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Biogeochemical Fate of Sediment-Associated PAH: Effect of Animal Processing

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BIOGEOCHEMICAL FATE OF SEDIMENT-ASSOCIATED PAH: EFFECT OF ANIMAL PROCESSING

By

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An Abstract of the Thesis Presented
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Biotransformation and fate of polycyclic aromatic hydrocarbons (PAHs) in marine invertebrates and sediment have been studied. Invertebrates can accumulate and metabolize sediment-associated PAHs to polar and aqueous PAH-derived compounds. The objectives of this study are to identify metabolites of PAHs in species of deposit-feeding polychaetes and to examine biogeochemical fate and microbial degradation of the identified metabolites. Two metabolites, 1-hydroxypyrene and 1-hydroxypyrene glucuronide, were identified as the primary phase I and phase II metabolites of the tetra-cyclic PAH pyrene in *Nereis diversicolor*. Identification was performed using high pressure liquid chromatography with diode array and fluorescence detection (HPLC/DAD/F) and an ion-trap mass spectrometer for positive identification of 1-hydroxypyrene glucuronide. A fast synchronous fluorescence spectrometry (SFS) method was developed for detection of pyrene metabolites in polychaete tissue. A good correlation between 1-hydroxypyrene measured by SFS and HPLC/F was observed. 1-hydroxypyrene was identified as the single phase I metabolite in tissue of three additional polychaete species *Nereis virens*, *Arenicola marina*, and *Capitella* sp.I. A

tentative aqueous metabolite identification scheme indicates that Nereid polychaetes predominantly make use of glucuronide conjugation whereas *Capitella* sp. I. and *Arenicola marina* appear to utilize sulfate and/or glucoside conjugation. Gut fluid from *Nereis virens*, *Arenicola brasiliensis*, and *Arenicola marina* and *Parastichopus californicus* could catalyze oxidative coupling of 1-hydroxypyrene in an apparent enzymatic reaction. Oxidative coupling will reduce subsequent bioavailability, toxicity, and transport of PAH metabolites in marine environments by formation of stable covalent bonds. An antioxidant enzyme, presumably a peroxidase, capable of oxidative coupling and with high oxyradical scavenging capacity was tentatively identified in gut fluid from *Nereis virens*. *Nereis virens* gut fluid could also catalyse formation of dityrosine, a marker of oxidative damage in proteins. Oxidative coupling of PAHs represents a new sink for organic contaminants in marine sediments and suggests a biological mechanism for the formation of aquatic humic material in general. Production of aqueous and polar metabolites by marine invertebrates does not enhance microbial degradation of pyrene either directly or in co-metabolic processes. The evidence suggests that enhanced degradation of larger PAHs in marine sediments is primarily due to bioturbation and irrigation processes of infauna.

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1. GENERAL INTRODUCTION

Organic contaminants consist of a large group of chemicals that have very different chemical properties, behavior, toxicity and fate when released into the marine environment. One of the best known groups of organic contaminants is polycyclic aromatic hydrocarbons (PAHs). PAHs are hydrocarbons composed of two or more fused aromatic (benzene) rings. Due to their low aqueous solubility and hydrophobic character, PAHs are readily adsorbed to particulate matter and solid surfaces and are therefore ubiquitous contaminants in marine sediments (Hites et al. 1980; Neff 1985). PAHs are mainly formed as products from the incomplete combustion of fossil fuels but are also natural components of unaltered fossil fuels. The most important sources of PAHs to the marine environment are through atmospheric fallout, spillage and seepage of petroleum and oil products, and industrial and domestic sewage.

Whether sediment-associated PAHs become buried in the seabed, degrade, enter food webs or are released to the water column, depends to some extent upon the activities of macrobenthos inhabiting the sediment. Marine invertebrates are known to accumulate PAHs from both particulate and dissolved phases (Meador et al. 1995) but less is known about their ability to metabolize PAHs and the subsequent fate of eliminated PAH-derived compounds. Furthermore, knowledge of the chemical identity of the metabolites produced by marine invertebrates is limited and their environmental fate and potential toxicity is unknown.

The main theme of this dissertation is to what extent animal metabolism controls the biogeochemical fate of PAHs when released from the animals. Once released from the animal, PAH-derived compounds may be reaccumulated by another animal, or

become incorporated in to the organic matter matrix and perhaps utilized by sedimentary microbes as a carbon and energy source.

PAHs themselves are relatively inert molecules and it is generally accepted that toxic and carcinogenic effects of PAHs are caused by their metabolites rather than parent compounds (Livingstone 1993). This dissertation will lead off with identification of intermediate and aqueous metabolites of pyrene (a tetracyclic PAH) in select species of deposit-feeding polychaetes (Chapters 2-4). Polychaetes are richly abundant within sediment and are present in all oceanic regions of the world. Polychaetes have been reported to constitute up 50% of macrobenthic species by number in sediments regardless of depth and are widely used in toxicological testing (Reish 1979; Reish and Gerlinger 1997). Deposit-feeding polychaetes constitute the dominant macrofauna in environments that tend to be depositional centers for organic matter and contaminants (Jumars et al. 1990). They are thus subject to an almost continuous exposure to PAHs because they ingest up to several times their own body weight of sediments per day (Cammen 1980a). Furthermore, parts of the work presented in this dissertation have been performed in Denmark and the polychaetes used (*Nereis diversicolor, Nereis virens*, and *Arenicola marina*) can be captured both in the intertidal zone of the coast of Maine and in shallow Danish Fjords and are easy to maintain in laboratory culture. Metabolite identification is followed by Chapter 5 which deals with oxidative coupling reactions in gut fluid and introduces a novel biogeochemical fate of PAHs in marine sediments, and Chapter 6 is a study of the microbial degradation potential of the identified pyrene metabolites.

PAH METABOLISM BY MARINE INVERTEBRATES

The ability of marine polychaetes to accumulate and metabolize PAHs has been documented for a few species (e.g. Christensen et al. 2002a; Forbes et al. 2001; Kane Driscoll and McElroy 1996; McElroy et al. 2000). In marine invertebrates the ability to metabolize PAH varies widely within and among phyla, though at rates which are much lower than those observed in vertebrates (Livingstone 1994; Stegeman and Hahn 1993). Because several PAHs have been identified as potent procarcinogens (Gelbroin 1980; Ramakrishna et al. 1992), routine PAH analysis has been incorporated into environmental monitoring and risk assessment programs.

Most enzymes responsible for biotransformation of xenobiotics in mammals have also been identified in invertebrates, although the mechanism by which their genetic expression is regulated in invertebrates is still poorly understood (Hahn 1998). In both prokaryotic and eukaryotic organisms, metabolism of PAHs proceeds through the addition of oxygen to the aromatic rings. Whereas prokaryotic organisms are able to cleave the aromatic ring and eventually mineralize the PAHs to CO_2, eukaryotic organisms convert hydrophobic, lipid-soluble, parent PAHs to water soluble conjugates in a two phase process.

Figure 1.1 shows a simplified eukaryotic biotransformation scheme for pyrene. The enzymes of phase I introduce a functional group (e.g. -OH) into the PAH, to which phase II enzymes attach a large polar moiety (e.g. carbohydrate or sulfate) so that the resulting conjugate can be readily excreted from the animal.

