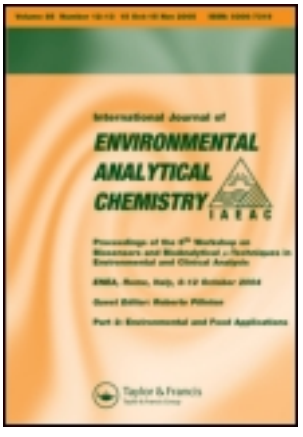

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In the marine environment, organisms can be exposed to oxidised forms of polycyclic aromatic hydrocarbons. Bioaccumulation and biotransformation of these derivatives has rarely been investigated and would lead to a better understanding of the overall fate of polycyclic aromatic hydrocarbons and that of other phenolic contaminants. The marine whelk *Neptunea lyrata* was exposed to 1-hydroxypyrene through its diet over 35 days. Extracts from the muscle and visceral mass of each animal were analysed by liquid chromatography with fluorescence detection. The quantified compounds included 1-hydroxpyrene and the phase II metabolites pyrene sulphate, pyrene glucuronide and one isomer of pyrenediol disulphate. The hydroxylated hydrocarbon was highly retained with 78% of the exposure amount recovered primarily from the visceral mass of the whelks, while the muscle accounted for 4% of the body burden. Whelks efficiently biotransformed 1-hydroxypyrene with a mean of 81% of the compound detected as phase II metabolites. The novel biotransformation product, pyrenediol disulphate, accounted for the largest proportion of the 1-hydroxypyrene derivatives detected at body burdens below 200 ng. At higher body burdens, bioaccumulation increased. Control animals showed trace levels of pyrene derivatives with 76% represented by metabolites. This study highlights the importance of investigating the multiple fates of reactive chemicals in order to interpret exposure.

Keywords: PAH; biotransformation; metabolite; bioaccumulation; whelk; pyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants with a complex environmental fate. Their sources, distribution and reactivity in the environment continue to be of interest for both human and environmental health [1,2]. It is the combination of bioaccumulation, biotransformation and binding to endogenous macromolecules that determine the fate of PAH within an organism. Bioaccumulation will typically occur with hydrophobic PAH accumulating in lipid rich tissues of organisms. Biotransformation is the process by which compounds are converted to more hydrophilic forms to ultimately enable their excretion.

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The biotransformation products of PAH are of interest because of their importance in the environmental fate and toxicity of PAH. They have been routinely used as biomarkers of contaminant exposure in humans and aquatic species [1]. Biotransformation affects the physical and chemical properties of PAH, altering their reactivity within organisms and in the environment. Phase I biotransformation results in only a relatively moderate increase in aqueous solubility over the parent PAH while the reactivity of the compound is substantially increased. Phase II metabolism greatly increases the aqueous solubility of the PAH derivatives, compared to phase I metabolites, enabling their excretion from organisms and can be viewed as a mechanism of chemical defence. This increase in polarity will also lead to an increased mobility in the aquatic environment.

Pyrene (PY) is a widespread tetracyclic PAH commonly used as a model compound in biotransformation studies because of its comparatively simple oxidation and its abundant co-occurrence with other PAH homologues. In vertebrates PY is first biotransformed to 1-hydroxypyrene (PYOH) followed by phase II conjugation to form pyrene-1-glucuronide (PYOG) and pyrene-1-sulphate (PYOS) as the major and minor phase II metabolites, respectively [1]. In invertebrates the biotransformation products of PY are species specific and consist primarily of PYOS, PYOG and pyrene-1-glucoside. Other metabolites have been detected in only a single species of invertebrate, such as pyrenediol monosulphate in clams [3]. In general, sulphation seems to be a more predominant fate for PAH in invertebrates than in vertebrates. This is due, at least in part, to the abundance of the sulphate ion in the marine environment, where many of the invertebrates studied to date reside [4]. The dominance of sulphation and the presence of diconjugated metabolites suggests that invertebrates' biotransformation of PY has similarities to not only vertebrate metabolism but also to fungal degradation [5].

Like many PAH, PY has shown mutagenic and carcinogenic effects [6] and impacts on the growth and reproduction of vertebrates and invertebrates, particularly in early life stages [7]. In the presence of UV light, PY exhibits one of the largest enhancements of toxicity of any PAH [8]. This photo-induced toxicity can be attributed to both the production of reactive oxygen intermediates and of more stable oxidation products [9].

The reactivity of the phase I metabolites is associated with pathways leading to the toxicity of many PAH which include DNA damage [10] and endocrine disruption. The phenolic moiety of hydroxylated PAH makes them structural analogues to estrogens and other emerging endocrine disrupting contaminants. Hydroxylated PAH including PYOH have shown anti-oestrogenic activity and the ability to bind to oestrogen receptors [11].

Once in the environment, parent PAH can become oxidised either chemically or biochemically. Those bioavailable to humans can also be excreted as metabolites and have been shown to persist throughout the sewage treatment process. The oxidised forms are detected both in influents and effluents at levels exceeding those of PAH [12]. The excretion of phase II metabolites by organisms is likely to represent a major input of contaminants in urban areas and a source of toxic phase I intermediates deriving from the hydrolysis of the conjugates [13]. Accumulation and biotransformation of PAH by benthic invertebrates can lead to transfer of oxidised PAH to higher trophic levels such as finfish [14,15]. Both UV radiation and increased temperature can lead to chemical oxidation of PAH [16]. In our warming climate, currently experiencing elevated levels of UV radiation, chemical oxidation of PAH could represent an increasing source of oxidised PAH. Oxidised PAH such as PYOH can also enter the environment directly as combustion products [11].

