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Differences in PAH tolerance between *Capitella* species: Underlying biochemical mechanisms

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Differences in PAH tolerance between *Capitella* species: Underlying biochemical mechanisms

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Abstract
The polychaete *Capitella capitata* consists of a species complex within which differences in tolerance to toxicants have been observed. For example, it has been shown that *Capitella* sp. S is more sensitive (e.g., in terms of survival, growth and reproduction) to PAH and other stressors than the more opportunistic *Capitella* sp. I, which is able to take up and biotransform the PAH fluoranthene (Flu). In the present study, an investigation was performed to examine whether differences in tolerance between *Capitella* species sp. I and sp. S are due to differences in biotransformation, measured as the amount of Flu-metabolites produced by worms. We exposed both sibling species to sediment contaminated with 21 and 26 μg Flu/g dry weight sed for 10–15 days. We found that *Capitella* sp. I took up more Flu from the sediment than sp. S (346 μg Flu eq./g dry weight worm versus 219 μg Flu eq./g dry weight worm, respectively), but as sp. I was much more effective at biotransforming this PAH (62% versus 11%, respectively of total Flu), the net amounts of parent Flu accumulated by the two species were similar. We found significant differences in the subcellular distribution of Flu and its metabolites between sibling species, with sp. I accumulating mostly in the cytosol and sp. S accumulating mostly in the membrane fraction. A previous study by our group showed Flu to be genotoxic to sp. I upon biotransformation. In the present study, we found no detectable genotoxicity in sp. S following Flu exposure. Our results demonstrate that DNA damage is tightly coupled to biotransformation ability and that other aspects of PAH toxicity (e.g., membrane disruption) are more relevant than DNA damage for predicting tolerance differences between these species.

Keywords: benthos, biomarker, polycyclic aromatic hydrocarbons, biotransformation, subcellular distribution, comet assay

1. Introduction

The polychaete originally referred to as *Capitella capitata* (Fabricius 1780) has been demonstrated to consist of a cryptic species complex of distinct sibling species (Grassle and Grassle, 1974; Gamenick et al., 1998). The sibling species are so morphologically similar that the complex is not separated by proper taxonomic species descriptions; nevertheless they are distinguished by a variety of genetic, developmental and reproductive features (Eckelbarger and Grassle, 1983, 1987; Pearson and Pearson, 1991; Wu et al., 1991). Furthermore, differences in ecophysiological characteristics, such as toler-

ance to abiotic factors, have been described for the species complex (Gamenick et al., 1998; Linke-Gamenick et al., 2000a). As a result of specific life-history traits (e.g., rapid growth, early maturation, widespread dispersal ability and rapid population growth) plus a generally high tolerance to stress factors in some of the sibling species, *Capitella* is successful in disturbed and organically polluted areas. In addition, some of the species in the complex are among the first invertebrates to recolonize areas following oil spills (Grassle and Grassle, 1974; Sanders et al., 1980). Although *Capitella* has been described as pollution tolerant, more recent work has shown marked differences in tolerance among sibling species (Gamenick and Giere, 1994; Linke-Gamenick et al., 2000a, 2000b).

In the marine environment, hydrophobic oil components such as high molecular weight PAHs rapidly associate to organic matter and sediment particles and, once incorporated into the sediment, persist and may have long-term effects on organisms living in the exposed areas. One of the most abundant PAHs in marine sediments is fluoranthene (Flu) (Baumard et al., 1998), and concentrations are found in the range of tens to hundreds of µg Flu/g dry weight of sediment (Shiaris and Jambard-Sweet, 1986). Flu has been found to be highly toxic to benthic invertebrates at concentrations down to 10 µg/g dry weight of sediment (Swartz et al., 1990). It may also possess genotoxic (mutagenic and carcinogenic) properties, though these effects are not associated directly with the parent compound, but arise largely as a result of biotransformation processes that lead to the formation of reactive intermediates (Rastetter et al., 1982; Babson et al., 1986).

As a result of living in polluted areas, *Capitella* is continually exposed to sediment-associated toxicants, e.g., Flu, which it is likely to accumulate via dietary exposure and to a lesser extent through the epidermis from the surrounding pore water (Forbes et al., 1996; Selck et al., 2003a). Previous studies in our laboratory have shown physiological differences between *Capitella* species in response to Flu. *Capitella* sp. I was found to be physiologically most tolerant to Flu-contaminated sediments compared to other sibling species, whereas *Capitella* sp. S was found to be least tolerant and showed increased metabolic rates and reduced food intake when exposed to 100 µg Flu/g dry weight sed (Linke-Gamenick et al., 2000a). For *Capitella* sp. S, but not sp. I, Flu-exposure (95 µg Flu/g dry weight sed) resulted in complete inhibition of reproduction and increased mortality compared to controls (Linke-Gamenick et al., 2000b). Despite evidence for differences in Flu-tolerance between the two species, little is known about the mechanisms behind these differences. However, Linke-Gamenick et al. (2000a) suggested that the higher tolerance of *Capitella* sp. I could be partly due to a more efficient detoxification system compared to sp. S. Forbes et al. (1996, 2001) and Selck et al. (2003b) studied the biotransformation ability of this species and showed a very extensive ability of *Capitella* sp. I to take up and biotransform Flu. However, nothing to date has been reported about the biotransformation ability of the less tolerant species, *Capitella* sp. S. Lee and Singer (1980) found a concentration-dependent increase in cytochrome P450 mixed-function oxygenase (MFO) activity in *Capitella* spp. after exposure to crude Kuwait oil or the PAH, benzo[*a*] anthracene, and it is expected that Flu would be detoxified via the same metabolic pathway.