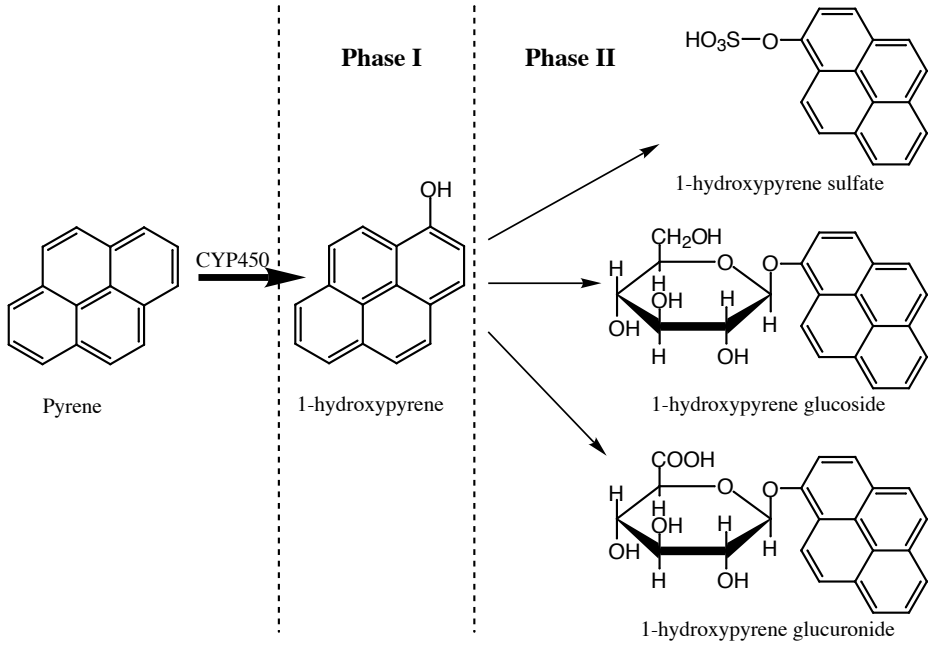

Figure 1.1 Simplified biotransformation scheme of pyrene.

Phase I biotransformation enzymes (CYP450) oxidize pyrene to 1-hydroxypyrene which is then conjugated by phase II enzymes to a carbohydrate or sulfate and excreted from the animal.

Phase I enzymes, like cytochrome P450, have been found or indicated in most phyla of marine invertebrates (Brown et al. 1998; Den Besten 1998; James and Boyle 1998; Lee 1998), and glucoside and sulphate conjugation appear to predominate over glucuronide conjugation as the preferred phase II metabolic route (James 1987; Livingstone 1998). Several of the PAHs used in environmental studies (e.g. fluoranthene, benz[a]anthracene, and benzo[a]pyrene) have complex metabolite patterns (e.g. Forbes et al. 2001; McElroy et al. 2000) and lack of commercially available standards makes studies on PAH metabolism and fate of metabolic products difficult. Benzo[a]pyrene for example have more than 25 known metabolites (Gelbroin 1980). These problems can be overcome by choosing the tetra-cyclic PAH pyrene as a marker for PAH exposure. Pyrene is a key member of the PAH family and measurement of 1-hydroxypyrene in excretory products of animals and humans has gained considerable attention as a potential biomarker for PAH exposure (Levin 1995). Although 1-hydroxypyrene is a metabolic intermediate, its occurrence can reflect both PAH exposure as well as the metabolic capacity of the species under investigation. Detection of 1-hydroxypyrene also provides insight into the actual accumulation of pyrene, integrated over all uptake routes, and taking into account bioavailability and thus information on species-specific bioaccumulation and biotransformation capacities can be obtained.

There have been numerous reports on the use of 1-hydroxypyrene as a biomarker for PAH exposure in both marine and terrestrial environments (e.g. Ariese et al. 1993c; Krahn et al. 1987; Lin et al. 1996; Stroomberg et al. 1999; Van der Oost et al. 1994) but none for marine invertebrates.

I have identified 1-hydroxypyrene in tissue samples of 4 deposit-feeding polychaetes *Nereis diversicolor*, *Nereis virens*, *Capitella sp. I.* and *Arenicola marina* (Chapters 2 and 4). A single dominant aqueous metabolite, 1-hydroxypyrene glucuronide, was observed and identified in both tissue and gut fluid from *Nereis diversicolor* (Chapters 2 and 3, Giessing and Lund 2002). A tentative aqueous metabolite identification scheme, based on pyrene-metabolite pattern observed in *Nereis diversicolor*, suggests that nereid polychaetes predominately make use of glucuronide conjugation whereas *Capitella* and *Arenicola marina* appear to utilize predominately sulfate and/or glucoside conjugation (Chapter 4). Thus, as for mammals and fish, large species-specific differences in phase II metabolism are observed among invertebrates and identification of 1-hydroxypyrene glucuronide as the major aqueous metabolite in *Nereis diversicolor* further emphasizes this commonly observed trend (Livingstone 1998).

The gut of a deposit-feeder is assumed to be both the primary route of uptake and elimination of organic contaminants (Forbes et al. 1998; Forbes et al. 1996; Mayer et al. 1996) and elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion. Identification of 1-hydroxypyrene and conjugates thereof in gut fluid (Chapter 2) further emphasizes the gut as an important reaction zone in determining both uptake and elimination of organic contaminants and metals in marine deposit-feeding invertebrates (Chen and Mayer 1998; Mayer et al. 1996; Voparil and Mayer 2000; Weston and Mayer 1998).

BIOGEOCHEMICAL FATE OF PAH METABOLITES

Infaunal macrobenthos are known to alter sediment properties by feeding, irrigating, and by constructing tubes and burrows, which cause changes in sediment parameters such as porosity, particle size, reaction rate distributions, fluxes and diffusion rates (Aller 1982; Aller et al. 2001; Krantzberg 1985; Rhoads 1974). This biological mixing, collectively called bioturbation, increases both vertical and horizontal transport of both solutes and particles (Aller 1982). Furthermore, most burrow constructors maintain contact with the overlying water by ventilating water through their burrow system, thus increasing the transport of ions and gases (e.g., O_2) across the sediment-water interface (Kristensen 1985). In the same way, burrowing activity may also alter PAH concentrations in sediments, causing PAHs to become buried with long-lasting effects on the marine ecosystem. Bioturbation by both small and larger polychaetes (e.g. *Capitella capitata* and *Arenicola marina*) is known to bury PAHs into anoxic zones of the sediment (Forbes and Kure 1997; Holmer et al. 1997; Madsen et al. 1997), which has significant implications for the fate of PAHs because microbial degradation of these compounds is redox sensitive. Reworking of sediment by macrofauna allows aerobic degradation of PAHs by distributing oxygen further into the sediment subsurface layers (Aller and Yingst 1985; Fenchel 1996) and repetitive oscillating redox conditions relative to stable conditions have been shown to stimulate organic matter degradation (Aller 1994). Furthermore, PAH degradation potentials of burrow sediment are generally higher than degradation potentials for non-burrow sediment (Chung and King 1999). Also, exposure to organic contaminants is known to induce changes in animal behavior such decreased feeding rates and lowered animal activity in general (Gilbert et al. 1997;

Prouse and Gordon 1976). Thus the biogeochemical fate of PAHs in marine sediments is not straightforward and is determined by complex interactions between infauna, bacterial populations, and PAHs with both positive and negative feedbacks.

The general hypothesis of my research is that animal conversion of non-polar parent compounds to more polar metabolites leads to altered mobility and increased microbial degradation of sediment-associated PAHs. Animal metabolism results in functionalized aromatic compounds which are chemically more reactive than their precursors (e.g. 1-hydroxypyrene). These compounds may react with dissolved organic material during gut passage to form chemically bound PAH residues within the organic matter matrix thereby reducing bioavailability and toxicity. Furthermore, animal processing of sediment increases solubility of sediment-associated PAH by formation of metabolic conjugates, enhances desorptive-diffusive loss as well as determining the spatial distribution (i.e. flushing of metabolites and parent PAHs from burrows) of PAHs.

