RESEARCH REPORT

Fate and distribution of pyrene in *Ilyanassa obsoleta* exposed through the diet

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Abstract

The ability of Eastern mud snails to bioaccumulate and biotransform a model polycyclic aromatic hydrocarbon (PAH), pyrene (PY), was investigated. The contaminant was added to fish at levels ranging from 2 to 5,000 ng/animal fed 20 mg/each and fed to snails. A linear relationship (p=0.03) was observed between the level of PY and the sum of metabolites consisting predominantly of 1-hydroxypyrene and pyrene-1-sulfate detected in soft tissues of snails. In healthy animals, more PY than metabolites was detected, with more biotransformation relative to the parent compound apparent at lower levels of exposure. In ten snails fed together, the body burden of PY-related compounds displayed 49% variability as well as a similar mean and median. One snail within that group had five times more metabolites than PY and was retracted inside the shell, indicating that the animal was stressed. Radio-labelled PY was detected in the largest proportion in the kidney of the animals. Three snails that died during the exposure had also greater than five times more PY metabolites relative to live counterparts. This study is unique in the links that it establishes between stress and the balance of fates of anthropogenic chemicals in biota.

Key Words: body burden; bioaccumulation; biotransformation; pyrene; dose-response; health

Introduction

The bioavailability and bioaccessibility of chemicals labelled as priority pollutants is an area of research that generates continual interest (Thorsen *et al.*, 2004; Johnsen *et al.*, 2006). Such interest results from population expansion that has enhanced the production of these anthropogenic compounds and from a heightened awareness of the associated deleterious effects. An assessment of the presence of contaminants in the abiotic environment provides a means of comparing the actual state of various locations with published guidelines for an acceptable or ideal background state (Chapman *et al.*, 1987; OSPAR, 2009). The latter publication states that "the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances". Questions about the potential impact of exposure on organisms' health may then be asked. The investigation of the fate of organic compounds in biota reflects the uptake and potential risk caused by exposure to contaminants which bioaccumulate and biotransform. This body burden information can provide the means to link the presence of anthropogenic chemicals to the probability of generating toxic effects (Meador *et al.*, 2008).

The effects of exposure to contaminants on the health of organisms can cover a broad range of end points. The challenge is to interpret the implications from simple chemical and biochemical levels of organisation, to the more complex of populations, ecosystems and communities (Hinton, 1993). Contaminants' mechanism of action differs with dose, where lethality represents an acute endpoint, and does not readily reflect the outcome of exposures. This reality raises the need for additional studies of effects. A balanced assessment based on the "weight of evidence" approach can be achieved (Chapman, 2007) by combining knowledge generated in laboratory investigations with the results of field observations. Environmental studies aim to discover the state of a site and its inhabitants relative to other or to past situations (Myers, *et al.*, 2003; Leung *et al.*, 2005). The goal of Canada's Oceans Strategy is "to ensure healthy and prosperous oceans for the benefit of current and

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future generations of Canadians". Our research has pursued the further development of tools which are the basis of efforts to monitor and assess sediment quality.

Polycyclic aromatic hydrocarbons (PAH) are recognized priority pollutants and pyrene (PY) is a commonly abundant PAH found in air, freshwater, rain, snow, seawater, sediments and soil (Garrigues, Narbonne, Lafaurie, Ribera, Lemaire, Raoux et al., 1993; Lane, Leithead, Baroi, Lee, Graham, 2008; Lima, Farrington, Reddy, 2005; Williams, Meares, Brooks, Watts, Lemieux, 1994). It mainly derives from combustion sources, such as the burning of coal, wood, food and garbage, or the presence of industries involving aluminum smelters, coke ovens, carbon or graphite-electrodes, as well as from natural events such as forest fires and volcanic eruptions. This tetracyclic molecule is always present in complex mixtures with numerous PAH and various additional chemicals, especially if sampling is near sewage effluents (Soclo et al., 2000; Hellou et al., 2002). The physical-chemical properties of PY lead to a relatively long residence time in organisms during depuration (Hellou and Leonard, 2004; Hellou, et al., 2009b). In the environment, PY has a slower degradation rate than smaller PAH, and in sediments, its concentration correlates to that of other large pyrogenic PAH associated with mutagenicity and carcinogenicity. These characteristics, along with the availability of a couple of metabolite standards, contribute to choosing PY as a model PAH for inter-species comparisons of fate (Grainger et al., 2005; Santella et al., 1994).

In the aquatic environment, PAH with an octanol water partition coefficient (log K_{ow} representing the amount in octanol/amount in water) values of <3.0 reside preferentially in water (McKim, 1994). Those with a log K_{ow} of 3.0 to 6.0 partition between water and particles, with an increasing tendency to associate with the latter with increasing log K_{ow} (Mackay et al., 1992). Particle-bound PAH with log K_{ow} >6.0 will be available from lipophilic material such as food. Since PY has a log K_{ow} of 5.2 under steady state conditions, it will be found in small proportion in water and more so in dietary items of the aquatic food web. Once taken up by biota, this reactive chemical can undergo a one or two step enzymatic oxidation to 1-hydoxypyrene (PYOH) and it can further conjugate with a biogenic moiety (Hellou and Leonard 2004; Pesch et al., 2007).