The biotransformation process is generally initiated by the oxidative introduction of an OH-group to the PAH (phase I), which involves the MFO system. This oxidation renders the products more reactive for further transformation during phase II, in which the oxidized phase I products are easily conjugated to various endogenous cell compounds, such as sugar derivatives, peptides or sulphates. Whereas phase I reactions increase the water-solubility of the compound only slightly, the solubility increases extensively as a result of phase II, thereby facilitating the excretion of the compound. However, some of the phase I products such as diols and phenols can be further oxidized to diol-epoxides and phenol-epoxides, which are highly reactive and can bind directly to DNA, consequently forming DNA-adducts. Other phase I products, particularly those producing free radicals and/or reactive oxygen species (ROS) may, for example, form alkali-labile sites on DNA (gaps on the DNA sugar backbone due to loss of nucleotide bases), and in both cases DNA strand breaks are likely to result (Shugart, 1988). Palmqvist et al. (2003) showed that Flu-exposure of *Capitella* sp. I initially caused DNA damage, but that the damage declined after 4 days despite continued exposure, suggesting induction of DNA repair activity.

The objectives of this study were: (1) to examine whether the difference in tolerance to Flu that

has been observed between sp. I and sp. S is due to differences in biotransformation ability; (2) to investigate whether differences in biotransformation ability are reflected in the cellular distribution of Flu and/or Flu-metabolites which may provide evidence of a target site of the toxic compound; (3) to determine whether the sensitivity to Flu of sp. S is related to genotoxicity (i.e., DNA damage) of Flu and/or a less efficient DNA repair compared to that known for sp. I.

2. Materials and methods

2.1. Worms

Capitella sp. I were collected in Setauket Harbour, Long Island Sound, New York, USA in 1984, and identified as species type I by J.P. Grassle. *Capitella* sp. S was collected from oxygen-rich, organic-poor intertidal flat sediments of the island of Sylt, North Sea, Germany, by Gamenick and Giere (1994). These species of *Capitella* reproduce readily in culture and have been reared in our laboratory for many generations. Separate populations of *Capitella* (sp. I and sp. S) were cultured in plastic aquaria containing a 3–4 cm layer of sediment (<250 μm) and aerated filtered seawater (<0.2 μm, 31‰ salinity) at room temperature (ca. 21 °C). The sediment was regularly supplemented with a mixture of ground commercial fish food (Tetra Min©, Tetra Werke, Melle, Germany), dried baby cereal (Milpo©, Milupa, Hoersholm, Denmark), and dried spinach in equal ratios.

2.2. Sediment

All sediments were collected from a shallow subtidal area in Roskilde Fjord distant from any point sources of contamination (Store Havelse; 55°54′N, 12°02′E), Denmark. The top few centimeters of the surface sediment were scraped off and sieved to a grain size of <63 μm using tap water to kill any remaining meiofauna. The sieved sediment was allowed to settle and the overlying tap water replaced with seawater before freezing to −20 °C. The resulting sediment had a water content of 70.9 ± 0.18% (n = 3), determined by weight loss at 110 °C for 24 h, and an organic content of 11.9 ± 0.48% (n = 3), determined by weight loss on ignition at 550 °C for 24 h. Radioactively labelled Flu (^{14}C-Flu) was used to determine the amount of Flu (i.e., unmetabolised parent Flu) and Flu-metabolites (i.e., aqueous and polar Flu-metabolites and unextractable Flu-residues) in worm tissues in both the biotransformation experiment and the subcellular distribution experiment. It was assumed that radioactive Flu behaves identically to non-radioactive Flu. Flu-contaminated sediments (30 μg Flu/g dry weight sed) were produced by mixing a stock solution of Flu (crystalline fluoranthene, 98% GC grade, Sigma–Aldrich, Copenhagen, Denmark) dissolved in acetone (2.9 mg Flu/ml) with a stock solution (100 μCi/ml methanol) of ^{14}C-labelled fluoranthene (^{14}C-Flu, 250 μCi/ml methanol (F6147), Sigma–Aldrich) in a glass container, so that 3 ml of the stock solutions were added per kilogram wet weight sediment. The unlabelled Flu and the labelled Flu were added to a final ratio of 2.7 × 10^{-5} μg Flu/dpm. For the DNA damage experiment, a stock solution of non-labelled Flu was prepared by dissolving crystalline Flu (98% GC grade, Sigma–Aldrich) in acetone (2.9 mg Flu/ml), and 3 ml of the solution was added per kilogram wet weight sediment to a glass container.

Flu was mixed with the sediment in the dark by coating the Flu onto the inner surface of the glass containers, which were left on a shaking table until the solvents had evaporated (approximately 2 h). A known volume of sediment (<63 μm) was added, and the glass containers were left on a shaking table for another 48 h in the dark at room temperature. Control sediments were prepared similarly by addition of solvents alone to the glass containers. The sediments were subsequently frozen (−20 °C) until use.

To quantify the actual Flu-concentration in the experimental sediment we used a method described by Linke-Gamenick et al. (1999), in which two replicate sediment samples were taken from each Flu-spiked experimental sediment portion and extracted with methanol and ethyl-acetate and subsequently analyzed by HPLC. All sediment spiking and further work with Flu was carried out under dimmed light to minimize photo-degradation of Flu.

2.3. Experimental setup

One day prior to the start of all experiments (day^{-1}), worms (*Capitella* sp. I and sp. S) were sieved from laboratory cultures and left individually in clean

seawater in multi-well dishes (GibcoBRL, Life Technologies, Taastrup, Denmark) until the following day. On the same day, equal volumes (3.2 ml) of either control or Flu-contaminated sediments were transferred to plastic beakers (20 ml, Nunc, Life Technologies) to which 10 ml of clean filtered (<0.2 μm) seawater was added. The beakers were left to stand for 24 h in the dark before addition of the worms. Worm size was estimated as body volume at the start of the experiments and on each sampling occasion. On day 0 (the start of the experiments), the overlying water was carefully drained and fresh overlying water was added before worms were individually transferred to the beakers. The beakers were covered with aluminium foil and wet paper towels to minimize photo-degradation of Flu and water evaporation. The experiments were conducted in the dark at room temperature (ca. 21 °C), and 7 ml of the overlying water was renewed every second day during the experiments, to avoid oxygen deficiency.

