Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and potentially harmful contaminants of the coastal and marine environment. Studies of their bioavailability, disposition and metabolism in marine organisms are therefore important for environmental monitoring purposes. Detecting PAH compounds in the biological fluids of marine organisms provides a measure of their environmental exposure to PAHs. In the present study, the shore crab *Carcinus maenas* was exposed to waterborne pyrene for 48 h. Urine and haemolymph samples were analysed by direct fluorimetry utilising both fixed wavelength (FF) and synchronous scanning fluorescence (SFS) techniques. Samples from exposed crabs exhibited fluorescence due to 1-OH pyrene equivalents, whilst samples from control crabs did not. Levels of equivalents were exposure dependent. Urine was shown to be a more suitable medium for the analysis of PAH equivalents. In a separate experiment, depuration of pyrene equivalents in urine was monitored over time. Urinary levels reached a maximum 2–4 days after initial exposure and decreased steadily thereafter. No unchanged parent pyrene was detected in samples from exposed crabs. While fluorimetric techniques could discriminate between 1-OH pyrene equivalents and parent pyrene, identification of specific metabolites was only possible with HPLC/F analysis. This revealed crabs had bio-transformed pyrene into 3 major conjugates of 1-OH pyrene, which were excreted in the urine. While such biotransformation of PAH is well documented in fish and several crustaceans, this is the first study to use direct fluorimetry to detect PAH equivalents in exposed crustacean urine. Fluorimetric results correlated well with those obtained by HPLC/F and ELISA techniques. The technique has great potential as a rapid, inexpensive and non-destructive technique for field biomonitoring of PAH exposure in crustaceans.
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1. Introduction

Marine waters and sediments, particularly those receiving anthropogenic inputs, contain a multitude of

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chemical contaminants that are potentially toxic to aquatic organisms (Krahn et al., 1984). One group of particular concern are the polycyclic aromatic hydrocarbons (PAHs). These are highly lipophilic chemicals, ubiquitous in soils, sediments, air and water (Lin et al., 1994). PAHs in the aquatic environment are derived largely from inputs of petroleum and its products, from sewage effluents, runoff and atmospheric deposition from the incomplete combustion of organic matter (Law et al., 1994). Industrial activities such as metal smelting (Naes et al., 1995; Beyer et al., 1996) and the electrolytic production of aluminium (Beyer et al., 1998) also release PAHs into rivers, estuaries and inshore waters. Coastal waters additionally receive substantial amounts of PAHs from products such as creosote, coal tar and coal tar pitch which are used as preservatives and antifouling agents (Uthe and Musial, 1986).

The conventional assessment of impacts involves measurements of contaminant concentrations in water and sediments. PAHs are sparingly soluble however, and their concentrations in water are very low (Lin et al., 1994). Consequently, they are difficult to detect. Large water volumes are needed for analysis, and their hydrophobicity/lipophilicity results in preferential partitioning into sediments (Lin et al., 1994). Analysis of sediments is equally laborious and time-consuming, involving numerous clean up and extraction steps. Furthermore, water/sediment-based measurements provide little information about the potential for contaminants to reach organisms and cause deleterious effects (i.e. bioavailability and exposure are not addressed). To assess exposure to PAHs (and other contaminants) more accurately, it is pertinent to detect them in the tissues and biological fluids of aquatic organisms.

Tissues such as liver, hepatopancreas, kidney, muscle and gills, and body fluids (blood/haemolymph, bile and urine) have been analysed previously to determine the concentrations and types of PAHs that enter biota. However, differences in the ability of aquatic species to metabolize contaminants affects their suitability as biological indicators. In organisms capable of metabolizing PAHs, measuring metabolites instead of simply quantifying parent compounds avoids underestimation of true uptake (McElroy et al., 1989). Measurement of parent PAH residues is more suited to species such as bivalve molluscs,

which have limited ability for biotransformation of PAHs and tend to accumulate these compounds in their tissues (Hufnagle et al., 1999; McElroy et al., 1989). Determination of PAH metabolites is particularly relevant in fish (Stegeman and Lech, 1991) as it is the biotransformed, active metabolites that exert significant toxic, mutagenic and carcinogenic effects (James, 1989; Stroomberg et al., 1996).

The development of easy-to-use, accurate and cost-effective techniques for the measurement of PAHs and their metabolites (and other contaminants) in the various matrices of aquatic biota is becoming increasingly relevant, as it provides useful information regarding contaminant exposure and adverse effects in populations in situ (Depledge, 2000; Wells et al., 2001). This information can then be used in the rapid assessment of the pollution status of the ecosystem and of the potential risk to humans who consume PAH contaminated animals. For example, the rapid assessment of marine pollution (RAMP) approach utilises a wide array of simple, inexpensive techniques to provide an indication of the extent of exposure of marine organisms to contaminants as well as evidence of effects (Depledge, 2000; Wells et al., 2001; Galloway et al., 2002). This information can then be used in setting priorities for more detailed and costly studies utilising more sophisticated techniques.

In the present study, a simple technique for the spectrophotometric analysis of urine and haemolymph from shore crabs has been developed and evaluated as a means of assessing exposure to polyaromatic hydrocarbons.

2. Materials and methods

2.1. Collection of experimental animals and laboratory conditions

Green, male, intermoult shore crabs, *Carcinus maenas* (carapace width 50–72 mm) were collected on incoming tides from Jenkins Quay on the Avon estuary at Bantham, South Devon, UK on three occasions between the months of April and July 2000. On return to the laboratory, they were maintained in holding tanks containing filtered (10 μm carbon filtered), well aerated 34 ppt, 15 ± 1 °C seawater,

under a 12 h light: 12 h dark regime for 1 week to permit acclimation. Crabs were fed twice weekly with irradiated cockles. Water was changed within 12 h of feeding.

2.2. Chemicals

Pyrene (98%, cat. no. 18, 551-5) and 1-OH pyrene (98%, cat. no. 36, 151-8) were purchased from Sigma–Aldrich (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK). Pyrene-d_{10} was acquired from Promochem (Welwyn Garden, UK). Ethanol (GPR^{TM}, 96%, v/v) and acetone (GPR^{TM}) were obtained from BDH Laboratory Supplies (Poole, BH15 1TD, UK).

2.3. Exposure–response exposure experiment

Crabs were transferred to glass aquaria containing 10 l of filtered (10 μm carbon filtered), well-aerated seawater (34 ppt, $15 \pm 1\,^{\circ}$C), under a 12 h: 12 h light: dark regime. After measurement of carapace width, the animals were assigned to one of 14 groups of 4 in separate aquaria, with groups of 8 crabs exposed to one of seven treatments, for 48 h. For exposed crabs, pyrene was added to the water in an acetone carrier (at a ratio of 1:1, w/v, pyrene/acetone) to increase solubility. Pyrene exposure was at five nominal concentrations (200, 100, 50, 25 and 10 μg l^{-1}). Acetone controls were exposed to acetone only and eight crabs were held in seawater alone (seawater controls). Animals were not fed during the exposure period. The crabs remained in the aquaria for the duration of the exposure, whereupon they were transferred to clean seawater to depurate for up to 3 weeks, during which time feeding was resumed.