Metabolites represent markers of exposure and effects (Perera , et al., 2005). The purpose of biotransformation is to produce more polar and easily eliminated derivatives and represents a biochemical defense mechanism. However intermediate PAH products formed during biotransformation such as quinones are toxic (Zielinska-Park et al., 2004). Livingstone (1998) described four types of studies where the role of biotransformation by invertebrates and vertebrates would be of interest, such as in research regarding animal ecology and evolution, in modelling, as biomarkers and in toxicity tests. Since less is known about the ability of gastropods to biotransform reactive molecules, our research recently centered on two species of offshore large snails, *Neptunea lyrata* and *Buccinum undatum,* a poisonous and an

edible species, respectively (Beach et al., 2010, in press; Beach and Hellou, 2010, in press). An analytical approach was developed for the extraction, separation and identification of bioaccumulation and biotransformation products present in soft tissues of invertebrates exposed to PY and PYOH (Beach et al., 2009). The accumulation and transformation of these two chemicals yielded up to eight phase I (oxidation) and phase II (conjugation) metabolites.

A smaller readily available related species, the Eastern mud snail, *Ilyanassa obsoleta,* is seen at low tide on estuarine beaches where it occupies a variety of habitat surfaces, including fine grain sediments, marsh grasses, pilings or rocks. This mollusc is reported to feed on lettuce, spinach, shrimp or fish when held in tanks, where animals can be easily maintained for experimentation. These snails are found in densities reaching thousands of individuals/m^2 (Cranford, 1988). They tolerate highly variable weather conditions, surviving temperatures of 40-45 °C for up to 30-90 min and a salinity of 0‰ for more than two days. Being surface deposit feeders, as grazers they ingest microalgae and diatoms as well as carrion, where small particles are enriched in lipophilic contaminants. Because of their abundance, *Ilyanassa obsoleta* could be used to monitor the availability of contaminants in field, mesocosm or laboratory experiments. The behavioural response of this species towards contaminated sediments and seawater was previously examined in our laboratory (Hellou et al., 2009a; Marklevitz et al., 2008a, b). In the present study, our goal was to study the fate of PY in snails exposed to food containing a range of PY levels. The behaviour of the animals was also noted in conjunction to exposure in order to examine potential differences between the behavioural response and fate. The long-term goal of our research aims to support studies evaluating the richness and abundance of fish species from an integrated ecosystem management perspective. Such research would also help to link toxicity and contaminants when assessing environmental quality with triad studies (Chapman, 1987; 2007) combining toxicity tests, chemical analyses and examining the benthic community.

Materials and Methods

Care and use of Ilyanassa obsoleta
Snails were collected in summer-early fall from an inter-tidal mud flat in Hantsport, Nova Scotia, Canada. Sediment and snails were obtained by scraping the upper 5-10 mm surface of the beach and they were transported to the laboratory in half-filled buckets with a couple of cm of additional seawater. Upon arrival (1 h drive), animals and sediments were separated. Sediments were placed in bags and frozen for future use. Our previous work had determined that the grain size of the mud was very fine <50 μm. Animals were housed in 1 m^2 tanks with flowing seawater maintained at a regulated temperature 15 ± 2 °C, with aeration and some sediment (<1cm) placed on the bottom. Snails were fed lettuce every other week, sediment was added every month and some was removed to maintain a depth of <1cm in the tank. A smaller 7 l aquaria covered with plastic wrap with pinholes was

placed in the holding tank to maintain snails deprived of food for 2.5 days prior to the beginning of experimentation.

To select animals for exposures performed in the fall, snails measuring 17-21 mm were removed from the tank, placed in a second one with seawater to determine whether they would take an upright position. They were then distributed randomly among the required 1 l beakers, placing ten per beaker. Animals that did not emerge from the shell and begin moving within a few minutes were replaced with active ones. Beakers were covered with plastic wrap and punctured with pinholes.

Behavior of snails

All chemical exposures were performed at 19-20 °C and snails were counted for their position, on non-immersed glass or in seawater in the 1l beakers containing 800 ml of seawater. Experiments were performed over a single season, fall, because of changes previously observed with time. To examine behaviour relative to food preference, exposures were done in the same size beakers with 200 ml of seawater. Animals were observed for being upright; in a reversed position lying on their shell with soft tissue extended, "distressed"; or completely within the shell with the operculum covering the soft tissue "retracted" (Harry and Aldrich, 1963).

Exposure to spiked food

Food preference was examined by comparing the consumption of algae, soft tissue of shrimp and filets of whitefish. When fish was offered, the material disappeared more quickly and snails remained on the offered food for a shorter period of time than with the other items. This behavior would indicate less potential loss of contaminant. Snails were then fed small pieces of the latter, and it was estimated that after 2.5 days without food, each snail would consume on average 20 mg of fish. Therefore, the 200 mg of food required for 10 snails was spiked evenly with <100 μl of an acetone solution while placed in a glass dish, the solvent was evaporated for 10 min and the food was introduced in the beaker and pulled into small pieces with tweezers. In all exposures, animals began to feed readily and consumed most of the food within 15 min, with no traces of fish visible after <60 min. Spiking levels were of 100, 1,000, 10,000 and 250,000 ng/g of PY in food and represent a dose of 2, 20, 200 and 5,000 ng of PY per snail in each beaker.

Dissection

In most cases, because of chemical analyses and the trace levels used in the exposures, animals from one beaker were pooled for analyses, except for any that died during the study, and except at the highest exposure level where each of ten individuals were analyzed separately. For the lipid and chemical extractions, whole animals were frozen until ready for processing. The material was then thawed, the operculum removed and the retractor muscle severed using pointed tweezers. In most cases the snail could then be pulled intact from the shell. When this was not possible, a small screw clamp was used to carefully crack open the shell. Prior to weighing, any parts of the shell remaining on the soft tissue were removed. It was important to weigh the snails soon after removing the shells because the moisture evaporated readily and the wet weight became measurably less within minutes of removing the shell. Dissection of snails revealed a soft tissue weight of 300-600 mg (wet), with nearly equal weights of muscle and visceral mass. These parts were not divided when processing samples, but only for preliminary physiological determinations.