2.4. Worm volume and dry weight

In order to facilitate comparisons with previous experiments and to allow calculation of size-specific Flu-concentrations, worm body volumes and dry weights were measured. A video camera mounted on a dissection microscope was used to grab pictures of live worms, and from each picture using SigmaScan Pro software (ver 5.0.0, SPSS®, Chicago, USA), individual body length (L, mm) and area (A, mm^2) were measured. Assuming that worms are cylindrical in shape (Self and Jumars, 1978), the body volumes (BV, mm^3) were estimated: $BV = [(\pi \times A^2)/(4 \times L)]$. The worm size estimate used in the analysis was the mean of two independent replicate volume determinations.

Worm dry weight was estimated as follows: a number of unexposed individual worms of both *Capitella* sp. I and sp. S were videotaped for measurement of body volume according to the method described above after giving worms 24 h to clear their guts of sediment. Individual worms were placed on a small piece of pre-weighed foil and left to dry at 50 °C for 2 h and then reweighed. A pilot experiment confirmed that worms reached dryness under these conditions. Measured worm dry weights (μg) were plotted against body volumes (mm^3) for each species separately, and the resulting equations used to calculate worm dry weight from measured volume.

2.5. Biotransformation experiment

The ability of *Capitella* sp. I and sp. S to biotransform Flu was estimated after in vivo exposure of the worms to Flu-spiked sediment by extracting worm tissue homogenates into four fractions: parent Flu (untransformed), polar Flu-metabolites, aqueous Flu-metabolites and Flu tissue residues. As polar Flu-metabolites are considered phase I products, aqueous Flu-metabolites phase II products and tissue residue Flu (unextractable Flu most likely consisting of some parent Flu but also some phase I products that are further oxidized to epoxides and bound to DNA, RNA and proteins), it was possible to measure the amounts of metabolites produced. The produced metabolites were compared to the amounts of Flu taken up, thus giving an estimation of the biotransformation ability.

2.5.1. Sampling

For measurement of biotransformation ability, worms were gently sieved out of the sediment at each sampling time. As a similar study by our group was recently conducted using sp. I (Selck et al., 2003b), we reduced the number of sampling times for sp. I to 3, 7, 10 and 15 days, while sampling times for sp. S were 0.25, 1–5, 7, 10 and 15 days of exposure. At each sampling occasion worms were videotaped for determination of final body volumes and left in clean filtered seawater (4–6 h) in order to empty their guts and to be cleaned of adsorbing Flu contaminated sediment. We chose a relatively short gut emptying time to minimize the loss of Flu and its metabolites by excretion (Selck et al., 2003b), while still allowing worms to empty their guts of sediment-adsorbed Flu. Visual observations confirmed that no fecal pellets were visible in the worms at the end of the gut emptying time. Finally, the worms (three replicates of four worms of sp. I or five worms of sp. S) were transferred to eppendorf tubes and frozen at −20 °C until analysis.

2.5.2. Flu and Flu-metabolite analysis

Extracts of Flu-exposed worms were fractionated into four groups following Selck et al. (2003b): un-

transformed parent compound (P-F), aqueous Flu-metabolites (conjugates) (A-M), polar Flu-metabolites (P-M) and residual Flu (unextractable from tissue) (T-R). Previous work has shown this technique separates fractions with a high efficiency and has a high overall recovery (Christensen et al., 2002; Driscoll and McElroy, 1996). Due to the small size and morphology of the *Capitella* species it was not possible to separate the reactive tissue (i.e., the chloragogen tissue) for analysis; therefore whole worm homogenates were used. Briefly, the extraction was initiated by addition of milliQ-water, methanol and chloroform to the worm homogenate. Samples were mixed, ultrasonically treated and subsequently centrifuged which resulted in a two phase solution, i.e., water/methanol phase (A-M) and chloroform phase (P-F and P-M), and a pellet. The extraction from the pellet was repeated twice, whereupon a tissue solubilizer was added to the pelleted tissue (T-R). To separate the chloroform phase into P-F and P-M, an extraction, including DMSO and hexane, was performed. The extraction was repeated twice, and after centrifugation the DMSO phase consisted of polar Flu-metabolites (P-M), whereas the hexane phase consisted of parent Flu (P-F). Radioactivity associated with each of the four phases was quantified by liquid scintillation counting and corrected for quench by the external standards ratio method after subtracting background. The concentrations of Flu and Flu-metabolites were calculated from the ratio between Flu and ^{14}C-Flu (2.7×10^{-5} µg Flu/dpm) and the data presented as Flu equivalents (Flu eq.).

2.6. Subcellular distribution experiment

The subcellular distribution experiment was performed on worms exposed in parallel to the biotransformation experiment and was based on a differential centrifugation technique which fractionates worm homogenates into four fractions: tissue debris-, mitochondrial-, microsomal- and cytosolic fractions.

2.6.1. Sampling

On each sampling occasion (i.e., after 0.25, 1, 3 and 10 days of exposure) worms were gently sieved out of the sediment, videotaped and left in clean water (4–6 h) in order to empty their guts and be cleaned of adsorbing Flu. Worms (four replicates of seven worms of sp. I or eight worms of sp. S) were transferred to eppendorf tubes and placed in liquid nitrogen for ca. 0.5 min to disrupt the cell membrane and frozen at −80 °C until analysis.