2.4. Time response exposure experiment

In a separate experiment, groups of eight crabs were exposed, under the conditions described above, to waterborne pyrene at the nominal concentrations of 400 and 200 μg l^{-1}. An acetone control group and a seawater control group, each comprising eight individuals, were also included as described above. Individuals were exposed and kept in their respective aquaria for a period of 10 days. After this time, they were removed and held in clean aerated seawa-

ter. For repeat sampling, individuals were identified by labels super-glued onto the dorsal surface of the carapace.

2.5. Urine and haemolymph sampling

In the exposure–response experiment, urine and haemolymph samples were taken from each crab after 48 h exposure using the technique described by Bamber and Naylor (1997). Briefly, crabs were removed from their aquaria and placed in a bucket containing clean seawater. After being drained of residual seawater, they were restrained with the ventral surface uppermost on a plastic board using rubber bands. The third maxillipeds were moved aside and kept apart by inserting absorbant paper between the base of the appendage and top of the sternum. The epistome was dried (to prevent seawater contaminating the urine) and the crab placed under a dissecting microscope (10×). The operculum of each antennal gland bladder was lifted using a hooked seeker, causing urine to flow from the bladder, through the opercula, where it was collected using a 200 μl Gilson$^{©}$ pipette fitted with a flexible flat tip. Samples (20–400 μl per crab) were then transferred to siliconized microcentrifuge tubes, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until analysis.

With the crab still restrained, the absorbant paper holding the maxillipeds was removed and a haemolymph sample was taken from a suitable arthrodial membrane at the base of a walking leg. The membrane was first gently punctured using a fine bore needle and then a fine capillary, with plunger, was used to draw out 200 μl of haemolymph. Samples were then expelled into siliconized microcentrifuge tubes, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until analysis. Test animals were returned to their respective aquaria immediately after sampling,

Urine and haemolymph samples were taken in this way wherever possible, but occasionally urine could not be obtained since the urinary bladders of some crabs were empty. It was necessary therefore, to repeat the above exposure and sampling procedure three times, to obtain sufficient replicates ($n > 10$) of urine samples. For the time–response experiment, urine samples were taken from all crabs (as described above) after 12, 24, 48 and 96 h, and 8 and 10 days.

2.6. Direct fluorescence spectrophotometry (fluorimetry)

2.6.1. Analysis of standards and urine samples

Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer, coupled to a software package operated by Windows 3.1. Fifty percent ethanol was the solvent used for samples, standards and blanks. Measurements were carried out in a 3.5 ml quartz fluorescence cuvette. All FF and SFS measurements were performed with excitation and emission slit widths of 2.5 nm. Resulting FF and SFS spectra were smoothed and fluorescence intensity measured at a fixed excitation and emission wavelength pair for solvent blanks, standards and samples alike.

2.6.2. Determination of wavelength pairs

Various authors have reported differing wavelength pairs for the detection of pyrenes in fish bile, including 341/383 nm (Aas et al., 1998; Camus et al., 1998; Beyer et al., 1998), 345/395 nm (Ariese et al., 1993; Escartin and Porte, 1999) and 349/391 nm (Sundt and Goksoyr, 1998). For synchronous fluorescence analyses of fish bile, wavelength differences of 34 nm (Lin et al., 1994) 37 nm (Ariese et al., 1993; Lin et al., 1994) and 42 nm (Aas and Klungsoyr, 1998; Aas et al., 2000a,b) have been used.

The optimum wavelength pairs for the detection of "pyrenes" (the parent compound and its metabolites) in the present study were selected after first analysing a subset of samples and several pyrene and 1-OH pyrene standards using a range of different excitation and emission wavelengths.

2.6.3. Preliminary analysis of standards and samples

Various concentrations (200, 100, 75, 50, 25, 10, 5 μg l^{-1}) of pyrene and 1-OH pyrene were prepared in 50% ethanol and analysed to determine the optimal wavelength pair for these compounds. The standards were analysed using fixed fluorescence (FF) at a range of excitation wavelengths (including 341, 345 and 349 nm, according to the papers reported above), with emission spectra taken from 330 to 500 nm. For synchronous fluorescence (SFS) analysis, $\Delta\lambda$'s of 34, 37 and 42 nm were tested, with excitation/emission spectra taken from 240 to 500 nm.

Several representative urine and haemolymph samples from each treatment were also analysed. A 20/50 μl aliquot of urine/haemolymph was dissolved in 1980/950 μl of 50% ethanol in the cuvette, to yield a 1:100/1:20 dilution, respectively. Analyses were performed using the above wavelength regimes for detection of pyrenes.

FF at Ex345 nm produced the largest and most clearly resolved peaks for standards and samples. For synchronous fluorescence spectrometry (SFS) analysis, 37 nm was the optimal $\Delta\lambda$. The predominant urinary peaks more closely approximated those produced by the 1-OH pyrene standards than the parent standards. Therefore, analysis of samples and 1-OH pyrene standards was undertaken with a fixed excitation wavelength of 345 nm and synchronous analysis was performed with a $\Delta\lambda$ of 37 nm.

For operational purposes, the fluorescence value assigned to individual standards and samples was the intensity measured at a fixed wavelength pair rather than at the maximum peak height of the dominant peak on the emission spectra. In all cases this value was within 5% of the peak maxima. The fixed wavelength pair was determined by calculating the mean emission wavelength of the dominant peak on both standard and sample spectra. For analyses, this was Ex345/Em387 nm (standards) and Ex345/Em382 nm (samples). For SFS analyses, wavelengths were Em387 nm (standards) and Em381.4 nm (samples).

2.6.4. Quantification of sample peaks

To take into account instrumental drift over the lifetime of the experiment and to allow all samples to be compared despite fluctuations in instrumental response, sample peaks were quantified with respect to a series of 1-OH pyrene standards (200, 100, 75, 50, 25, 10, 5 μg l^{-1} for urine samples and 10, 5, 2, 1, 0.5 μg l^{-1} for haemolymph samples), analysed at the fixed wavelength pair of Ex345/Em387 nm (FF) and Em387 nm (SFS). The use of 1-OH pyrene was favoured over the parent compound as it more closely approximates the peaks seen in the samples. 1-OH pyrene does not serve to quantify sample peaks in terms of μg l^{-1} of 1-OH pyrene as the peaks seen in samples are blue shifted by 5 nm and are unlikely to be the pure standard. Results are therefore reported in terms of μg l^{-1} of 1-OH pyrene "equivalents".

2.6.5. Urine and haemolymph samples

Urine (diluted 1:100) and haemolymph (diluted 1:20) samples from exposed and control crabs were analysed for pyrenes as described above. Fluorescence intensity was measured at the assigned wavelength pair of Ex345/Em382 nm (FF) and Em381.4 nm (SFS) and expressed as $\mu g\,l^{-1}$ of 1-OH pyrene equivalents following comparison to standards. Before analysis of either standards or samples, a blank of 50% ethanol was scanned by FF and SFS. This provided a value for the background fluorescence contribution of the solvent, for comparison with samples and standards.