Lipid and moisture content

Lipid content was determined by air-drying tissue in the fume hood overnight with a sub-sample placed at 70 °C in order to confirm the moisture in the air-dried tissue. Moisture represents the difference in weight before and after oven drying, expressed as a percentage of wet weight. Portions of 200-500 mg of air-dried tissue were weighed into centrifuge tubes and crushed using a manual homogenizing pestle. Lipids were extracted using three 10 ml portions of a solution of 1:1 hexane:dichloromethane. This latter solvent was also used to rinse residues off equipment to prevent the loss of material. For each extraction, the tissue and solvent were mixed by vortexing for 30 s and then sonicated for 3 min. After mixing, samples were centrifuged at 2000 rpm for 5-8 min, and the supernatant collected before adding the next aliquot of solvent. Combined extract was treated with oven-dried sodium sulphate (60-80 °C, continuously), the solution was filtered after 5 min and it was evaporated to dryness. The residue was then transferred to a pre-weighed vial with repetitive rinses of dichloromethane. Further evaporation using nitrogen and gravimetric determination of the lipid content was expressed as percent of dry tissue.

Analysis of soft tissue

Soft tissue was placed in a pre-weighed aluminum dish using ten animals from each exposure level that were extracted together. The detailed method has been published by Beach *et al.* (2009) and consists of a stepwise methanol extraction repeated four times. Ten snails provided 3.9 to 4.7 g of wet tissue. However, smaller samples such as three dead animals of 0.230 g total mass were extracted separately from the remaining ones. The tissue was spiked with 51 ng of 2-hydroxyfluorene (FlOH) using 100 μl of a 0.51 μg/ml solution in acetone and left for 10 min to allow the solvent to evaporate. This phenolic compound represented a surrogate standard to determine the loss of material during processing.

Tissue was transferred to a centrifuge tube and the Petri dish rinsed into a centrifuge tube with 10ml dichloromethane and then 10ml methanol. Scoops of dry sodium sulphate were added to ensure that the tissue would deposit in the tube. The mixture was homogenized using a Polytron blender, then vortexed for 30 s and sonicated for 3 min. Instruments were rinsed with methanol and scraped into the tube to minimize the loss of material. The mixture was centrifuged at 2000 rpm for 5-8 min and the supernatant transferred to a round-bottomed flask. The extraction step was repeated three more times: twice with 10ml methanol and once with 10ml dichloromethane and the extract evaporated using a rotary evaporator at 34-42 °C.

The flask was carefully rinsed with three 1 ml portions of methanol, blown down under nitrogen,

then rinsed again with 2 ml dichloromethane and blown down again. The residue was suspended in 2 ml methanol and filtered through glass wool into another calibrated test tube, rinsing with more methanol. The solvent was evaporated and the residue weighed. A measured amount of methanol was added to give the desired final volume depending on the concentration of PY. A sub-sample of the liquid was transferred to a 2 ml amber HPLC vial using a syringe with a filter tip (Waters Millex HV 4.5 μm) to remove any remaining particles. The extract was analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection, and diluted and re-analyzed as necessary to give a concentration within the range of the calibration curves.

Quality assurance/quality control

Snail tissue spiked with PY, PYOH, pyrene-1-sulfate (PYOS) and pyrene-1-glucuronide (PYOGlcA), as well as FlOH as surrogate standard, were extracted as described above and detailed in Beach *et al.* (2009). A snail matrix spiked with 10-100 ng of each of the available standards and processed to examine the efficiency of the approach yielded generally >70% for PY and PYOH, while the amount of the more polar compounds was always underestimated. Each processed sample was spiked with FlOH to examine variations in the processing. FlOH recoveries were above 60% and reaching up to 96%. The concentrations of analytes were therefore adjusted for losses. Concentrations are expressed in terms of available standards and there is a need for isotopically labeled standards that could be used to examine the recoveries of the more polar compounds during the work up of each sample.

Exposure to ^{14}C labeled PY and sample preparation

A spiking solution was prepared by mixing 22 μl of a 100 μCi/ml ^{14}C labeled PY in methanol with 35 μl (of the 992 μg/ml of PY). The solution was blown dry with a gentle stream of N_2 and dissolved in 100 μl of acetone. This amount was applied dropwise to 200 mg of whitefish and allowed to dry for 10 min. The food was given to ten *Illyanassa* held in a 1 l beaker as described above and animals sampled 72 h later.

The operculum was removed from live shellfish, they were then cracked with a screw clamp and the soft tissue was removed. Each of the animals was slowly lowered into liquid nitrogen with a spoon and held there for 1-2 min. The frozen animal was transferred with forceps to a 50 ml beaker containing 40 ml of carboxymethylcellulose solution (26 g/l). Larger forceps were then used to dip the beaker halfway into liquid nitrogen and hold it there until the gel became thoroughly white (5-10 min). As the freezing procedure began, a micro-spatula was used to prevent the tissue from floating to the gel's surface. Finally, the beakers were placed in ziplock bags and stored in the freezer until they were shipped to another location. Snails were then processed as described by Frouin, *et al.* (2007) with more than 30 slices generated for each snail, where images were further examined for radio-labeling.

