyls | (Fossi et al. 1996, 1997, 2000) |
| Fish | Mosquitofish, *Gambusia affinis* | Whole body homogenates | Produced water[a] from an offshore oil platform | (Casini et al. 2006) |
| | Blind cave fish, *Astyanax fasciatus* | Liver | Agrochemicals, (e.g. pesticides), heavy metals, polycyclic aromatic hydrocarbons | (CarrascoLetelier et al. 2006) |
| Birds | Brown pelican, *Pelecanus occidentalis thagus;* neotropic cormorant, *Phalacrocorax olivaceus;* kelp gull, *Larus dominicanus* | Liver, kidney, faeces | Not specified | (Casini et al. 2001) |
| Marine mammals | South American sea lion, *Otaria flavescens* | Faeces, blood, fur | Not specified | (Casini et al. 2002) |

[a]Produced water (PW) is the water extracted from the subsurface during oil, gas and coal extraction. PW may include water that is naturally trapped in the reservoirs and/or water that has been injected into the formation to facilitate the oil/gas/coal recovery.

of exposure (De Matteis & Lim 1994, Fossi et al. 2000, Chaufan et al. 2002).

The analysis of porphyrins in blood, urine and faeces is commonly used in the clinical diagnosis of porphyrias, inherited or acquired diseases due to enzyme deficiencies in the haem pathway (Lim 1991), as porphyrins can be detected spectrofluorometrically at very low concentrations (<10 μg $l^{-1}$) (Schwartz et al. 1976, Grandchamp et al. 1980, Valcarel et al. 1987, Westerlund et al. 1988).

In clinical diagnosis these two tests are usually performed on human urine samples; this has the advantage of not involving invasive techniques to collect a sample and allows repeatable sampling. In addition, analysing urine samples does not involve further separation, purification or other laborious pretreatment steps in contrast to most other biological tissues or fluids (e.g. blood, faeces), and they can be stored at −80°C until analysis. Thus, performing these tests on urinary samples from crabs has major advantages compared with previously used techniques, because: (1) samples can be collected non-destructively allowing repeatable sampling from individual animals; also, there is no need for transportation of animals because the sampling procedure only takes 1–2 min per individual and can be performed in the field; (2) in addition to cost-effectiveness and ease-of-use, the major advantages of the two assays proposed is that they can be conducted on a single sample, with the additional possibility of measuring PAH metabolites as a biomarker of exposure in the same sample; (3) pretreatment of samples is less extensive than for other tissues, thus facilitating the adaptation of the method from humans to crustaceans; the more steps that are required to prepare samples prior to analysis, the more likely it is that further modifications of the procedures are necessary due to differences in body tissue composition between humans and crabs.

The aim of this work was, first, to determine whether or not TBARS and porphyrins are present in *Cancer novaezelandiae* crab urine and could be measured using available technologies adopted from human medical toxicology tests. Second, provided that these urinary tests can be applied to decapod crabs, this experiment aimed to investigate the potential correlation between these measurements and environmental pollution levels to test the validity of these assays as ecotoxicological biomarkers in environmental risk assessment.

## Materials and methods

### *Animal sampling and acclimatization*

Thirty male pie-crust crabs (*C. novaezelandiae*) (75–145 mm carapace width), were collected offshore at 10–20 m, approximately 5 km northwest off the Otago Harbour entrance (Taiaroa Head), New Zealand, using baited pots. Crabs were transported to the Portobello Marine Laboratory and allocated to six 50-l tanks (five crabs per tank) according to size range (mean size 113 ± 5 mm for each tank) and held at 15 ± 1°C, 35 ± 1 psu, under natural light/dark cycle conditions in tanks with running filtered seawater. The crabs were allowed to acclimatize to these conditions for a week prior to the experiment and were fed 10 g each of cockles from Aramoana (control site). Tanks were cleaned before every feeding session.

A feeding experiment with cockles collected from a presumably impacted and reference field site was conducted to simulate the exposure to environmentally realistic concentrations of chemical contaminants encountered by these animals in their natural habitat. Furthermore, monitoring the effects of the dietary uptake of contaminants probably best reflects the natural uptake route of contaminants for epi-benthic omnivores such as decapod crustaceans. Also, contaminant concentrations are generally lower in seawater than in sediments and in marine fauna, due to their high affinity for particles and organic matter and as a result of bioaccumulation and biomagnification, respectively.

Cockles, *Austrovenus stutchburyi,* were collected by hand from two sites: Aramoana and Portobello Road Outfall (Figure 1), Otago Harbour, New Zealand, and kept frozen at -20°C until use. Frozen cockles from Aramoana and Portobello Road were homogenized and analysed for PAH content at Hill Laboratories (Hamilton, New Zealand) (Table 2). PAH compounds analysed include all 16 priority pollutants listed by the US Environmental Protection Agency (EPA 1987).

Sites were chosen to represent a pollution gradient. Aramoana, which is located at the entrance of Otago Harbour, is considered a 'clean' site, with no major pollution sources in the vicinity. In contrast, Portobello Road is located at the head of Otago Harbour (Upper Harbour) and is subject to elevated contamination input from residential and industrial activities in Dunedin city. Previous studies have shown elevated total PAH levels in much of the sediments in the Upper Harbour, mostly varying between 5 and 20 μg $g^{-1}$ dry weight (Depree & Ahrens, unpublished results, 2006, Stewart 2007), and exceeding the Australian and New Zealand Environment Conservation Council (ANZECC 2000) low trigger value of 4 μg $g^{-1}$ dry weight. PAH levels are particularly high in the vicinity of the Portobello Road drain (436.27 μg $g^{-1}$ dry weight) (Stewart 2007), clearly exceeding the ANZECC (2000) high trigger value (45 μg $g^{-1}$ dry weight) by one order of magnitude. The sedimentary PAH profile at Portobello Road comprises conspicuously high concentrations of

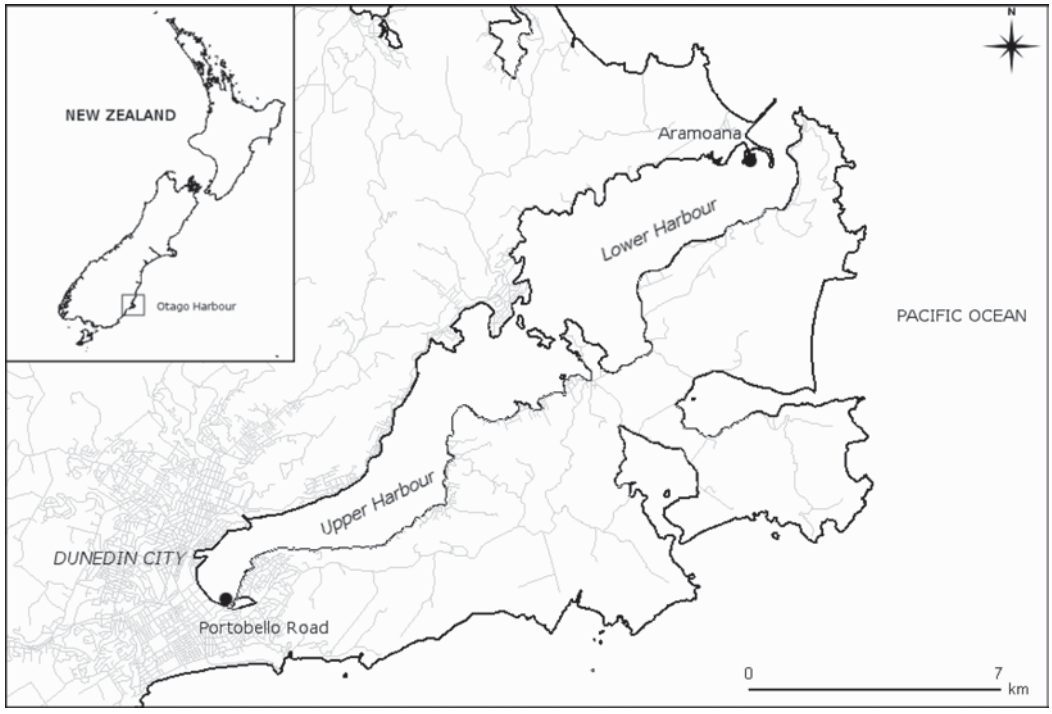


**Figure 1.** Location of the two collection sites for cockles (*Austrovenus stutchburyi*) in Otago Harbour, South Island, New Zealand.