2.6.6. Statistical analysis

Data were analysed using the software package Statgraphics Plus 5.1 and are presented using notched box and whisker plots. The rectangular part of the plot ("box") extends from the lower quartile to the upper quartile, covering the middle 50% of data values (interquartile range). The centre lines within each box show the position of the medians, whilst the plus signs show the position of the mean. The "whiskers" extend from the upper and lower quartiles to maximum and minimum values lying within 1.5 times the interquartile range. Outliers are shown as individual points. Also included are notches covering a distance either side of the median line, representing the 95% confidence interval for the median. Where notches for any pair of medians overlap, there is no statistically significant difference between medians at the 95% confidence level. Where no overlap occurs, the difference is significant at the 95% confidence level.

All urine and haemolymph data were variance checked for homogeneity of variance between groups and found to be not normally distributed (a statistically significant difference amongst the S.D., $P \leq 0.05$). This large variability in the data (more than a 3 to 1 difference between the smallest S.D. and the largest) invalidates an analysis of variance, which assumes that S.D. at all treatments is equal. All urinary data were therefore log-transformed for comparison of means by one-way ANOVA, using Fisher's least significant difference (LSD) procedure to discriminate between means at the 95% confidence level. Haemolymph data, when variance checked, were found to be not normally distributed, even following log transforma-

tion. Data were therefore subject to a Kruskal–Wallis test to compare medians at the 95% confidence level.

2.6.7. GC/MS analysis of water samples

To investigate the uptake of waterborne pyrene by exposed crabs, a series of tanks were set up in duplicate (one containing crabs, the other without). Pyrene was added to the water at the same nominal concentrations as for the exposure experiment and water samples taken at 0, 2, 6, 12, 24, 48 and 96 h. Samples were analysed for parent PAH using GC/MS analyses (Hewlett-Packard Model 5890 II Plus GC and a 5972 mass selective detector (MSD) (Palo Alto, CA)). Internal standard spiked (pyrene-d_{10}) water samples (100–500 ml) were concentrated using C_{18} cartridges (IST, Hengoed, UK), which were subsequently eluted (three times) using 3 ml of ethyl acetate. The eluent was then concentrated down to 1 ml before analyses by GC/MS.

2.6.8. Inner filter effects and matrix effects

Provided the total absorbance of a sample is low, a linear relationship exists between the intensity of fluorescence emitted by a compound within the sample, and its concentration. If the sample absorbs more than 5% of the incident light, fluorescence intensity will be reduced by any compound in the sample which absorbs either the excitation or emission light. This is known as "inner filter effect" (UNEP/IOC/IAEA, 1992). To test for inner filter effects, representative urine samples from each treatment were serially diluted in 50% ethanol (1:100, 1:50, 1:25, 1:10, 1:5) and analysed as above (FF Ex345/Em382 nm). Fluorescence intensity results were then plotted against the relative concentration of the sample to establish the relationship between dilution and fluorescence signal.

To determine whether the 5 nm difference between the standard peak and the urinary peak was not due to a urinary matrix effect, an additional experiment was carried out. Urine from control crabs was spiked with known amounts of 1-OH pyrene standard (yielding final concentrations of 25, 50, 100 and 200 $\mu g\,l^{-1}$). These spiked samples were then analysed as described above and the spectral position of the resulting peak was compared to the peak seen in urine from pyrene exposed crabs.

2.6.9. HPLC/F identification of urinary metabolites

HPLC/F analysis on selected urine samples from control and exposed crabs was kindly carried out by Dr. Mike Howsam, Vrije Universiteit, The Netherlands. Methodology and results are reported in full in Fillmann et al. (2003).

HPLC was performed using an ion pair elution system under acidic conditions on a reversed phase C_{18} analytical column (Vydac 201TP54 column (250 mm × 4.6 mm), Hesperia, CA, USA). The column temperature was maintained at 30 °C in a column oven. An elution gradient of acetonitrile (CH_3CN) and aqueous buffer (10 mM ammonium acetate, adjusted to ∼pH 5 with acetic acid/l buffer) was used at a flow rate of $0.5 \, ml \, min^{-1}$ ($t = 0$ min, 5% CH_3CN; $t = 40$ min, 90% CH_3CN; isocratic at 90% CH_3CN for 10 min). The instrument used consisted of two Spectroflow 400 pumps (Applied Biosystems), a Spark-Holland PROMIS II autosampler (20 µl injection loop), a GT-103 in-line degasser (Separations, The Netherlands) and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). Fluorescence detection was carried out at $\lambda_{ex/em} = 346/384$ nm for pyrene metabolites (slit widths $\lambda_{ex/em} = 18/40$ nm) (modified from Stroomberg et al., 1999).

Samples were diluted 1:30 with ethanol (modified with $5 \, mg \, ml^{-1}$ ascorbic acid) and stored at −20 °C. Peaks were identified according to their retention times (Stroomberg et al., 1999), and confirmed for the system used in this study (Howsam, personal communication). Quantification of 1-OH pyrene was performed using a series of 1-OH pyrene external standards, while conjugates were quantified using their relative fluorescence efficiencies compared to 1-OH pyrene; pyrene-1-glucoside = 2.0 ± 0.31, pyrene-1-sulphate = 1.23 ± 0.09, pyrene-1-'conjugate' = 1.75 ± 0.18 (Stroomberg et al., 2003).

2.6.10. Comparison of fluorescence results with other analytical techniques

To validate the direct fluorimetric technique, levels of urinary equivalents in selected samples were analysed using HPLC/F and an immunoassay technique and then compared to fluorescence results. Samples were analysed with a PAH-competitive tube format ELISA kit (PAH RaPID Assay®, SDI Europe, Alton, UK) shown to be highly suitable for detecting PAH in biological samples (Fillmann et al., 2002). The ELISA was calibrated using 1-OH-pyrene.

3. Results

3.1. Pyrene and 1-OH pyrene standards

FF analysis of pyrene standards with an excitation wavelength of 243 nm produced a predominant peak at Em372–373 nm, with an additional smaller peak at Em392 nm. Ex345 nm produced no peaks. SFS with a $\Delta\lambda$ of 37 nm yielded a sharp peak at Em 372 nm (Fig. 1c). FF analysis of 1-OH pyrene standards yielded three peaks, with the largest and most clearly resolved at 386–387 nm emission (see Fig. 1a). SFS ($\Delta\lambda$37 nm) of the same standards yielded a large sharp peak at the wavelength pair of Ex349–350/Em386–387 nm (Fig. 1c).

3.2. Urine samples

FF analysis of urine samples from exposed crabs (from both dose response and time response experiments) produced three clear peaks on the emission spectrum, with the largest of these being at approximately 381–383 nm (see Fig. 1a). These peaks were conspicuously absent from control samples. SFS reduced these peaks to a single emission band, with a large sharp peak around Em381–383 nm in "exposed" samples (see Fig. 1c), with controls lacking this peak. The position of the dominant emission peaks seen in FF and SFS is shifted approximately 5 nm from that of the largest peak seen in the hydroxylated standards (∼Em387 nm). This has important implications for quantitation and metabolite identification, which will be discussed below. The position of the SFS peak also strongly suggests that the fluorescence signal is produced almost exclusively by pyrenes other than the parent compound.