Results and Discussion

Lipid content

The morphometrics and the amount of neutral lipids measured in snails are presented in order to enable comparison by other researchers in future endeavors (Table 1). Mature animals were chosen according to shell length within a restricted size range having 7% variability (standard deviation/mean). The soft tissue weights of the visceral mass and muscle displayed a wider range of results with 22 and 26% variability, respectively. Mean lipid content was 5.8% and varied by 47% over that period. The fish used to feed the snails contained a similar amount of lipid, 5.8%. The mean moisture content of snails and fish was also similar, 83 and 80%, respectively.

PY food exposure

The food preference of the snails was determined by comparing their attraction to, and the time taken to deplete offered items, i.e., algae, shrimp and fish. Experiments determined that animals not fed over a period of 2.5 days would be readily attracted to and consume fish more rapidly than other substances. This preliminary conditioning was previously needed in experiments which examined the behavior of snails placed in tanks containing two choices of sediments (Marklevitz *et al.*, 2008a, b). This standardization prior to exposure helped to interpret the avoidance/preference response of snails relative to spiked seawater or to single spiked sediment (Hellou *et al.*, 2009a). Under these holding conditions, an average mature snail readily ingested 20 mg of fish within a few minutes.

Table 1 Morphometric data of some of the snails (n=22) sampled between September and December.

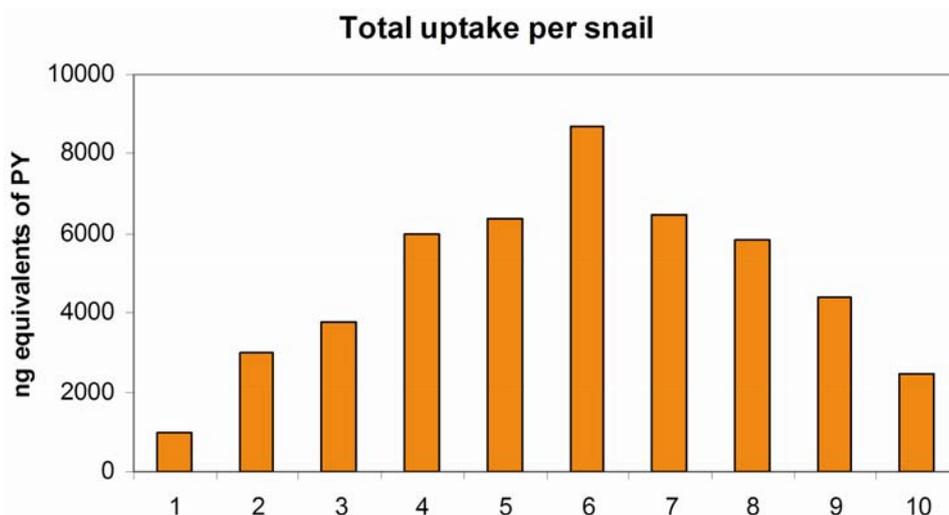|	Shell length (mm)	Shell width (mm)	Visceral mass (mg)	Muscle mass (mg)	Index V/M	Moisture content %	Lipid content %
Mean	481	19	11	261	1.1	83	5.8
SD	139	1	74	58	0.2	4	2.7
Minimum	204	17	9	156	0.6	78	3.4
Maximum	861	21	11	431	1.6	87	10.5

Total uptake per snail

Fig. 1 Body burden of ten snails exposed to the highest level of pyrene (PY) spiked in food and representing 5,000 ng per snail for a total of 50,000 ng in 200 mg of fish or 250,000 ng/g fish.

Behavior towards spiked food

Behavior was examined because avoidance represents a stress response towards chemical or physical disturbances like spillage of oil or changes in temperature and wind conditions. Interest in behavioral research using fish and invertebrates has recently broadened (Roudez *et al.*, 2008; Robinson, 2009). The stress stages that can develop in snails have been described by Harry and Aldrich (1963). They consist initially of an attraction-or-avoidance reaction. Thereafter animals may be observed to appear "distressed", soft tissue protruding as they lie on their shells, and eventually to have completely "retracted" soft tissue within their shells. These stages reflect the quality of the snails' environment and have been detected in exposures to contaminated environments (Burris *et al.*, 1990; Marklevitz *et al.*, 2008a, b; Hellou *et al.*, 2009a).

Animals were observed during feeding and at intervals during the three-day experiments. None of the offered food was avoided. Some variability was detected with time, but without a statistically significant difference in stress (better than 5% level) between exposure levels. In contrast to this behavior towards spiked food, animals exposed to harbor sediments and to sediment spiked with a mixture of seven abundant PAH avoided that matrix (Marklevitz *et al.*, 2008b). Although PY has been associated with a range of toxic and therapeutic endpoints (Long *et al.*, 1995; Law and Klungsoyr, 2000; Jensen and Sverdrup, 2003; Clément *et al.*, 2005; Culotta *et al.*, 2007) stress was not observed during feeding.

Variability in contaminants' uptake:

Prior to analyzing pools of soft tissue from snails exposed to low levels of PY, it was important to determine the distribution in the uptake that would be detected in ten snails exposed together to spiked food. Animals exposed to an expected, calculated 5,000 ng of PY/animal were therefore analyzed individually. This group of snails was of a restricted

mean mass 0.53 g ($\pm$0.09 g). Assuming that the spiked food was homogenous in PY content, since the solvent spike was added uniformly to the food and with great care, then the lowest and highest consumed amount of food interpreted from the body burden consisting of summed PY and derivatives was 20 and 170% (Fig. 1). The mean and median of the body burden were nearly equal (4,800 $\pm$ 2,300 and 5,100 ng equivalents of PY/animal) and displayed a normal distribution with five animals with concentrations above and below the mean. The inter-individual variability of 49% likely reflects the competition between snails or a snail's need for food. This investigation provided the standard deviation to be expected with the analysis of pooled tissue of snails exposed to lower levels of PY that could not be analyzed individually due to the available instrumentation. Concentrations differing by less than 50% between exposure groups would be deemed similar.