Oxidative coupling of PAH metabolites

The association of organic pollutants with macromolecular organic matter has been suggested to be a major sink for organic pollutants in soils (Bollag and Loll 1983). Functionalized metabolites of PAHs can chemically react with functional groups of other organic matter by condensation processes to form stable chemical bonds (Richnow et al. 1994). Essential constituents in the formation of terrestrial humic matter originating from plants (i.e. lignin) or other organic material, possess phenolic functionality and because degradation products of many pollutants also have phenolic characteristics they may be incorporated into organic matter during terrestrial humification processes. Some typical

metabolites of PAHs, including 1-hydroxypyrene, have been shown to be covalently bound to soil and riverine humic substances (Richnow et al. 1994; Richnow et al. 1997) by oxidative coupling. Oxidative coupling reactions can be catalyzed or caused by various oxidoreductive enzymes, inorganic chemicals, clay and soil extracts, or it can take place by auto-oxidative processes (Bollag 1983; Bollag 1992).

Figure 1.2 shows the possible interactions of metabolites of PAHs and organic matter in sediment (modified after Richnow et al. 1994). Sorption of hydrophobic organic chemicals to sediments is frequently described as fast and reversible (Karickhoff and Morris 1985) and the 'bonds' that are made between the sorbate (i.e. PAH) and organic matter are through relatively weak, non-covalent, intermolecular attractions (e.g. hydrogen bonds and weak charge transfer complexes). Covalent binding (i.e. ester, ether and C-C bonds) of PAH metabolites with macromolecular organic matter, on the other hand has significant implications for their subsequent fate and is believed to reduce their toxicity and bioavailability and alter transport processes, especially in aquatic systems.

The formation of covalent bonds between 1-hydroxypyrene and sedimentary organic matter is examined in Chapter 5. A significant increase in 1-hydroxypyrene extractability from fecal matter from pyrene-exposed worms was observed after acid hydrolysis, suggesting that 1-hydroxypyrene was bound covalently, through ester bonds, to the organic matter matrix. Although total extractable 1-hydroxypyrene constituted less than 1% of total extractable PAH, covalent binding of PAH metabolites to organic matter during gut passage represents a new sink for organic contaminants in marine sediments.

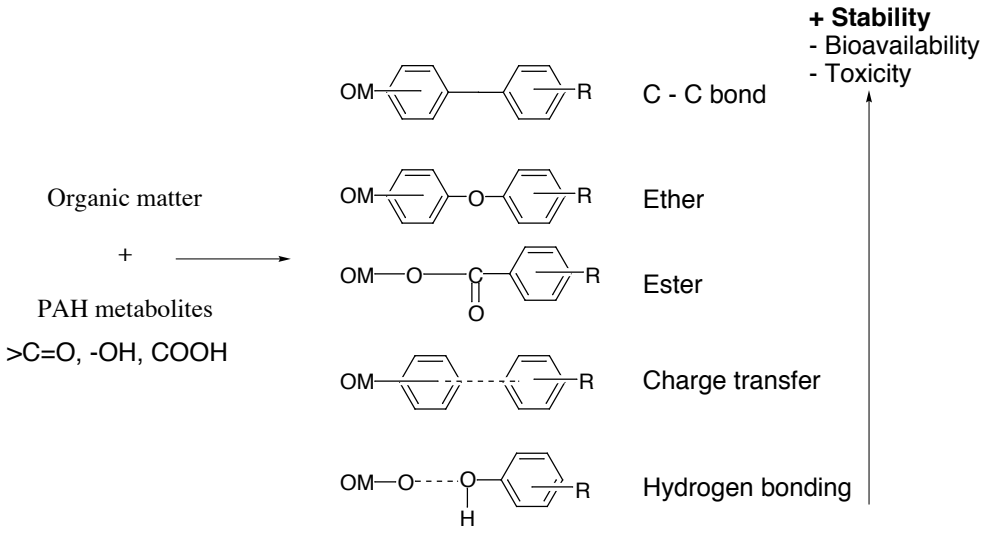

Figure 1.2 Types of associations between PAH metabolites and organic matter in sediments.

The gut of a deposit feeder is a place of intense enzymatic activity, with high concentrations of dissolved organic matter and metals compared to ambient sediment (Chen et al. 2000; Mayer et al. 1997), making the gut an ideal environment for oxidative coupling reactions. I have identified high oxidative coupling potential in gut fluid from three polychaetes and one holothuroid (Chapter 5). The high oxidative coupling potential of polychaete gut fluid is accompanied by high oxygen radical scavenging capacity indicating that significant amounts of reactive oxygen species, such as lipid peroxides and the very reactive hydroxyl radical, are generated in the gut environment. These reactive oxygen species are generated either as a consequence of normal digestive processes and/or due to the ingestion of sediment rich in redox-cycling metals like iron, manganese and copper. The observed oxidative coupling potential in polychaete gut fluid is in part catalyzed by oxidoreductive enzymes, presumably a peroxidase, though the enzyme was not isolated or its origin identified.

The oxidative enzymes in gut fluid were also able to catalyze the formation of dityrosine, the product of oxidative coupling of the essential amino acid tyrosine. Tyrosine coupling is believed to be a prime marker of oxidative stress in artherosclerosis, inflammatory conditions, and neurodegenerative disease (Leeuwenburgh et al. 1999; Souza et al. 2000). Identification of dityrosine formation catalyzed by gut fluid provides strong evidence for oxidative coupling reactions of naturally occurring compounds during gut passage and might prove to be a valuable marker for oxidative coupling reactions in natural systems.

MICROBIAL DEGRADATION OF PAH METABOLITES FROM INVERTEBRATES

Rates of microbial degradation of PAHs in marine sediments are typically slow and are thought to be limited by the concentration of PAHs solubilized in the aqueous phase (Bosma et al. 1997; Harms and Bosma 1997). It is well known that PAHs can be degraded aerobically. Low-molecular weight PAHs (≤ three fused benzene rings) degrade readily in sediments whereas high-molecular weight PAH (≥ four fused benzene rings) appear more resistant to microbial attack (Cerniglia 1991; Kanaly and Harayama 2000), even in aquatic ecosystems which are chronically exposed to petrogenic hydrocarbons (Heitkamp and Cerniglla 1987). A few studies have reported anaerobic microbial degradation of PAHs (e.g. Coates et al. 1996; Coates et al. 1997). However, basic catabolic microbial processes quickly deplete oxygen and generally the rate of oxygen consumption in the intertidal zone exceeds the rate with which oxygen can be replenished within the sediment. Marine invertebrates are known to stimulate microbial mineralization rates of sediment-associated contaminants as well as of natural organic

matter in marine sediments (e.g. Aller and Yingst 1985; Hansen et al. 1998; Holmer et al. 1997; Kristensen and Blackburn 1987). Worm burrows increase the surface area of sediment exposed to overlaying water and thereby provide more area for diffusion of oxygen. In the case of contaminants, increased mineralization rates are most likely due to higher availability of oxygen and irrigation activities of worms will flush burrows of parent compound and metabolites and maintain a concentration gradient favoring desorption of PAH into oxygenated burrow wall and burrow water. Lack of accumulation of oxygenated intermediates of microbial degradation in sediments containing larger PAHs (e.g. Augenfeld et al. 1982; McElroy et al. 1990) supports the hypothesis that the rate limiting steps in aerobic degradation of PAHs is the initial ring-oxygenation and that intermediate intracellular metabolites are further oxidized almost as rapidly as they are produced (Heitkamp and Cerniglla 1987; Herbes and Schwall 1978). Increased bioavailability of PAHs due to animal processing could increase the microbial degradation potential of sediment-associated PAHs by increasing the fraction available for microbial attack either, by enhancing diffusion and/or by providing PAHs in a more readily degradable form.