**Table 2.** Concentrations (mg $kg^{-1}$ wet weight) of 16 polycyclic aromatic hydrocarbons (PAHs) in cockle flesh from Aramoana and Portobello Road outfall.

| PAH compound | Aramoana cockles | Portobello Road cockles |
|---|---|---|
| Acenaphthene | <0.00050 | 0.0096 |
| Acenaphthylene | <0.00050 | 0.043 |
| Anthracene | <0.00020 | 0.059 |
| Benz[a]anthracene | <0.00020 | 0.21 |
| Benzo[a]pyrene | <0.00020 | 0.23 |
| Benzo[b]fluoranthene | <0.00020 | 0.28 |
| Benzo[g,h,i]perylene | <0.00020 | 0.088 |
| Benzo[k]fluoranthene | <0.00020 | 0.11 |
| Chrysene | 0.00035 | 0.21 |
| Dibenz[ah]anthracene | <0.00020 | 0.048 |
| Fluoranthene | <0.00020 | 0.46 |
| Fluorene | <0.00020 | 0.024 |
| Indeno[1,2,3-cd] pyrene | <0.00020 | 0.12 |
| Naphthalene | <0.00500 | 0.029 |
| Phenanthrene | 0.0016 | 0.18 |
| Pyrene | <0.00050 | 0.43 |

high-molecular-weight PAHs, with pyrene accounting for more than 10% of the total PAH concentration in storm water (Brown & Peake 2006) and sediments (Depree and Ahrens unpublished results, 2006), most likely from the PAH-rich sludge and tar from a discontinued gasworks (Brown & Peake 2006). Similarly, cockle flesh has been shown to contain very high PAH levels at the Portobello Road site (4.912 mg $kg^{-1}$ wet weight), with concentrations between 2 and 3 orders of magnitude higher than those measured for cockles from adjacent sites (Kitchener Street 0.009 mg $kg^{-1}$; Orari Street 0.018 mg $kg^{-1}$) (Stewart 2007). In particular, benzo[a]pyrene, a mutagenic and highly carcinogenic PAH, occurs in such high concentrations (0.25 mg $kg^{-1}$) in cockles from Portobello Road that only 0.8 g of cockle flesh would be considered safe for human consumption on a weekly basis (according to British Columbian guidelines for B[a]P concentrations in shellfish for human consumption) (Stewart 2007).

### *Experimental design*

After the acclimatization period, the six tanks were randomly divided into two groups with three replicates for each treatment (3 × 5 individuals per treatment). Each group was fed cockles (60–80 g per tank) three times weekly. Cockles were collected from Aramoana (80 g of flesh ~ 5–8 deshelled cockles) and Portobello Road Outfall (80 g of flesh ~ 100 deshelled cockles) for the control and the treatment groups, respectively.

### *Urine sampling*

Urine samples were collected from each crab after 24 h, 1 week and 2 weeks exposure following a urine sampling procedure adapted from Bamber and Naylor (1997). Briefly, the crabs were restrained ventrally on a plastic backboard using elastic bands running parallel

to the pereopods and the third maxillipeds were moved aside and kept apart using forceps. The epistome was then dried using absorbent paper and the apertures of the branchial exhalant chambers were covered with absorbent paper to prevent dilution with water expelled from the chambers. The operculum of each antennal gland bladder was lifted and urine was collected using a variable volume pipette with a flexible microcapillary (25 µm) tip. Samples were transferred to 1.7-ml microcentrifuge tubes and immediately frozen at -80°C until analysis. Correct animal ethical procedures were followed throughout the procedure (Otago University Animal Ethics Committee approval 82/07).

Fluorescence analyses were performed using a LS 50B Perkin-Elmer fluorescence spectrophotometer with a 3.5 ml quartz fluorescence cuvette for all three assays (TBARS, porphyrin, PAH metabolites).



### *TBARS assay*

The amount of TBARS in urine samples was determined using the OXItek TBARS Kit (ZeptoMetrix Corporation, Buffalo, NY, USA; No. 0801192) according to the manufacturer's instructions as follows: one vial containing 0.53 g of TBA was dissolved in 50 ml of TBARS diluent 1 and 50 ml of TBARS diluent 2 and mixed until the TBA was completely dissolved. A series of six standards (0–100 nmol MDA $ml^{-1}$) was prepared by diluting the MDA standard stock solution, containing 100 nmol $ml^{-1}$ of malondialdehyde bis (dimethyl acetal), in MDA diluent. One-hundred microlitres of urine sample or standard was mixed with 100 µl of SDS solution in a 5-ml polypropylene test tube with a screw top lid and the content mixed by vortexing. Then, 2.5 ml of TBA/buffer reagent was added to each tube and the tubes incubated at 95°C for 60 min. Tubes were cooled to room temperature in an ice bath for 10 min before centrifugation at 3000 rpm for 15 min. After centrifugation, 2 ml of supernatant was removed from samples and transferred to another 5-ml polypropylene test tube containing 1.5 ml of distilled water, to load the 3.5 ml cuvette.

Fluorescence emission was monitored at 550 nm, with the excitation wavelength set at 530 nm, with slit widths of 5 nm and the scan rate set at 60 nm $min^{-1}$. The detection limit was determined as the mean of three blank measurements plus three standard deviations. Peak fluorescence emission signals at 550 nm were quantified against the standard curve and results are presented as MDA equivalents in nmol $ml^{-1}$.

### *Porphyrin assay*

All solutions were prepared according to Westerlund et al. (1988) as follows. A 3 mol $l^{-1}$ hydrochloric acid (HCl) solution was prepared by adding one part (250 ml) of HCl (AR grade) to three parts (750 ml) Milli-Q water. A 0.3 mol $l^{-1}$ stock solution of iodine was prepared by dissolving 3.8 g of iodine in 100 ml of ethanol (LR grade). Prior to each analysis, a HCL-iodine stock solution was prepared by mixing 500 µl of the 0.3 mol $l^{-1}$ iodine stock solution with 50 ml of the 3 mol $l^{-1}$ HCl reagent. In addition, a 0.45 nmol $l^{-1}$ sodium-thiosulfate solution was prepared by dissolving 35.5 mg of sodium thiosulfate in 500 ml of Milli-Q water.

Porphyrinogens are the products of porphyrin metabolism *in vivo* and can comprise half of the porphyrins present in urine. These compounds are colourless and non-fluorescent and therefore require oxidation to fluorescent porphyrins before analysis. Consequently, 100 µl of urine (100 µl of Milli-Q water for blank) was mixed with 1 ml of the freshly prepared HCl-iodine reagent and incubated for 30 min at room temperature (Valcarel et al. 1987, Westerlund et al. 1988). After the oxidation period, samples were decolourized by adding 5 ml of thiosulfate solution to each sample and scanned within 5 min (Westerlund et al. 1988).

Urine samples were analysed spectrofluorometrically monitoring the fixed emission at 650 nm with a 20 nm bandwidth while exciting from 350 nm to 430 nm with a 5 nm bandwidth, with the scan rate set at 60 nm $min^{-1}$ (Martinez & Mills 1971, Schwartz et al. 1976, Westerlund et al. 1988). Fluorescence signals were recorded at the Soret band (i.e. absorption band in the blue region of all porphyrins) peak $\lambda_{exc} = 400$ nm (Martinez & Mills 1971, Schwartz et al. 1976, Westerlund et al. 1988, Pudek et al. 1991) and corrected for background fluorescence by subtracting the corresponding values determined for the blank measurements. The detection limit was determined as the mean of three blank measurements plus three standard deviations. Data are reported as fluorescence intensities (i.e. peak heights).