Results are reported in terms of $\mu g \, l^{-1}$ of 1-OH pyrene equivalents, determined by interpolation of sample fluoresence intensities (at FF Ex345/Em382 nm and SFS Em381.4 nm) from a 1-OH pyrene standard curve (using intensities at FF/SFS Em387 nm). No significant differences ($P > 0.05$) in fluorescence signal were seen between acetone control and seawater control groups, so these data were combined and are presented simply as "controls".
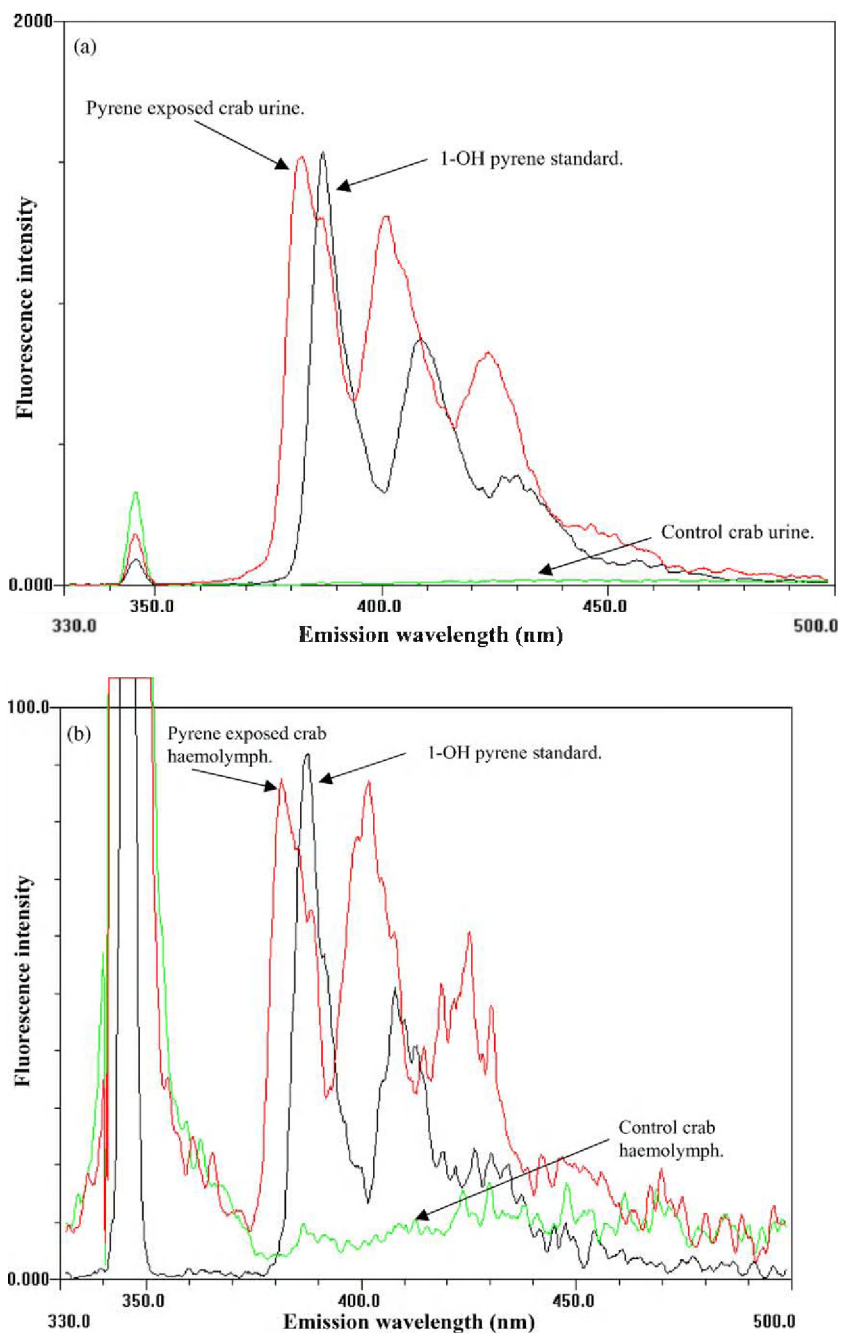
Fig. 1. (a) Fluorescence (FF, Ex345 nm) spectra from $200\,\mu g\,l^{-1}$ pyrene exposed crab urine, control crab urine and $100\,\mu g\,l^{-1}$ 1-OH pyrene standard. Urine samples diluted 1:100. (b) Fluorescence (FF, Ex345 nm) spectra from $200\,\mu g\,l^{-1}$ pyrene exposed crab haemolymph, control crab haemolymph and $5\,\mu g\,l^{-1}$ 1-OH pyrene standard. Haemolymph samples diluted 1:20.
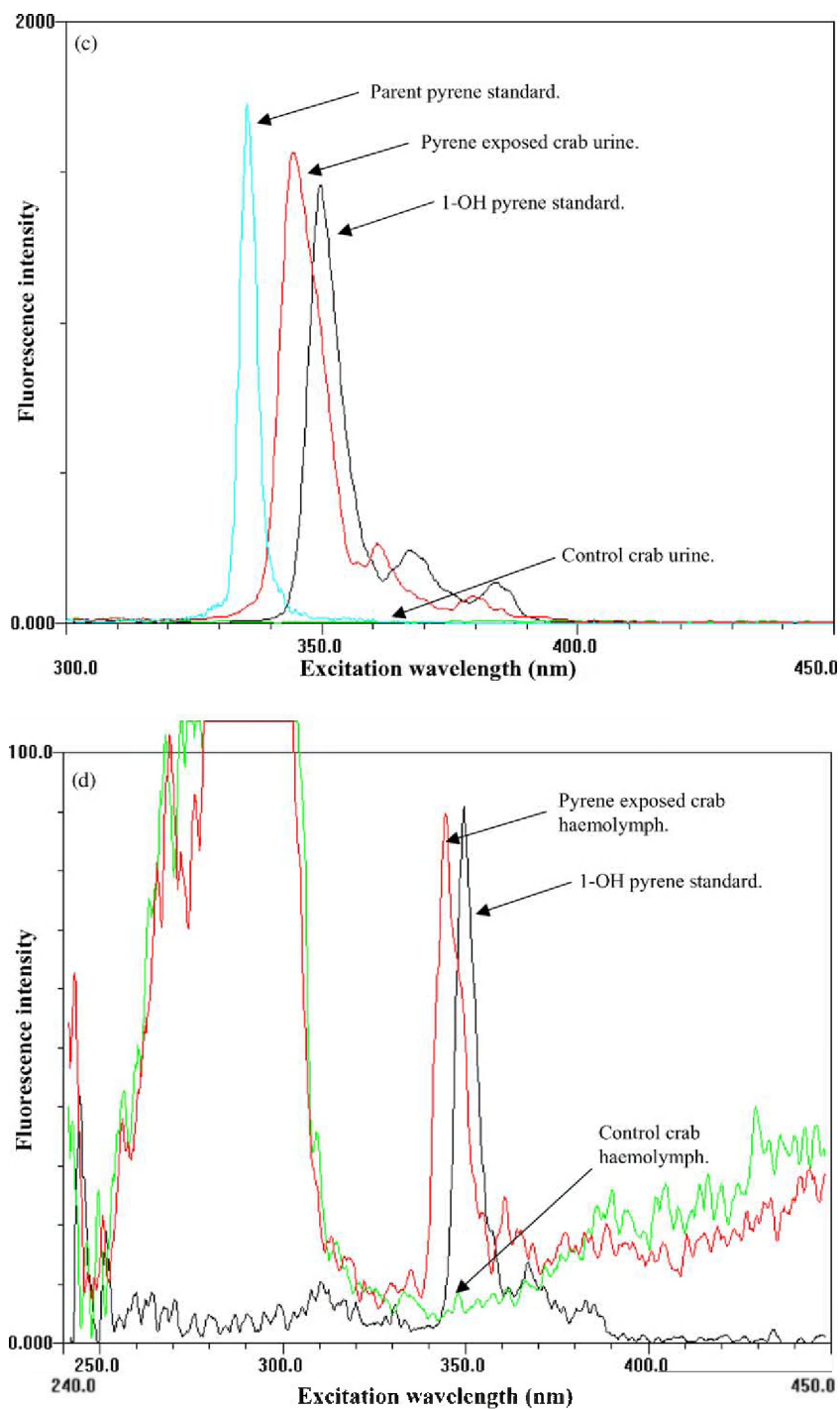
Fig. 1(c) Fluorescence (SFS, $\Delta\lambda$ 37 nm) spectra from $200\,\mu g\,l^{-1}$ pyrene exposed crab urine, control crab urine, $100\,\mu g\,l^{-1}$ 1-OH pyrene and $100\,\mu g\,l^{-1}$ pyrene standards. Urine samples diluted 1:100. (d) Fluorescence (SFS, $\Delta\lambda$ 37 nm) spectra from $200\,\mu g\,l^{-1}$ pyrene exposed crab haemolymph, control crab haemolymph and $5\,\mu g\,l^{-1}$ 1-OH pyrene standard. Haemolymph samples diluted 1:20.