Initial experiments of microbial degradation of PAH metabolites (Chapter 6) show that an increase in degradation potential due to animal processing might not be the case, at least not for pyrene metabolites. Neither pyrene nor pyrene metabolites were substantially mineralized by microbial populations in sediment from a pristine Danish Fjord. Though degradation, measured as total $^{14}CO_2$ evolved, of ^{14}C-metabolites was higher than ^{14}C-pyrene in a pristine sediment after 175 d, pre-exposure of the same sediment to pyrene for 60 d failed to increase degradation rates of ^{14}C-metabolites

compared to ^{14}C-pyrene. Mineralization of ^{14}C-pyrene and ^{14}C-pyrene metabolites were also reduced by amendment of crude oil and in naturally aged oil contaminated sediment, indicating a dominant role of competitive inhibition by more labile oil components. Furthermore, individual bacterial strains of known pyrene degraders were not able to utilize 1-hydroxypyrene as a sole source of carbon end energy. In fact, the results in Chapter 6 suggests that 1-hydroxypyrene might be toxic to these microorganisms due to higher aqueous solubility of 1-hydroxypyrene compared to pyrene. Thus increased solubility and hence bioavailability due to animal processing does not seem to increase the microbial biodegradation potential of pyrene. The results presented in Chapter 6 strongly suggests that enhanced degradation of larger PAHs in marine sediments is primarily due to bioturbation and irrigation processes of infauna. Whether limited microbial degradation potential of metabolites applies to larger PAHs with more than one intermediate metabolite is at present unknown.

There are numerous questions that arose during my experimental work that I have been unable to address due to time constraints of the project and the need for collaboration with experienced colleagues. For example, I would like to expand the pyrene metabolite identification to include marine invertebrate taxa other than polychaetes. A field survey correlating 1-hydroxypyrene concentrations in deposit-feeding invertebrates with total PAH concentrations in sediment would also be needed to establish 1-hydroxypyrene as a biomarker for PAH exposure in marine invertebrates as proposed in Chapter 4. To establish if the enzymatic oxidative coupling reactions identified in Chapter 5 play a role in marine humification processes, the involved

enzymes need to be isolated and purified. This isolation will allow detailed product studies using substrates of marine origin (e.g. brown algae phlorotannins) and reveal if the enzymes are indeed actively secreted into the gut by the animal or are incidental due to continuous lysis of gut or prey cells. Further development of my dityrosine analysis method is needed for extraction and identification of dityrosine from natural sediments to establish if oxidative coupling of tyrosine in important in preserving proteins in marine sediments. More microbial degradation studies using individual bacterial strains are underway but could not be included due to time constraints. The microbial fate or other fate of 1-hydroxypyrene glucuronide remains an open question.

2. 1-HYDROXYPYRENE GLUCURONIDE AS THE MAJOR AQUEOUS PYRENE METABOLITE IN TISSUE AND GUT FLUID FROM THE MARINE DEPOSIT-FEEDING POLYCHAETE *NEREIS DIVERSICOLOR*

ABSTRACT

1-hydroxypyrene and 1-hydroxypyrene glucuronide are identified as the primary phase I and phase II metabolites of the four ringed polycyclic aromatic hydrocarbon pyrene in the marine deposit-feeding polychaete *Nereis diversicolor*. Identification of pyrene and primary metabolites was performed using high pressure liquid chromatography with diode array and fluorescence detection (HPLC/DAD/F) and an ion-trap mass spectrometer for positive identification of 1-hydroxypyrene glucuronide. Besides 1-hydroxypyrene and 1-hydroxypyrene glucuronide, the HPLC/F trace of tissue samples from pyrene-exposed worms showed three additional low intensity peaks that may be related to pyrene metabolism based on similar excitation/emission wavelengths. The peaks were all too low in intensity to be positively identified. Of the total PAH in tissue, 1-hydroxypyrene glucuronide, 1-hydroxypyrene and pyrene constituted 73%, 2%, and 25% respectively. Gut elimination of metabolic products is supported by the identification of 1-hydroxypyrene and 1-hydroxypyrene glucuronide in both gut fluid and defecation water. Being the only phase I metabolite of pyrene, 1-hydroxypyrene becomes a useful marker for PAH exposure, and it may serve as a valuable model compound for assessing species-specific PAH metabolic capabilities.

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) constitute a broad class of environmentally persistent organic compounds that are ubiquitous in both marine and terrestrial environments (Neff 1985). Because several PAHs have been identified as potent procarcinogens, PAH analysis has been incorporated into environmental monitoring and risk assessment programs. PAHs themselves are relatively inert molecules and it is generally accepted that toxic and carcinogenic effects of PAHs are caused by their metabolites rather than the parent compounds (James and Kleinow 1993; Livingstone 1993). Detection and quantification of PAH metabolites in conjunction with their parent compounds might therefore provide a better method for environmental risk assessment.

The ability of marine polychaetes to accumulate and metabolize PAHs has been documented for a few species (e.g. Forbes et al. 2001; Kane Driscoll and McElroy 1996; McElroy et al. 2000). In marine invertebrates, in general, the ability to metabolize PAHs varies widely within and among phyla, though at rates which are much lower than those observed in vertebrates (Livingstone 1994). Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P450 enzyme suite although the mechanism by which their gene expression is regulated is still poorly understood (Hahn 1998). The general function of P450 enzymes is to convert hydrophobic lipid-soluble parent PAH to water-soluble metabolites in a two-phase process. The enzymes of phase I metabolism introduce or modify a functional group (-OH, -COOH, NO_2 etc.) into the PAH to which glucuronosyltransferase or other phase II enzymes attach a large polar moiety (glucuronic

acid, glutathione, sulfate, amino acid, etc.), which is then readily excreted from the animal.

The present study investigates formation of phase I and II metabolites of pyrene in the deposit-feeding marine polychaete *Nereis diversicolor*. *N. diversicolor* has known PAH metabolic capacity (Christensen et al. 2002a; Kane Driscoll and McElroy 1996; Kane Driscoll and McElroy 1997), but direct identification of phase II biotransformation products in a marine polychaete has, to our knowledge, not yet been published. Most enzymes responsible for biotransformation of xenobiotics in mammals have also been identified in invertebrates. Phase I enzymes, like cytochrome P450, have been found or indicated in annelids, crustaceans, echinoderms and molluscs (Brown et al. 1998; Den Besten 1998; James and Boyle 1998; Lee 1998). In the case of phase II metabolism, glucose and sulphate conjugation predominates over glucuronic acid conjugation in marine invertebrates (Livingstone 1998). However, as for mammals and fish, large species-specific differences in phase II metabolism are observed among invertebrates. Consistent with this, Hryk et al. (1992) found formation of glucoside but not glucuronide conjugates of phenol in the terrestrial snail *Capaea nemoralis* and Michel et al. (1995) found sulphate and glucuronide conjugates of benzo[a]pyrene (B[a]P) in the mollusc *Mytilus galloprovincialis*. This species-specific difference in phase II metabolism is also evident in metabolism of pentachlorophenol in an oyster (*Crassostrea gigas*) and two species of abalone (*Haliotis fulgens* and *H. rufescens*). All three species formed sulphated conjugates whereas only the abalone formed glucoside conjugates of pentachlorophenol (Shofer and Tjeerdema 1993; Tjeerdema and Crosby 1992).

Pyrene is not known for its toxicity, so more attention has usually been given to B[a]P which is known to be an extremely potent carcinogen after metabolic activation (Gelbroin 1980). However, measurement of pyrene metabolites, primarily 1-hydroxypyrene, in excretory products of animals and humans has gained considerable attention as a potential biomarker for PAH exposure (Levin 1995). 1-hydroxypyrene was the only observed phase I metabolite of pyrene metabolism when administered to pigs, rats, rabbits and a terrestrial isopod (Boyland and Sims 1964; Jacob et al. 1989; Keimig et al. 1983; Stroomberg et al. 1999), thereby limiting the number of potential phase II metabolites. There have been several reports on the use of 1-hydroxypyrene as a biomarker for PAH exposure in both marine (primarily in fish) and terrestrial environments. Stroomberg et al. reported glucoside and sulfate conjugates of 1-hydroxypyrene in the hepatopancreas of terrestrial isopod (1999) and both glucuronide- and sulfated-conjugated 1-hydroxypyrene have been observed in fish (Krahn et al. 1987).

