



Trophic transfer of pyrene metabolites between aquatic invertebrates

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ABSTRACT

The trophic transfer of pyrene metabolites was studied using *Gammarus setosus* as a predator and the invertebrates *Lumbriculus variegatus* and *Chironomus riparius* as prey. The results obtained by liquid scintillation counting confirmed that the pyrene metabolites produced by the aquatic invertebrates *L. variegatus* and *C. riparius* were transferred to *G. setosus* through the diet. More detailed analyses by liquid chromatography discovered that two of the metabolites produced by *C. riparius* appeared in the chromatograms of *G. setosus* tissue extracts, proving their trophic transfer. These metabolites were not present in chromatograms of *G. setosus* exclusively exposed to pyrene. The present study supports the trophic transfer of PAH metabolites between benthic macroinvertebrates and common species of an arctic amphipod. As some PAH metabolites are more toxic than the parent compounds, the present study raises concerns about the consequences of their trophic transfer and the fate and effects of PAHs in natural environments.

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1. Introduction

One of the most important groups of Persistent Organic Pollutants (POPs) are the polycyclic aromatic hydrocarbons (PAHs), present ubiquitously in the aquatic environment, mainly due to anthropogenic activities. Although it has been demonstrated that the concentration of some POPs, such as PCBs and total DDTs increase along the trophic chain (biomagnification) (Hoekstra et al., 2003; Nfon et al., 2008), PAHs do not biomagnify (Nfon et al., 2008; Wan et al., 2007), although can be transferred between prey and predator (Filipowicz et al., 2007). The reported greater biotransformation and excretion capabilities of organisms that belong to higher trophic levels (e.g. fish and mammals) explain this fact (Broman et al., 1990). Although the trophic transfer of PAH metabolites in higher trophic levels may be low, it may be important between invertebrates, as excretion of PAHs or their metabolites is not as efficient as in vertebrates (Landrum et al., 1996).

Although PAHs provoke their most toxic effects after biotransformation reactions (Stegeman and Lech, 1991), the existing literature regarding the trophic transfer of their metabolites to predators is scarce, with only few studies reported (McElroy and Sisson, 1989; Palmqvist et al., 2006). There is an increasing

concern about the toxicological risks and impacts that biotransformation products may cause (McElroy et al., 2011; Ng et al., 2011; van Zelm et al., 2010). Some PAHs derivatives are also a threat due to their persistence (defined as the propensity of a certain chemical to remain in the environment, taking into account the different environmental media and the irreversibility of the chemical's degradation reactions; Webster et al., 1998; Rodan, 2002). For example, fungal phase II metabolites are not mineralized to CO₂ (Schmidt et al., 2010) and oxy-PAHs have been found in similar or greater concentrations than PAHs in several environmental matrices (Layshock et al., 2010).

One of the most commonly used PAHs in laboratory tests is pyrene, which is included in the US-EPA list of priority pollutants (<http://www.epa.gov/waterscience/methods/pollutants.htm>).

Although pyrene is not as potentially carcinogenic or toxic as some five ring PAH as dibenzo (a, l) pyrene, it is able to cause other types of toxicity in certain species and conditions. For example, at realistic environmental concentration, pyrene exerted toxicity to benthic microalgae (Petersen et al., 2008) toxicity that increased with the presence of UV light. Also, the blood vessel pulse rate decreased with time in *L. variegatus* in a continuous exposure to pyrene in water, what was suggested as a sign of narcosis (Mäenpää et al., 2009). Clement et al. (2005) described a decrease in survival and growth in the amphipod *H. azteca* in two different sediments, but at relatively high concentrations.

Pyrene is an excellent model compound due to its simple biotransformation pathway. In eukaryotic organisms, the biotransformation of pyrene results in 1-hydroxy-pyrene as its main phase I

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metabolite (Giessing and Johnsen, 2005), processes performed by isoform(s) of the cytochrome P-450 (CYP) family (Ikenaka et al., 2006).

A step further in the biotransformation of PAHs is the phase II, which consists in the conjugation of the phase I metabolite(s) with molecules such as glucose, glucuronic acid, sulphate or other more complicated structures (Beach et al., 2010; Carrasco Navarro et al., 2011; Ikenaka et al., 2006, 2007; Stroomborg et al., 2004; Ueda et al., 2011).