3.3. Exposure–response experiment

Untransformed data are presented in Fig. 2a and b; Table 1. A moderately strong exposure–response relationship can be seen with median and mean levels of urinary 1-OH pyrene equivalents increasing with waterborne pyrene concentration (FF, $r^2 = 68.23\%$, correlation coefficient $= 0.826$, SFS, $r^2 = 66.12$, cc $= 0.813$) (Fig. 2a and b). Mean values determined by SFS are marginally higher than those determined by FF. There was a statistically significant difference (KW, $P < 0.05$) between the medians at each treatment, whether determined by FF or SFS, with the notable exception of the medians from the 100 and $200\,\mu g\,l^{-1}$ treatment groups (SFS). Analysis of variance of log-transformed data revealed statistically significant differences in mean fluorescence between treatments at the 95% confidence level (***$P = 0.00$).

Baseline fluorescence at Ex345/Em382 nm and Em381.4 nm is reported for controls (as these samples lack a peak at the wavelengths), and was very low compared to exposed samples. The control level of fluorescence at these wavelengths was comparable to that seen in the solvent blank (data not presented).

The data from both methods showed considerable inter-individual variability. Variability increased with exposure and was most marked in the 100 and $200\,\mu g\,l^{-1}$ treatment groups.

3.4. Haemolymph samples

Results are reported in terms of $\mu g\,l^{-1}$ of 1-OH pyrene equivalents, determined as above. There were no significant differences (KW, $P > 0.05$) in fluorescence signal between acetone control and seawater control groups, so these data were combined and are presented simply as "controls".
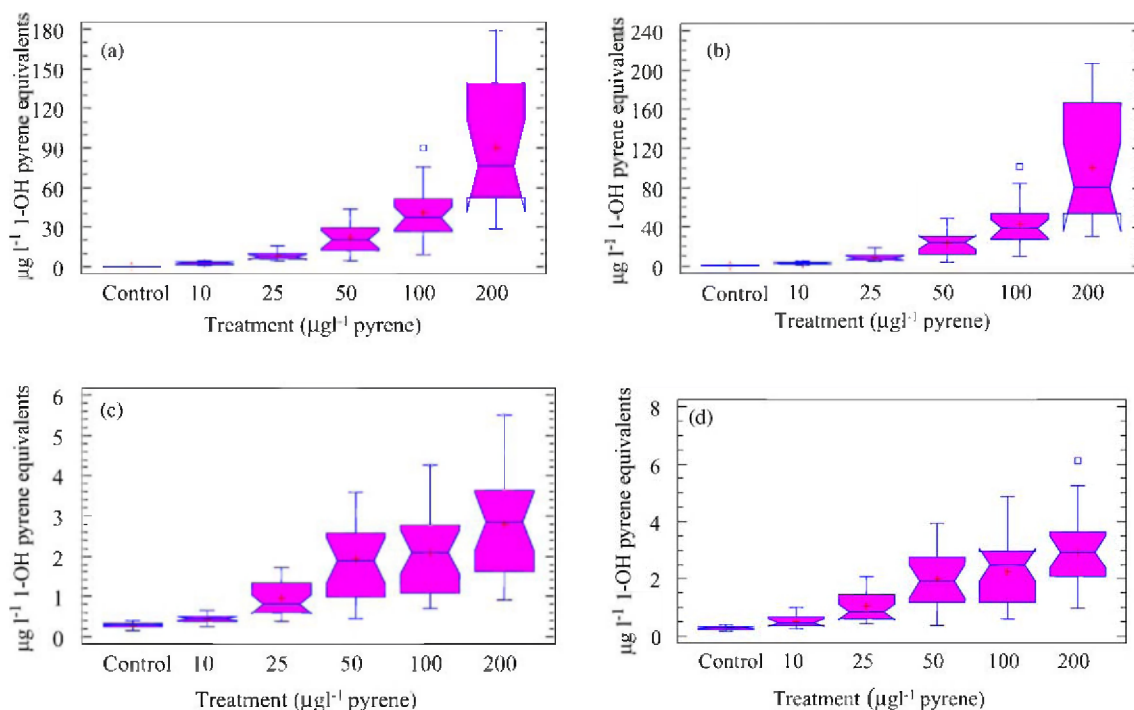

Fig. 2. (a) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by FF. (b) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by SFS. (c) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by FF. (d) Notched box and whisker plot of concentration ($\mu g\,l^{-1}$) of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by SFS.

Table 1

Mean concentrations of 1-OH pyrene equivalents in diluted (1:100) urine of pyrene exposed *C. maenas*, determined by FF and SFS

Treatment ($\mu g \, l^{-1}$ pyrene)	$\mu g \, l^{-1}$ 1-OH pyrene equivalents (FF @ Ex345/Em382 nm)	$\mu g \, l^{-1}$ 1-OH pyrene equivalents (SFS @ Em381.4 nm)
200	89.77 ± 47.40 (15)	100.44 ± 55.69 (15)
100	40.58 ± 20.70 (19)	43.75 ± 23.49 (19)
50	21.50 ± 11.66 (16)	24.27 ± 13.15 (16)
25	8.10 ± 3.21 (15)	8.93 ± 4.01 (15)
10	2.59 ± 1.00 (12)	2.92 ± 1.22 (12)
Controls	0.38 ± 0.21 (20)	0.44 ± 0.26 (20)

Data are mean ± S.D. Numbers in parentheses indicate the number of samples analysed.