The investigation of PAH exposure through the diet has received more attention in human populations where food represents a major exposure route in non cigarette smoking individuals [1,17]. Studies of PAH uptake in marine organisms have largely investigated exposure through sediment and water and less often from dietary sources, an important uptake route from an ecosystem perspective. PY and PYOH have calculated $\log K_{ow}$ values of 4.9 and 4.5,[1] respectively, while PYOH is much more water soluble than PY (0.13 mg/L vs 3.5 mg/L). Phenolic compounds such as PYOH remain unionised in seawater and can be considered non-ionic lipophilic contaminants [18]. Non-ionic lipophilic chemicals with $\log K_{ow}$ values between 3.5 and 5.5 are known to partition in water and lipid enriched particles, henceforth potentially be available from both media, the latter including dietary items. In accordance with these properties, oxidised PAH are detected in both seawater [19] and marine sediment [20].

Marine snails consume small particles such as detritus and are exposed to contaminants through multiple routes. Snails have been used as model organisms in the study of the environmental impact of tributlytin and rarely for organic contaminants [21]. Gastropods are abundant and long lived, with many residing in close contact with sediment. These factors, along with a susceptibility to endocrine disrupting contaminants, make uptake and metabolism of organic contaminants in these animals important to pursue [7]. Species which spend much of their time in direct contact with sediment will be at particular risk to exposure to PY and PYOH through multiple sources.

Studies involving gastropods are scarce. The biotransformation of PAH has been briefly investigated in a smaller species of whelks, *Ilyanassa obsoleta*, which was shown to biotransform close to 50% of the benzo[a]pyrene accumulated from sediment spiked with 230 ng of radioisotope labelled benzo[a]pyrene [22]. Another marine snail, *Hydrobia ulvae*, was shown to bioaccumulate PY through dietary uptake of sediment organic matter, but biotransformation was not investigated [23].

Neptunea lyrata is a species of large marine snail, referred to hereafter as whelk, which is found in the North Atlantic. The species is known to produce the toxin tetramethylammonium to immobilise its prey and is also toxic to humans who consume this species [24]. However, whelks are preyed on by ground fish such as cod, and crustaceans such as crab and lobster, all of commercial importance [25]. Other species of whelks which co-occur with *N. lyrata* are edible and of interest to the Canadian Department of Fisheries and Oceans as a target for expanded fishery. Whelk populations are vulnerable to stress from heavy fishing and pollution which has resulted in localised extinctions in the past [26]. The bioaccumulation and biotransformation of organic contaminants by *N. lyrata* has yet to be investigated.

Despite their numerous inputs into the environment and their toxicity, the bioaccumulation and biotransformation of oxidised PAH has rarely been studied in organisms exposed directly to these compounds [3,14]. Our study explores the fates of PYOH in *N. lyrata* exposed through their diet, the snails *Ilyanassa obsoleta*. It follows on one performed on a different large whelk species, *Buccinum undatu,* comparing the fate of PY and PYOH [27]. The details of the analytical method developed for the extraction of metabolites in soft tissues of invertebrates and their identification by liquid chromatography with mass spectrometry detection (LC-MS) have been presented elsewhere [28]. Results of the present study will lead to a better understanding of the fate of this hydroxy PAH and other phenolic contaminants in the environment.

2. Experimental

2.1 *Chemicals and reagents*

Pyrene (98%), 1-hydroxypyrene (98%), 2-hydroxyfluorene (98%) and ammonium acetate (99.999%) were purchased from Sigma Aldrich (Oakville, ON). Pyrene-1-sulphate potassium salt was purchased from the National Cancer Institute (Kansas City, Missouri). Water, methanol, dichloromethane, acetone and acetonitrile were all of HPLC grade or better and were used without additional purification. A qualitative sample of 1,6-pyrene disulphate (1,6-PYDS) was obtained from Dr Haidrun Anke of the Institute of Biotechnology and Drug Research (Kaiserslautern, Germany) and was characterised by ^{1}H-NMR and high resolution mass spectrometry as part of a previous study [5].

PYOG was obtained from EQ Laboratories (Atlanta, GA) as a 10 ng μL^{-1} solution of pyrene-1-glucuronide. The purchased material was identified as a methylated derivative of pyrene glucuronide by LC-FLD retention time and LC-MS/MS analysis ($m/z = 407$). As described by Beach *et al*. [27], hydrolysis was accomplished by treatment with excess KOH achieving complete conversion to PYOG as determined by LC/FLD.

2.2 *Animal collection and maintenance*

Whelks were collected opportunistically during a survey by the Canadian Department of Fisheries and Oceans (DFO) in summer 2007. *Neptunea lyrata* were collected in the Bay of Fundy around Cape Split and Cape Chignecto, Nova Scotia, Canada. Animals were maintained in a 91 cm × 91 cm × 46 cm holding tank filled 37 cm high with running, aerated, sand filtered sea water from a deep area in the Bedford Basin, Halifax, Nova Scotia. The tank contained a layer of reference sediment from Hantsport Beach, Nova Scotia, also on the Bay of Fundy. As commonly done with crustaceans maintained in our labs, animals were fed a diet of whitefish for two months before the beginning of the exposure. During this period the water temperature remained at 8°C.

The 24 whelks had a mean length, width and soft tissue mass (±standard deviation) of 65 ± 8 mm, 38 ± 5 mm and 10 ± 4 g, respectively. Mature males were identified by the presence of a penis. The 10 animals identified as males did not differ significantly in dimensions from the remaining animals. The sex of the remaining animals remained unassigned as animals without a visible penis could either be females or immature males.

