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BIOACCUMULATION AND BIOTRANSFORMATION OF PYRENE AND 1-HYDROXYPYRENE BY THE MARINE WHELK *BUCCINUM SPP.*

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Abstract – Despite well documented toxicity and abundant sources, bioaccumulation and biotransformation in organisms exposed directly to oxidized polycyclic aromatic hydrocarbons have rarely been studied. The fates of pyrene and 1-hydroxypyrene were compared in *Buccinum spp.* Two groups of whelks were exposed to equimolar amounts of the contaminants through dietary exposure over 15 days. Tissue extracts from the muscle and visceral mass were analyzed by liquid chromatography with fluorescence and mass spectrometry detection. Nine biotransformation products were detected in animals from both exposures. These included 1-hydroxypyrene, pyrene-1-sulfate, pyrene-1-glucuronide, pyrene glucose sulfate, two isomers each of pyrenediol sulfate and pyrenediol disulfate, and one isomer of pyrenediol glucuronide sulfate. These compounds represent a more complex metabolic pathway for pyrene than is typically reported. Di-conjugated metabolites were preferentially formed in animals exposed to 1-hydroxypyrene compared to pyrene. *Buccinum spp.* accumulated pyrene in their soft tissue more readily than 1-hydroxypyrene due to their increased ability to metabolize 1-hydroxypyrene. The analytical methods were sensitive enough to detect biotransformation products both in

laboratory control whelks and in those sampled offshore. Throughout the study, the visceral mass contained a much higher concentration of contaminants than the muscle. The tissue distribution of ^{14}C labelled PY was also studied using autoradiography. Radioactivity was present primarily in the digestive and excretory system of the whelks and not in the gonad or muscle tissue. Radioactivity accumulated in the gill and sensory organ of the whelks could have resulted from solubilisation and respiration of the contaminant during feeding.

Keywords – dietary uptake, polycyclic aromatic hydrocarbons, metabolite, phenolic, invertebrate

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are present as mixtures and widespread in the environment due to their numerous natural and anthropogenic sources. The former are represented by forest fires, volcanic eruptions and underwater petroleum seeps, and the latter are discharged in cigarette smoke, vehicles exhaust and during industrial activities involving combustion and the use of fossil fuels.

Biotransformation of PAH significantly alters their chemical properties and their reactivity, impacting the compounds fate both within an organism and in the environment. Phase I biotransformation results in a moderate increase in hydrophilicity and a significant increase in reactivity. Phase II biotransformation results in a significant increase in the solubility of contaminants ultimately enabling their excretion. It is their oxidized forms which are associated with the toxicity of PAH. These can include endocrine disrupting properties [1], developmental effects [2], mutagenicity and carcinogenicity [3]. PAH exposure has also been linked to community level toxic effects such as reduced viability and food web disruption [4].

Marine organisms can be exposed directly to oxidized as well as parent PAH. Sources of these chemicals to the marine environment include direct input from combustion [1] and sewage effluents [5]. Parent PAH released into the environment can also undergo oxidation as a result of thermal and UV irradiation [6]. As well, organisms exposed to parent PAH can represent a source of phenolic PAH through excretion of phase I or phase II metabolites, the latter can then undergo hydrolysis back to phenolic compounds [7]. As a result of these numerous sources, oxidized PAH have been detected in water and marine sediment [8, 9]. PAH and metabolites that bioaccumulate in species low on the food chain can then be taken up by higher trophic levels through dietary exposure [10]. The tendency for such trophic transfer to occur depends on numerous factors including the chemical properties of the contaminant, the food source as well as its residence time in and the pH of the gut of the organism [11]. These factors all affect the bioavailability of a contaminant from a food source to the consumer.

When taken up by an organism, PAH and their metabolites then become distributed throughout the different organs. It is now accepted that the measured concentration of a toxic compound in the target tissue represents the most meaningful dose metric for toxicity bioassays [12]. Work with fish and crabs have examined the tissue distribution of parent PAH and metabolites. In trout, parent PAH were preferentially accumulated in the lipid enriched liver, while metabolites accumulated in the gallbladder bile before excretion [13]. In crabs, PAH metabolites accumulated in the hepatopancreas and epidermis while the muscle mass, gills and the gonad accumulated parent PAH only [14]. The tissue distribution in clams exposed to PAH varied significantly with the length of time after exposure [15].

Exposure to radioisotope labelled PAH and autoradiographic imaging of tissue sections is an effective way of studying tissue distribution [15]. This technique offers a very fine level of detail

of organs, especially in animals difficult to dissect. However, tissue specific chemical extraction is still required to identify structural differences between chemicals differently distributed in tissues such as parent PAH or their metabolites.

Polar metabolites of PAH accumulate in bile and are eliminated in urine and feces [16]. Because of the ease of analysis of metabolites in biofluids, this approach has been adopted as a biomarker of exposure and effects [17, 18]. In smaller species of invertebrates, the absence of an easily accessible bio-fluid makes tissue analysis necessary to detect metabolites. Since whole body analysis in these organisms will result in a dilution of tissue which concentrates PAH or their metabolites, a tissue selective dissection can be used to increase the sensitivity of these analyses.