### *PAH metabolites*

The level of pyrene metabolites was quantified in the same urine samples to complement the findings of the two assays described above, as PAH exposure is known to affect the urinary excretion of TBARS and porphyrins in humans. The presence of urinary PAH metabolites provides evidence of exposure, which is interesting as not all crabs are expected to feed equally and metabolize the pollutants at the same rate, and also serves as a precursor of the TBARS and porphyrin assay responses and gives insight into the dose-, as well as time-related correlation between exposure and effects.

Urine samples were screened for pyrene metabolites following the procedure as described in Watson et al. (2004). Briefly, an aliquot of 100 µl of urine sample was mixed with 3.4 ml of 50% ethanol, resulting in a

1:35 dilution. Samples and a series of 1-hydroxypyrene standards (0–200 μg $l^{-1}$) were analysed by fixed excitation fluorescence (FF) technique, setting the excitation wavelength at 345 nm and monitoring the emission from 290–500 nm at a scan rate of 400 nm $min^{-1}$, with excitation and emission slit widths of 5 nm. Fluorescence peak heights were recorded for samples and standards at wavelengths 382 nm and 387 nm, respectively, and corrected for background fluorescence contributed by the solvent, by subtracting the corresponding values determined for the blank of 50% ethanol. The detection limit was determined as the mean of three blank measurements plus three standard deviations and peak heights were quantified against the standard curve. Data are presented as 1-OH pyrene (i.e. hydroxypyrene) equivalents (μg $l^{-1}$) after correction for dilution.

### *Statistical analysis*

Data were analysed using the software packages JMP 7.0 (SAS Institute, Cary, NC, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All data were tested for normality using the Shapiro–Wilk's test and for homogeneity of variance according to Levene's test. As the data did not satisfy the assumptions of normality and homogeneity of variance, even after transformation, a non-parametric Kruskal–Wallis single factor analysis of variance by mean ranks was performed, using the Friedman's test for multiple comparisons of mean ranks within treatment over time (Zar 1984). Friedman's test was used because samples within treatment are dependent over time, as the same individuals were sampled at three time intervals. Differences at the 5% significance level were considered significant. Spearman's rank correlation coefficient was used to analyse the correlation between the three assays.

To investigate potential differences in variability between the two groups, an index of multivariate dispersion (IMD) was computed, as well as the average squared Euclidean distance for each treatment (i.e. distance among samples within treatment based on Euclidean dissimilarity matrix) using the SIMPER routine in PRIMER (Plymouth Routines in Marine Environmental Research) statistical software package v6.0 (Plymouth Marine Laboratory, U.K.) (Warwick & Clarke 1993, Clarke & Warwick 2001).

## Results

### *Cockle analyses*

The concentration of 16 PAH compounds in cockle flesh from Aramoana and Portobello Road outfall indicated a strong PAH contamination gradient between the two sites (Table 2). The PAH levels in the cockles from Aramoana were almost all below detection levels, except chrysene and phenanthrene, which were present in very low concentrations. In contrast, cockles from Portobello Road outfall contained conspicuously high amounts of high-molecular-weight compounds, including pyrene, fluoranthene, benz[a]anthracene, benz[a]pyrene, benz[b]fluoranthene and chrysene, in accordance with the sedimentary data mentioned previously. Thus, these results indicate a clear contaminant gradient between control (Aramoana) and treatment (Portobello Road) groups in this experiment.

### *Pyrene metabolites*

The screening of urine samples for pyrene metabolites exhibited a significant difference between mean ranks at the 95% confidence level between control (Aramoana, ARA) and treatment (Portobello Road, PBR) groups at all time intervals (24 h, 1 week, and 2 weeks) (Table 3, Figure 2). The within-treatment variability remained approximately the same for the ARA group and values ranged between 1 and 11 μg $l^{-1}$ 1-hydroxypyrene at all three sampling occasions (Table 3). The Friedman's test however revealed a significantly higher level of pyrene metabolites in ARA after 1 week ($\chi^2_{(3,\ 10,\ 0.05)} = 7.2$, $p = 0.025$) compared with the values recorded at 24 h and 2 weeks, respectively. For the PBR group, the coefficient of variation increased from 30% to 56% after the first measurement at 24 h while the maximum level of pyrene metabolites recorded increased almost threefold during that time (Table 3). However, there was no significant difference over time for pyrene metabolite levels in the PBR group.

### *MDA*

The fluorescence spectra recorded for the TBARS assay clearly indicated the presence of MDA-type compounds in urine samples, which were absent in all blank measurements (see Appendix – Figure 5).

The level of MDA excreted in urine did not differ significantly at the 95% confidence level between PBR and ARA at any sampling periods (Table 3). Similarly, there was no significant difference within treatment groups over time, although there was an increasing trend in MDA levels for the PBR group after 1 week, followed by an apparent decrease at the 2-week sampling period (Figure 3). Furthermore, the interindividual variability, expressed as the coefficient of variation, decreased in the ARA group, while it increased in the PBR group during the feeding experiment (Table 3). Similarly, the range of values showed that maximum MDA levels were twice as high for the PBR group compared with ARA at the 1-week and 2-week time intervals (Table 3).

**Table 3.** Mean values ± standard deviation of urinary pyrene metabolites, malondialdehyde (MDA) levels and porphyrin fluorescence intensities recorded for pie-crust crabs (*Cancer novaezelandiae*) fed on cockles from Aramoana (ARA, control) and Portobello Road (PBR, treatment) after 24 h, 1 week and 2 weeks of exposure. Ranges of values are in parentheses. Details are given for statistical significant differences at the 95% confidence level between treatment groups.

| | Pyrene metabolites | | MDA | | Porphyrin | |
|---|---|---|---|---|---|---|
| Time | ARA | PBR | ARA | PBR | ARA | PBR |
| 24 h | 4.87 ± 1.92 (2.92–9.33) | 7.50 ± 2.23 (4.09–10.03) | 0.33 ± 0.17 (0.13–0.65) | 0.36 ± 0.18 (0.11–0.85) | 0.44 ± 0.11 (0.35–0.77) | 0.58 ± 0.25 (0.38–1.15) |
| | $\chi^2_{(1,26)} = 8.8281$, $p = 0.003$ | | N/D | | N/D | |
| 1 week | 6.44 ± 2.37 (3.69–11.11) | 10.61 ± 5.93 (5.65–27.13) | 0.39 ± 0.17 (0.17–0.77) | 0.54 ± 0.34 (0.22–1.43) | 0.32 ± 0.11 (0.15–0.48) | 0.67 ± 0.68 (0.26–2.84) |
| | $\chi^2_{(1,25)} = 6.3673$, $p = 0.0116$ | | N/D | | $\chi^2_{(1,23)} = 5.4154$, $p = 0.02$ | |
| 2 weeks | 4.53 ± 2.36 (1.146–0.14) | 10.25 ± 5.87 (2.98–23.08) | 0.29 ± 0.10 (0.17–0.51) | 0.41 ± 0.29 (0.17–1.25) | 0.28 ± 0.08 (0.15–0.37) | 0.49 ± 0.41 (0.23–1.87) |
| | $\chi^2_{(1,22)} = 9.2517$, $p = 0.0024$ | | N/D | | $\chi^2_{(1,23)} = 7.6460$, $p = 0.0057$ | |

N/D, no significant difference detected between treatments.