The purpose of this study was to investigate formation of pyrene metabolites in *N. diversicolor* with special attention given to the detection and identification of phase II biotransformation products in both tissue and gut fluid. The gut is assumed to the primary route of both uptake and elimination of organic contaminants in deposit-feeding polychaetes (Forbes et al. 1998; Forbes et al. 1996; Mayer et al. 1996) and elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion. Metabolites were studied using high pressure liquid chromatography (HLPC) with fluorescence and UV/vis detection and a liquid chromatography mass spectrometry (LC/MS) system was used for structural identification of the dominant phase II metabolite.

MATERIALS AND METHODS

Pyrene (98%), 1-hydroxypyrene (98%), and ammonium acetate (>99%) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Acetonitrile and methanol (ChromAR® HPLC grade) were purchased from Mallinckrodt (Paris, KN, USA). 100% Ethanol was obtained from Quantum Chemical Corporation (Tuscola, IL, USA). Glucuronidase-arylsulfatase (from *Helix pomatia*, EC 3.2.1.31 and EC 3.1.6.1., 30 and 60 U/ml respectively) was supplied by ICN Biochemicals (Aurora, OH, USA). All chemicals were used as received. All water was Milli-Q quality and was filtered through a 0.22 μm filter (Durapore PVDF membrane, Millipore, MA, USA) prior to use.

TEST ANIMALS AND SEDIMENT SPIKING

The test animals used in the experiments, *N. diversicolor*, were collected on an intertidal sand flat in the Damariscotta River, South Bristol, Maine, USA in July of 2000. Gravid females were avoided and all animals were kept in culture in sieved sediment (<1 mm) and with 0.45 μm-filtered seawater for a minimum of 7 days prior to use.

Sediment was collected at the same time as worms and was sieved (<1 mm) and frozen (-20˚C) prior to use. Thawed sediment was spiked by adding the desired amount of pyrene, dissolved in a minimum of acetone, to a slurry of sediment and 0.45 μm filtered seawater to give a nominal concentration of 25 μg pyrene g^{-1} sediment (dry weight, dw). Seawater used in the experiments was always filtered unless otherwise stated. The slurry was placed on a stand with a power drill fitted with a mud slinger and was mixed continuously for a minimum of 24 h. The slurry was then allowed to settle, the

water was decanted, and the sediment was allowed to sit at 5°C for one week prior to use in microcosms.

METABOLITE IDENTIFICATION EXPERIMENT

In a separate experiment, accumulation of pyrene and induction of pyrene metabolism in *N. diversicolor* was studied by exposing worms to 136 μg pyrene g^{-1} sediment (dw). Worms were sampled daily and pyrene and 1-hydroxypyrene were quantified using synchronous fluorescence spectroscopy (see Chapter 4). Accumulation of pyrene and formation of 1-hydroxypyrene in *N. diversicolor* reached quasi-steady state tissue concentrations after approximately 3 days of exposure. Subsequently, an exposure period of 5 days was chosen in later experiments.

Total metabolite pattern was studied by exposing worms to 25 μg pyrene g^{-1} sediment (dw) in a microcosm setup fitted with an airstone, changing overlying water daily during the five day exposure period. On day five, 5 worms were used for tissue analysis and 5 were taken for gut fluid extraction. Unexposed worms were kept separate in an identical microcosm and sampled at the end of the experiment. Pyrene, 1-hydroxypyrene and phase II metabolites were identified using high pressure liquid chromatography (HPLC) with diode array (HPLC/DAD) and fluorescence detection (HPLC/F). Fractions of aqueous phase II metabolites were collected, stored at –80°C and reinjected on a liquid chromatograph coupled with a mass spectrometer (LC/MS) at a later time.

SAMPLING AND SAMPLE PREPARATION

Worms were collected after the five day exposure period and allowed to purge their guts in individual petri dishes filled with 10 ml seawater for a maximum of 12 hours prior to extraction - a time sufficient to empty their guts of sediment. Tissue extraction was performed by homogenizing whole worms in 1 ml methanol using a Tissue-Tearor™ (Biospec Products, Inc. Bartlesville, OK, USA) at full speed for 1 minute and subsequently sonicating samples for 10 minutes. Samples were then centrifuged at 420 g (IEC Centra MP4R centrifuge fitted with a 49119 rotor head) for 10 minutes to precipitate any debris and 500 μL of supernatant was filtered through a 0.22 μm syringe filter and transferred directly to brown HLPC vials without any further sample preparation.

Gut fluid from *N. diversicolor* was removed under a microscope, by carefully cutting open the body wall and inserting a small gauge hypodermic needle mounted on a micromanipulator fitted with a syringe. Each worm yielded only several μL of gut fluid and therefore gut fluid extracted from 5 worms was combined (approx. 30 μL total) by rinsing the needle and syringe using the same 500 μL of methanol. The mixture was filtered through a 0.22 μm syringe filter and analyzed using HLPC/F.

ENZYMATIC HYDROLYSIS OF CONJUGATED METABOLITES

The presence of glucuronic acid and sulphate conjugates of 1-hydroxypyrene in tissue samples was studied by enzymatic hydrolysis of manually collected chromatographic peaks. Glucuronidase-arylsulfatase enzymes effectively hydrolyse the conjugates to give free 1-hydroxypyrene. 100 μL aliquots of a collected fraction was

diluted to 500 μL with deionized water (DI) and incubated for 2 hours at 37˚C with 10 μL of glucuronidase-arylsulfatase solution. After addition of 500 μL ice-cold 100% ethanol the samples were mixed and centrifuged at 420 g for 5 minutes to precipitate denatured protein. The supernatant was transferred to brown HPLC vials and analyzed using HLPC/F.

CHROMATOGRAPHY

Total metabolite pattern was analyzed on a Hitachi D-7000 HPLC using a method optimized for pyrene metabolites provided by Dr. Gerard Stroomberg (AquaSense, Amsterdam, NL). A Supelcosil LC-PAH column, 25cm x 4.6 mm, 5μm (Supelco, Bellefonte, PA, USA) was used and the acetonitrile/water (v/v) gradient profile went from 5/95 linearly to 90/10 over 40 min, and then held at 90/10 for 10 min at a flow rate of 0.8 ml min^{-1}. Detection was by absorbance at 339 nm and fluorescence at $\lambda_{EX/EM}$ = 346/384 nm for metabolites/conjugates and $\lambda_{EX/EM}$ = 333/384 nm for parent compound. Injection volume was 100 μL and column temperature was kept at 28˚C. Peaks were identified with a Hitachi L-7450 Diode Array Detector (DAD) by collecting spectra in the 190-370 nm range.

LC/MS^N

An Iontrap LCQ Deca (ThermoFinnigan, San Jose, CA, USA), fitted with an atmospheric pressure chemical ionization (APCI) probe connected to a HPLC/DAD system (TSP Spectra Systems), was used to identify the collected fraction of aqueous metabolites. The method is described in detail in Chapter 2 and Giessing and Lund

(2002). Briefly, the chromatographic method used was identical to the one described above. Interfacing an HPLC with a mass spectrometer requires the use of volatile, mobile phase additives. Adding 10 mM ammonium acetate to the eluent and adjusting pH to 5 was chosen for this purpose. Due to the acidic characteristics of the supposed glucuronide conjugate, samples were analyzed using APCI in negative ionization mode ($APCI^-$). The mass spectrometer used in this experiment is equipped with an ion trap which allows for isolation of specific product ions and performing subsequent multiple MS experiments (MS^n) across a single chromatographic peak in real time.

RESULTS

PYRENE METABOLITES IN TISSUE

An HPLC/UV absorption trace of a methanol extract of tissue of a single *N. diversicolor* exposed to 25 μg pyrene g^{-1} sediment (dw) for 5 days (Figure 2.1, top), shows 3 peaks marked I, II, III, at 8.9, 32.9 and 42.2 minutes respectively. These peaks were not present in the chromatogram of an unexposed worm (dotted line). The high absorption band in the first 5 minutes of the chromatogram is due to polar endogenous compounds (i.e. protein and carbohydrates) from polychaete tissue and is not related to pyrene metabolism. The 339 nm HPLC/UV chromatograms of all tissue samples from exposed *N. diversicolor* were identical to the one shown in Figure 2.1. The corrected DAD UV absorption spectra of peaks I, II, and III are presented in the middle panel of Figure 2.1. Peaks II and III are readily identified by their retention times (32.8 min and 42.2 min respectively) and their DAD absorption spectra as 1-hydroxypyrene and pyrene respectively.