Pyrene (PY) is a commonly studied tetracyclic PAH. It is often used as a model compound in studies of metabolism because of its relatively simple oxidation, a result of the molecule's symmetry. Initial chemical and biochemical oxidation of PY predominantly produces a single compound, 1-hydroxypyrene (PYOH). Because PY typically makes up a large proportion of the pyrogenic PAH with which it co-occurs, PYOH has often been used as an environmental or occupational marker of exposure to these compounds [17]. In addition to being good model compounds and abundant in the environment, PY and PYOH also exhibit a range of toxicities including mutagenicity [19], developmental effects [20], endocrine disrupting properties [1] and food web level effects [4].

The common northern whelk, *Buccinum undatum* is found throughout the North Atlantic both in North America and Europe between the low tide mark and over 50 m depth. This carnivorous, benthic species is preyed on by groundfish and crustaceans of commercial importance. *Buccinum*

undatum itself is also fished commercially and is considered a species of interest for fishery expansion by the Canadian Department of Fisheries and Oceans (DFO).

Environmental exposure of *Buccinum undatum* to polychlorinated biphenyls has been documented throughout Europe [21]. Because of their sensitivity to endocrine disrupting effects, they have also been used to monitor the environmental impact of tributyltin in the marine environment [22]. The slow growth and the relative immobility of whelk populations make them vulnerable to the impacts of overharvesting and pollution [23]. The bioaccumulation and biotransformation of PAH has yet to be reported for *Buccinum sp.*

The development of methods for the extraction and analysis of PAH metabolites from the tissue of whelks has been described by Beach et al. [24]. Given the lack of authentic standards for PAH metabolites, the structural information provided by LC-MS/MS was used for the identification of metabolites. On the other hand, the similar fluorescence response of compounds with a PY moiety made LC-FLD the preferred method for quantitative and semi-quantitative analysis of the identified compounds. The bioaccumulation and biotransformation of PYOH in another whelk species is described by Beach and Hellou [25].

Using a similar analytical protocol, the present study makes a direct comparison between the bioavailability, bioaccumulation and biotransformation of PY and PYOH by *Buccinum spp.* This comparison will be important in understanding the difference between the fates of traditional hydrophobic lipophilic contaminants in an organism to that of phenolic contaminants.

EXPERIMENTAL

Chemicals and Reagents

Pyrene (98%), 4, 5, 9, 10-¹⁴C pyrene (100 μ Ci/mL in methanol), 1-hydroxypyrene (98%), 2-hydroxyfluorene (98%) and ammonium acetate (99.999%) were purchased from Sigma Aldrich (Oakville, ON). Pyrene-1-sulfate (PYOS) was purchased as its potassium salt from the National Cancer Institute (Kansas City, Missouri). Water, methanol, dichloromethane, acetone and acetonitrile were all of HPLC grade. A qualitative sample of 1,6-pyrenediol disulfate (1,6-PYdOdS) was obtained from Dr. Haidrun Anke of the Institute of Biotechnology and Drug Research (Kaiserslautern, Germany). The sample was purified and characterized by ¹H-NMR and high resolution mass spectrometry as part of a study of the fungal degradation of PY [26]. A 10 ng/ μ L solution of pyrene-1-glucuronide (PYOGlcA) was obtained from EQ Laboratories (Atlanta, GA). The purchased material was determined to be a methylated derivative of PYOGlcA. Hydrolysis to PYOGlcA was accomplished by treatment with excess KOH [24].

Animal collection and maintenance

Whelks were collected opportunistically during the summer of 2008 by the Canadian Department of Fisheries and Oceans (DFO). *Buccinum spp.* were collected around Campobello Island in the Bay of Fundy, New Brunswick, Canada. The exposed whelks and those used as a laboratory control were held in tanks at sea before being brought to the Bedford Institute of Oceanography, Nova Scotia, Canada. Five additional whelks collected from the same area were frozen at sea immediately following sampling. Animals were maintained in a 91 cm x 91 cm x 46 cm holding tank filled 37 cm high with running, aerated, sand filtered sea water at 6°C from a deep area in the Bedford Basin, Halifax, Nova Scotia, Canada. Whelks were fed crushed soft-shell clams

obtained from a consumer grocery store for two months leading up to the experiment. During part of this time, the tank contained a layer of reference sediment from Hantsport Beach, Nova Scotia, also on the Bay of Fundy. The tank was cleaned and this sediment was removed approximately one month prior to the exposure.

Whelks had a mean soft tissue mass of 11 ± 4 g and belonged to the genus *Buccinum*. The animals were initially identified as the common whelk, *Buccinum undatum*, but some uncertainty remained as to whether or not other similar species were also part of the group. Mature males were identified by the presence of a penis. Since animals without a visible penis could either have been females or immature males their sex remained unassigned. The 5 animals identified as males did not differ significantly in dimensions from the remaining animals.

Laboratory exposure

Buccinum spp. were exposed to PYOH through their diet which consisted of soft shelled clams. The exposure tank was identical to the holding tank but included a hard plastic grating dividing it into two equal halves. Five animals were placed into each half of the tank and were allowed to acclimatize without feeding for one week prior to exposure.

Contaminated clams were prepared by injecting 50 μ L of an acetone solution into the foot muscle of the clam immediately before feeding. The equimolar spikes of PY and PYOH had a nominal value of 49.7 μ g and 52.5 μ g, respectively. Using the mean mass of the clams, these spikes gave a concentration of 5.4 μ g/g and 5.7 μ g/g wet weight, respectively.