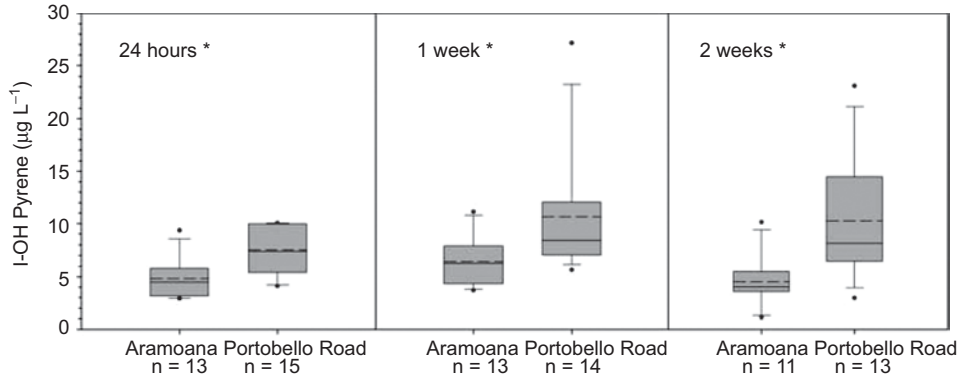


**Figure 2.** Box and whisker plots of 1-OH pyrene concentrations (µg $l^{-1}$) in urine samples of pie-crust crabs (*Cancer novaezelandiae*) for the treatment (Portobello Road) and the control (Aramoana) group after 24 h, 1 week and 2 weeks exposure times. *Significant difference between treatment groups; solid black line, median; dashed line, mean. The box is drawn from the lower quartile (25th percentile) to the upper quartile (75th percentile); whiskers indicate the 10th and the 90th percentile of the sample data.

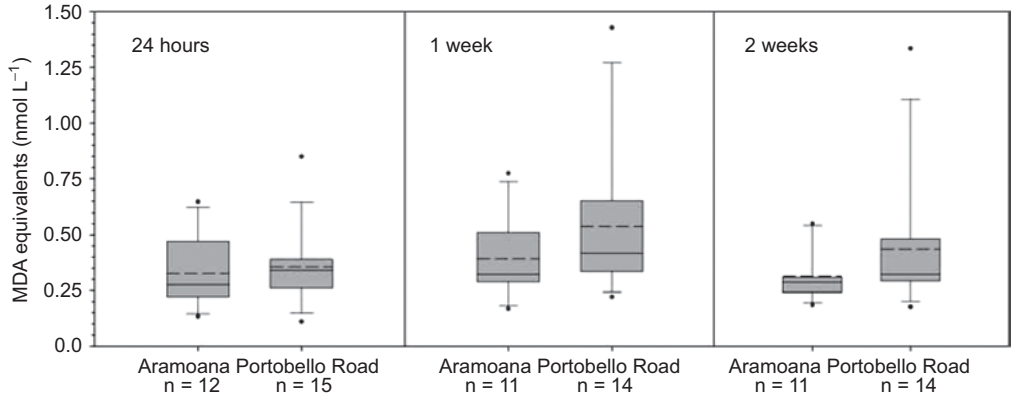


**Figure 3.** Box and whisker plots of malondialdehyde (MDA) concentrations (µg $l^{-1}$) in urine samples of pie-crust crabs (*Cancer novaezelandiae*) for the treatment (Portobello Road) and the control (Aramoana) group after 24 h, 1 week and 2 weeks of exposure. Solid black line, median; dashed line, mean. The box is drawn from the lower quartile (25th percentile) to the upper quartile (75th percentile); whiskers indicate the 10th and the 90th percentile of the sample data.

### *Porphyrin*

The fluorescence spectra recorded for porphyrin analysis of urine samples exhibited a clear peak at the expected wavelength, which was absent in all blank measurements (see Appendix – Figure 6).

The screening for porphyrins in urine exhibited a significant difference between the control (ARA) and the treatment (PBR) group after 1 week of exposure, which remained significant after 2 weeks (Table 3, Figure 4). In particular, the maximum fluorescence signal recorded for the porphyrin analysis at 1 week and 2 weeks is approximately 6 times higher at PBR compared with the highest value recorded in ARA urine samples (Table 3). Additionally, the interindividual variance within the PBR group was even more prominent than for the pyrene or the TBARS test, with coefficients of variation for 1-week and 2-week measurements being 102% and 84%, respectively, and individual responses within treatment varying by one order of magnitude (Table 3). Furthermore, the mean peak fluorescence signals recorded for the porphyrin analysis showed a decreasing trend over time for both treatments (Figure 4), with values at 24 h differing significantly from the values measured after 1 week and 2 weeks for ARA ($\chi^2_{(3, 10, 0.05)} = 13.3$, $p = 0.001$) and PBR ($\chi^2_{(3, 14, 0.05)} = 12.0$, $p = 0.002$). However, it should be noted that for PBR the mean value is higher after 1 week compared with 24 h, but the median, as well as mean rank, used in the non-parametric Friedman test, are lower (see Figure 4). This apparently contradictory effect can be explained by the presence of a few extremely high values.

### *Correlation between three assays*

The Spearman's rank test for all data combined (i.e. both treatments and three time intervals combined) exhibited a significant correlation between all three assays, although the Spearman coefficient ($\rho = 0.5$, $p < 0.0001$) indicated that there is only a moderate correlation. However, when analysing the correlation between the three assays for both treatment groups separately (three time intervals combined), only a slight correlation between porphyrin and pyrene metabolites ($\rho = 0.33$, $p = 0.049$) existed within the control group. This trend did not intensify when examining the correlation between variables for each time interval within the ARA treatment separately.

In contrast, there was a relatively strong correlation between all three assays for the PBR treatment group ($\rho > 0.50$, $p < 0.001$). Furthermore, for the PBR group the correlation between the three variables at different time intervals showed that after 24 h there was only a significant correlation between porphyrin and TBARS levels ($\rho = 0.58$, $p = 0.025$), but after 1 week there was a significant correlation between all three assays ($\rho > 0.56$, $p < 0.050$), which further intensified after 2 weeks ($\rho > 0.64$, $p < 0.020$). In particular, a very strong relationship was detected between porphyrins and pyrene metabolites after 1 week, which was more pronounced after 2 weeks, with a Spearman's coefficient $\rho$ of 0.81 ($p = 0.0005$) and 0.92 ($p < 0.0001$), respectively.

### *Variability within treatment*

The multivariate dispersion (MVDISP) analysis revealed a decreasing dispersion among samples for the control (ARA) group over time, in contrast to an increase in dispersion among samples within the treatment (PBR) group. Correspondingly, the indices of IMD between the PBR and the ARA group all give positive values and increase with time (Table 4). Similarly, the average squared Euclidean distance is higher between samples from the PBR at all three time intervals, with substantial differences in within-treatment dissimilarity between the two treatments after 1 week and 2 weeks (Table 4).

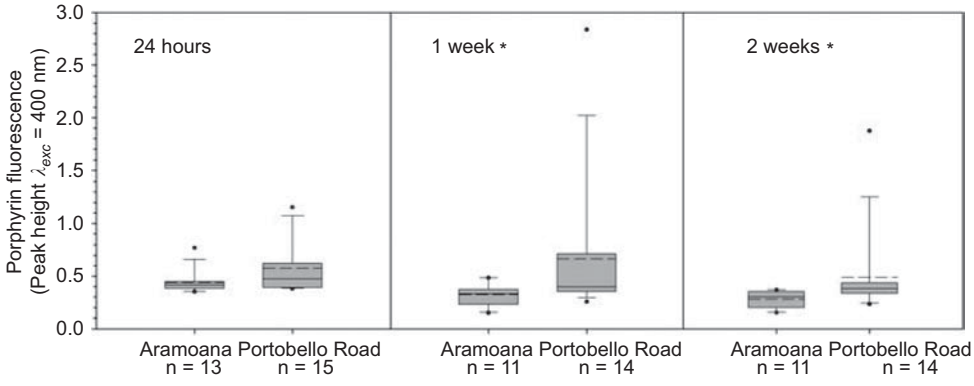


**Figure 4.** Box and whisker plots of fluorescence intensities of porphyrins (peak height at $\lambda_{excitation} = 400$ nm) in urine samples of pie-crust crabs (*Cancer novaezelandiae*) for the treatment (Portobello Road) and the control (Aramoana) group after 24 h, 1 week and 2 weeks of exposure. *Significant difference between treatment groups; solid black line, median; dashed line, mean. The box is drawn from the lower quartile (25th percentile) to the upper quartile (75th percentile); whiskers indicate the 10th and the 90th percentile of the sample data.