3.5. Exposure–response experiment

Untransformed data are presented in Fig. 2c and d; Table 2. FF and SFS analysis of haemolymph samples produced peaks in the same positions as those seen in the urine samples (Fig. 1b and d) but with greatly reduced intensities (see Table 2). Median and mean levels of 1-OH pyrene equivalents, determined by both FF and SFS, increase in a concentration dependent manner with waterborne pyrene concentration (FF, $r^2 = 49.79\%$, cc $= 0.706$ and SFS, $r^2 = 48.64$, cc $= 0.697$) (Fig. 2c and d). Mean values determined by SFS were marginally higher than those determined by FF.

FF and SFS data showed large variability, which increased with exposure concentration and was most pronounced in the 50, 100 and 200 $\mu g \, l^{-1}$ groups. This variability was greater than that seen in the urine samples from the same crabs. Despite log-transformation, the standard deviations of the FF and SFS data at each treatment differed significantly at the 95% confidence level (*$P < 0.05$), excluding an analysis of variance. Kruskal–Wallis analysis of FF and SFS data revealed a statistically significant difference (***$P = 0.00$) amongst the medians at each treatment. Medi-

ans from the control, 10 and 25 $\mu g \, l^{-1}$ groups differed significantly from each other and from the medians of the remaining treatment groups. The box and whisker plots show however, that there was no statistically significant difference between the 50, 100 and 200 $\mu g \, l^{-1}$ groups ($P > 0.05$).

3.6. Time response experiment

The time response data determined by both FF and SFS (Fig. 3a and b) showed a clear pattern of uptake and elimination of pyrene in *C. maenas*. Levels of equivalents in the urine of 200 and 400 $\mu g \, l^{-1}$ exposed crabs increased to reach a maximum after 2 and 3–4 days, respectively, and dropped steadily over the remainder of the exposure period. The maximum mean level seen in the 400 $\mu g \, l^{-1}$ exposed crab urine was approximately twice the level in the 200 $\mu g \, l^{-1}$ exposed crabs. Fluorescence at 1-OH pyrene equivalent wavelengths in control crab urine was not detectable above the background baseline for the entire experimental period. After 10 days, levels of equivalents in exposed crabs had decreased to levels similar to those after 12 h exposure, but was still significantly different to controls (KW, *$P < 0.05$).

Table 2

Mean concentrations of 1-OH pyrene equivalents in diluted (1:20) haemolymph of pyrene exposed *C. maenas*, determined by FF and SFS

Treatment ($\mu g \, l^{-1}$ pyrene)	$\mu g \, l^{-1}$ 1-OH pyrene equivalents (FF @ Ex345/Em382 nm)	$\mu g \, l^{-1}$ 1-OH pyrene equivalents (SFS @ Em381.4 nm)
200	2.79 ± 1.25 (19)	2.93 ± 1.32 (19)
100	2.08 ± 1.02 (23)	2.24 ± 1.15 (23)
50	1.93 ± 0.99 (17)	2.00 ± 1.01 (17)
25	0.97 ± 0.43 (24)	1.95 ± 0.52 (24)
10	0.44 ± 0.11 (13)	0.51 ± 0.21 (13)
Controls	0.29 ± 0.06 (26)	0.29 ± 0.06 (26)

Data are mean ± S.D. Numbers in parentheses indicate the number of samples analysed.
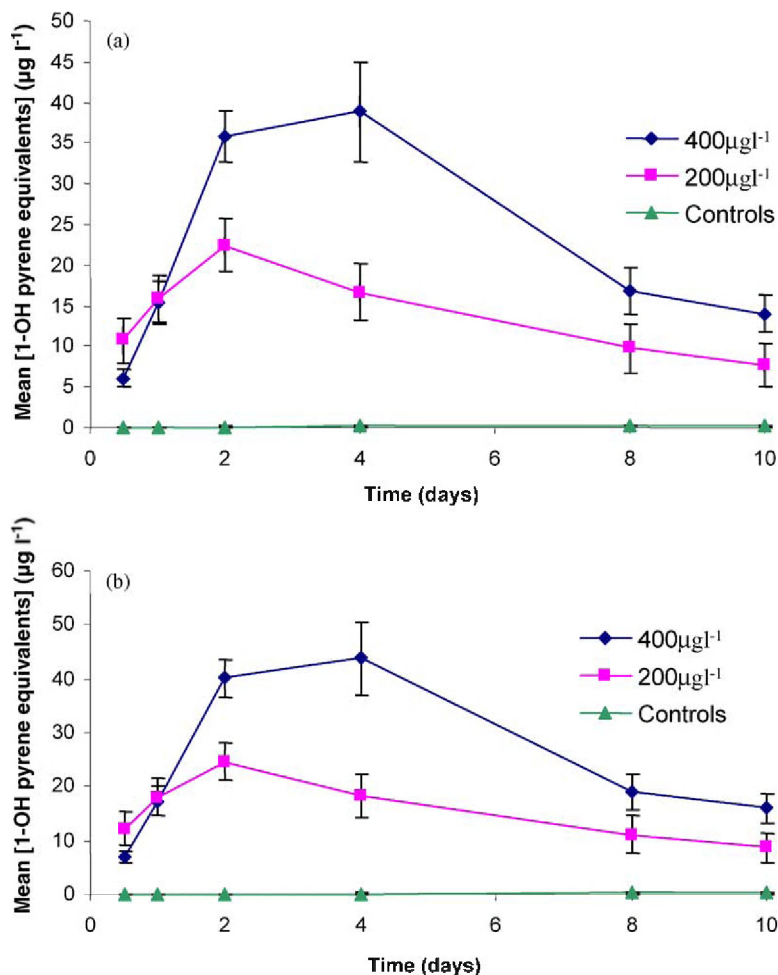
Fig. 3. (a) Temporal response of 1-OH pyrene equivalent levels in the urine (diluted 1:100) of pyrene exposed *C. maenas*, determined by FF. Values are mean ± S.E. 400 μg l^{-1}. $n = 7$: 200 μg l^{-1}, $n = 6$–7: controls $n = 3$–8. (b) Temporal response of 1-OH pyrene equivalent levels in the urine (diluted 1:100) of pyrene exposed *C. maenas*, determined by SFS. Values are mean ± S.E. 400 μg l^{-1}, $n = 7$: 200 μg l^{-1}, $n = 6$–7: controls $n = 3$–8.

3.7. GC/MS analysis of water samples

Recoveries were $95 \pm 3\%$ ($n = 18$). Measured concentrations confirmed that nominal values were within $\pm 12\%$. Results from tanks with and without crabs are presented in Fig. 4, revealing that whilst pyrene was lost from water without crabs (most likely through photo-oxidation and adsorption processes), its levels decreased much more rapidly in the water from tanks with crabs. For example, after 2 h, an average of 8% of the nominal concentrations of parent pyrene was lost from seawater alone, whilst 34% was lost from sea-

water being filtered over the gills of crabs. After 6 h of exposure, these losses increased to 37 and 52% of the initial concentration, respectively.