2.6.2. Flu analysis

The subcellular distribution of Flu was assessed by measuring the concentration of Flu and its metabolites in four cellular fractions, separated by differential centrifugation following Selck and Forbes (2004). Briefly, following centrifugation at 1000, 9000 and 100,000 × *g* four fractions were produced. The first pellet contains tissue fragments and other cellular debris (primarily membranes) and is referred to as the debris fraction. The second pellet, the mitochondrial fraction, contains mitochondria, peroxisomes and lysosomes. The last pellet, the microsomal fraction, contains small pieces of the plasma membrane, fragments of golgi apparatus, ribosomes and microsomes (disrupted endoplasmic reticulum forming closed vesicles), which contain several enzymes involved in xenobiotic metabolism including cytochrome P450 mixed function oxygenase. The fourth fraction is the cytoplasm (cytosol fraction), which contains a large number of enzymes and other proteins, and is thus a major site for cellular metabolism.

As for the biotransformation experiment, radioactivity was quantified by liquid scintillation counting and data presented as Flu equivalents.

2.7. DNA damage experiment

DNA damage induced by Flu was assessed by the comet assay applied to a mixture of cells from in vivo Flu-exposed sp. S worms. The comet assay is a rapid and sensitive method for detecting alkali labile sites as well as single- and double stranded breaks in the DNA, which are mainly the result of genotoxic events and may lead to mutations or cell death. As a previous study performed by our group recently demonstrated genotoxic effects of 30 µg Flu/g dry weight sed in sp. I (Palmqvist et al., 2003), we did not repeat the comet assay on Flu-exposed sp. I. However, to facilitate comparison in reaction patterns between the two species, the cell preparation and the comet assay were performed using the same protocol as Palmqvist et al. (2003).

2.7.1. Sampling

Flu-induced DNA damage in *Capitella* sp. S was measured by applying the comet assay after 0–3, 5, 7, 10, 15 and 28 days of exposure with four replicates for the Flu-exposed group, three replicates for the control group and a positive control (treatment with hydrogen peroxide). Each replicate was a pool of four worms. At each sampling time, worms were gently sieved out of the sediment and left in clean seawater (4–6 h) in order to empty their guts and be cleaned of adsorbing Flu-contaminated sediment.

2.7.2. Preparation of worm cells

Preparation of cell suspensions and performance of the comet assay (until the end of electrophoresis) was conducted under yellow light or in the dark to reduce any photo-oxidative DNA damage to the cells. The cell suspension was prepared by placing a replicate (i.e., four worms) in an eppendorf tube with EDTA-buffer and cutting the worm into small pieces using scissors. Following centrifugation the supernatant was replaced by a trypsin/EDTA-buffer, and the cells were carefully dispersed. The sample was centrifuged and the pellet resuspended in PBS buffer. For the positive control, the pellet was resuspended in 0.5 ml 50 mM H_2O_2 for 7 min, the sample was centrifuged, and then the pellet was resuspended in PBS buffer. The comet assay was carried out immediately after preparation of the cell suspensions.

2.7.3. Comet assay procedure

The comet assay was performed following Palmqvist et al. (2003) until scoring of the comets. From each replicate (cell suspension) three subsamples were taken and mixed with low melting point agarose and cast on a GelBond film. The gel was allowed to set and the gel immersed in cold lysis buffer. The gels were then washed with milliQ-water and submersed in electrophoresis buffer (pH >13) in an electrophoresis tank for 40 min to allow DNA unwinding followed by electrophoresis at 25 V, ~300 mA for 10 min. The slides were washed in neutralization buffer and in milliQ-water, fixed in ethanol and left overnight to air dry. Prior to analysis, gels were marked blindly before staining with SYBRGold and analyzed under a fluorescence microscope (Dialux, 22 EB, Leica). On each gel, 40 randomly selected cells (i.e., each of three sub-replicate) were analyzed for tail extent moment (tail extent moment = tail length × % DNA in tail/100) using the software application Kinetic Imaging (Komet 3.1, 1996, Kinetic Imaging Ltd., Liverpool, UK). The DNA damage was recorded as the mean tail extent moment from analysis of 120 individual cells. The levels of DNA damage were compared to a negative control (worms unexposed to Flu) and a positive control (unexposed worm cells treated with H_2O_2).

2.8. Statistics

Relationships between worm dry weight and volume were estimated by linear regression of $\log_{10}$-transformed data. Differences in worm dry weight/worm volume (μg/mm^3) relationships between species were tested by an analysis of covariance (ANCOVA) on $\log_{10}$-transformed data.

For the biotransformation experiment, correlations between Flu-concentrations (μg Flu eq./g dry weight worm) in the four metabolite fractions (i.e., P-F, A-M, P-M and T-R) and exposure time, as well as correlations between worm volume and exposure time were tested with Spearman's rank order correlation coefficient. Differences in total Flu body-burden as well as fractional concentrations (μg Flu eq./g dry weight worm) in P-F, A-M, P-M and T-R between sp. I and sp. S at the end of exposure were tested by analysis of variance (ANOVA).

For the subcellular distribution experiment, effects of exposure time on Flu-concentrations in the subcellular fractions (i.e., debris, mitochondria, microsomal and cytosol), differences in Flu-concentration in each fraction at the end of the exposure period between species, and differences in total final body-burden between species were all tested by ANOVA. For the DNA damage experiment, a two-way analysis of variance was used to test for differences in DNA damage with treatment and exposure time as factors. When the two-way ANOVA indicated significant effects, a Tukey's HSD test was performed to test for pairwise differences between groups.

Prior to all analyses, assumptions of normality and homogeneity of variances were verified by graphical inspection of the data. Significance was defined as $p \leq 0.05$ and marginal significance as $0.05 < p \leq 0.1$. All statistical analyses were conducted using Statistica (ver 6., StatSoft Inc., USA).

3. Results

3.1. Sediment Flu-concentrations

The measured initial Flu-concentrations in contaminated sediments were 26.4 ± 1.4 (n = 2) µg Flu/g dry weight of sediment in the biotransformation and subcellular distribution experiments, and 21.4 ± 2.7 (n = 2) µg Flu/g dry weight of sediment in the sediment used for the DNA damage experiment. For simplicity, the results that follow are expressed in terms of the nominal Flu-concentration of 30 µg Flu/g dry weight of sediment.