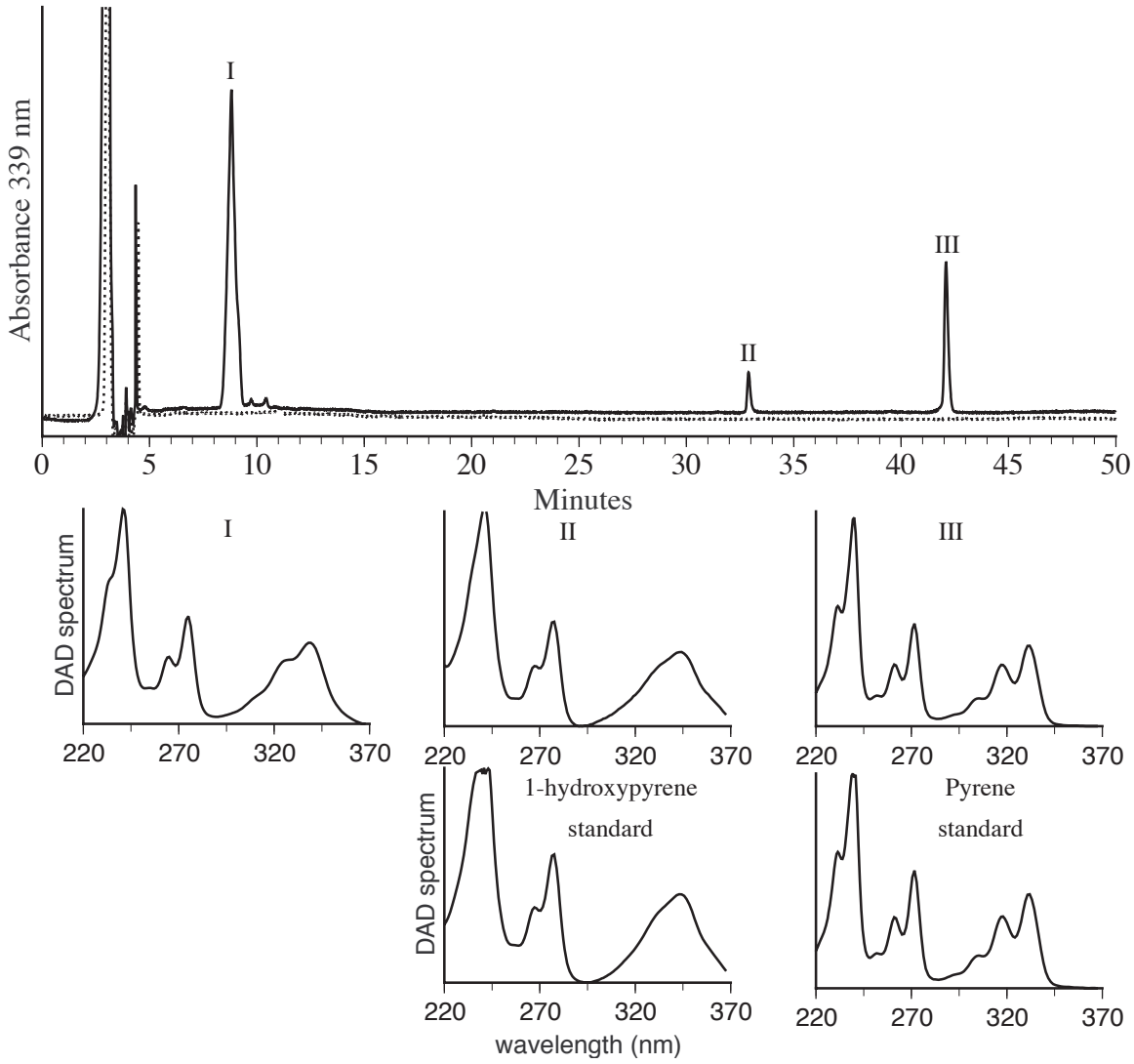

Figure 2.1 HPLC 339 nm UV chromatogram of a single *Nereis diversicolor* exposed to sediment-associated pyrene.

Top: 339 nm chromatogram. Peaks not present in 339 nm UV trace from an unexposed worm are marked I, II, and III. Bottom: DAD absorption spectra of peak I, II, and III and spectra of 1-hydroxypyrene and pyrene standards.

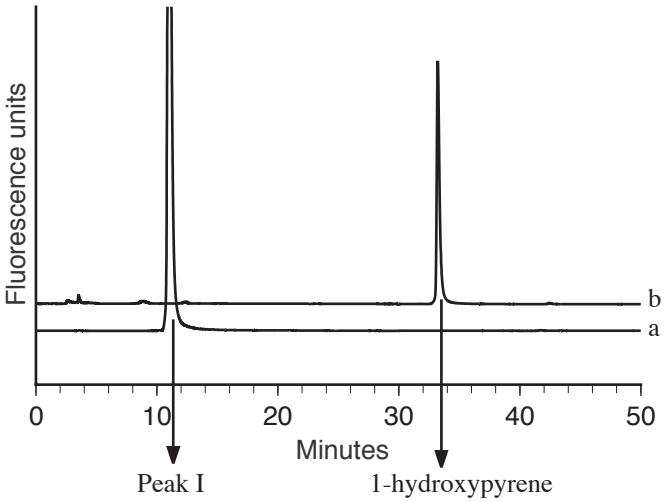

Figure 2.2 The effect of glucuronidase-arylsulfatase treatment of unidentified aqueous metabolite.

HPLC/F detected chromatograms ($\lambda_{EX/EM}$ = 346/384 nm) of peak I manually collected from the sample presented in Figure 2.1. Chromatogram **a** shows the unhydrolyzed conjugate after fractionation and chromatogram **b** shows the same sample after hydrolysis, featuring 1-hydroxypyrene.

The more hydrophilic Peak I with a retention time of 8.9 minutes has a UV spectrum similar to 1-hydroxypyrene and was collected manually for enzymatic hydrolysis. Figure 2.2 shows the effect of glucuronidase-arylsulfatase treatment of peak I. The lower chromatogram (a) is the fluorescence trace ($\lambda_{EX/EM}$ = 346/384 nm) of the manually collected fraction of peak I and the upper chromatogram (b) is the fluorescence trace of the same sample after glucuronidase-arylsulfatase hydrolysis. The original peak at 8.9 minutes has disappeared and 1-hydroxypyrene is identified at 32.8 min indicating either a glucuronide or sulfate conjugate. All fractions of peak I (n = 5) collected from *N. diversicolor* tissue samples that were treated with glucuronidase-arylsulfatase gave identical chromatograms after enzymatic hydrolysis. Complete enzymatic hydrolysis of peak I allows for the quantification of conjugated 1-hydroxypyrene in tissue samples by measuring the increase in 1-hydroxypyrene in the sample after glucuronidase-arylsulfatase treatment. Total PAH is defined as the sum of pyrene, 1-hydroxypyrene and hydrolysable conjugate in a sample. Of the total PAH in tissue, 1-hydroxypyrene glucuronide, 1-hydroxypyrene and pyrene constituted 73% ± 23, 2% ± 1, and 25% ± 23 (average ± SD, n = 5) respectively.

Figure 2.3 shows the fluorescence trace of the tissue extract of the same sample presented in Figure 1. Peak I, II, and III in the fluorescence spectrum are readily identified by comparing retention times to the 339 nm UV trace in Figure 2.1. The fluorescence chromatogram shows three additional peaks marked IV, V, and VI between 20 and 25 minutes which are not present in the 339 nm UV trace.