A high variability has been observed when analyzing animals exposed in the laboratory to spiked food. For example, a wide standard deviation has been reported in studies involving terrestrial and aquatic invertebrates (Stroomberg *et al.*, 2004; Dam *et al.*, 2006; Granberg and Forbes, 2006). Rates of feeding, excretion and metabolism differ between members of a species even when individuals of a restricted size range are chosen in an experiment in order to minimize the morphometric differences associated with age.

Fingerprint of uptake

The fingerprint of the extracts demonstrated the complexity of the biotransformation process taking place in this species (Fig. 2). The proposed structures of the metabolites are based on earlier studies performed in our laboratory and involving larger gastropods, *N. lyrata* and *B. undatum* (Beach, *et al.*, in press, 2010; Beach and Hellou, in press, 2010). The presently detected metabolites were identified by comparing the chromatograms of the
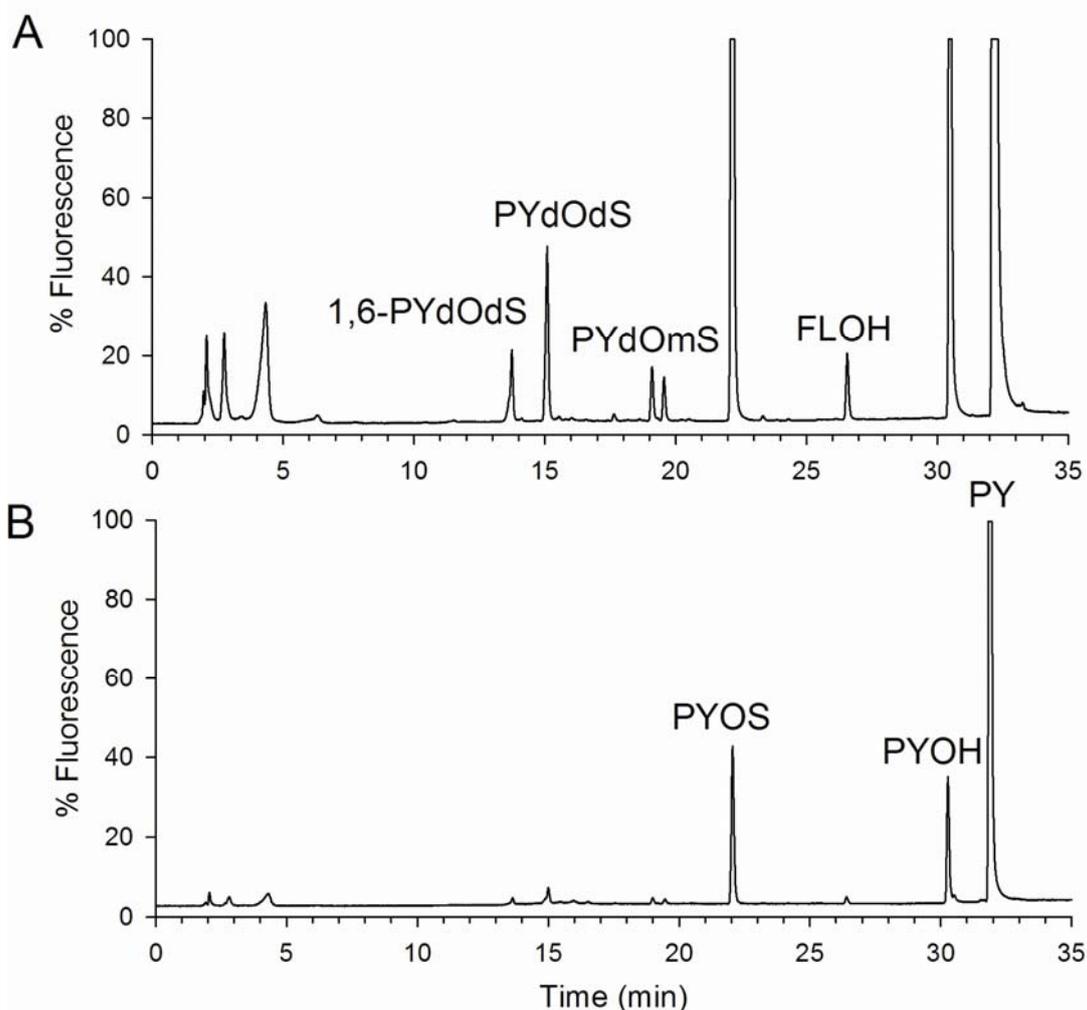
Fig. 2 High performance liquid chromatography (HPLC) fluorescence chromatograms displayed on two scales to highlight the fine fingerprint, with pyrene (PY) derivatives detected in tissue extracts, with 1-hydroxypyrene (PYOH), fluorenol (FLOH) representing the surrogate standard, two isomers of pyrene diol monosulfate (PYdOmS) are followed by two isomers of pyrene diol disulfate (PYdOdS). Chromatogram A represents a sample that is more concentrated and injected from a smaller volume of solvent (2 ml), while B is more dilute and injected from a larger volume of solvent (20 ml).

larger and smaller species that were injected on the HPLC using a common protocol, i.e., same extraction method, guard column and column, solvent gradient, as well as temperature, with injections performed on the same day. The identity of the peaks was previously determined (Beach, *et al.*, 2009; Beach and Hellou, in press, 2010) in part by comparing the retention times and fluorescence spectra of detected peaks to available standards, by performing enzymatic hydrolyses and especially by using a hyphenated chromatography-spectroscopy technique (LC-MS) on an extract fractionated by solid phase extraction.