3.2. Worm volume and dry weight

The initial volumes of *Capitella* sp. I ranged from 0.07 to 2.43 mm^3 (mean = 0.48 ± 0.40 mm^3, n = 112). Although ANOVA (on log-transformed data) revealed a significant difference (p = 0.009; r^2 = 0.106) in the initial volumes among groups, a Tukey's HSD test showed only a significant difference between the groups harvested on days 1 and 3, and the difference in mean volume was 38%. The initial volumes of *Capitella* sp. S ranged from 0.05 to 0.91 mm^3 (mean = 0.29 ± 0.18 mm^3, n = 128), and no significant difference (two-way ANOVA) in body volume was found among time-groups (p = 0.57) or fluoranthene-treatments (p = 0.47). ANCOVA detected no difference between species in dry weight to volume relationships (p = 0.59). Both species followed the equation: log DW = 2.26 (±0.01) + 0.86 (±0.05) × log V (r^2 = 0.80, n = 98), where DW is worm dry weight in µg and V is worm volume in mm^3.

3.3. Biotransformation experiment

During the exposure period, *Capitella* sp. I increased its body-burden of parent Flu (P-F), aqueous Flu-metabolites (A-M), polar Flu-metabolites (P-M) and tissue residues (T-R) to different degrees (Figure 1). The body-burden of P-F increased slowly up to day 7, increased markedly between days 7 and 10 and then dropped to about 50% by day 15, and was by the end of the experiment only marginally different from the body-burden at the first sampling time (day 3) (ANOVA: p = 0.09). For all metabolite groups (A-M, P-M, T-R) there was a significant positive correlation between body-burden and exposure time (Spearman's rank order correlation coefficient: all p-values <0.05). However, the body-burden of T-R increased only slightly during the exposure period. Untransformed Flu (P-F) constituted about one third (37.6%) of the total body-burden, whereas A-M made up the highest proportion of metabolites produced (37.8%), followed by a considerable amount of P-M (20.6%). The tissue residues only contributed a few percent (4.0%) (i.e., P-F = A-M > P-M > T-R). *Capitella* sp. S showed an increased body-burden of parent Flu and Flu-metabolites (i.e., A-M, P-M and T-R) from the start to the end of the exposure period. The body-burdens of the four fractions were markedly different (Figure 1). A positive correlation was evident between concentration (µg Flu eq./g dry weight worm) and

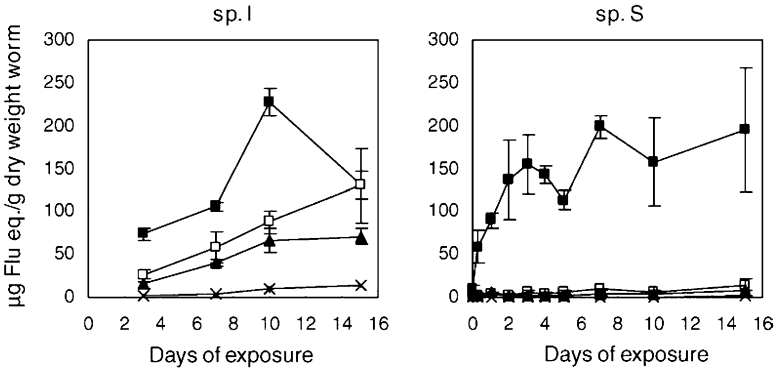

Figure 1. Relationship between exposure time and mean (±S.D., n = 3) tissue concentration of fluoranthene equivalents (µg Flu eq./g dry weight worm). The body-burden was fractionated into: (■) parent Flu (P-F), (□) aqueous Flu-metabolites (A-M), (▲) polar Flu-metabolites (P-M) and (×) tissue residues (T-R) in *Capitella* sp. I and sp. S following exposure to 30 µg Flu/g dry weight sediment.

exposure time for the P-F, A-M, P-M and T-R fractions (Spearman's rank order correlation coefficient: all p-values <0.05). The rate of net uptake (defined as the actual uptake minus elimination) for the P-F was initially rapid but reduced from about day 3. At the end of the experiment (day 15), the total body-burden consisted primarily of untransformed parent Flu (P-F) (89.2%), with only a small proportion of metabolites (A-M: 6.7%, P-M: 3.3% and T-R: 0.8%).

The absolute amounts of metabolites (A-M, P-M and T-R) produced during the course of the exposure period were higher in *Capitella* sp. I compared to the amounts produced by sp. S (ANOVA: all p-values <0.001), though there was no significant difference in the final body-burden of parent Flu (P-F) between *Capitella* sp. I and sp. S at the end of the experiment (day 15) (ANOVA: $p = 0.25$). The amount of Flu taken up during the experiment was reflected in the total body-burden (parent + metabolites). There was a significant difference in the total body-burden between the two species (ANOVA: $p = 0.042$) as *Capitella* sp. I reached a total body-burden of 346.4 µg Flu eq./g dry weight worm (S.D. = 35.3, $n = 3$), while the total body-burden of sp. S was 219.0 µg Flu eq./g dry weight worm (S.D. = 65.9, $n = 3$).