3.8. Inner filter effects and matrix effects

A linear relationship between fluorescence intensity and sample concentration (dilution factor) indicates there is no significant inner filter effect, permitting any measurements to be used for quantification (UNEP/IOC/IAEA, 1992). This result also signified that fluorescence from the compounds of
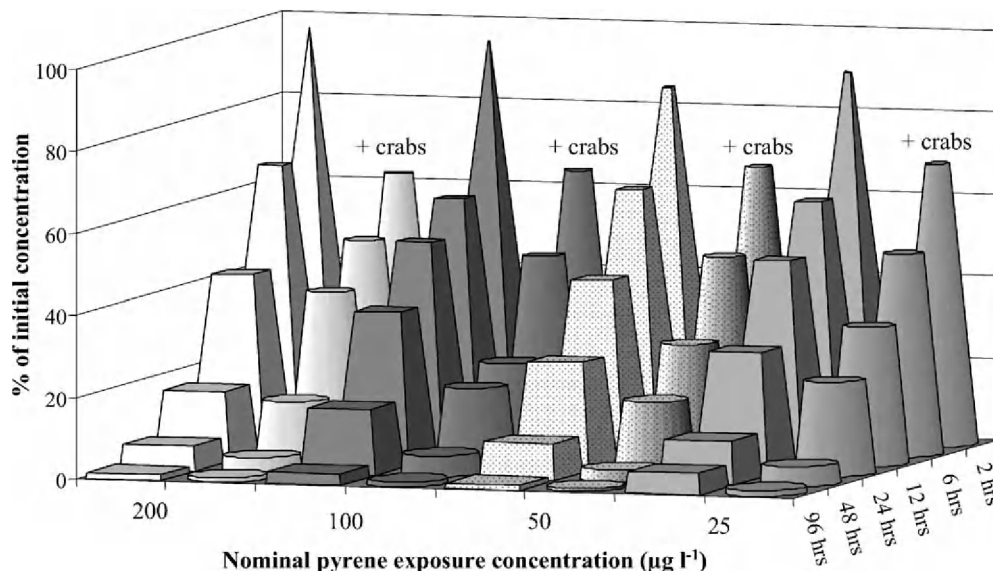
Fig. 4. Levels of pyrene in exposure water over the experimental period, as a percentage of the initial concentration (y-axis). Circular and square columns indicate levels of pyrene in tanks with and without crabs, respectively.

interest increases in a linear fashion with their concentration, allowing values for undiluted samples to be extrapolated from diluted values.

In each spiked sample, the peak observed was that of the 1-OH standard (~Em387 nm), with no shift to ~Em382 nm. The urinary matrix therefore does not alter the fluorescence characteristics of 1-OH pyrene. This suggests the peak seen in exposed crab urine is not the free-hydroxylated metabolite but is due to a compound or compounds with subtly different fluoresecent characteristics.

3.9. HPLC/F identification of urinary metabolites

HPLC/F analysis revealed conjugation of 1-hydroxypyrene into three major metabolites. Two of the conjugates were identified as pyrene-1-glucoside (elution time 28.0 min) and pyrene-1-sulphate (26.3 min). The relative distributions of conjugates show that pyrene-1-glucoside and an as yet unidentified pyrene-1-"conjugate" (25.3 min) dominated. Comparing the relative amounts of 1-OH-pyrene and its conjugates, it was found that an average of 38.9% is present as pyrene-1-glucoside, 9.7% as pyrene-1-sulphate, 47.7% as the unknown conjugate and 3.7% as non-conjugated 1-hydroxy-pyrene

(39.1 min). The unknown pyrene-1-"conjugate" is not a glucuronide conjugate nor (derived from) a glutathione conjugate (M. Howsam, personal communication). Parent pyrene (47.7 min) was shown to be negligible in exposed samples. Results can be found in full in Fillmann et al. (2003).

3.10. Comparison of fluorescence results with other analytical techniques

Comparison of urinary equivalent levels determined by HPLC/F with those of direct fluorescence showed a strong correlation ($r^2 = 0.91$) (Fillmann et al., 2003). Results obtained from the ELISA also correlated well with fluorescence results ($r^2 = 0.88$) (Fillmann et al., 2002).

4. Discussion

The aim of the present study was to develop and evaluate a rapid, simple and inexpensive technique to measure PAH exposure in decapod crustacea. Results have shown that direct fluorometric detection of PAH metabolites in biological fluids of the shore crab (*C. maenas*) is a reliable means of doing so. Compelling

evidence is presented that exposure of crabs to pyrene can be assessed by measuring pyrene "equivalents" in their urine and to a lesser extent, their haemolymph. Urine and haemolymph from control crabs was conspicuously free of peaks at Em381–383 nm. Parent pyrene was not detected in the urine with the present method, providing evidence that biotransformation of pyrene has occurred. Fluorescence spectrophotometry has proven to be a valuable method for establishing proof of exposure and metabolism, and has potential for use in environmental assessments of hydrocarbon impacts. Comparison of fluorometric data with that from HPLC/F and immunochemical analysis of the same samples shows good agreement, reinforcing the use of the fluorometric screen as a biomonitoring tool. Immunoassay techniques can also be applied in conjunction with fluorimetry to rapidly screen crustaceans for PAH exposure (Fillmann et al., 2002). The presence of urinary metabolites not only provides evidence of exposure but also highlights the potential for deleterious effects, as biotransformation products are often more toxic and bioactive than their parent compounds.

Urinary levels of PAH equivalents were exposure concentration-dependent, a finding which may help in prioritising contaminated sites according to PAH exposure and for detecting gradients of pollution in estuaries and coastal ecosystems. In a similar study on fish bile, Aas et al. (2000a) state that "a prerequisite for application of the FF technique for monitoring purposes is the establishment of a reasonable dose–response relationship". A simple regression analysis of mean urinary levels over the range of exposure concentrations produces a correlation coefficient of 0.83, indicating a moderately strong relationship. The data presented here therefore fulfil the above requirement. A field exposure level equivalent to the $10 \mu g l^{-1}$ exposure concentration in the present study should be considered the minimum detectable by this method, since pyrene equivalent peaks become obscured by the baseline fluorescence below this level. Levels of pyrene equivalents in the haemolymph are also exposure-dependent, although this relationship is not as strong as in urine. The haemolymph fluorescence intensities are, on average, much lower than those in urine and the variability of the data much greater, with the three highest treatment groups showing no significant differences ($P > 0.05$). Additionally, as a result of ultrafiltration in the anten-

nal gland, urine is free from high molecular weight compounds which may interfere with the analysis. It needs no extraction or clean-up before analysis and the only preparation required is dissolution in the appropriate solvent (ethanol). Urine is therefore a much more suitable biological medium to analyse than haemolymph, when available.