2.3 *Laboratory exposure*

N. lyrata were exposed to PYOH added to their diet which consisted of the soft tissue from the snail *I. obsoleta*. The contaminated food was prepared by applying a 5 mL acetone solution containing 300 µg of PYOH to the tissue of 100 snails in a Petri dish. This food was stored in a sealed glass jar at −12°C until needed and thawed before each subsample was removed. After the exposure was complete, a concentration of 680 ng PYOH/ g wet tissue was measured in the spiked food and represents the actual exposure concentration. No degradation was observed during an additional investigation with sub-sampling performed after several freeze-thaw steps. Correspondingly, each whelk was exposed to a mean of 205 ng of PYOH per week.

The group of whelks used in the exposure, 24 *N. lyrata*, were moved to a tank with similar dimensions to the holding tank but a layer of 20–30 mesh Ottawa sand (Fisher Scientific) was placed at the bottom of the tank instead of sediment. The initial

temperature of the filtered sea water was 8°C. Since the exposure was carried out between October and November, the deep sea water flowing into the tank decreased in temperature throughout the course of the exposure reaching a final temperature of 4°C.

The animals transferred to the exposure tank were starved for one week prior to the exposure. Afterwards, four unexposed animals were collected at time 0. The 20 remaining animals were then fed 0.30 g of food per exposed animal per week. Seven days after feeding, four animals were sampled. The remaining animals were fed similar doses of contaminated food per animal for the following five weeks. Four animals were collected one week after each exposure. All animals were immediately frozen in jars at −12°C after sampling.

2.4 *Potential 1-hydroxypyrene degradation*

The preparation of contaminated snail tissue was replicated on a third of the scale of the original treatment to check on the concentrations that were measured at the end of the above experiment. In a small Petri dish, the tissue from 40 snails (12 g) was treated with 160 µg of PYOH in 2 mL of acetone. The material was transferred to a glass jar, left open for 30 min and then stored at −12°C. Subsamples of about 1 g of this spiked tissue were taken before freezing and after different numbers of freeze/thaw cycles and at different lengths of time after the treatment.

Samples taken directly following treatment showed a 5% decrease in PYOH concentration relative to the recovery corrected nominal spike. Storage of the spiked snail tissue for 35 days at −12°C including six freeze/thaw cycles corresponded to the level of PYOH present at the end of the whelk exposure. Throughout this period, the levels of PYOH in the spiked snail tissue did not differ significantly from that of the tissue extracted directly after treatment. Extraction of the dish used for treatment showed that $<0.5\%$ of the nominal spike remained on the glassware. Extraction of the jar showed 3% of the nominal spike present in this material.

2.5 *Sample preparation and analysis*

The development of the extraction and analysis method for PY metabolites in tissues from various species of whelks is described in detail elsewhere [28]. Frozen whelks were thawed for 30 minutes before dissection. The tissue of each animal was divided into muscle and visceral mass and treated separately. Muscle mass included the foot, head and tissue surrounding the proboscis as well as the penis in sexually mature males. Visceral mass included the digestive mass, liver, gonad and red gut tissue inside the proboscis. Each tissue sample was cut up into small pieces, mixed and spiked with a surrogate standard. In the cases when the total muscle or visceral mass of an animal amounted to less than 4 g, the entire amount was used in the analysis. The tissue was homogenised in methanol using a tissue grinder probe and ultrasonic homogeniser, and analysed by HPLC-FLD.

In the study of potential PYOH degradation on snail tissue, all of the subsamples analysed were of 1 g. They were extracted immediately with three 5 mL aliquots of methanol using the same procedure as for whelk tissue. The recovery of PYOH extracted from snail tissue was determined by spiking duplicate samples with 12 µg of PYOH

in 10 μL of acetone, stirring and extracting immediately. In all cases, including the recovery experiment, duplicate samples showed good agreement (< 20% difference).

All analyses were carried out using a Hewlett Packard 1090 HPLC with an HP 1046A fluorescence detector using conditions adapted from previous work [29]. A Waters X-Terra RP18 3.5 μm, 2.1 mm × 150 mm column with a 10 mm guard column of the same stationary phase were used. The mobile phases consisted of A: water and B: methanol : acetonitrile : water (38 : 57 : 5 v/v/v) both with a 10 mM ammonium acetate buffer (pH 6.5 for A). The elution gradient was 35 minutes in length and varied between 10% and 100% organic with a 5 μL injection volume and a flow rate of 200 μL min^{-1}.

Fluorescence wavelength pairs were chosen as the most intense maxima from the excitation and emission spectra collected with the detector. Detection was carried out at an excitation/emission wavelength of 235/388 nm for PYOH and its metabolites. The surrogate standard FLOH was detected at 260/333 nm ex/em. For comparison, the visceral extracts of control animals were also screened using a higher excitation wavelength, 335 nm, which corresponded to the second maximum in the FLD excitation spectrum of PYOS.

2.6 *Quantitative and semi-quantitative analysis*

Quantitative analysis was carried out using an external calibration curve for PYOH, PYOS, PYOG and the surrogate standard 2-hydroxyfluorene (FLOH). In the absence of a quantitative standard, the concentrations of the most abundant isomer of pyrenedisulphate (x-PYDS) were estimated using semi-quantitative analysis. Since relative fluorescence response data is not available for this compound, the molar fluorescence response of PYOS was used. The less abundant isomer 1,6-PYDS was not present in the extracts at levels detectable by LC-FLD and was not quantified. The method detection limits (S/N = 3 for a 4 g tissue sample) for PYOH, PYOS and PYOG were 0.1, 0.01 and 0.06 ng g^{-1} wet tissue, respectively. The method limits of quantification (S/N = 10 for a 4 g tissue sample) for PYOH, PYOS and PYOG were 0.3, 0.03 and 0.3 ng g^{-1} wet tissue, respectively.