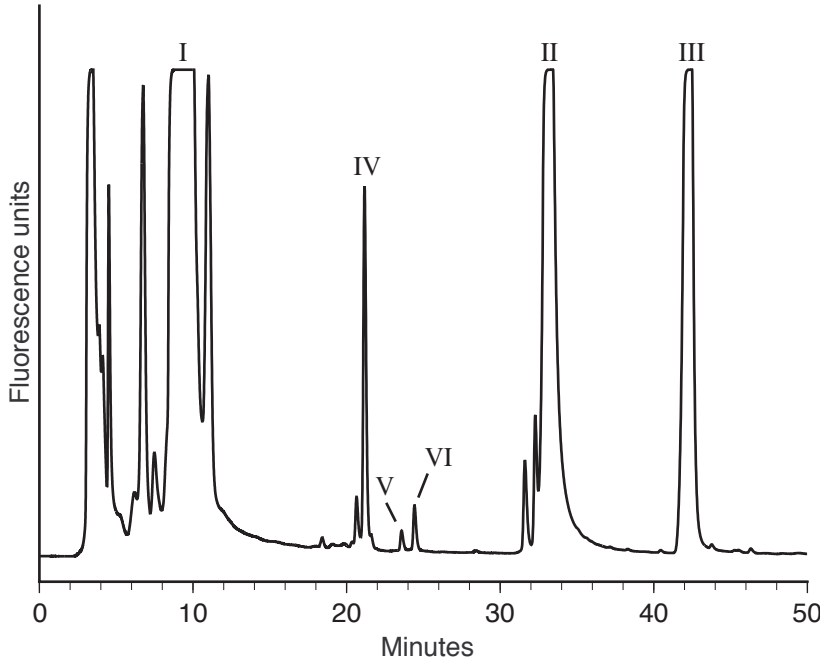

Figure 2.3 HPLC/F chromatogram of the tissue sample presented in Figure 2.1. Beside peaks I-III, at least three additional unidentified peaks, marked IV through VI, are present in the fluorescence trace.

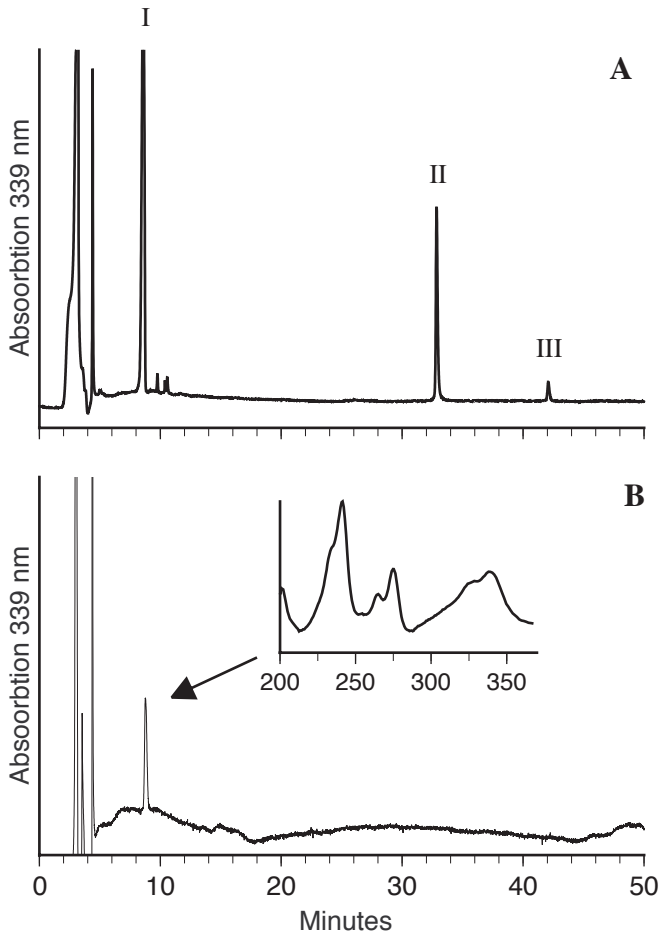

Figure 2.4 HPLC chromatograms of gut fluid and water from defecation dish.

A HPLC 339 nm UV chromatogram of gut fluid taken from five exposed *Nereis diversicolor*. Peaks marked I, II, and III can be identified by comparing retention time and DAD spectrum as identical to those shown in Figure 2.1. **B** HPLC 339 nm UV chromatogram and DAD (insert) of water taken from the defecation dish of an exposed worm. Retention time and DAD spectrum of the small peak is identical to peak I.

These peaks could not be identified. The large peak at 21.5 minutes (peak IV on Figure 2.3) was collected manually and its fluorescence spectra (em = 430 nm) was similar to the fluorescence spectra of peak I (data not shown), indicating a second 1-hydroxypyrene conjugate in the sample. The remaining peaks were present with varying intensity in all samples of pyrene-exposed worms, but their intensity in collected fractions were too low to allow identification.

PYRENE METABOLITES IN GUT FLUID

The 339 nm UV trace of the diluted gut fluid sample taken from pyrene-exposed worms (Figure 2.4A) shows the 1-hydroxypyrene conjugate, 1-hydroxypyrene, and pyrene at 8.7, 32.8 and 42.2 minutes, respectively, which was not present in gut fluid from unexposed worms. DAD spectra of the 3 small peaks at 10 minutes had no spectroscopic features that could be related to pyrene or 1-hydroxypyrene (data not shown) and are presumably not related to pyrene metabolism. Of total PAH in gut fluid, 1-hydroxypyrene glucuronide, 1-hydroxypyrene and pyrene constituted 90%, 9%, and 1% respectively, based on a triplicate measurement of the pooled gut fluid sample. The relative distribution among chemical forms in gut fluid versus tissue was not significantly different (t-test, $p > 0.05$).

Prior to homogenization, all worms were placed in 10 ml seawater and allowed to purge their guts for up to 12 hours. A small sample (1 ml) of this water was passed through a 0.22 μm syringe filter and analyzed using HPLC/DAD (Figure 2.4B). The DAD spectrum of the small peak at 8.7 minutes (insert) is identical to that of the

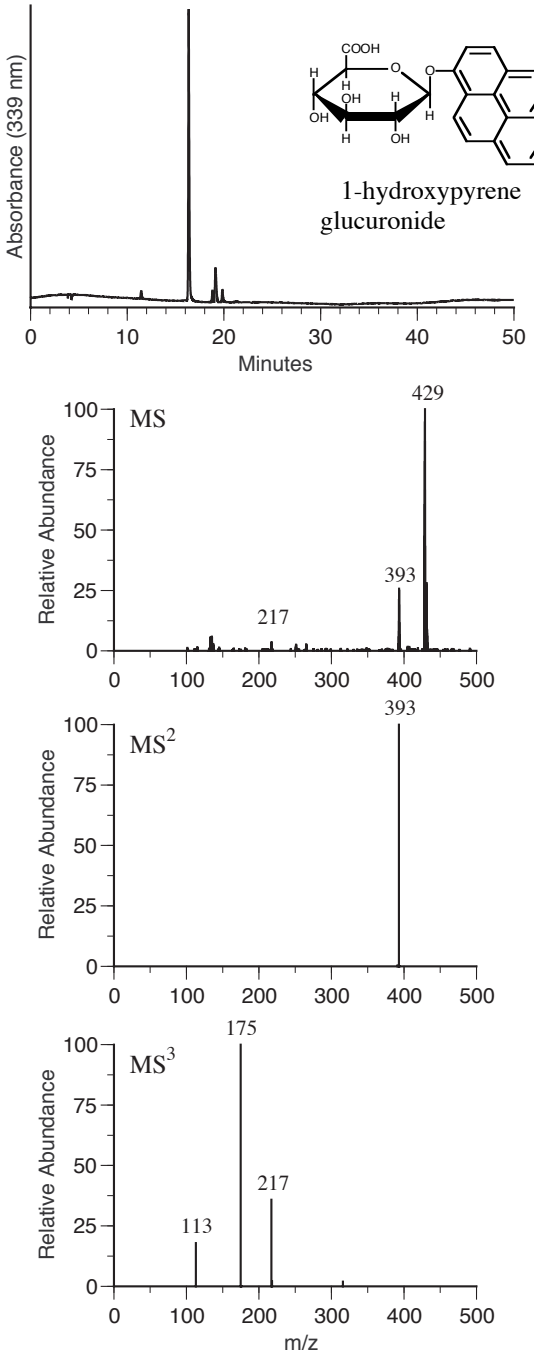

Figure 2.5 LC/MS of unidentified peak from Figure 2.1.