The exposure lasted 15 days and consisted of feedings on days 1, 4, 8 and 11 with animals sampled on day 15. At each feeding, two clams spiked with PY were offered to the whelks in one

tank and two clams spiked with PYOH were offered to the whelks in the adjacent tank. In all cases the soft tissue of the clams was consumed completely within a few hours of feeding. This corresponded to a mean feeding rate to 8% of the wet tissue mass of the whelks per day. Since whelks were offered contaminated food as a group, each individual may not have consumed the same amount of food. The total PY or PYOH added to each tank over the course of the exposure was 398 µg and 430 µg, respectively. On day 15 all the exposed whelks were sampled and frozen at -12°C until dissection. At this time, five unexposed *Buccinum spp.* from the holding tank were also sampled as a laboratory control group.

Sample preparation

Frozen whelks were thawed for 30 min before dissection. The soft tissue was then removed from the shell and divided into muscle and visceral mass. Muscle mass included the foot, head and muscular sheath of the proboscis as well as the penis when present. Visceral mass included the digestive mass, nephridium, gonad, the red gut tissue inside the proboscis and the contents of the mantle cavity. After dissection, each tissue mass was stored in a scintillation vial at -12°C until extraction and analysis.

The development of the extraction and analysis method for PYOH metabolites in whelk tissue is described in detail elsewhere [24]. Briefly, after thawing each tissue sample was cut up into small pieces, mixed well and then sub-sampled. Each 2 g sub-sample was then spiked with a surrogate standard and tissue was homogenized and extracted with methanol. Extracts were then concentrated and analyzed by HPLC-FLD. This same extraction method was also used for whole soft shelled clams.

LC-FLD and LC-MS analysis

All analyses were carried out using a Hewlett Packard 1090 HPLC with an HP 1046A fluorescence. A Waters X-Terra RP18 3.5 μm , 2.1 mm x 150 mm column with a 10 mm guard column of the same phase were used. The mobile phases consisted of water and methanol:acetonitrile:water (38:57:5 v/v/v), both with 10 mM ammonium acetate. The solvent gradient was 35 min in length with the organic phase varying from 10% to 100% over 27 minutes. An injection volume of 5 μL , a flow rate of 200 $\mu\text{L}/\text{min}$ and a column temperature of 40°C were used throughout. A second HPLC gradient, previously reported by Ikenaka *et al.* [27] to separate PYOS, pyrene glucoside (PYOGlc) and pyrene glucoside sulfate (PYOGlcS) was used to confirm the results obtained by LC-MS. This method consisted of an acetonitrile organic phase, a 20% organic initial mobile phase and the same ammonium acetate solution as the aqueous mobile phase.

Fluorescence wavelength pairs corresponding to the most intense excitation and emission maxima from spectra collected with the HP 1046 detector were used for analysis. These consisted of excitation/emission wavelength pairs of 235/388 nm for PYOH and its metabolites. The surrogate standard FLOH was detected at 260/333 nm during a period between 25.5 min and 28 minutes where no peaks were observed at 235/388 nm. As part of the identification of PY derivatives, fluorescence excitation and emission spectra were collected using an Agilent 1100 series HPLC equipped with an Agilent 1200 series fluorescence detector. The same column and chromatographic conditions used for LC-FLD analysis were used and spectra were collected with a 5 nm step size in separate runs scanning excitation or emission spectra.

LC-MS analysis was carried out using an Agilent 1200 series LC system and an API4000 QTRAP triple quadrupole linear ion trap mass spectrometer equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, ON, Canada) operated in negative ion mode. Nitrogen gas at 50 Torr (GS1, GS2), a spray voltage of -4.5 kV and a source temperature of 600°C were used in all experiments. All multiple reaction monitoring (MRM) experiments used a declustering potential of -60 V. Transitions and collision energies (CE) (Table 1) were obtained from literature values and previous LC-MS/MS analysis in our lab [24]. All LC parameters remained the same as in LC-FLD analysis.

¹⁴C PY exposure and autoradiography

The exposure of six *Buccinum spp.* to ¹⁴C PY was carried out using a similar procedure to that used in the exposures of animals to unlabelled PY. A spiking solution was prepared by mixing solutions of ¹⁴C labelled and unlabeled PY to give a final concentration of 1200 µg PY/mL with an activity of 60 µCi/mL. Clams were injected in the foot muscle with 100 µL of the spiking solution. Each of the four exposures consisted of one clam offered to 6 whelks on days 1, 4, 8 and 11 with all whelks collected on day 15.

Immediately after collection, the soft tissues of each whelk were removed from the shell, frozen in liquid nitrogen and embedded in a 5% carboxymethylcellulose gel. The gel was then also frozen in liquid nitrogen. Samples were then kept frozen until analysis. For each whelk, 24 to 64 pairs of 20 µm thick sections were prepared with a cryomi-crotome (Leica CM3600). Sections were then freeze-dried and placed onto phosphor screens for 163-170 hours. After exposure, the screens were scanned with a Cyclone Phosphor ImagerTM (Packard Bioscience). The autoradiographic images were analyzed using the software Optiquant (Canberra-Packard).