Values obtained between the limits of quantification and limits of detection were substituted with the limit of detection for the purpose of sums and means. Any value below the limit of detection was substituted by a value of 0. Samples containing levels of compounds which produced an off-scale response were diluted with methanol to obtain a measurement on the calibration curve.

Levels of contaminants and metabolites in this study are reported as body burden, defined as the sum of all PYOH derivatives in an animal's soft tissue expressed as ng PYOH. One of the challenges in comparing results between different studies of bioaccumulation and biotransformation is the difference in units used to express results. The important characteristics of the tissues and a sample conversion using mean values are presented (Table 1) to simplify comparison to results expressed in other common units.

One exposed animal, sampled on the last day of the exposure, was extracted using a different extraction method as part of the method development. After the muscle and visceral masses were dissected and cut into small pieces, each sample was spread out in a Petri dish and dried overnight in a fume hood. The tissue was then scraped into the centrifuge tube and homogenised using a pestle. The remainder of the extraction followed the procedure used for wet tissue. Because the PYOH spiked food was not shared equally

Table 1. The sum of PYOH derivatives expressed in a number of common units and the physical properties of soft tissue of *N. lyrata* required for the calculations.

Tissue characteristics	Visceral mass	Muscle mass	Total soft tissue
Tissue index (tissue mass/soft tissue)	0.47	0.53	–
% moisture content[a]	70	75	73
% lipid content[a]	10	0.8	9
Sample conversions – ΣPYOX in a 10 g whelk			
Body (tissue) burden (ng)	229	3	232
Wet concentration (ng g^{-1} wet tissue)	49	0.6	23
Dry concentration (ng g^{-1} dry tissue)	162	2	86
Lipid normalised (ng g^{-1} lipid)	487	71	258

[a]Moisture and lipid content of *N. lyrata* tissue masses determined by Beach *et al.* [27].

between the animals in the exposure, it was important to include the levels of PYOH and phase II metabolites detected in the tissue of this animal in the discussion of results. When spiked onto whelk tissue, the dry extraction method gave lower and more variable recoveries of PYOH, PYOS and PYOG than the wet extraction method. Therefore the mean body burden presented for the final point in time is underestimated, but less so than if the results were omitted.

2.7 *Quality assurance and quality control*

All labware was carefully rinsed with water, acetone and dichloromethane before use. Each instrumental sequence included a blank injection and a standard used to ensure a clean, calibrated system. In cases where the blank contained a signal, as was the case for PYOH in some samples, the integrated value was subtracted from the samples in that batch. Procedural blanks incorporating all of the extraction steps were processed along with tissue samples, one for every 10 samples. These consistently showed that no cross contamination was occurring between samples.

To ensure consistency of extraction and to monitor for sample loss, each extracted tissue sample was spiked with a surrogate standard of FLOH. The mean recoveries (±standard deviation, $n = 23$) of these spikes were 101 ± 6 % and 97 ± 7% for visceral and muscle samples. Therefore reported concentrations are not adjusted for recoveries.

To ensure tissue subsamples were representative of the whole tissue, two samples of visceral mass from exposed animals were analysed in duplicate. Duplicates showed good agreement, differing by 0.3% to 20% depending on the compound and sample. The values for PYOG showed the largest difference between duplicates.

3. Results and discussion

3.1 *Feeding preference of whelks*

Results from a preliminary investigation of whelk feeding preference showed that snail meat was more appealing to the whelk than whitefish. To ensure that all of the contaminated food would be consumed during an exposure lasting several weeks, half of the value determined in the feeding study, 3% of the mean tissue mass of the whelks,

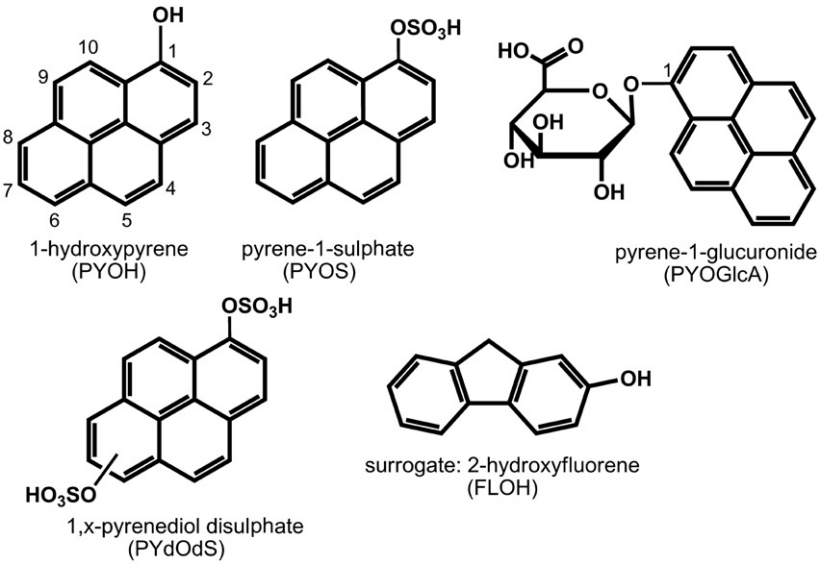

Figure 1. Compounds measured by LC-FLD in tissue extracts of *N. lyrata*.

was chosen as the portion size. This corresponded to 0.3 g of snail meat, offered per whelk every week. Throughout the exposure, this portion of food was consistently consumed within the first few hours after feeding. Faeces were visible on the bottom of the exposure tank one day after feeding. Since animals were exposed as a group in a single tank it was not possible to tell if all animals were eating similar amounts of spiked food.