For the present study, we chose freshwater and marine species that have a wide distribution and are key organisms in their respective food webs. As prey, *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Diptera: Chironomidae) were used. They are recommended for the testing of chemicals (OECD, 2007, 2010, respectively) and also represent groups that are an important part of the diet for larger invertebrates and small fish (Leversee et al., 1982).

As predator, the marine arctic amphipod *Gammarus setosus* (Amphipoda) is a common organism present in arctic intertidal and subtidal zones on the Svalbard East coast (Olsen et al., 2007) and was chosen as a predator because a model animal with opportunistic feeding behaviour was required for the test design. Other members of the *Gammarus* genus are also commonly used in ecotoxicological tests (Gaskell et al., 2007).

The principal objective of the present study was to assess the trophic transfer of pyrene metabolites produced by *L. variegatus* and *C. riparius* to *G. setosus*. As far as we know, this is the first study that evaluates the trophic transfer of pyrene and its metabolites between aquatic invertebrates.

2. Materials and methods

2.1. Test organisms

The marine arctic amphipod *Gammarus setosus* (Amphipoda) were collected at the Svalbard shoreline in August 2008, transported in coolers and kept at the University of Eastern Finland in 30 L water tanks filled with Artificial Sea Water (ASW); 32 practical salinity units (psu). ASW was made by mixing Instant Ocean® synthetic Sea Salt (Aquarium Systems, Sarrebourg, France) with deionized tap water. Organisms were kept in the dark at $2 \pm 1^\circ\text{C}$ with constant aeration. Change of ASW and feeding (crushed Tetramin®) was performed twice a week.

Lumbriculus variegatus (Oligochaeta) were originally obtained from the US-EPA, Duluth, MI, USA, and reared in artificial freshwater (AFW) at $20 \pm 1^\circ\text{C}$ in a 16 h:8 h light:dark cycle at the same location as amphipods. Powdered Tetramin® was used as food source three days per week and pre soaked paper towels as a substrate.

The nonbiting midge *Chironomus riparius* (Diptera: Chironomidae) were reared as described by Ristola et al. (1999). Eggs laid by adults were collected and placed in a 200 ml beaker filled with AFW until they hatched and developed into 4th instar larvae.

2.2. Chemicals

Pyrene (98%) and radiolabeled pyrene (specific activity $58.7\text{ mCi mmol}^{-1}$) and triethylamine (TEA; 99%) were purchased from Sigma–Aldrich, St. Louis, MO, USA; 1-hydroxy-pyrene and its glucuronide conjugate from Dr. Ehrenstorfer (Augsburg, Germany); ammonium acetate (puriss. p.a. for HPLC) from Fluka (Buchs, Switzerland). Solvents (hexane, methanol, acetone, ethanol and acetonitrile) were all reagent or High Performance Liquid Chromatography (HPLC) grade. If not specified otherwise, water used in the experiments was purified Milli-Q water (Millipore Co, Billerica, MA, USA).

2.3. Trophic transfer experiment

2.3.1. Feeding of *G. setosus* with *L. variegatus*

Approximately 1500 *L. variegatus* (Oligochaeta) were exposed to AFW borne pyrene ($20\text{ }\mu\text{g L}^{-1}$) approximately for two months at 20°C . Exposure waters were renewed once a week and feeding (powdered Tetramin®) was provided twice per week. After the two months exposure, a large group of worms was placed in clean Lake Höytiäinen ($62^\circ 41'21''\text{ N}$, $29^\circ 23'49''\text{ E}$) sediment for 96 h and then in clean AFW until their use as a prey. These were called depurated worms. As worms eliminate pyrene faster than metabolites (Leppänen and Kukkonen, 2000), this treatment was used to decrease the parent pyrene body burdens. The whole depuration process was performed similarly as described in Carrasco Navarro et al. (2012).

One day prior to the start of the feeding test, *G. setosus* were added to 400 ml beaker (one organism per beaker), filled with 350 ml of ASW (32 psu) made as described above. Water was not changed during the whole experiment. Aeration was provided constantly and water temperature was $1.5 \pm 1^\circ\text{C}$.

In two different treatments, depurated and control (unexposed) worms were fed to two-days-fasted *G. setosus* ($n = 8$ and $n = 4$, respectively) in the dark. The daily ingestion was recorded. Worms that were not ingested were removed from the beakers.

The feeding test was designed to last 168 h, but the test was interrupted approximately at 150 h due to the low feeding rate of the organisms during the last days. *G. setosus* were sampled, rinsed with deionized water, dried on paper towels, weighed and stored at -20°C .