Quantitative and semi-quantitative analysis

Compounds for which quantitative authentic standards were available, PY, PYOH, PYOS and PYOGlcA, were quantified using a seven point external calibration curve. The concentration of compounds with no quantitative standards available, the two isomers of PYdOdS, the two isomers of pyrenediol monosulfate (PYdOS) and one isomer of pyrenediol glucuronide sulfate (PYdOGS) were estimated using semi-quantitative analysis. In the absence of standards and relative fluorescence response data for these compounds, the molar fluorescence response of PYOS was used.

The method detection limits ($S/N = 3$ for a 2 g tissue sample) for PY, PYOH, PYOS and PYOGlcA were 0.1, 0.3, 0.02 and 0.1 ng/g wet tissue, respectively. The method limits of quantification ($S/N = 10$ for a 2 g tissue sample) for PY, PYOH, PYOS and PYOGlcA were 0.7, 0.58, 0.06 and 0.6 ng/g 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 zero. Samples containing levels of compounds which produced an off-scale response were diluted with methanol to obtain a measurement on the calibration line.

Values for PAH in whelk tissue are represented as body burden, the sum of PY derivatives in the soft tissue of a whelk expressed in ng PY. There are a number of other common ways of expressing such values making the comparison of results between studies in this field challenging. Using the mean values for whelk mass, tissue index, moisture content and lipid content it is possible to convert results in this study between different units to allow for easier comparison with other studies (Table 2).

Quality assurance and quality control

Water, acetone and dichloromethane and hexane were used to wash all labware carefully before use. Each sample batch included a blank injection and a mixed standard used to ensure a clean, calibrated system. The calibration series was re-run whenever the measurement of any compound in this standard deviated from the expected value by more than 10%. In such cases where the blank run contained a signal, the integrated value was subtracted from the samples in that batch. Because of a roofing project on a nearby building, blank samples typically contained trace levels of PY. 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.

Samples of muscle and visceral mass from control *Buccinum spp.* were spiked with an in-house prepared standard solution of PY, PYOH, FLOH, PYOS and PYOGlcA to determine the recoveries of compounds extracted from whelk tissue. The mean recovery values for these compounds in five replicates processed throughout the extractions were $98 \pm 1\%$, $83 \pm 7\%$, $98 \pm 1\%$, $91 \pm 11\%$ and $67 \pm 3\%$ for visceral mass and $95 \pm 1\%$, $78 \pm 6\%$, $95 \pm 1\%$, $93 \pm 2\%$ and $79 \pm 1\%$ for muscle samples, respectively. Given the high values of these recoveries, measured concentrations were not corrected. These results suggest that values for PYOH and particularly PYOGlcA may be underestimated compared to PY and PYOS. The lower recoveries for PYOGlcA suggest that lower recoveries may also be observed for more polar compounds such as the conjugates of pyrenediol for which no standards were available.

To ensure consistency of extraction and to monitor for sample loss, each extracted tissue sample was spiked with 2-hydroxyfluorene (FLOH) as a surrogate standard. The mean recoveries for the

20 spikes were $94 \pm 10\%$ and $95 \pm 2\%$ for visceral and muscle samples. These recoveries were not used to correct the reported values, only to ensure consistency in extractions.

RESULTS AND DISCUSSION

Identification of PY metabolites

Analysis of exposed whelk tissue extracts by LC-FLD showed peaks matching in retention time and fluorescence excitation spectrum (not presented) to standards for PY, PYOH, PYOS, PYOGlcA, 1,6-PYdOdS and the surrogate standards FLOH (Fig. 1). Six other peaks in the LC-FLD chromatograms possessed excitation spectra similar to the other PY derivatives and represented targets for LC-MS/MS identification.

Qualitative LC-MS/MS analysis was used to confirm the identification of the compounds detected by LC-FLD and as a tool for the structural elucidation of peaks with fluorescence excitation spectra characteristic of PY metabolites. This was accomplished by using tandem mass spectrometry in multiple reaction monitoring (MRM) mode to screen samples for a number of transitions for known and postulated metabolites of PY and PYOH (Table 1) [24]. Since conjugates of pyrenediol are known in fungal metabolism of PY, and PYdOS has been identified in clams as a metabolite of PY and PYOH [28], other conjugates of pyrenediol were also included. At least two transitions for each conjugate of interest were monitored to ensure correct identification. The presence of un-conjugated PYOH was monitored using a pseudo single ion monitoring (SIM) transition of m/z 217>217 to detect the PYO^- ion.

Results of MRM screening of tissue extracts supported the identification of PYOH, PYOS, PYOGlcA and 1,6-PYdOdS made by LC-FLD (Fig. 2). Of the other six peaks which showed excitation spectra similar to the PY derivatives, four were identified by LC-MS/MS. These include the identification of peaks G and H as two isomers of PYdOS, peak I as an isomer of

PYdOdS other than 1,6- and peak J as PYdOGS. All of these compounds were detected in the tissue extracts of both PY and PYOH exposed animals.

With the exception of 1,6-PYdOdS for which a qualitative standard was available, the identification of conjugates of pyrenediol were not isomer specific. In all cases in the literature where conjugates of pyrenediol have been reported, it is exclusively the 1,6- and 1,8- isomers which have been identified [26, 28, 29]. It is therefore probable that these are the isomers of PYdOdS formed by *Buccinum spp.* Isolation of a larger quantity of these compounds than available in this study would be required for analysis by nuclear magnetic resonance spectroscopy (NMR) and an unequivocal structural elucidation.