3.4. Subcellular distribution experiment

Analysis of the subcellular distribution of Flu (Figure 2) showed that exposure time had an effect on Flu-concentrations in the subcellular fractions (i.e., debris, mitochondria, microsomal and cytosol) (ANOVA: all p-values <0.05). Throughout the experiment sp. I and sp. S accumulated almost equal amounts of Flu in the debris fraction, and on day 10 both species contained an accumulated Flu eq. concentration of ca. 80 µg Flu eq./g dry weight worm. The Flu eq. content in the mitochondria and microsomal fractions showed that *Capitella* sp. I accumulated higher amounts than sp. S after 10 days of exposure. Thus, the level of Flu on day 10 in the mitochondria fraction was up to ca. 90 µg Flu eq./g dry weight worm for sp. I and ca. 40 for sp. S, whereas in the microsomal fraction

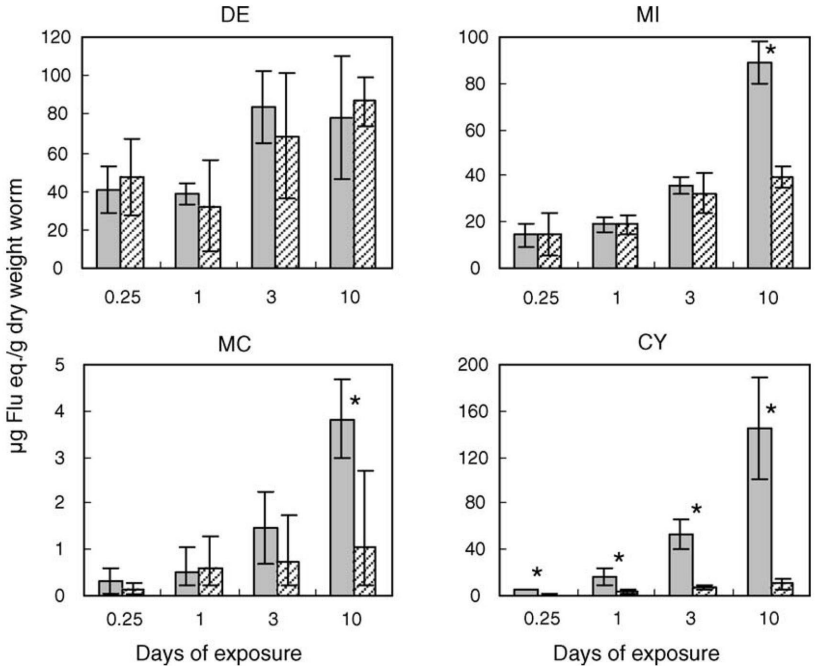

Figure 2. Distribution of total fluoranthene (parent + metabolites; Flu) among debris (DE), mitochondria (MI), microsomes (MC) and cytosol (CY) in cells from *Capitella* sp. I (■) and sp. S (▨) exposed to 30 µg Flu/g dry weight sediment over a period of 10 days. Data are presented as the mean ± S.D. (µg Flu eq./g dry weight worm) of four replicates of 7/8 worms. Asterisks show significant differences between species.

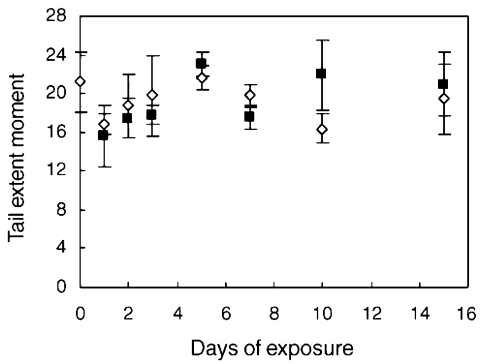

Figure 3. DNA damage in *Capitella* sp. S cells in (◊) control groups (n = 3) and in (■) Flu-exposed groups (n = 4) (30 µg Flu/g dry weight sed) over a time period of 15 days. Mean DNA damage (±S.D.) is measured as tail extent moment using the comet assay.

the Flu-concentration only reached ca. 4 µg Flu eq./g dry weight worm for sp. I and ca. 1 µg Flu eq./g dry weight worm for sp. S. The distribution of Flu in the cytosolic fraction at the end of exposure showed, however, the largest difference between *Capitella* sp. I and sp. S (ANOVA: $p < 0.001$). Whereas *Capitella* sp. I had increased the amount of Flu up to ca. 145 µg Flu eq./g dry weight worm after 10 days, sp. S only showed a Flu accumulation up to ca. 10 µg Flu eq./g dry weight worm. At the end of the experiment, the percent distribution of Flu in the four fractions for sp. I was: debris: 24.8%, mitochondria: 28.1%, microsomal: 1.2% and cytosol: 45.9% and for sp. S: debris: 63.2%, mitochondria: 28.5%, microsomal: 0.8% and cytosol: 7.5% As in the biotransformation experiment, there was a significant difference (ANOVA: $p = 0.003$) in the total body-burden between the two species as *Capitella* sp. I reached a total body-burden of 326.7 µg Flu eq./g dry weight worm (S.D. = 74.8, n = 4), while the total body-burden of sp. S was 137.2 µg Flu eq./g dry weight worm (S.D. = 17.7, n = 4).

3.5. DNA damage experiment

Overall there was no significant effect (ANOVA: $p = 0.75$) of treatment (Flu-exposed versus control worms) in DNA damage measured as tail extent moment on *Capitella* sp. S (Figure 3). Even though an effect of time was detected (ANOVA: $p = 0.005$), the only observed difference was in the Flu-exposed group between days 1 and 5 (Tukey's HSD test: $p = 0.01$), but since there were no differences between Flu-exposed and control worms on either day, this cannot be assigned to an effect of Flu exposure. Also, there was no significant interaction between time and treatment (ANOVA). The positive control showed a significant difference in tail extent moment (ANOVA: all p-values <0.05) (data not shown) compared to the control group, demonstrating that the assay was performed correctly.