3.2 *Identification of PYOH metabolites*

The tissue extracts of *N. lyrata* exposed to PYOH in the present study have also been analysed by LC-MS [28]. A number of biotransformation products of PYOH were identified in both muscle and visceral masses of the animals (Figure 1). In addition to unmetabolised PYOH, the known metabolites PYOS, PYOG and 1,6-PYDS were identified. Two novel metabolites, pyrenediol glucuronide sulphate and a second isomer of pyrenediol disulphate (x-PYDS), were also identified by LC-MS. Of the compounds which were identified, PYOH, PYOS, PYOG and x-PYDS were present in many of the tissue extracts at quantifiable levels, above $S/N = 10$ by LC-FLD (Figure 2). These four compounds were measured in tissue extracts from control and exposed whelks.

3.3 *Control animals, time 0*

The four animals collected at time 0 of the exposure represent the state of the whelks prior to exposure. Trace levels of PYOH and its metabolites were detected in the visceral mass of all control animals and at even lower levels in muscle mass (Figures 2a and 2b). The body burden in the whelks is defined as the sum of all the PYOH derivatives in an individual represented in ng PYOH. In control animals the mean $\pm$ SD body burden corresponded to 38 ± 23 ng PYOH (6.7 ± 4.1 ng g^{-1} wet weight), with 76% represented

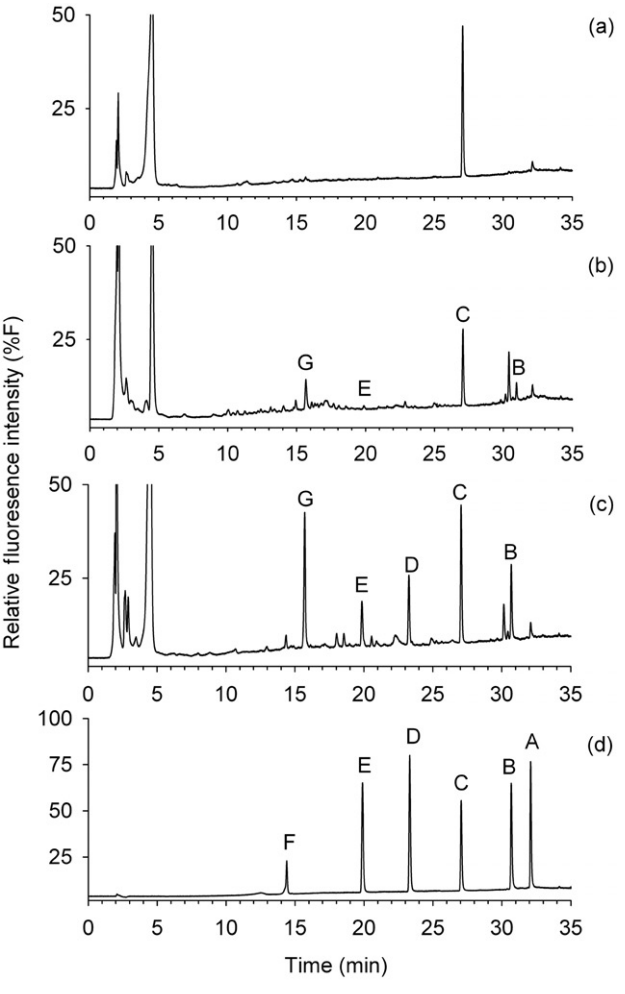

Figure 2. LC-FLD chromatograms of tissue extracts of (a) – control animal muscle, (b) – control animal visceral mass, (c) – exposed animal visceral mass and (d) – available chemical standards for PY derivatives. Labelled peaks represent PY (A), PYOH (B), FLOH spike (C), PYOS (D), PYOG (E), 1,6-PYDS (F) and x-PYDS (G).

by the metabolite x-PYDS. The presence of these compounds can perhaps be attributed to trace levels of PY detected in the sediment of the whelk holding tank since PY was not detected in the whitefish of the preliminary diet [30]. This sediment, obtained from an undeveloped beach in the Bay of Fundy, has been used as reference sediment in earlier studies. Previously, low levels of several PAH including PY were detected in sediment from this beach that were consistent with the background levels in fine grain sediment of beaches away from urban areas [30]. No PYOH or phase II metabolites of PY were detected in this sediment. The metabolites detected in the reference whelks likely resulted from exposure to PY and not PYOH meaning that PYOS, PYOG and x-PYDS can also be considered PY metabolites of *N. lyrata*.

The fact that biotransformation products of PY were detected in the tissue of whelks living on reference sediment illustrates the sensitivity of the analytical method employed

in this study. For example, a higher excitation wavelength maximum 335 nm, more common in the analysis of PY metabolites [31,32], was not sensitive enough to detect these compounds in all of the control animals. The presence of the metabolites identified near the detection limits in the tissues of control animals was also qualitatively verified by LC-MS. This was important to confirm that peaks present in the LC-FLD chromatograms did not result from interfering endogenous fluorescent compounds. The sensitivity of the analytical method employed makes it valuable as a tool for the analysis of environmental samples.