Two peaks in the FLD chromatograms (Fig. 1, peaks x and y) were not identified by LC-MS. Their retention times relative to PYOH and PYOS are consistent with what would be expected from 1,6- and 1,8-pyrenediol or their auto-oxidation products 1,6- and 1,8- pyrenequinone [26, 29, 30]. Since a number of different conjugates of pyrenediol were detected in tissue extracts, each with two isomers, two isomers of pyrenediol could also be expected as biotransformation products. A pseudo-SIM transition similar to that used to detect PYOH, m/z 233>233, did not show a signal for pyrenediol in preliminary experiments and was therefore not included in the final MRM method.

Signals were detected for the two transitions monitored for PYOGlcS, m/z 459>241 and 459>97 [27]. Peaks for these transitions were detected 0.1 minutes before, and overlapped partially with, peaks for PYOS in the transitions m/z 297>217 and 297>189. The difference in retention time means that these signals are not adduct ions of PYOS. The intensity of these signals never exceeded 0.05% of that of the PYOS peak in the visceral mass extracts. Although ESI-MS

response can be highly variable between compounds, these weak signals are not likely to represent a detectable peak in LC-FLD chromatograms. This was confirmed by changing the LC gradient to one previously reported to separate PYOS and PYOGlcS [27]. This analysis did not show peaks above the limit of quantification ($S/N = 10$) at the expected retention time of PYOGlcS, confirming that partial overlap with PYOS would not impact the measurement of either compound by LC-FLD.

Two transitions were monitored for PYOGlc, m/z 379>217 and 439>217, reported as a metabolite of PY in invertebrates [27]. These correspond to the loss of anhydro glucose from $[M-H]^-$ and $[M+CH_3COO]^-$, respectively (Table 1). Both transitions showed strong signals in whelk tissue extracts with identical retention times to peaks for PYOS which was present in concentrations as high as 4.5 $\mu\text{g/mL}$ in the tissue extracts. These signals can be attributed to the adduct ions of PYOS $[M-H+Na+CH_3OO]^-$ for m/z 379>217 and $[M-H+Na+CH_3COOH+CH_3COO]^-$ for m/z 439>217. An LC gradient previously reported to separate PYOS and PYOGlc [27] was used to confirm that adduct ions were the cause of these signals. LC-FLD analysis using this gradient did not show a peak near the expected retention time of PYOGlc.

The formation of these adduct ions could result in the false identification of PYOGlc by mass spectrometry in samples containing PYOS. Care should be taken to ensure that chromatographic methods used for the MS study of PY metabolites are able to separate sulfate and glucoside conjugates to ensure that PYOGlc is not mistaken for an adduct of PYOS.

The lack of an authentic standard for PYOGlc precludes the use of a single analytical technique to study the fate of PY in invertebrates. It is therefore necessary to use techniques such as LC-

MS/MS for structural elucidation and LC-FLD for semi-quantitative analysis. True quantitative analysis of individual conjugates in complex samples without the use of authentic standards can only be achieved by fractionations followed by enzymatic hydrolysis. Unequivocal structural elucidation, particularly of structural isomers of the same metabolite is typically only achieved using NMR [28].

These techniques have very different sensitivities and detection limits which can restrict their use. Eelectrospray LC-MS/MS operated in MRM mode can be more sensitive than LC-FLD for easily ionizable conjugates such as PYOGlcS and PYOGlcA but much less sensitive to hydroxylated phase I conjugates and insensitive to parent PAH [24]. LC-FLD offers relatively similar sensitivity for all PY related compounds. Structural elucidation by NMR is typically only possible with larger quantities of highly purified material compared with ng quantities necessary for LC-ESI-MS/MS and LC-FLD [24].

A metabolic pathway is proposed for PY and PYOH in *Buccinum spp.* based on the metabolites detected (Fig. 3). Of the compounds identified, PYdOS and PYOGlcS have both previously been reported in only a single species and were considered specific to the clams and *Daphnia sp.*, respectively [27, 28]. The only metabolite of PY previously identified in a marine invertebrate that was not detected in *Buccinum spp.* was PYOGlc. The occurrence of a number of different di-conjugated metabolites of PY, each with two different isomers, has also not been observed in macro organisms. The identified derivatives portray a more complex metabolism of PY than is typically reported. The detection of nine different metabolites of PY is also more than has been reported for any single aquatic species studied to date.

The same two isomers of PYdOdS as well as PYdOGS have also been identified as metabolites of PYOH in another species of whelk, *Neptunea lyrata* exposed to PYOH through their diet [25]. It is possible that the formation of the di-conjugated metabolites identified in this study is more widespread than in marine whelks. A number of previous studies have presented unidentified chromatographic peaks that could represent these metabolites [27, 31, 32]. Most notably, in cases where mass spectrometry has been used to identify PYOGlcA with m/z 393, isobaric PYdOdS represents a likely structure of unidentified signals.

Laboratory control and deep water reference whelks

Whelks exposed to PY and PYOH were part of a larger group maintained in tanks of flowing seawater over the course of three months before the exposure. These animals were fed similar amounts of the same food used in the experiment, soft-shell clams, during the time leading to the exposure. Five *Buccinum spp.* from this group were sampled at the same time as the exposed animals. This control group represented the state of the animals without the addition of PY or PYOH to their diet.