4. Discussion

Tolerance to toxicants is dependent on a combination of uptake (uptake routes), intracellular fate (e.g., biotransformation), gut retention time of the toxicant, excretion, kind and degree of damage caused by the toxic agent and the degree to which the organism is able to deal with the damage (e.g., repair mechanisms). Our results suggest that the efficiency of biotransformation ability may have a major impact on the toxicity of Flu to *Capitella* species. Biotransformation of PAHs is common in marine invertebrates, but the efficiency of biotransformation varies greatly, and until now there has been limited knowledge about the influence of biotransformation ability on observed differences in species sensitivity. This study focused on two sibling species, *Capitella* sp. I and sp. S, which have shown marked differences in tolerance to Flu exposure. We found that *Capitella* sp. I contained a significantly higher amount of Flu-metabolites compared to sp. S, demonstrating a higher biotransformation ability of *Capitella* sp. I. In a similar study, Selck et al. (2003b) reported that Flu-metabolites constituted 75% of the total PAH body-burden in *Capitella* sp. I after 5 days of exposure to 30 µg Flu/g dry weight sed. Like Selck et al. (2003b), we found low amounts of polar metabolites (P-M) compared with aqueous metabolites (A-M) in *Capitella* sp. I, which probably reflects the speed with which the initial polar metabolites are further conjugated to more aqueous metabolites (A-M fraction). As suggested by Selck et al. (2003b), *Capitella* sp. I may depurate large amounts of Flu in the form of aqueous metabolites, which indicates that sp. I in this study may have had an even higher uptake of parent Flu than we estimated. Although the total Flu eq. in sp. I increased as a function of time, the accumulated concentration of parent Flu at the end of the experiment (day 10) was not different from the concentration at the first sampling time (day 3). Similarly the net uptake ki-

netics of parent Flu by sp. S was initially rapid followed by a reduced accumulation rate, but in contrast to sp. I this pattern was closely reflected in the accumulation of total Flu. Previous experiments using an identical setup suggest that degradation/loss of Flu in the sediment was less than 5% during the time period of this experiment and that worms did not deplete ingestible sediment particles (Selck et al., 2003b). Thus, neither of these factors provides a likely explanation for the reduced net uptake rates. Neither is it likely that this reduction in net accumulation rate was due to decreased sediment processing, since Méndez et al. (2001) found no effect of Flu (10 and 90 µg/g dry weight sed) on sediment processing by either sp. I or sp. S. The latter corresponds well with the fact that we found no effect of 30 ppm Flu on growth rate for sp. S (data not shown), and that Selck et al. (2003a) likewise found no effect of Flu on growth rate in sp. I.

It is known that biotransformation efficiency varies greatly among species, even among species that are closely related. The observed differences in biotransformation efficiency between *Capitella* species may be due to several factors. Recently, Palmqvist (unpublished) found that Flu-metabolites constituted ca. 35% of total Flu (i.e., Flu-metabolites and parent Flu) in *Capitella* sp. S after 4 days of exposure to 100 µg Flu/g dry weight sed, whereas *Capitella* sp. I was found to biotransform up to 66% Flu. In relation to the present study, (exposure to 30 µg Flu/g dry weight sed) this suggests that the biotransformation system in *Capitella* sp. S may be inducible at higher exposure concentrations, although it does not reach the high activity found for sp. I. The mechanism of induction of CYP-genes in invertebrates is not yet clear. In mammals, PAHs enter the cell through the plasma membrane, whereupon some of the PAH may bind to the cytosolic Ah receptor protein. The Ah receptor–ligand complex is transformed into a form that can migrate into the nucleus and combine with one or more regulatory sites on *CYP1A1* and other genes, bringing about increased transcription, followed by increased protein synthesis (Hahn, 2002). However, such an Ah receptor has, to our knowledge, not been identified in marine invertebrates, and attempts using photoaffinity labelling to find evidence for the receptor in some marine invertebrates such as echinoderms, arthropods, molluscs and annelids have failed (Hahn and Stegeman, 1992). Yet indications of the existence of an Ah receptor homologue have been found in several organisms including the nematode, *C. elegans* (Powell-Coffman et al., 1998), the clam, *Mercenaria mercenaria* (Brown et al., 1995) and other mussels, though the exact role of this homologue is still under discussion [see, Hahn (2002) for review]. The observed difference in biotransformation between *Capitella* sp. I and sp. S in the present study may be a result of lower levels of activity of an Ah receptor homologue in *Capitella* sp. S compared to sp. I, which could result in less transcription of the involved CYP-gene and subsequently less biotransformation. Li et al. (2004) did not detect the *CYP3A* (*CYP331A1*) and *CYP4A* (*CYP4AT1*) transcripts in unexposed *Capitella* sp. S using RT-PCR, whereas both transcripts were detected in *Capitella* sp. I. Also, the *CYP3A* was induced (up to two-fold) after exposure to different concentrations of Flu in sp. I, though the induction (2.5-fold up-regulation) was more pronounced following exposure to benzo[*a*] pyrene. However, according to the data obtained by Palmqvist (unpublished) it is clear that sp. S is able to biotransform Flu to some degree, which suggests that either the genes investigated by Li et al. (2004) are inducible in sp. S and that the basal level is too low to be detected by RT-PCR or that other CYP-genes are responsible for the biotransformation of Flu. The differences in biotransformation ability are reflected in the differences in subcellular distribution pattern of Flu in the four fractions (i.e., debris, mitochondria, microsomal and cytosol) between *Capitella* sp. I and sp. *S. Capitella* sp. S did not biotransform very efficiently, whereas sp. I had a high biotransformation ability. In organisms that are able to biotransform it is expected that the metabolites are found in the non-membrane cell compartments due to the less lipophilic character of the metabolites. In contrast, the untransformed parent compound would be located in the different membranes (primarily the plasma-membrane but also in the membranes of organelles such as mitochondria, lysosomes and ER) in organisms both with high and with low biotransformation ability. Lowe and Pipe (1994) found increased membrane fluidity in the lysosomes of mussel digestive cells measured as increased probe loss (neutral red) from the lysosomes to the cytosol after exposure to Flu, and it is known that much of the damage to the lysosomes is a consequence of their ability to concentrate a wide range of contaminants, resulting in increased permeabil-

ity of their membranes and loss of the acid hydrolase into the cytosol, causing cellular damage (Lowe et al., 1995). However, Moore and Willows (1998) hypothesized that lysosomes (mitochondrial fraction) are pre-adapted to stress and that animals with highly developed cellular lysosomal systems are more tolerant to pollutants. In the present study, *Capitella* sp. I showed much higher levels of Flu in the mitochondrial fraction than sp. S, which could be explained by the above-mentioned hypothesis. However, there is also the possibility that the Flu equivalents in the mitochondria fraction consist of biotransformed Flu, most likely in the form of polar Flu-metabolites.