**Table 4.** Multivariate dispersion (MVDISP) analysis of biomarker responses in *Cancer novaezelandiae* and averaged squared Euclidean distance (based on Euclidean similarity matrix) among samples for crabs fed on cockles from Aramoana (ARA, control) and Portobello Road (PBR, treatment) after 24 h, 1 week and 2 weeks of exposure.

| | Dispersion | | Index of multivariate dispersion (IMD) | Average squared Euclidean distance | |
|---|---|---|---|---|---|
| Time | ARA | PBR | | ARA | PBR |
| 24 h | 0.904 | 1.061 | 0.158 | 3.68 | 5.06 |
| 1 week | 0.848 | 1.092 | 0.245 | 5.18 | 35.79 |
| 2 weeks | 0.730 | 1.190 | 0.463 | 5.57 | 34.69 |

## Discussion

The results have shown that *C. novaezelandiae* crabs absorb pyrene as a result of ingestion of contaminated food, with pyrene metabolites being detectable in urine only 24 h after dietary exposure. Significantly higher levels of urinary pyrene metabolites in crabs fed cockles from the Portobello Road outlet is in accordance with the higher PAH concentrations in cockle flesh from this site.

The significantly higher level of pyrene metabolites detected for the ARA group after 1 week probably reflects the natural variability in fluorescence measurements for *C. novaezelandiae* urine samples.

Although there was no significant difference between pyrene metabolite concentrations in urine over time for the PBR treatment group, there was an increase in the excretion of pyrene metabolites after the initial 24 h, with the maximum value recorded after 1 week being almost three times higher than the maximum value recorded after 24 h. However, due to the large variation within the PBR treatment group this trend was not detectable at the 5% significance level. Similarly, the 1-OH pyrene concentration in urine did not seem to increase any further after the first week, with a similar mean value and value range recorded after 2 weeks of exposure. This trend suggests that the excretion rate of PAH metabolites reached its maximum within only a few days and remained constant thereafter when exposure conditions remained unchanged. Practically, this means that *C. novaezelandiae* (and potentially other crab species) could be used to assess the PAH contamination at specific sites by conducting transplant experiments over a very short period of time (about 1 week).

The high variability among individuals within the PBR treatment group could be explained by the kinetics of PAH uptake and metabolism in crustaceans. Previous studies have shown that the elimination rate of PAH metabolites in crustaceans generally peaks after about 48 h postexposure and decreases steadily thereafter (Lee et al. 1976, James et al. 1995, Fillmann et al. 2004, Watson et al. 2004). Thus, in this case the urinary 1-OH hydroxypyrene concentration would strongly depend on the amount of cockles individual crabs have ingested about 48 h prior to the sample collection. Furthermore, the high variation among crabs may result from the fact that crabs were not eating equal amounts on each of the three weekly feeding occasions.

Even though urinary MDA levels did not differ significantly between the two treatment groups (ARA and PBR) at any time, the range of values suggests that overall oxidative stress was higher for crabs that fed on cockles from Portobello Road (PBR). This trend was probably masked by the increasing variability over time among individuals within the PBR group. Additionally, the fact that MDA levels did not differ after 24 h between the two treatments indicates that the dietary exposure does not have an immediate effect on the TBA reacting substances excretion profile. In particular, considering that pyrene metabolite levels differed significantly between treatments after only 24 h suggests that PAH-induced lipid peroxidation only occurs after a few days following the dietary uptake of contaminants.

The decrease in MDA levels in urine for the PBR group in the second week could be attributed to an increase in antioxidant enzyme activity (Chaufan et al. 2006). While testing the toxicity of hexachlorobenzene (HCB) for *Chasmagnathus granulatus* crabs, Chaufan et al. (2006) found that after an initial increase in MDA levels, as a result of toxicant-induced lipid peroxidation, antioxidant defences would activate after about 1 week and cause a transient reduction of MDA levels. This trend was reversed after about 4 weeks, when the accumulation of HCB caused a pro-oxidant effect that overwhelmed antioxidant response mechanisms (Chaufan et al. 2006). In addition, shellfish meat is an important source of antioxidants for humans because of its high levels of vitamin E and selenium (Egeland & Middaugh 1997), which in this case might have also contributed to an interim reduction of lipid peroxidation.

Furthermore, water-borne naphthalene, which is a low-molecular-weight PAH, has been shown to increase TBARS levels in the hepatopancreas, haemolymph and reproductive tissues of the estuarine crab *Scylla serrata* (Vijayavel et al. 2004, 2005). However, the first of these studies used a relatively high concentration of 100 mg $l^{-1}$ of naphthalene considering the previously determined $LC_{50}$ concentration of 180 mg $l^{-1}$ and the level of TBARS was only evaluated after 96 h of exposure (Vijayavel et al. 2004). Moreover, in the second study where the crabs were exposed to a lower concentration of naphthalene (10 mg $l^{-1}$), lipid peroxidation was only evaluated after 30 days of exposure, with no measurements recorded in between (Vijayavel et al. 2005).

Consequently, the duration of this experiment might not have been long enough to detect statistically significant differences among the two treatment groups, potentially due to the activation of antioxidant defence mechanisms

after an initial increase in oxidative stress. However, the trends seen in this experiment suggest that the uptake of PAHs through ingestion can result in an increase in the generation of ROS in decapod crabs and may potentially lead to adverse effects due to oxidative stress.

The significant difference between control and treatment groups after 1 week strongly suggests that the dietary exposure to xenobiotics has had an adverse effect on the porphyrin excretion profile of crabs that fed on cockles from Portobello Road. However, the extremely high interindividual variance within the PBR treatment group indicates that not all crabs show a response as a result of contaminant uptake. Chaufan et al. (2006) observed a similar pattern in their study, where only a portion of the crabs exposed to HCB showed signs (30–45% deviation from control) of uroporphyrinogen decarboxylase (UroD) enzyme activity inhibition (enzyme which catalyses haem biosynthesis), whereas the UroD activity in control groups remained homogeneous over time. Similar results have also been observed in studies with rats (Rios de Molina et al. 2002) and sea lions (Casini et al. 2002), suggesting that differences in enzyme activity susceptibility may be common among different taxa, which would explain the large variance in urinary porphyrin levels observed in PBR exposed crabs. In addition, in the current experiment, food competition among crabs within one tank could have caused differences in contaminant uptake among individuals.

The decrease in the temporal response of urinary porphyrins for both treatment groups after 1 week could potentially result from a beneficial effect of the experimental conditions on the health status of the control group. In human medicine, improved nutrition is part of the prevention and treatment of porphyria (Robert et al. 1994). Similarly, in this experiment, an improved nutritional status, as a consequence of the abundant amount of feed that crabs were given, could have resulted in reduced porphyrin excretion.