3.4 *Bioaccumulation and biotransformation of PYOH*

Exposed *N. lyrata* showed bioaccumulation of PYOH, highest in the final two weeks of the exposure (Figure 3). Throughout the exposure a significant proportion of the PYOH taken up by the animals was biotransformed into phase II metabolites (Figure 3). The mean value of the proportion of PYOH detected as phase II metabolites in the 24 animals of the present study was 81 +/− 14% (SD). This relatively high proportion is consistent with the previous suggestion by McElroy *et al.* [22] that phase I biotransformation is the rate limiting step in the PAH metabolism of many marine invertebrates. The results of phase II metabolism of PYOH in this study can therefore be considered comparable to the phase II component of the biotransformation of PY.

Because the tendency for PAH to bioaccumulate is inversely proportional to the ability of a species to biotransform PAH [32–34], the high proportion of PYOH biotransformed by *N. lyrata* demonstrates that bioaccumulation is not the major fate at the present exposure conditions. Using the concentration of PYOH measured in the spiked food at the end of the exposure (680 ng g^{-1}) the bioaccumulation factor (BAF) can be calculated for exposed animals. The BAF is defined as the ratio of the concentration of PYOH in whelk tissue to that in the spiked food, both in wet weight. The log BAF calculated for exposed *N. lyrata* is −1.7, comparable to that of polychaetes known

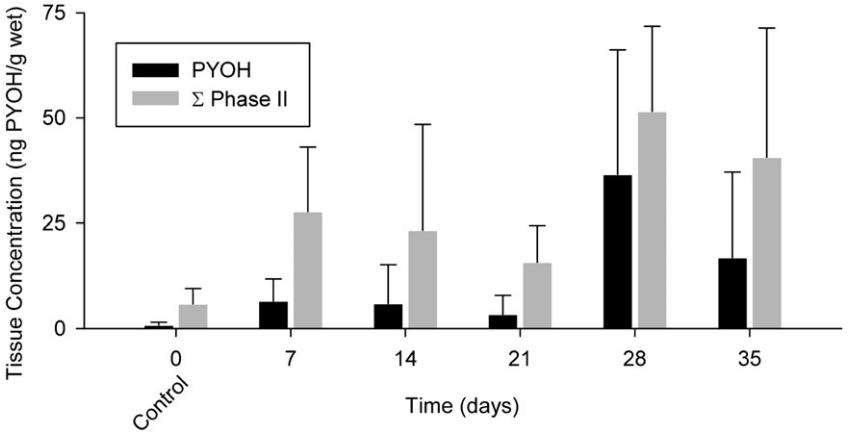

Figure 3. Bioaccumulation and biotransformation of PYOH in *N. lyrata*. Σ Phase II represents the sum of PYOG, PYOS and x-PYDS in whole whelk in units of ng PYOH/ g wet tissue. Values represent the mean values obtained from the analysis of the four animals collected at each time point. Error bars show standard deviation.

to effectively biotransform PAH [35]. In this experiment, a relatively small amount of food was offered to ensure complete consumption. As a result, the availability of contaminated food may have been the limiting factor in the bioaccumulation of PYOH. The BAF values presented here represent the proportion of offered PYOH which is taken up by a whelk and accumulated as unmetabolised PYOH. In this context, the value differs somewhat from the $\log K_{ow}$ correlated log BAF values calculated for non-ionic lipophilic contaminants [18] because of the ability of the whelks to biotransform PYOH and possibly the availability of contaminated food. In a recent study performed by the authors, the dietary uptake of PY and PYOH was examined in *Buccinum undatum*, another large whelk species [27]. Fate examined two weeks post exposure demonstrated the presence of $>90\%$ biotransformation products in tissue extracts, with common derivatives identified but in different proportions, where PYOS was the most abundant. Mean log BAF for PY and PYOH were of -1.4 and -1.7, with wide standard deviations, similar to the present results.

The assimilation efficiency is the proportion of PYOH in food that is taken up by each whelk during digestion. It is defined as the ratio of the body burden in each animal to the total PYOH offered to each animal. Because the whelks were exposed as a group, the contaminated food would not have been divided equally among all the animals. Because of this variability in feeding, the total mass balance of the experiment is a better representation of the overall assimilation efficiency than a mean value. Using the measured concentration in the contaminated food at the end of the experiment, the sum of all of the PYOH derivatives detected in exposed animals, less the body burden of control animals, indicates that 78% of PYOH offered to the whelks was recovered as bioaccumulated PYOH or its biotransformation products. This relatively high value demonstrates that PYOH is highly bioavailable from spiked food. It could also represent the retention as opposed to excretion of conjugates as well as PYOH.

The variability ($\text{SD/mean} \times 100 = \text{RSD}$) in the body burden measured in control animals was 64%. The variability between animals sampled at each time point did not show a trend throughout the exposure and ranged from 56% to 134% (Figure 4). Variability of this magnitude can be expected in a study of dietary uptake with relatively few animals per data point and is consistent with inter-individual variability observed in other studies of dietary uptake of PAH [36]. Dietary uptake leads to high variability even when 10 animals are analysed per sampling time [37]. Our exposure was designed with infrequent feedings in order to ensure that all of the food would be consumed. The period of time between exposure and sampling could be responsible for the detection of inter-individual differences in capacity to biotransform PYOH and excrete metabolites. Because animals were fed as a group, contaminated food was not divided evenly between animals. The bioaccumulation of contaminants taken up from the diet will depend on the rates of feeding and digestion [23] as well as life stage and physiological condition of the animals [38]. Since these are all factors that could vary between individual whelks, they represent possible factors contributing to the observed variability. No correlation was observed between the size or sex of the whelks and the body burdens of PYOH and phase II metabolites. It is likely that subtle differences in uptake and elimination of contaminants between sexes or sizes would be obscured by the overall variability in feeding.