The time response data showed urinary levels of pyrene equivalents reaching a maximum after 48–96 h in exposed crabs and steadily declining for a further 6 days to levels similar to those at 12 h. Similar kinetics are seen in fish (Aas et al., 2000a; Camus et al., 1998) and various crustaceans (James et al., 1995; Solbakken et al., 1980; Lee et al., 1976; Sundt and Goksoyr, 1998). Time dependent changes in urinary levels of PAH equivalents may prove useful for monitoring episodic pollution incidents, e.g. oil spills (Johnston and Baumann, 1989), in the same way that concentrations of PAH equivalents in fish bile indicate levels of exposure at the time of sampling.

Several authors have identified metabolites of various PAHs in the biological fluids of aquatic species (fish, crustaceans) following exposure, and of the phase I biotransformation products, the phenolic (monohydroxy) and dihydrodiol derivatives appear to be the dominant class of metabolites identified (Lee et al., 1976; Solbakken et al., 1980; Pritchard and Bend, 1991; Law et al., 1994; Sundt and Goksoyr, 1998). Phase II conjugation of PAH in aquatic species is also extensive (James, 1987). The majority of metabolites detected in the bile and urine of fish exposed to PAH are found in their conjugated forms (Ariese et al., 1993; Aas and Klungsoyr, 1998; Aas et al., 1998) with sulphates and glucuronides being the most common type (Law et al., 1994; Solbakken et al., 1980). Despite occurring at a slower rate and to a lesser extent than in fish species, conjugation of PAH metabolites does occur in a wide variety of crustaceans including the edible crab, *Cancer pagurus* (Sundt and Goksoyr, 1998), the lobster *Homarus americanus* (Li and James, 1993) and *C. maenas* (McElroy and Colarusso, 1987).

Other authors report that conjugation of 1-OH pyrene is highly likely in *C. maenas* and that glucosides or sulphates are the most likely candidates (M.O. James, personal communication). Ariese et al. (1993) report the emission spectrum of conjugated 1-OH pyrene is blue shifted by 5 nm and more

intense, which accounts for the 5 nm peak shift seen in exposed urine samples and supports the above assumptions.

The spiking experiment revealed that the urinary matrix is not responsible for any peak shift, suggesting that the peak seen in urinary spectra is distinct from the free hydroxylated peak. Final confirmation of this was provided by the HPLC analysis, which showed that *C. maenas* is capable of conjugating the intermediate phase 1 metabolite 1-hydroxypyrene into three major metabolites (pyrene 1-glucoside, pyrene 1-sulphate and an "unknown"). These findings concur with suggestions made above regarding phase II biotransformation of the 1-OH pyrene intermediate. It is now clear that these compounds contribute the bulk of the observed fluorescence at ~Em382 nm on the urinary spectra. Since non-conjugated 1-OH pyrene only accounts for 3.7% of the equivalents eliminated in the urine, it is clear that the conjugation rate is high, at least in this excretory pathway and accounts for the lack of a distinct peak at Em387 nm. The ratio of glucoside to sulphate conjugate illustrates that glucosidation is more extensive than sulphation. The metabolite profile suggests glucuronidation is not occurring to any significant degree. Whilst glucuronides are common phase II metabolites in fish (Ariese et al., 1993; Aas et al., 1998; Law et al., 1994; Solbakken et al., 1980), they are seemingly absent in crustaceans. Concurrent with fluorometric results, levels of parent pyrene are negligible and suggest complete biotransformation of pyrene through this excretory route.

It should be noted however, that identification of specific metabolites, while useful, is not a necessary requirement for PAH exposure monitoring. Direct fluorometric (FF and SFS) techniques are perfectly adequate for discriminating between organisms exposed to PAHs and those that are not exposed, as demonstrated by the present study and many others using fish bile. Certain limitations associated with these techniques must be considered, however. As both FF and SFS lack any kind of separation, it possible that the fluorescent signals observed at specific wavelength pairs or $\Delta\lambda$'s are made up of contributions from a range of PAHs, even if these wavelengths are not optimal for the individual compound (Aas et al., 2000a; Camus et al., 1998; Lin et al., 1996; Vahakangas et al., 1985). Also, direct fluorimetry cannot accurately discriminate between compounds with overlapping excitation and emission spectra (Lin et al., 1994). Whilst this phenomenon is not problematic for single contaminant exposures, it is potentially important in field trials, where organisms are exposed to a multitude of contaminants, including numerous PAHs. It is also possible that certain compounds, produced by normal metabolic processes and excreted in the urine, might also fluoresce at the wavelengths used. Hellou and Upshall (1995) suggest that biogenic compounds such as cholesterol, progesterone and tesosterone can contribute fluorescence which can interfere with the PAH signal. However, the likelihood of such compounds doing so in crustaceans is small.

For the purposes of rapid environmental screening, direct fluorimetry analyses of crustacean urine can determine PAH exposure with sufficient resolution to aid decision-making. The technique can categorise crab populations in terms of their level of exposure to certain classes of PAH. Validation of fluorometric data using HPLC/F and ELISA, in the present work and in accompanying studies (Fillmann et al., 2002, 2003), greatly strengthens the case for this rapid technique to be used for assessing PAH exposure in crustacea.

It is worth noting here that the present study has dealt strictly with exposure following an acute dose of waterborne pyrene. Whilst analysis of urine has established proof of exposure via this route, waterborne levels of PAH from even severely impacted sites are often very low, given their affinity to bind to sediments or suspended organic material (Onuska, 1989; Burns et al., 1990; Lin et al., 1994; Woodhead et al., 1999). Ingestion of sediments and suspended particles containing adsorbed PAH, or of PAH contaminated prey, can result in PAH being absorbed from the stomach and intestine (James et al., 1995). This route is especially important in epi-benthic omnivores such as lobsters and crabs. Therefore, determination of urinary/haemolymph PAH equivalents following dietary and/or sediment exposure to parent compounds is desirable.

The urine and haemolymph data from the dose response experiment showed considerable inter-individual variability, particularly at the higher exposure concentrations. Variations in the rate and degree to which individual crabs take up, metabolise and excrete waterborne pyrene may explain this. Variation in rate and degree of metabolism will be largely due to the level of induction and efficacy of

the appropriate p450 enzymes and subtle differences in moult stage between crabs. Large inter-individual variation in PAH metabolite levels has been observed in field populations of fish, as observed by Krahn et al. (1986) and Lin et al. (1994).

Whilst the present technique can determine exposure to PAHs such as pyrene, it tells us nothing about the potential for deleterious effects such exposure might have. Biotransformation of a parent compound, such as in the present study, can result in its metabolites becoming more toxic and bioactive. Therefore, to fully understand the risks posed to organisms whose biological fluids contain detectable levels of PAH metabolites, it is necessary to combine the present technique with suitable measures of physiological, histopathological and biochemical parameters. Parameters commonly used in fish, which correlate with elevated PAH metabolites in the bile, include incidence of hepatic neoplasia (Krahn et al., 1984, 1986) and CYP1A induction (Beyer et al., 1996; Miller et al., 1999).

The primary aim of the present technique is application to field situations. To identify impacted sites by analysis of the urinary PAH profile of its crustacean populations is a step towards this method being employed as a robust technique for widespread coastal and estuarine monitoring. It is the present author's intent to employ this technique in several impacted and reference field sites to fully test its effectiveness for exposure screening.

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