The overall correlation between the three assays for all data combined is significant, although the Spearman's rank correlation indicates that there is only a moderate correlation between assays. However, when analysing the correlation between the three assays for both treatment groups separately, it is apparent that there is a strong correlation between all three assays for the PBR treatment group in contrast to the very low correlation between assays for the ARA control group. As the ARA treatment group is the 'unpolluted' group, the crabs fed on cockles from ARA have not been exposed to any, or only negligible concentrations of contaminants. Thus, no correlation is expected. Conversely, in the PBR treatment group crabs were exposed to a range of contaminants through their diet, including relatively high PAH concentrations, which are known to affect the haem biosynthesis and cause oxidative stress (Marks 1985, Marks et al. 1988, Winston & Di Giulio 1991, Livingstone 2001, Casini et al. 2003). The strong relationship between the three variables for the PBR group therefore provides strong evidence that urinary TBARS and porphyrin excretion profiles are affected by pyrene exposure in *C. novaezelandiae*. In particular, this argument is reinforced by the fact that all three assays exhibited elevated levels in particular individuals.

Furthermore, the lack of correlation between urinary pyrene metabolite levels and porphyrin and TBARS levels for PBR crabs after 24 h, and the fact that after 24 h TBARS and porphyrin levels did not differ between 'polluted' and 'unpolluted' treatments, indicate there was no detectable exposure effect on TBARS and porphyrin excretion profiles after 24 h. However, the level of pyrene metabolites was already significantly higher for the PBR group, suggesting that there is a time delay between pyrene uptake and the consequent response in the porphyrin and TBARS tests. In particular, the correlation between the three assays increases after 1 week and even further after 2 weeks, showing that pyrene exposure has had a clear impact on the response seen for the porphyrin and the TBARS assay, with a very strong correlation between 1-OH pyrene concentrations and MDA levels as well as porphyrins in urine.

Warwick and Clarke (1993) noted that in numerous environmental impact studies the variability in community structure and species abundance patterns among samples collected from polluted areas is often substantially higher than for samples from control sites, a phenomenon which has been observed in various biomarker studies (e.g. Depledge & Lundebye 1996, Casini et al. 2001, Fillmann et al. 2004, Watson et al. 2004). Thus, Warwick and Clarke (1993) suggested that the variability itself may be an indicator of perturbed conditions, which could be added to the suite of community/biomarker responses currently used for assessing the impact of anthropogenic disturbances on the marine environment.

The IMD (a proxy of variability) supported this trend, whereby variability increased with exposure time for the PBR group, indicating a correlation between within-treatment variance and pollution level. In particular, a further increase of the IMD between the first and the second week of the experiment suggests that the pollution-induced effect on the health status of the exposed crabs further intensified. However, this trend cannot be derived from the univariate analysis, indicating that the IMD potentially represents a more sensitive approach to detect pollution-induced adverse effects. The Euclidean distance exhibited a similar trend, which is consistent with the fact that the PBR group represents the 'polluted' group and ARA is considered a control group.

Consequently, the increase in variability among individuals with increasing levels of stress seen in this

study further proves that a pollution-related increase in variability is a widespread and general phenomenon in environmental impact studies. However, the application of the comparative IMD still lacks any obvious statistical framework that would allow testing hypotheses of comparable variability between groups (Warwick & Clarke 1993, Clarke & Warwick 2001).

In conclusion, the recorded fluorescence spectra exhibited clear peaks at the expected wavelengths, indicating that TBARS as well as porphyrins are present and measurable in decapod crab urine. However, further work would be necessary to help determine the sensitivity and accuracy of the methods by assessing the correlation between HPLC and fluorescence techniques and analysing the recovery using spiked urine samples.

Thus the results of this study indicate that the urinary TBARS and porphyrin assays commonly used in human medicine can be readily applied to decapod crabs. The consistency of the direction of change between assays, whereby the levels recorded for all three assays increased in the treatment (PBR) group and the strong correlation between tests, provide strong evidence that changes in the TBARS and porphyrin excretion profile reflect an increase in pollution disturbance. Thus, the current work provides preliminary validation for these two assays as non-destructive biomarkers of pollution. In particular, the fact that this experiment consisted of the dietary exposure of crabs to environmentally realistic levels of contaminants facilitates the practical application of these methods in future field studies.

However, stress-induced responses occurred in only a few individuals resulting in large within-treatment variability, which precluded a clear separation between the control and the impacted group. Even though the current work further proves that increasing variability itself can potentially serve as an indicator of pollution, the implementation of this analysis tool still lacks any statistical framework. Hence, this drawback has to be taken into account when applying these tests in field studies and different analysis techniques should be considered when comparing the anthropogenic impact among different sites.



## Acknowledgements

The authors gratefully acknowledge the departments of Marine Science and Chemistry, University of Otago, New Zealand, for funding and use of facilities. This work was partially funded by the Ecology Research Group at the University of Otago. Moreover, we thank the University of Otago for a Postgraduate Publishing Bursary that facilitated write up of this research as a manuscript. We also thank the anonymous reviewers whose comments improved the manuscript.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

Almroth BC, Sturve J, Berglund A, Forlin L. (2005). Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquatic Toxicol 73:171–80.

Aloisio Torres M, Pires Testa C, Gaspari C, Beatriz Masutti M, Maria Neves Panitz C, Curi-Pedrosa R, Alves de Almeida E, Di Mascio P, Wilhelm Filho D. (2002). Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Marine Pollution Bull 44:923–32.

Ando S, Yanagida K. (1999). Susceptibility to oxidation of copper-induced plasma lipoproteins from Japanese eel: protective effect of vitellogenin on the oxidation of very low density lipoprotein. Comp Biochem Physiol C Toxicol Pharmacol 123:1–7.

ANZECC. (2000). Aquatic ecosystems. Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Australian and New Zealand Environment and Conservation Council, Canberra.

Bamber SD, Naylor E. (1997). Sites of release of putative sex pheromone and sexual behaviour in female *Carcinus maenas* (Crustacea: Decapoda). Estuarine Coastal Shelf Sci 44:195–202.

Brock V. (1992). Effects of mercury on the biosynthesis of porphyrins in bivalve molluscs (*Cerastoderma edule* (L.) and *C. lamarcki* (Reeve)). J Exp Marine Biol Ecol 164:17–29.

Brown JN, Peake BM. (2006). Sources of heavy metals and polycyclic aromatic hydrocarbons in urban stormwater runoff. Sci Total Environ 359:145–55.

Camejo G, Wallin B, Enojarvi M. (1998). Analysis of oxidation and antioxidants using microtiter plates. In: Armstrong D, editor. *Methods in Molecular Biology*, vol. 108: Free Radical and Antioxidant Protocols. Totowa, NJ: Humana Press. p. 377–87.

Carrasco-Letelier L, Eguren G, de Mello FT, Groves PA. (2006). Preliminary field study of hepatic porphyrin profiles of *Astyanax fasciatus* (Teleostei, Characiformes) to define anthropogenic pollution. Chemosphere 62:1245–52.

Casini S, Fossi MC, Gavilan JF, Barra R, Parra O, Leonzio C, Focardi S. (2001). Porphyrin levels in excreta of sea birds of the Chilean coasts as nondestructive biomarker of exposure to environmental pollutants. Arch Environ Contam Toxicol 41:65–72.

Casini S, Fossi MC, Cavallaro K, Marsili L, Lorenzani J. (2002). The use of porphyrins as a non-destructive biomarker of exposure to contaminants in two sea lion populations. Marine Environ Res 54:769–73.

Casini S, Fossi MC, Leonzio C, Renzoni A. (2003). Review. Porphyrins as biomarkers for hazard assessment of bird populations: destructive and non-destructive use. Ecotoxicology 12:297–305.

Casini S, Marsili L, Fossi MC, Mori G, Bucalossi D, Porcelloni S, Caliani I, Stefanini G, Ferraro M, di Catenaja CA. (2006). Use of biomarkers to investigate toxicological effects of produced water treated with conventional and innovative methods. Marine Environ Res 62:S347–51.

Chaufan G, Corvi M, Armesto A, de Viale LCS, Luquet C, de Molina MCR. (2002). Comparison between crab hepatopancreas and rat liver uroporphyrinogen decarboxylase. Comp Biochem Physiol B Biochem Mol Biol 133:251–6.