At the highest exposure level, PY represented between 61 and 93% of the sum of compounds. The next two more detectable derivatives were PYOH and PYOS, followed by two isomers of disulfate derivatives representing between 0.2 and 1.2% of the sum of compounds. Two additional diols present as monosulfates were detected at lower levels. PYGlcA was detected at trace levels. Animals with more bioaccumulated PY also produced more metabolites, with a significant linear regression between these values (p=0.005, r^2=0.6623, with n=9, Fig. 3). The exception was for one snail with 1,500 ng of PY detected along with 965 ng of metabolites (as PY equivalents), where PYOH and PYOS were of equivalent proportion. The regression would predict about five times less metabolites or nearly 200 ng. This animal's behavior differed from that of the others as its body was extended in a manner that is symptomatic of stress.

The sum of PY and metabolites extracted from this group of snails represented 96% of the amount spiked in food. This result confirmed that the experimental setup was optimal because most of
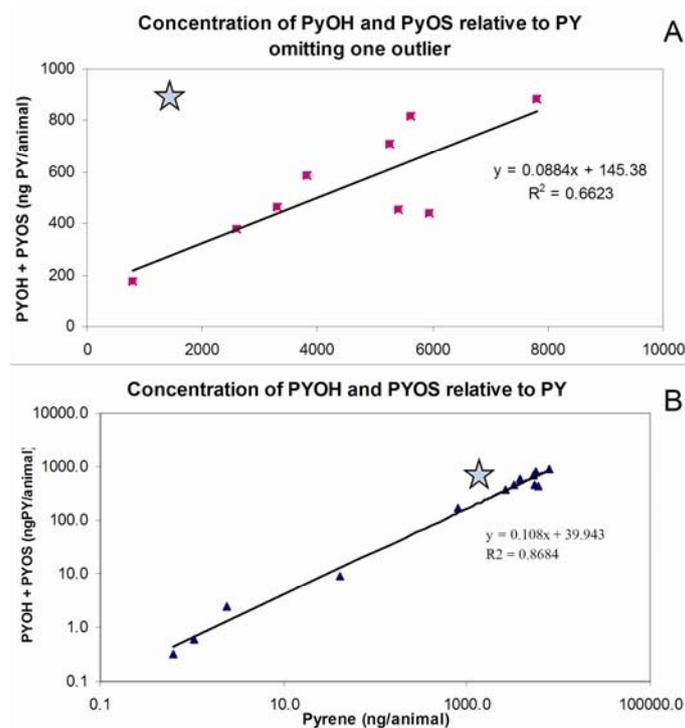
Fig. 3 A. Linear regression obtained from the analysis of nine healthy individual snails at the higher pyrene exposure, with the star representing the tenth outlier snail. B. Linear regression obtained from the analysis of healthy individual or pooled snails (including those in Fig. A).

the food was consumed. Little discharge was apparent in the beakers over the 72 h exposure, reflecting retention of the food or re-ingestion of eliminated material. This has also been observed by Beach and Hellou (in press, 2010) exposing larger marine gastropods and by Dindal and Wurzingerref (1971) studying terrestrial snails. It highlights the importance of the recycling of contaminants in the environment with potential trophic transfer by animals consuming detritus.

Lower levels of exposure

Three lower exposure levels offering the same amount of white fish with 2, 20 and 200 ng of PY per animal were performed. The control and 2 ng group displayed close body burdens (Fig. 4). Since body burden could be expressed in various units, results obtained for reference animals highlight these differences for an average animal weighing 0.5 g (Fig. 4). These body burdens indicate that a similar amount of PY was present in the dietary intake of control snails ingesting small particles and animals from the low dietary exposure. In extracts from control snails and the lowest exposure, nearly half, 45-55% of the anthropogenic material, detected in an animal was present as parent PY (Fig. 4).

In extracts from snails exposed to 20 and 200 ng/animal, PY represented 65 and 80% of the body burden, respectively, with PYOS and PYOH representing the abundant metabolites, and PYOS displaying twice the concentration of PYOH. Therefore, more biotransformation took place at the

lower exposure levels. In healthy animals, tissue extracts contained from 21 to 76% of the PY spiked on food. Recovery rates of PY equivalents were 76 and 96% in the lowest and highest exposures, respectively. Therefore, in the middle exposure, either the spike remained in uneaten minute food particles, or PY leached out prior to food consumption. The present species handles low levels of xenobiotics through efficient biotransformation better than higher levels (Fig. 3).