Some of the whelks offered PYOH contaminated food showed levels of PYOH and phase II metabolites similar to the control animals (Figure 4). These individuals, one each collected on days 14 and 21, showed body burdens within one standard deviation of control animals and can be said not to have consumed contaminated food

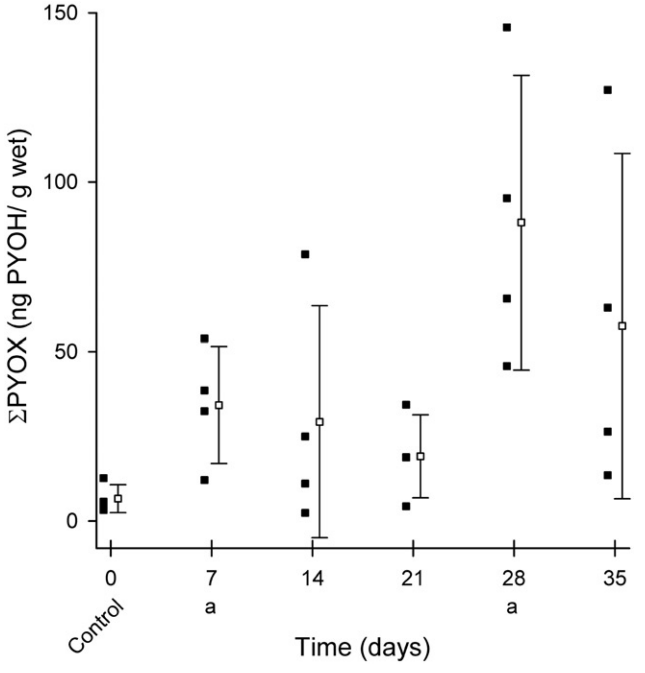

Figure 4. The sum of PYOH and phase II metabolites in *N. lyrata* from each time point. Mean values for each week and standard deviation are given for each time point. a = significantly higher than day 0 control ($p < 0.055$).

during the exposure. In contrast, some animals sampled on days 14, 28 and 35 represent individuals that could have consumed the extra food.

Differences in the amount of food consumed could be due to feeding preference, health of the individuals or competition between individuals. The resulting variability would mirror the type of variability that could be expected in the field where trophic transfer from a contaminated food source would represent the primary mode of uptake [37]. This differs somewhat from results expected if the uptake was by respiration or contact with contaminated sediment. Such an exposure would lead to a more uniform uptake between organisms and their environment.

It is also useful to examine the tissue burdens independently of knowledge of the exposure dose or sampling time (Figure 5). Metabolic profiles observed in control animals would have arisen from exposure to PY and not PYOH. For this reason, these animals as well as the two individuals that did not consume spiked food were not included in Figure 5. This scenario is analogous to environmental sampling when such knowledge is often not available and body burden or tissue residue are used to characterise exposure to a contaminant and risk to a population [39]. At body burdens below about 140–150 ng (Figure 5), little or no PYOH was present and phase II metabolites made up the body burden. This suggests that the whelks are able to effectively biotransform PYOH at these levels. As the total body burden increases, bioaccumulation of unmetabolised PYOH became a more significant fate. This could be interpreted as saturation of the phase II enzyme system at higher tissue burdens. This finding also suggests that under the conditions of this experiment, at lower body burdens PYOH would not be the most

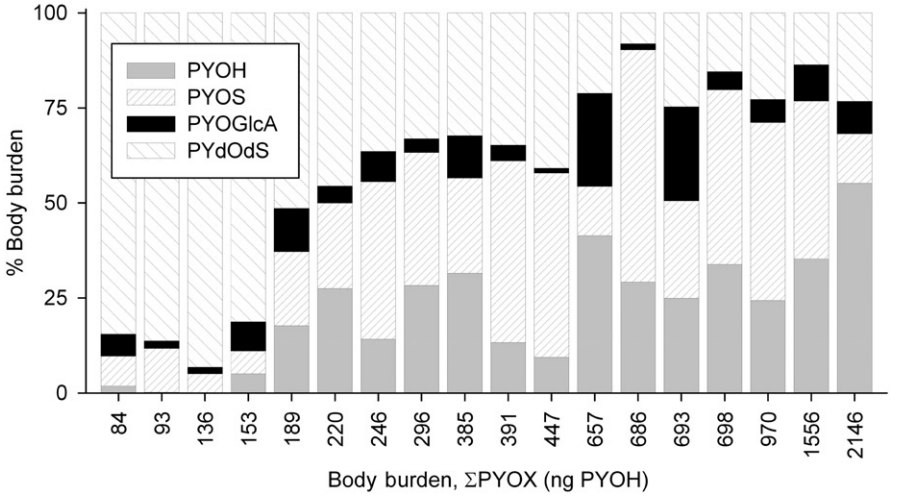

Figure 5. The contribution of each compound to the total body burden in *N. lyrata* that consumed contaminated food.

sensitive choice of biomarker of exposure. In fact, at body burdens below about 190–220 ng x-PYDS makes up more than half of the total body burden. A positive relationship between exposure dose and production of metabolites is more commonly observed in the literature [40].