In order to determine the pyrene and metabolite body burdens, a pool of depurated worms ($115.3 \pm 3.72\text{ mg}$; $n = 3$) were sampled during the second day of feeding for later extractions and analyses by HPLC.

Additionally, four *G. setosus* were set at the same conditions with a tea infuser containing two pyrene exposed but not depurated worms. This treatment was set in order to evaluate whether the pyrene equivalents leaked from *L. variegatus* were available to amphipods directly via water.

2.3.2. Feeding of *G. setosus* with *C. riparius*

C. riparius (Diptera: Chironomidae) were exposed for 24 h to a nominal water concentration of pyrene of $1.5\text{ }\mu\text{g L}^{-1}$ at their fourth instar larval stage. As feeding experiment to *G. setosus* lasted three days, three separate exposures of 24 h were set in three consecutive days, starting one day prior to the beginning of the test. Constant aeration was provided but no food was added. For further extractions, HPLC and LSC analyses, samples of 1–2 midges ($8.05 \pm 0.32\text{ mg}$; $n = 5$) were sampled per exposure day. Due to the low amount of organisms, the five midge samples were pooled resulting in a single sample for an analysis.

Two treatments were established, i.e. *G. setosus* ($n = 8$) fed 24 h exposed *C. riparius* and *G. setosus* controls (starved; $n = 2$). The conditions were the same as in the *L. variegatus* feeding test, but temperature was $4 \pm 1^\circ\text{C}$.

The test was started when one *C. riparius* larva was added and ingested by the amphipod. Another larva was added after 16 h. After following 24 h of starvation, (40 h after the start of the test), four replicates of amphipods fed *C. riparius* were sampled randomly, rinsed, dried, weighed and stored at -20°C . The remaining four replicates were fed with three larvae, at 1–2 h intervals, to make a total ingestion of five larvae. After approximately 16 h of starvation (timepoint 65 h), all the remaining replicates, fed *C. riparius* and controls, were sampled, rinsed, dried and stored at -20°C as described above. All the amphipods ingested the *C. riparius* larvae immediately.

Also in feeding experiments with *C. riparius*, *G. setosus* ($n = 4$) were exposed to prey caged in a tea infuser, in order to evaluate whether the pyrene equivalents leaked from the midge and were available to amphipods directly via water.

2.4. Exposure of *G. setosus* to pyrene in water

In order to determine the biotransformation pathway of pyrene by *G. setosus*, organisms were exposed to pyrene in ASW. These samples belonged to an experiment where the bioaccumulation, toxicokinetics and biotransformation of pyrene were evaluated at two different temperatures using *G. setosus* as test organism (Carrasco Navarro et al., in preparation). After exposure, organisms were extracted following the same method described in the present study and run on the HPLC. The resulting chromatograms were compared to the chromatograms of prey and *G. setosus* fed prey in order to discern what peaks were produced by prey or *G. setosus* and thus evaluate the trophic transfer.

2.5. Extractions of pyrene and metabolites

All organisms were extracted following a method described in Carrasco Navarro et al. (2011, 2012). In a first step, organisms were homogenized with a polystyrene pestle in mixture of hexane and acetone (2.5:2.5 ml) and sonicated for 20 min. Then two more extractions steps with their correspondent sonication (20 min) were performed: in the second step, a mixture of acetone and methanol (2.5:2.5 ml) was used and in the third step, methanol (3 ml) was used. After each of the sonications, the homogenates were centrifuged for 10 min (1200 g) and the three resulting supernatants collected and pooled in a test tube. Fifty μl of nonane was added to the pooled extracts and they were evaporated to near dryness with nitrogen flow. Methanol (500 μl) was added to resuspend the supernatants. The methanol resuspensions were collected, filtered in Spin-X® Centrifuge tube filters (Corning Incorporated, Corning, NY, USA) and centrifuged at approximately 540 g for one minute to facilitate the filtration process. This step was repeated twice (final volume $\sim 1500\text{ }\mu\text{l}$) and resuspensions were transferred to amber HPLC vials and stored at -20°C .

The final tissue residues were not further analysed.

2.6. HPLC analyses

Extracts were run on the HPLC system, that included the following apparatus: binary pump, degasser, automatic injector, column thermostat, diode array detector (DAD; set at 339 nm), fluorescence detector (FLD; set at 346/384 nm Ex/Em) and

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