Trace levels of PY, PYOH and PYOS were detected in the visceral mass of these animals as well as in the muscle mass of some animals. The mean body burden of the control animals was 32 ± 25 ng PY. The clams used as a food source did not contain detectable levels of these compounds. Their detection in control whelks could be attributed to exposure to PY in the sediment in the holding tank prior to the exposure. This sediment was used as reference sediment in previous work [25]. It was reported to contain low levels of a number of PAH including PY but PYOH or PYOS have not been detected in this sediment. It was also of interest to determine if the compounds detected in control whelks were a result of environmental exposure before being maintained in our facilities.

During the collection of whelks from deep waters of the Bay of Fundy, five *Buccinum spp.* were frozen immediately after they were brought on board. These deep water reference animals were analyzed to determine the environmental levels of PY and its metabolites in whelk tissue. The visceral mass of the deep water reference animals was found to contain detectable levels of PY, PYOH and PYOS corresponding to a mean body burden of 19 ± 17 ng PY. This value was not significantly different from the laboratory control animals (t-test, $p = 0.24$). This indicates that levels of PY, PYOH and PYOS detected in control animals originated primarily from environmental exposure to PY before animals were collected.

Represented as a wet tissue concentration, the level of PY in whelks from the Bay of Fundy was 0.5 ± 0.4 ng/g. It should be pointed out that PY nor its metabolites had previously been detected by GC-MS in whelks collected in the Northwest Atlantic [33]. In both the deep water reference and laboratory control animals, PYOS detected in the visceral mass of the animals was the most sensitive marker of exposure to PY. The detection of bioaccumulation and biotransformation of PY from exposure in the environment illustrates the sensitivity of the analytical methods used in this study.

The identification of metabolites of PY in control animals brings into question a common approach used for identifying metabolites. When chromatograms from control and exposed animals are compared to determine which signals represent metabolites, the presence of a signal in the control animals can be grounds for assigning it to an endogenous compound. The use of spectroscopic evidence for determining which signals represent metabolites is a more rigorous approach. This becomes especially important when analytical methods become sensitive enough to detect ubiquitous contaminants in control samples.

Bioaccumulation and Biotransformation

All whelks exposed to PY and PYOH through dietary exposure showed body burdens of PY and their metabolites more than 300 times higher than laboratory control animals. In all cases, PYOS was present in the animals at much higher concentrations than any other single compound. The di-conjugated metabolites detected in all exposed whelks were also sulfate conjugates. The tendency of marine invertebrates to biotransform PAH to sulfated metabolites is due in part to the abundance of the sulfate ion in the marine environment [34]. In vertebrates, sulfation represents a major pathway for steroid inactivation, which can either be induced or suppressed by the presence of xenobiotics [35]. Links between the immune system and the biotransformation of xenobiotics have also been drawn [36]. These represent possible areas for future investigation of effects in invertebrates highly efficient at biotransforming PAHs such as the whelks used in this study.

Within each sample group, no differences in bioaccumulation or biotransformation were observed between sexes or sizes of animals. In previous studies, differences have been noted between PAH metabolites as well as corresponding toxic effects detected in different sexes of fish [35]. In the present case, such differences could have been obscured by variability in the feeding of the whelks.

In PY exposed animals, singly conjugated phase II metabolites were much more abundant than doubly conjugated metabolites, each contributing 87% and 4% of the body burden, respectively. Biotransformation products of PY made up a total of 93% of the body burden with the remaining 7% present as parent PY. In contrast, conjugates of 1-hydroxypyrene and pyrenediol represented 64% and 35%, respectively, of the body burden in PYOH exposed animals. Biotransformation products of PYOH made up 98% of the body burden and PYOH the other 2%. This indicates that

PYOH offered at the same level as PY produced nearly 20 times more diol conjugates in whelks. The similar and low proportion of PYOH in both groups supports the suggestion that phase I biotransformation is the rate limiting step in the biotransformation of PY in benthic invertebrates [34].

The bioaccumulation factor (BAF) is defined as the ratio of the concentration of a contaminant in an organism to that in the medium through which it was exposed. In this case, the BAF is the ratio of PY or PYOH concentrations expressed as wet weight in the soft tissue of the whelks to the concentration in the spiked clams. The log BAF was calculated as -1.4 ± 1.4 for PY exposed animals. The log BAF of PYOH in animals exposed to this compound was of a similar but lower value of -1.7 ± 1.7 . Both these values and the high proportion of each compound which was metabolized are consistent with values reported for other benthic invertebrates which can effectively biotransform PAH [36]. Another species of whelk, *Neptunea lyrata*, exposed to PYOH through spiked food at lower levels also exhibited a mean log BAF of -1.7 [25].

Bioaccumulation in organisms exposed to PAH through dietary uptake is also a function of the assimilation efficiency of the organism. This is defined as the ratio of the body burden to the total dose of PY or PYOH in the spiked food, both in units of PY equivalents. The assimilation efficiencies of PY and PYOH by *Buccinum spp.* were 0.4 ± 0.1 and 0.8 ± 0.8 , respectively. This is reflected in the higher overall body burden observed in PYOH exposed animals (Fig. 4). It also demonstrates that PYOH is more bioavailable from a contaminated food source than PY. The mean assimilation efficiency in the exposure of *N. lyrata* to PYOH was found to also be 0.8 [25].