Dead animals

On a per animal basis, seven times more PYOH and PYOS were detected in three dead animals than in surviving animals from the 200ng/snail exposure (Fig. 4). These two metabolites were 30% more abundant as PY (0.56 vs 0.45 nmol/g), while in healthy animals metabolites represented 20% of the detected PY (0.08 vs 0.36 nmol/g). In this mid exposure, metabolites were produced in a somewhat similar opposite ratio as in the distressed animal at the highest food exposure. In the 5,000 ng/animal exposure, healthy animals had metabolites representing 12% of the parent PY, while the stressed snail had metabolites representing 60% of the level of PY (11 vs 17 nmol/g animal in the one animal compared to a mean of 5.1 vs 42 nmol/g animal in the others). Therefore, at least five times more metabolites were produced in animals experiencing toxicity. Whether this effect is due to specific derivatives such as quinones (Zielinska- *et al.*, 2004) remains to be investigated.
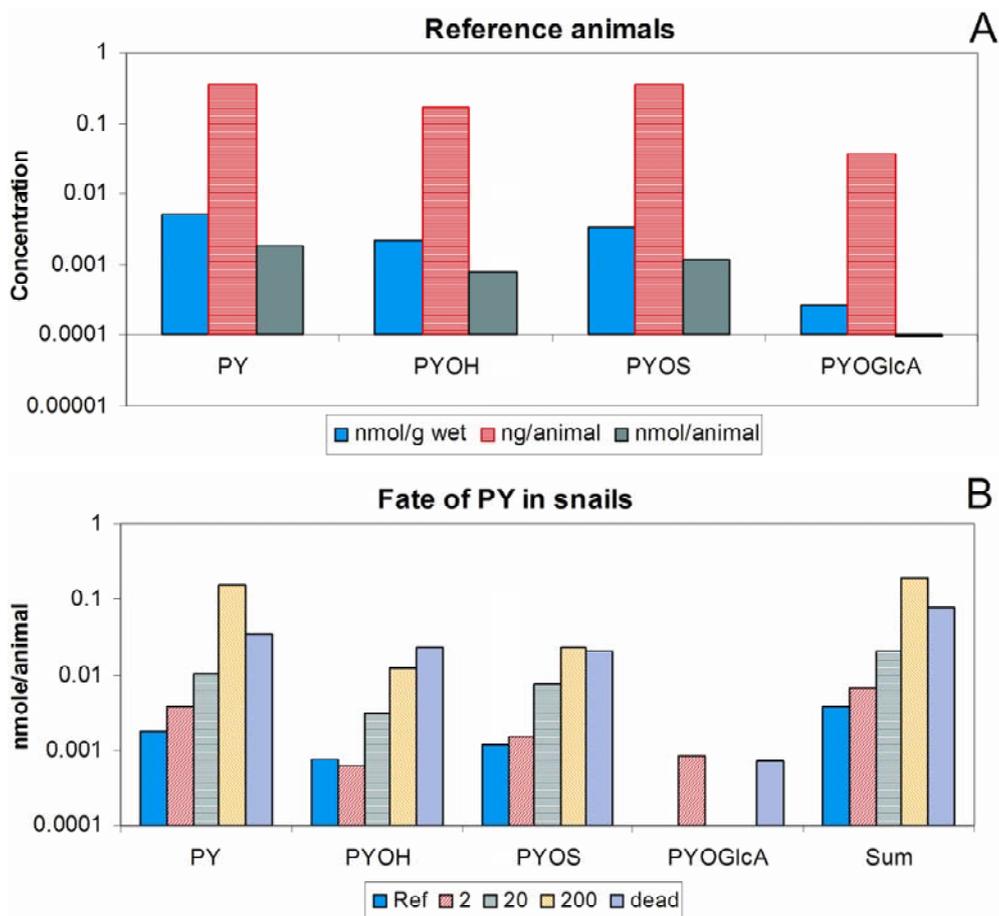
Fig. 4 A. Mean concentrations of pyrene (PY) derivatives expressed in different units and detected in reference snails, levels are reported for 1-hydroxypyrene (PYOH), pyrenesulfate (PYOS) and pyreneneglucuronide (PYGlcA). B. Concentration of PY derivatives detected in animals exposed to various concentrations of pyrene spiked in fish. Ref stands for reference, 2, 20 and 200 is the amount of spiked PY (ng) expected to be taken up per animal.

Comparing fate to that in other species

The fate of this combustion-derived contaminant has been investigated in the bile of many finfish species from freshwater and marine environments. Since the gall bladder bile represents a distinct relatively easy matrix to handle, many studies have examined metabolites in this liquid (Solbakken *et al.*, 1982; Escartin and Porta, 1999). In terrestrial vertebrates, the non-invasive urine analysis has been used most frequently (Grainger *et al.*, 2005; Walker *et al.*, 2006) with glucuronide and sulfate conjugates detected.

Interest in the uptake kinetics of PAH, in terms of concomitant bioaccumulation and biotransformation, has been relatively restricted. Some of the initial studies performed more than two decades ago demonstrated differences in the ability of finfish and shellfish to biotranform vs bioaccumulate PAH (Varanasi *et al.*, 1985; Varanasi *et al.*, 1986; McElroy, 1990). For example, only one of two species of amphipods was capable of transforming PY (Reichert *et al.*, 1985). Some of the