Chaufan G, Juarez A, Basack S, Ithuralde E, Sabatini SE, Genovese G, Oneto ML, Kesten E, Rios de Molina MdC. (2006). Toxicity of hexachlorobenzene and its transference from microalgae (*Chlorella kessleri*) to crabs (*Chasmagnathus granulatus*). Toxicology 227:262–70.

Clarke KR, Warwick RM. (2001). Changes in marine communities: an approach to statistical analysis and interpretation, 2nd edn. Plymouth, UK: PRIMER-E.

Damiens G, Gnassia-Barelli M, Loques F, Romeo M, Salbert V. (2007). Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere 66:574–583.

De Matteis F, Lim CK. (1994). Porphyrins as 'nondestructive' indicators of exposure to environmental pollutants. In: Fossi MC, Leonzio C, editors. *Nondestructive Biomarkers in Vertebrates*. Boca Raton, FL: CRC Press. p. 93–120.

De Zwart LL, Meerman JHN, Commandeur JNM, Vermeulen NPE. (1999). Biomarkers of free radical damage: applications in experimental animals and in humans. Free Rad Biol Med 26:202–26.

Depledge MH, Lundebye AK. (1996). Physiological monitoring of contaminant effects in individual rock crabs, *Hemigrapsus edwardsi*: the ecotoxicological significance of variability in response. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 113:277–82.

dos Santos APM, Mateus ML, Carvalho CML, Batoreu MCC. (2007). Biomarkers of exposure and effect as indicators of the interference of selenomethionine on methylmercury toxicity. Toxicol Lett 169:121–8.

Downs CA, Shigenaka G, Fauth JE, Robinson CE, Huang A. (2002). Cellular physiological assessment of bivalves after chronic exposure to spilled *Exxon Valdez* crude oil using a novel molecular diagnostic biotechnology. Environ Sci Technol 36:2987–93.

Egeland GM, Middaugh JP. (1997). Balancing fish consumption benefits with mercury exposure. Science 278:1904–5.

EPA. (1987). Quality Criteria for Water 1986. EPA 440/5-86-001. Washington, DC: US Environmental Protection Agency.

Fillmann G, Watson GM, Howsam M, Francioni E, Depledge MH, Readman JW. (2004). Urinary PAH metabolites as biomarkers of exposure in aquatic environments. Environ Sci Technol 38:2649–56.

Fossi MC, Lari L, Casini S, Mattei N, Savelli C, Sanchez-Hernandez JC, Castellani S, Depledge M, Bamber S, Walker C, Savva D, Sparagano O. (1996). Biochemical and genotoxic biomarkers in the Mediterranean crab *Carcinus aestuarii* experimentally exposed to polychlorobiphenyls, benzopyrene and methylmercury. Marine Environ Res 42:29–32.

Fossi MC, Savelli C, Casini S, Franchi E, Mattei N, Corsi I. (1997). Multi-response biomarker approach in the crab *Carcinus aestuarii* experimentally exposed to benzo(a)pyrene, polychlorobiphenyls and methyl-mercury. Biomarkers 2:311–19.

Fossi MC, Casini S, Savelli C, Corbelli C, Franchi E, Mattei N, Sanchez-Hernandez JC, Corsi I, Bamber S, Depledge MH. (2000). Biomarker responses at different levels of biological organisation in crabs (*Carcinus aestuarii*) experimentally exposed to benzo(alpha)pyrene. Chemosphere 40:861–74.

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Depledge MH, Jones MB. (2006). The ECOMAN project: a novel approach to defining sustainable ecosystem function. Marine Pollution Bull 53:186–94.

Gorbi S, Virno Lamberti C, Notti A, Benedetti M, Fattorini D, Moltedo G, Regoli F. (2008). An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea. Marine Environ Res 65:34–49.

Grandchamp B, Deybach JC, Grelier M, Deverneuil H, Nordmann Y. (1980). Studies of porphyrin synthesis in fibroblasts of patients with congenital erythropoietic porphyria and one patient with homozygous coproporphyria. Biochim Biophys Acta 629:577–86.

James MO, Altman AH, Li CLJ, Schell JD. (1995). Biotransformation, hepatopancreas DNA-binding and pharmacokinetics of benzo[a]pyrene after oral and parenteral administration to the American lobster, *Homarus americanus*. Chem-Biol Interact 95:141–60.

Lamola AA, Joselow O, Yamane T. (1975). Zinc protoporphyrin (ZPP): a simple, sensitive, fluorometric screening test for lead poisoning. Clin Chem 21:93–7.

Lee RF, Ryan C, Neuhauser ML. (1976). Fate of petroleum hydrocarbons taken up from food and water by blue-crab *Callinectes sapidus*. Marine Biol 37:363–70.

Lim CK. (1991). Porphyrins. In: Hanai T, editor. *Journal of Chromatography Library, vol. 80: Liquid Chromatography in Biomedical Analysis*. Amsterdam: Elsevier. p. 209–29.

Liu J, Yeo HC, Doniger SJ, Ames BN. (1997). Assay of aldehydes from lipid peroxidation: gas chromatography–mass spectrometry compared to thiobarbituric acid. Anal Biochem 245:161–6.

Livingstone DR. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bull 42:656–66.

Marks GS. (1985). Exposure to toxic agents: the heme biosynthetic pathway and hemoproteins as indicator. Crit Rev Toxicol 15:151–79.

Marks GS, Powles J, Lyon M, McCluskey S, Sutherland E, Zelt D. (1987). Patterns of porphyrin accumulation in response to xenobiotics parallels between results in chick embryo and rodentsa. Ann NY Acad Sci 514:113–27.

Marks GS, McCluskey SA, Mackie JE, Riddick DS, James CA. (1988). Disruption of hepatic heme-biosynthesis after interaction of xenobiotics with cytochrome-P-450. Faseb J 2:2774–83.

Martinez CA, Mills GC. (1971). Spectrofluorometric determination of porphyrins in urine. Clin Chem 17:199–205.

Ng JC, Wang JP, Zheng B, Zhai C, Maddalena R, Liu F, Moore MR. (2005). Urinary porphyrins as biomarkers for arsenic exposure among susceptible populations in Guizhou province, China. Toxicol Appl Pharmacol 206:176–84.

Oakes KD, Van Der Kraak GJ. (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquatic Toxicol 63:447–63.

Oakes KD, McMaster ME, Van Der Kraak GJ. (2004). Oxidative stress responses in longnose sucker (*Catostomus catostomus*) exposed to pulp and paper mill and municipal sewage effluents. Aquatic Toxicol 67:255–71.

Pudek MR, Schreiber WE, Jamani A. (1991). Quantitative fluorometric screening-test for fecal porphyrins. Clin Chem 37:826–31.

Regoli F, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S, Winston GW. (2002). Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Marine Environ Res 54:419–23.

Rios de Molina MCR, Mazzetti MB, Galigniana M, Aldonatti C, Tomio JM, de Viale LCS. (2002). The decrease in uroporphyrinogen decarboxylase activity induced by ethanol predisposes rats to the development of porphyria and accelerates xenobiotic-triggered porphyria, damage regardless of hepatic. Brazilian J Med Biol Res 35:1273–83.

Robert TL, Varella L, Meguid MM. (1994). Nutrition management of acute intermittent porphyria. Nutrition 10:551–5.

Romeo M, Hoarau P, Garello G, Gnassia-Barelli M, Girard JP. (2003). Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. Environ Pollution 122:369–78.

Schwartz S, Edmondson P, Stephenson B, Sarkar D, Freyholtz H. (1976). Direct spectrofluorophotometric determination of porphyrin in diluted urine. Ann Clin Res 8:156–61.

Sheu JY, Chen PH, Tseng WC, Chen CY, Tsai LY, Huang YL. (2003). Spectrophotometric determination of a thiobarbituric acid-reactive substance in human hair. Anal Sci 19:957–60.