These results illustrate how the interpretation of bioavailability would be confounded in cases where biotransformation was not taken into account and only the bioaccumulation factor was

used as a metric. While the lower bioaccumulation factor of PYOH could be interpreted as reduced bioavailability, in reality it represents the enhanced ability for biotransformation of PYOH compared to PY. Results also demonstrate the similarity in the metabolism of PYOH by the two species of marine whelk *N. lyrata* and *Buccinum spp.*

Because the whelks in each group were fed as a group, each individual can be expected to have consumed different amounts of clam tissue. This represents one of the major sources of variability in the body burdens detected within each group. Snails, which feed on detritus and carrion, are known to re-ingest their own feces [38]. In rats, where detailed studies of uptake, excretion and tissue distribution have been carried out, 17% of an oral dose of PY was identified as PYOH in feces [16]. The possibility that whelks in the PY exposure consumed PYOH from their feces should also be considered as a source for di-conjugated metabolites in PY exposed animals. It is also possible that variability in feeding resulted in the diets of individual whelk being made up variably of different proportions of spiked clam and feces, further compounding variability in feeding.

These results confirm that while oxidized PAH have a moderately lower tendency to accumulate in an organism than parent PAH, they are more bioavailable when considering all aspects of their fate. This also supports the suggestion that trophic transfer of metabolites as well as parent compounds should be considered as an exposure route to PAH [10]. In the future, the bioavailability of these compounds could need to be incorporated into calculations of trophic transfer of PAH in more components of aquatic systems. This is especially important since the toxicity of oxidized species has been reported as more substantial than that of the parent PAH [3, 20].

Tissue distribution

In all the exposed animals, the concentrations of PY and its metabolites in muscle tissue represented only a minor contribution to the total body burden. In animals exposed to PY, muscle tissue contained a mean of $3 \pm 1\%$ of the total body burden. The muscle tissue of animals exposed to PYOH contained a similar value of $2 \pm 1\%$. The ratio of PY, PYOH or phase II metabolites did not vary between muscle and visceral mass. Since whelk muscle tissue has a much lower lipid content than the visceral mass (Table 1), partitioning of PY or PYOH based on their lipophilicity would not be expected in the short term or under conditions without the saturation of enzymatic biotransformation enzymes.

As a complement to the extraction of tissue masses, six *Buccinum spp.* were exposed to ^{14}C labeled PY. Tissue sections of these animals were studied by autoradiography to determine the distribution of ^{14}C activity within the animals (Fig. 5a). In all cases, labeled PY was detected either exclusively or primarily in the visceral mass of the animals. This shows that the low concentrations of PY derivatives measured in muscle tissue represent the amount transferred from the internal organs and that binding to macromolecules in muscle tissue would not be an important fate in animals exposed to PY under the current experimental conditions.

Autoradiograms also showed finer detail within the visceral mass of the whelks (Fig. 5a). The accumulation of ^{14}C PY occurred predominantly in the digestive system of the animals and accumulation into the heart or the gonad was not observed. In general, concentrations of ^{14}C activity in the digestive system followed the trend of nephridium (kidney) $\geq$ stomach lining $>$ esophagus $\geq$ intestine/rectum $>$ digestive mass (liver). The gill and the osphradium (the sensory organ) both also showed accumulation of some radioactivity (Fig. 5b). Since both of these organs are exposed directly to respired water, it is possible that some of the ^{14}C PY was solubilize

during feeding and respired by the whelks as they ate. Elimination of PY metabolites through the gill after circulation in the blood stream represents a possible explanation for radioactivity in the gill. However, the osphradium, which does not receive high blood flow, would not be expected to be correspondingly labeled. These results suggest that in addition to dietary exposure, whelks in this study could have been exposed, at least to some degree, to PY and PYOH through respiration as they sensed and ate clam tissue.

In previous work, the tissue distribution of ^{14}C PY in clams was shown to vary widely over a 21 day period after a single exposure [15]. While whelks in the present study were collected at a single time point, exposures were conducted on days 1, 4, 7 and 11, prior to sampling on day 15. The observed tissue distribution therefore represents a composite of the tissue distributions after these four periods. Since the majority of PY, PYOH and their metabolites were recovered from the visceral mass of the animals, it may be possible to conduct similar studies using only the visceral mass as a biomarker for exposure. This would be similar to the analysis of PAH metabolites in the gallbladder bile of fish which is used to monitor PAH uptake from the environment.

CONCLUSIONS

The presented results illustrate the importance of biotransformation in understanding the fate of PAH in an organism. While *Buccinum spp.* was found to bioaccumulate PY more readily than PYOH, the mean assimilation efficiency of PYOH was higher than that of PY. The latter, which takes into account biotransformation products as well as the parent compound, suggests that PYOH is more bioavailable from dietary sources than is PY.

The identification of nine biotransformation products of PY represents the most complex metabolic pathway of this commonly studied contaminant reported to date in marine invertebrates. The presence of abundant di-conjugated metabolites of pyrene shows similarities between invertebrate and fungal metabolism of pyrene. The possibility that these compounds are more widespread as metabolites of PY than in marine whelks should be addressed in future work.

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Table 1. Mass spectral fragments and collision energy (CE) used in the multiple reaction monitoring method used in the detection of biotransformation products in whelk tissue extracts.