In this study, we found no genotoxic effect of Flu in the form of DNA damage in *Capitella* sp. S, as Flu has been shown to induce in sp. I. Palmqvist et al. (2003) compared fractions of Flu-metabolites (P-F, A-M, P-M and T-R) with DNA damage caused by Flu (30 ppm) in *Capitella* sp. I and found it most likely that the aqueous metabolites (A-M), or probably short-term precursors of the aqueous metabolites such as Flu epoxides or diol-epoxides, were responsible for the genotoxic effect. As the A-M fraction is considered to represent phase II products, which are polar phase I metabolites further conjugated to endogenous cell constituents, this fraction may therefore contain Flu epoxides and Flu diol-epoxides, which are both found to be genotoxic (Babson et al., 1986). In agreement with this, Forbes et al. (2001) investigated biotransformation and biotransformation products of Flu in *Capitella* sp. I by HPLC analysis, and proposed the existence of two metabolites, 3- and 8-hydroxyfluoranthene, formed by *Capitella* sp. I. Both metabolites could potentially be further biotransformed into the mutagenic 2,3-diol Flu and 7,8-diol Flu, which may form adducts to the nucleoside in the DNA molecule and may result in DNA helix distortion or in labile AP-sites (Carr and Hoekstra, 1995; Akcha et al., 2000; Palmqvist et al., 2003). Even though *Capitella* sp. S produced small amounts of potentially DNA-damaging metabolites, the worms may either have excreted these metabolites before they interacted with the DNA molecule and exerted damage, or they were capable of maintaining low levels of DNA damage as the result of an effective repair system. Another reason for not detecting any DNA damage may be due to the limitations of the comet assay. Flu or Flu-metabolites may have generated DNA damage that the comet assay is not designed to detect. This could be DNA damage for example in the form of bulky adducts that results in replication blockage and thereby cytotoxicity (Shugart, 2000).

DNA damage is often used as a biomarker of exposure to genotoxic chemicals at the whole organism level. However, the usefulness of this assay is debated (Kriek et al., 1998; Mitchelmore and Chipman, 1998; Akcha et al., 2000; Shugart, 2000; Ching et al., 2001; Palmqvist et al., 2003) and in relation to the findings of this study the recommendation of DNA damage as a relevant biomarker of exposure is dubious. In the present study, there was indication of a poorer survival in the sensitive *Capitella* sp. S when exposed to Flu, but this species showed no increase in DNA damage after Flu treatment. In a similar study, with exposure to the same Flu-concentration, Palmqvist et al. (2003) found elevated levels of DNA damage in the more tolerant species (sp. I), however, this response was only transient. That such closely related species give such contradictory responses demonstrates that DNA damage and the use of the comet assay is not sufficient as a biomarker of either exposure or effect.

Although Flu possibly does not have genotoxic effects in *Capitella* sp. S, it exerts other effects in this species. When Flu is not biotransformed it is highly lipophilic (log K_{ow} = 5.24), and tends to accumulate within the lipid-rich cell membranes. As a result of being able to biotransform, *Capitella* sp. I primarily accumulated Flu eq. in the cytosolic fraction, whereas the highest Flu equivalent concentration in sp. S was found in the membrane (debris fraction). Binding of Flu to the cell membranes may have serious costs for the cells. When sufficient amounts of a toxicant bind to the membrane, the function of the membrane becomes impaired and it is likely that Flu has such a narcotic mode of action (Lowe et al., 1995; Driscoll et al., 1997). Furthermore, a well-functioning cell-membrane is important for the homeostasis of the cell, and Yamaguchi et al. (1996) found that Flu triggered an influx of extracellular Ca^{2+} resulting in cell-dysfunction and cell death (apoptosis) in T-cells. Apoptosis is often a result of increased levels of intracellular Ca^{2+} as a result of dysfunction of the membrane-bound Ca^{2+}-pumps and gates in the plasma-, mitochondria- and ER-membranes leading to Ca^{2+}-influx from the extracellular region and release from intracellular stores (Kass and Orrenius, 1999).

Compared to many other marine benthic invertebrates, including *Capitella* sp. S, *Capitella* sp. I is an efficient biotransformer (Selck et al., 2005; Christensen et al., 2002). Together with its opportunistic life-history traits, this gives *Capitella* sp. I an advantage in competition with other sediment-dwelling organisms, and is probably an important contributor to the success of this species in organically polluted areas.

5. Conclusion

Understanding why some species are more tolerant than others to toxic chemicals, such as PAHs, and improving understanding of the cellular mechanisms that underlie these differences in tolerance are of central importance in ecotoxicology. In this study, we examined whether the difference in tolerance to Flu between *Capitella* sp. I and sp. S can be explained by differences in biotransformation ability. We found that *Capitella* sp. I took up more Flu from the sediment than sp. S, but was much more effective at biotransforming this PAH, such that the net amounts of parent Flu accumulated by the two species were similar. Flu is known to have the potential to become genotoxic upon biotransformation, and indeed, DNA damage has been used as a biomarker of exposure to and effects of PAH. Our results demonstrate that DNA damage is tightly coupled to biotransformation ability and that other aspects of PAH toxicity (e.g., membrane disruption) are more relevant than DNA damage for predicting tolerance differences between these species.

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