early studies used labeled material to determine the soluble nature of labeled extracts and enzymatic hydrolysis to further pursue the structure of conjugates (Collier *et al.*, 1978; Solbakken *et al.*, 1979; Cravedi and Tulliez, 1982; Solbakken *et al.*, 1978; McElroy, 1990). These investigations pursued a balance of fates and the publication by McElroy (1990) is an excellent example. Concern with invertebrates has expanded over the last ten years. A diversity of metabolites has been discovered (Eickoff *et al.*, 2003; Stromberg, *et al.*, 2004; Lee, 2005;) and reviewed in Beach *et al.* (2009). Briefly, monohydroxy and dihydroxy pyrene, phase I metabolites, along with glucuronide, sulfate, malonate, glucoside phase II products, have been identified in tissues of isopod, clam, crab and whelk. The largest number of identified PY related compounds was detected in a large gastropod, *B. undatum* (Beach *et al.*, 2010, in press). There are few studies of fates and effects that discuss the presence of metabolites relative to effects (Dam *et al.*, 2006). The present observations are unique.
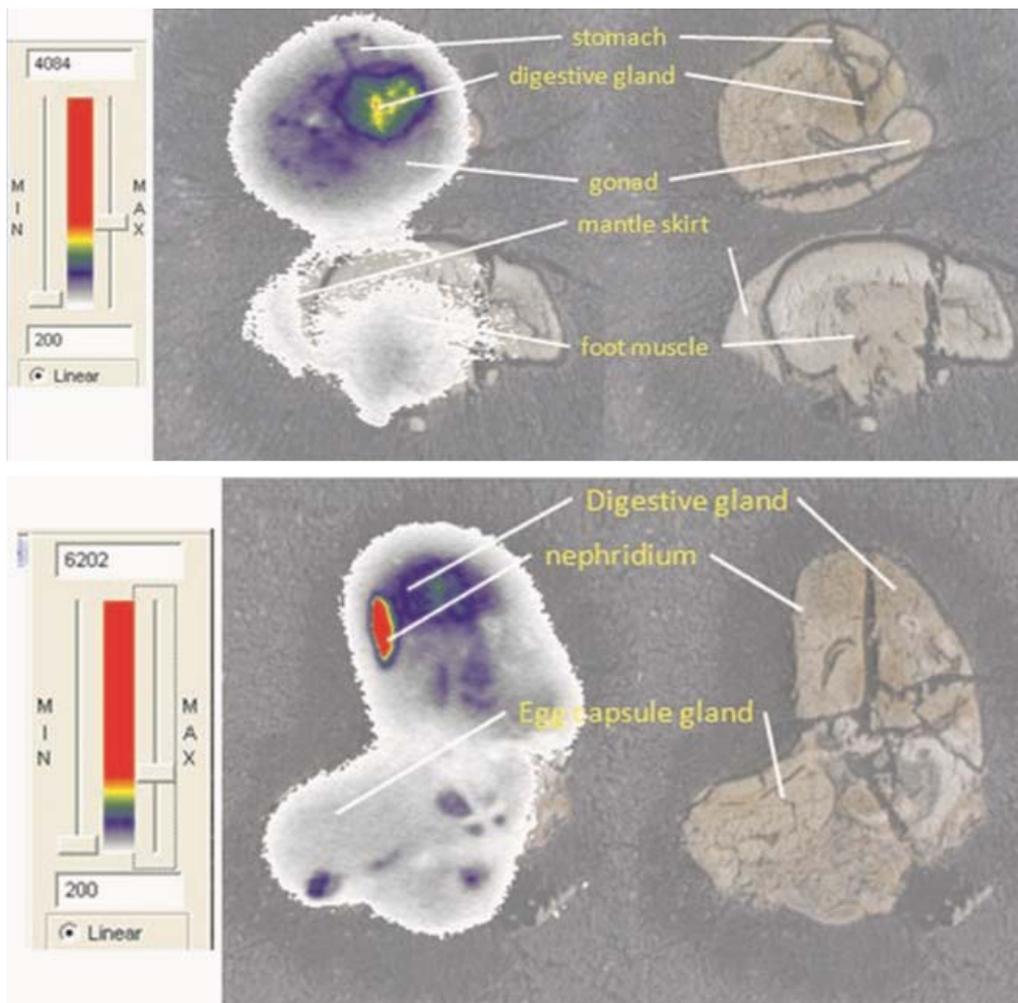
Fig. 5 Example of two images of radio labelled snail tissue with colour associated with the intensity of the labelling and highlighting identified organs.

Since animals were analyzed whole, an additional exposure to the highest level of PY was performed using [14][C] labeled PY in food, with identical experimental conditions as used for unlabelled PY. The interest was to discover the tissue-specific distribution of PY after a three day exposure. As observed with whelks (Beach *et al.*, 2010, in press), the most labeling was apparent in the nephridium/kidney (Fig. 5). A much lower proportion of labeling was detected in the rest of the digestive tract, particularly in the esophagus, as the images indicate. There was no evidence of labeling in the reproductive system, most likely because of the length of exposure. This species of snails reproduces during May-July under our climate conditions.

Conclusions and Perspectives

A linear relationship (p= 0.03) was observed between the level of PY accumulated and transformed in soft snail tissues. Results demonstrate the complex metabolic capacity of *I. obsoleta* exposed to realistic levels of food contamination. Animals starved for 2.5 days prior to exposure did not avoid PY-spiked food. They also did not display significant levels of stress over the following three days, except for one and three snails offered a mean of 5,000 and 200 ng of PY per food portion, respectively. These animals displayed more than five times the amount of metabolites detected in soft tissues of healthy members of the respective groups. The difference in the fates of PY points to the importance of pursuing the multi-faceted fates of PAH to understand the chemical-biological link between fates and effects. With more balanced research, the development of environmental assessment criteria (OSPAR, 2009) would benefit from the type of research described herein.

The Eastern mud snails could be used as model organisms for the production and comparison of aromatic metabolites difficult to purchase commercially. The potential presence of a specific PY metabolite that would be associated with the

observed stress needs to be pursued. More investigations should also be performed in order to investigate the link between the fate of other contaminants and stress in various animals.

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