Compound	Precursor>Product	Assignment	CE (V)	Peak
1-hydroxypyrene (PYOH)	217>217	[PYO] ⁺ >[PYO] ⁺	-60	B
pyrene-1-sulfate (PYOS)	297>217	[M-H] ⁺ >[PYO] ⁺	-60	D
	297>189	[M-H] ⁺ >[M-H-SO ₃ -CO] ⁺	-80	D
pyrene-1-glucuronide (PYOGlcA)	393>217	[M-H] ⁺ >[PYO] ⁺	-60	E
	393>189	[M-H] ⁺ >[M-H-C ₆ H ₈ O ₆ -CO] ⁺	-80	E
pyrene glucoside	379>217	[M-H] ⁺ >[PYO] ⁺	-60	-
	439>217	[M+CH ₃ CO ₂] ⁺ >[PYO] ⁺	-60	-
pyrene glucoside sulfate (PYOGlcS)	459>241	[M-H] ⁺ >[M-H-PYO] ⁺	-60	K
	459>97	[M-H] ⁺ >[HO ₃ SO] ⁺	-60	K
pyrenediol sulfate (PYdOS)	313>233	[M-H] ⁺ >[M-H-SO ₃] ⁺	-38	G,H
	313>81	[M-H] ⁺ >[SO ₃ H] ⁺	-38	G,H
pyrenediol disulfate (PYdOdS)	393>233	[M-H] ⁺ >[M-H-(SO ₃) ₂] ⁺	-38	F,I
	393>313	[M-H] ⁺ >[M-H-SO ₃] ⁺	-38	F
	313>81	[M-H-SO ₃] ⁺ >[SO ₃ H] ⁺	-38	F,I
	313>233	[M-H-SO ₃] ⁺ >[M-H-(SO ₃) ₂] ⁺	-38	F,I
pyrenediol glucuronide sulfate (PYdOGS)	489>233	[M-H] ⁺ >[M-H-SO ₃ -C ₆ H ₈ O ₆] ⁺	-38	J
	409>233	[M-H-SO ₃] ⁺ >[M-H-SO ₃ -C ₆ H ₈ O ₆] ⁺	-38	-
	489>313	[M-H] ⁺ >[M-H-C ₆ H ₈ O ₆] ⁺	-38	J
pyrenediol glucuronide (PYdOG)	409>233	[M-H] ⁺ >[M-H-C ₆ H ₈ O ₆] ⁺	-38	-
pyrenediol diglucuronide (PYdOdG)	585>409	[M-H] ⁺ >[M-H-C ₆ H ₈ O ₆] ⁺	-20	-

Table 2. Properties of soft tissue of *Buccinum spp.* exposed to PY to enable conversion to various common units used in other studies. Values represent the sum of PY and its biotransformation products in tissue.

<u>tissue characteristics</u>	<u>visceral mass</u>	<u>muscle mass</u>	<u>total soft tissue</u>
tissue index (tissue mass/soft tissue)	0.47	0.53	-
% moisture content *	60	72	66
% lipid content *	8	2	5
<u>sample conversions</u> – mean μg PY derivatives in a 11 g whelk (n=5)			
body (tissue) burden (μg)	27	1	28
wet concentration ($\mu\text{g/g}$ wet tissue)	5	0.2	2.5
dry concentration ($\mu\text{g/g}$ dry tissue)	13	0.6	7.7
lipid normalized ($\mu\text{g/g}$ lipid)	65	9	53

* Moisture and lipid content of *Buccinum spp.* tissue determined as detailed in Beach et al. [24]

Fig. 1. LC-FLD chromatograms of (a) visceral mass extract of *Buccinum* spp. exposed to PY (b) a mixture of available standards. Identified Compounds included A- PY, B- PYOH, C- FLOH (spiked surrogate), D- PYOS, E- PYOglcA, F- 1,6-PYdOdS, G/H- PYdOS, I- x-PYdOdS, J- PYGS. (c) FLD excitation spectra of peaks with no available standard. The excitation spectra of peaks x and y were similar to those of other PY metabolites but were not identified.

Fig. 2. MRM transitions for PY metabolites identified in tissue extracts of the visceral mass of *Buccinum spp.* exposed to PY and PYOH. Peak labels correspond to LC-FLD peak labels in Fig. 1. MRM transitions (Table 1) correspond to PYOH (a), PYdOGS (b), PYdOdS (c), PYdOS and PYdOdS (d), PYOGlcA (e), PYOGlcS (f) and PYOS (g).

Fig. 3. Proposed pathway for the metabolism of PY and PYOH by *Buccinum* spp. The reactions in the box, biosynthesis of PYOS, represents the major fate of PY and PYOH in *Buccinum* spp. Pyrenediol, in brackets was not identified in whelk extracts but is a known intermediate.

Fig. 4. Mean body burden for PY, PYOH and phase II metabolites in *Buccinum* spp. exposed to PY and PYOH

Fig. 5. Optical imaged (left) and autoradiograms (right) of tissue sections *Buccinum* spp. exposed to ^{14}C labeled PY. Axis labels indicate the anterior (A), posterior (P), left (L), right (R), dorsal (D) and ventral (V) sides of the animal. Pane (a) shows the distribution of ^{14}C activity between muscle and visceral mass and within the digestive system of the animal. Pane (b) shows labeling of the osphradium and the gill.