The large contribution of x-PYDS at lower tissue burdens has important implications for environmental assessment of PAH exposure. PY has traditionally been expected to have only one major hydroxylated isomer as a phase I metabolite, PYOH. However, phase II metabolites can also include conjugates of pyrenediol. Di-conjugated metabolites such as PYDS can each form more than one isomer, complicating metabolic profiles compared to the singly conjugated chemical species typically analysed as metabolites of PY. Enzymatic hydrolysis of these conjugates, commonly used to simplify metabolic profiles of PAH, would produce pyrenediol as opposed to PYOH, the latter being typically analysed in hydrolysed extracts. Pyrenediol has a reduced stability under laboratory conditions [41], degrading to pyrenequinone which has different fluorescence properties than singly conjugated PY metabolites [42]. It is therefore likely that LC-FLD methods used to quantify PYOH after enzymatic hydrolysis would not be as sensitive for detecting pyrenediol and pyrenequinone. The detection of multiple isomers of PYDS also means that multiple isomers of pyrenediol and pyrenequinone could be expected (Figure 1), further increasing detection limits compared to a single compound. In the fungal degradation of PY, where di-conjugated metabolites are well documented, it is exclusively the 1,6- and 1,8- isomers of pyrenediol, its conjugates and pyrenequinone which have been identified [5]. These are also the only two isomers of pyrenequinone identified in vertebrates [42].

PY metabolites conjugated with two large polar groups such as sulphuric or glucuronic acid are also more polar than singly conjugated metabolites (Figure 2). Tissue samples analysed by LC-FLD can show large peaks arising from endogenous compounds such as the abundant aromatic amino acids tyrosine, phenylalanine and tryptophan known to elute with a low percentage of organic mobile phase in reverse phase

liquid chromatography [43]. There is a risk of having highly polar PY metabolites such as PYDS and pyrenediol glucuronide sulphate co-eluting with this matrix material. Indeed, a chromatographic method with an initial mobile phase composition of 20% organic resulted in co-elution of PYDS with the matrix material appearing early in the run.

It is possible that the formation of PYDS as a metabolite of PY is more widespread than in *N. lyrata* and has simply gone undetected in other studies. Future work is required to investigate how widespread the occurrence of PYDS and other di-conjugated metabolites is and how significant they are as metabolites.

3.5 *Tissue distribution*

Low levels of PYOH and its biotransformation products were identified in many muscle samples. The mean sum (±SD) of PYOH and its metabolites in muscle of the 20 exposed animals was 12 ± 17 ng or 2 ± 2 ng g^{-1} wet tissue and did not vary significantly from that of control animals, 3 ± 3 ng or 0.7 ± 0.8 ng g^{-1} wet tissue. Samples with higher levels of contaminants could have resulted from cross-contamination from visceral mass during the dissection. However, no correlation was observed between the concentration of contaminants in the muscle and visceral mass of each animal. This correlation would be expected if there was systematic contamination of the muscle mass from the visceral mass during dissection.

Over the 35 days of the exposure with weekly feeding, PYOH and its biotransformation products accumulated primarily in the visceral mass of the animals. Throughout the exposure, the muscle mass contained $3 \pm 5\%$ (SD) of the total metabolites in the soft tissue of each animal. This result is consistent with previous work with fish and crabs which examined the tissue distribution of parent PAH and metabolites. In crabs, PAH metabolites have been shown to accumulate in the hepatopancreas and only parent PAH in the muscle mass [38]. In trout, PAH metabolites have been shown to accumulate in muscle tissue after a longer time period than in the internal organs [34]. However, this tissue distribution would also depend on the level of exposure and the ability of an organism to eliminate the contaminant through conjugation.

When the PAH content in the internal organs (analogous to visceral mass) and the outer carcass (mostly muscle) of feral finfish were compared, Hellou *et al.* [44] suggested that accumulation in the internal organs could represent the PAH content of the contaminated food source of the fish while accumulation into the carcass represented longer term uptake in the predator fish. In the context of this study, this hypothesis indicates that longer term biomagnification from a food source to consumer is not a major fate for PYOH under the experimental conditions investigated.

The muscle mass represented 53 +/−6% (SD) of the total soft tissue of the whelks. The dissection was effective at separating the visceral mass which accumulated the compounds of interest from the muscle that, in this case, did not. The dissection is important for achieving good detection limits and minimising sample workup in tissue analysis. The analysis of the soft tissue as one mass would have effectively doubled detection limits by diluting the concentrations of compounds in the visceral mass with muscle. This tissue specific analysis could be further improved by studying the distribution of these compounds in the organs within the visceral mass. Since the tissue specific dissection of internal organs in small invertebrates is challenging, knowledge could be

gained by studying the tissue distribution of radioisotope labelled PAH using autoradiography [45].

In future studies, it may be possible to conduct exposure studies with snails by only extracting the visceral mass of the organism. This could be considered analogous to the analysis of metabolites in gallbladder bile of fish. This targeted analysis would have the added advantage of also extracting parent PAH expected to accumulate in the visceral mass displaying a significantly higher lipid content (Table 1). Accumulation of PAH and their metabolites in the muscle mass over longer periods of time at higher exposure levels would have to be further investigated before these findings could be generalised to cases of chronic environmental exposure.

4. Conclusion

The relatively high value for assimilation efficiency reported in this study demonstrates that oxidised PAH were highly bioavailable from spiked food. The relatively low value reported for log BAF and the relatively high proportion of body burdens detected as phase II metabolites demonstrates that *N. lyrata* is able to effectively biotransform PYOH, limiting bioaccumulation. However, at increasing body burdens, bioaccumulation of PYOH becomes a more important fate than biotransformation. The high proportion of conjugated metabolites in control animals compared to those with higher body burdens demonstrates the importance of conjugation as a defence mechanism against the accumulation of contaminants. The large contribution of x-PYDS to the total body burden, especially at low body burdens, justifies future work to determine whether this is the case in other marine species.

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Note

1. Log K_{ow} and solubility values calculated using Advanced Chemistry Development (ACD/Labs) Software v8.14 © 1994–2007.

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