Stewart B. (2007). Stormwater discharges from Dunedin City: compliance monitoring (2007). Report prepared by Ryder Consulting Ltd. for Dunedin City Council.

Uchiyama M, Mihara M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–8.

Valcarel M, Gomez Hens A, Rubio S, Petidier A. (1987). Direct quantification of coproporhyrins and uroporphyrins in urine by derivative synchronous fluorescence spectroscopy. Clin Chem 33:1826–31.

van der Oost R, Beyer J, Vermeulen NPE. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149.

Viarengo A, Pertica M, Canesi L, Accomando R, Mancinelli G, Orunesu M. (1989). Lipid peroxidation and level of antioxidant compounds (GSH, vitamin E) in the digestive glands of mussels of three different age groups exposed to anaerobic and aerobic conditions. Marine Environ Res 28:291–5.

Viarengo A, Canesi L, Pertica M, Livingstone DR. (1991). Seasonal variations in the antioxidant defence systems and lipid peroxidation of the digestive gland of mussels. Comp Biochem Physiol C Comp Pharmacol 100:187–90.

Vijayavel K, Gomathi RD, Durgabhavani K, Balasubramanian MP. (2004). Sublethal effect of naphthalene on lipid peroxidation and antioxidant status in the edible marine crab Scylla serrata. Marine Pollution Bull 48:429–33.
Vijayavel K, Anbuselvam C, Balasubramanian MP. (2005). Naphthalene-induced hematological disturbances and oxidative stress in an estuarine edible crab, *Scylla serrata*. Environ Toxicol 20:464–6.
Warwick RM, Clarke KR. (1993). Increased variability as a symptom of stress in marine communities. J Exp Marine Biol Ecol 172:215–26.
Watson GM, Andersen OK, Galloway TS, Depledge MH. (2004). Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph. Aquatic Toxicol 67:127–42.
Westerlund J, Pudek M, Schreiber WE. (1988). A rapid and accurate spectrofluorometric method for quantification and screening of urinary porphyrins. Clin Chem 34:345–51.
Wilhelm Filho D, Tribess T, Gaspari C, Claudio FD, Torres MA, Magalhaes ARM. (2001). Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). Aquaculture 203:149–58.
Winston GW, Di Giulio RT. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. Aquatic Toxicol 19:137–61.
Winston GW, Moore MN, Kirchin MA, Soverchia C. (1996). Production of reactive oxygen species by hemocytes from the marine mussel, *Mytilus edulis*: lysosomal localization and effect of xenobiotics. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 113:221–9.
Woods JS. (1996). Altered porphyrin metabolism as a biomarker of mercury exposure and toxicity. Can J Physiol Pharmacol 74:210–15.
Zar JH. (1984). Biostatistical Analysis, 2nd edn. Englewood Cliffs, NJ: Prentice-Hall.

## Appendix

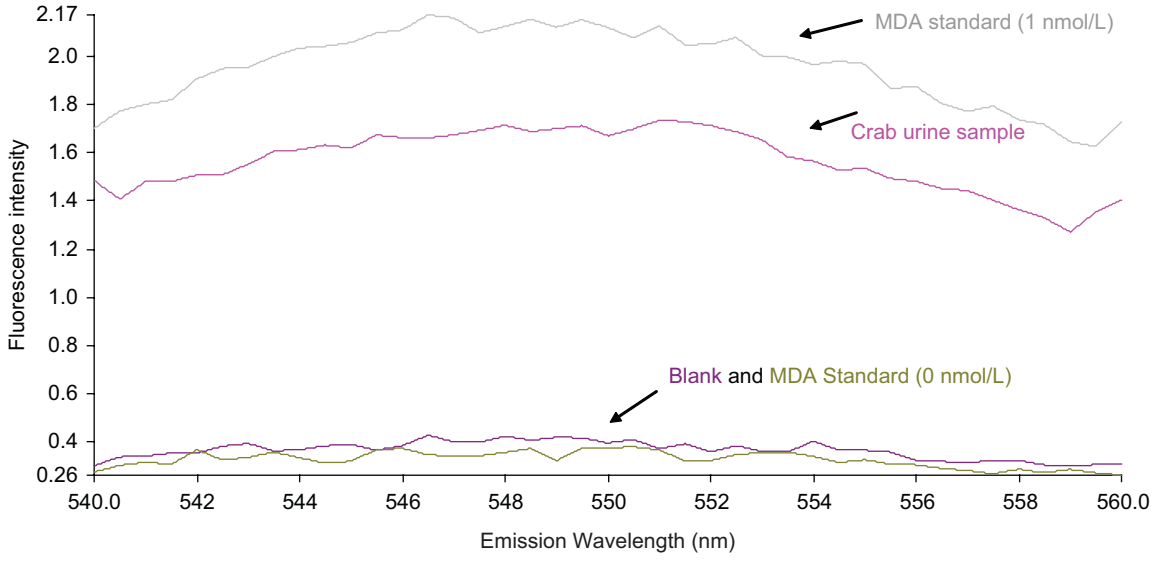


**Figure 5.** Fixed excitation fluorescence spectra ($\lambda_{excitation}$ = 530 nm) for diluted (1:47) crab urine sample, blank and 0 nmol $l^{-1}$ L MDA standards.

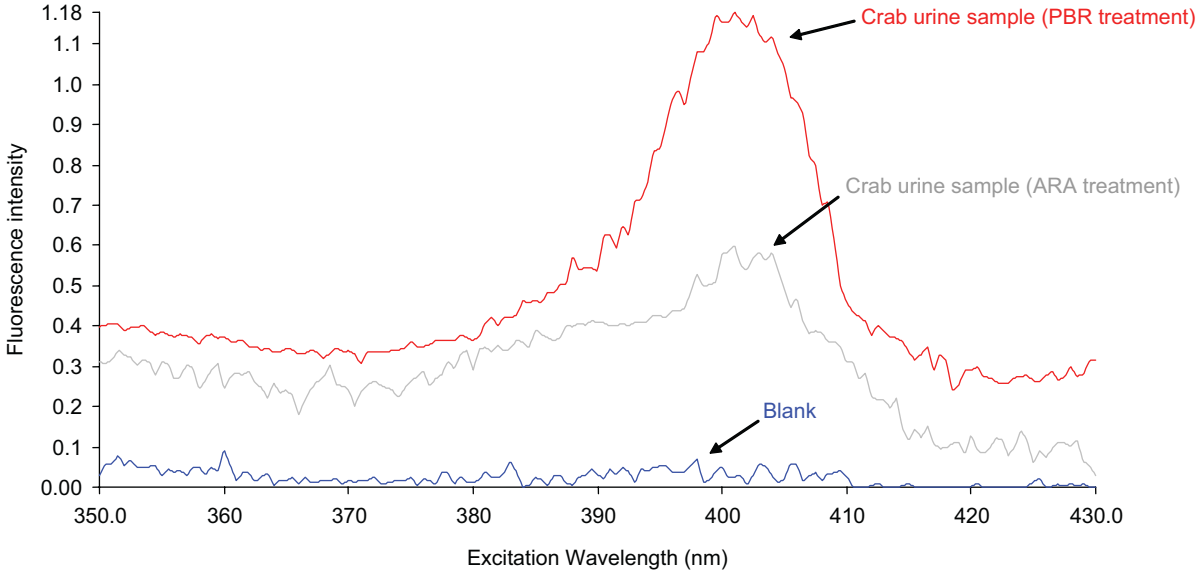


**Figure 6.** Fixed emission fluorescence spectra ($\lambda_{excitation}$ = 650 nm) for diluted (1:60) crab urine sample from two treatment groups (ARA and PBR) and a blank. The fluorescence spectrum of the crab urine sample reflects the typical shape of the porphyrin fluorescence signal in a human urine sample (see Westerlund *et al.* 1988, Fig. 5 p. 348).