(**Top**) 339 nm LC chromatogram and (**bottom**) APCI-MS^n product ion spectra. The peak is identified as 1-hydroxypyrene glucuronide (insert on chromatogram). See text for details.

1-hydroxypyrene-conjugate peak in tissue (Figure 2.1) and gut fluid chromatograms (Figure 2.4A).

LC/MS^N OF UNIDENTIFIED PEAK

APCI$^-$ mass spectrometric analysis of peak I from Figure 2.1 is presented in Figure 2.5. Retention time of the peak in the 339 nm chromatogram (Figure 5, top) shifted to 16.5 minutes after the application of the ammonium acetate buffer. The dominating mass m/z = 429 of the peak at 16.5 minutes corresponds to the molecular ion of 1-hydroxypyrene glucuronide plus eluent adducts, $[M - H + 2H_2O]^-$, m/z = 393 is the 1-hydroxypyrene glucuronide molecular ion $[M - H]^-$, and m/z = 217 corresponds to the 1-hydroxypyrene $[M - H - glucuronide]^-$ fragment ion. Ion-trap MS^n allows multiple MS experiments to be performed across an HPLC peak thereby affording more detailed structural information. To understand fragmentation pathways and connect them back to the parent molecule, MS^2 and MS^3 experiments were conducted by isolating the dominating mass of each subsequent MS experiment in the ion trap and applying 30% (arbitrary units) of the available chemical ionization energy. MS^2 experiments on m/z 429 yielded a simple product ion spectrum with a dominant ion at m/z 393, corresponding to the precursor ion with the loss of eluent adducts (Figure 2.5, middle). The product ion spectrum collected in the MS^3 experiments (Figure 2.5, bottom) for the ion at m/z 393 yielded a relative simple spectrum with ions at m/z 217, 175, and 113, corresponding to fragment ions of 1-hydroxypyrene (m/z = 217) and glucuronide (m/z = 175); m/z = 113 is a known fragment of m/z = 175 (Yang et al. 1999).

DISCUSSION

IDENTIFICATION OF PYRENE METABOLITES IN TISSUE

The purpose of the present study was to investigate formation of pyrene metabolites in the marine deposit-feeding polychaete *N. diversicolor*. Special attention was given to detection and identification of phase II biotransformation products and detection of 1-hydroxypyrene in gut fluid.

Besides pyrene, only two metabolites, 1-hydroxypyrene and a 1-hydroxypyrene glucuronide conjugate, were identified in the 339 nm UV trace from tissue extracts of *N. diversicolor* exposed to 25 μg g^{-1} sediment-associated pyrene (Figures 2.1, 2.2, and 2.5). To our knowledge, this identification of 1-hydroxypyrene glucuronide is the first direct identification of a phase II metabolic product in a marine deposit-feeding polychaete. Previous studies of PAH metabolism in marine polychaetes have been limited to separation into aqueous and polar metabolites based on three-phase extraction schemes and using ^{14}C-labeled parent compounds (i.e. Christensen et al. 2002a; Kane Driscoll and McElroy 1996; Kane Driscoll and McElroy 1997; McElroy 1990; McElroy et al. 2000). *N. diversicolor* has known PAH metabolic capability (Christensen et al. 2002a; Kane Driscoll and McElroy 1996; Kane Driscoll and McElroy 1997). Christensen et al. (2002a) recently identified 1-hydroxypyrene in tissue from *N. diversicolor* exposed to sediment-associated pyrene for two weeks. Their metabolite identification used a three-phase extraction scheme separating parent PAH, water-soluble and water-insoluble metabolites. However, the authors only reported HPLC/UV analysis of the water-insoluble fraction, thereby limiting their metabolite identification to 1-hydroxypyrene.

McElroy (1990) found that a congener species, *Nereis virens*, rapidly accumulated and metabolized radiolabeled benz[a]anthracene (BA) and that most radioactivity was either in the form of water soluble or unextractable, bound metabolites with very little remaining as BA. Their study also showed that levels of water soluble and bound fractions increased over time. Glucuronidase-arylsulfatase treatment of aqueous extracts could only convert 2-6% of radioactivity to non-polar, solvent extractable metabolites, indicating that the water soluble fraction is not conjugated to glucuronic acid or sulfate but to some other endogenous compound such as glucose which is not hydrolyzed by glucuronidase-arylsulfatase. In a recent study McElroy et al. (2000) surveyed *in vivo* metabolism of ^{3}H-B[a]P in 10 small marine invertebrates from 3 phyla, Annelida, Mollusca, and Arthropoda. Metabolite identification was done using a liquid:liquid extraction scheme of ^{3}H- B[a]P and HPLC radiochromatography of tissue extracts from a representative species of each phyla. High variability in metabolic capability within each phylum was observed, with polar metabolites ranging from 7% to 96% in annelids. The radiochromatograms presented indicated at least 4 identifiable, phase I, ^{3}H-B[a]P metabolites and 2, possibly 3, unidentified phase II metabolites in animal extracts from *Nereis succinea* (McElroy et al. 2000). In a study of PAH metabolism in the deposit-feeding polychaete *Capitella* sp. I, Forbes et al. (2001) tentatively identified at least two, hydroxylated, phase I metabolites of fluoranthene.

Chemicals with phenolic structure are substrates for both carbohydrate and sulfate conjugation. In vertebrates, the preferred carbohydrate cosubstrate is uridine 5'diphosphoglucuronic acid (UDP-glucuronic acid), while in marine invertebrates uridine 5'diphospho-glucose (UDP-glucose) is indicated to predominate over UDP-glucuronic

acid as the preferred carbohydrate cosubstrate (Livingstone 1998). However, foreign compounds are rarely metabolized in the same manner by even closely related species (Livingstone 1998) and identification of 1-hydroxypyrene glucuronide as the major phase II metabolite in *N. diversicolor* further emphasizes this commonly observed trend.

The fluorescence trace (Figure 2.3) revealed at least 3 additional peaks not detected in the UV trace (Figure 2.1) of the same tissue sample. Fluorescence spectra (em = 430 nm) measured from the fractionated peak IV from a single tissue sample resembled that of 1-hydroxypyrene glucuronide (data not shown). This similarity suggests that a second conjugate is present in the sample. However, the intensity of the peak was low and fractions from more samples would need to be pooled in order to determine the exact identity of this conjugate. The two additional peaks marked V and VI in the fluorescence spectrum appeared with varying intensity in all the examined worms. They were both too low in intensity to be reliably identified by the current method and are most likely insignificant in the mass balance. Giessing and Lund (2002) presented 339 nm UV evidence for two additional aqueous metabolites in tissue extracts from *N. diversicolor* exposed to 50 μg pyrene g^{-1} sediment (dw). The peaks were low in intensity and could not be identified by their MS spectra due to ion suppression by very abundant co-eluting worm tissue impurities. Deconjugation with glucuronidase-arylsulfatase enzymes is known to effectively hydrolyze one of the two peaks to 1-hydroxypyrene, indicating the presence of a sulfate conjugate (see Chapter 3). As mentioned earlier, even closely related animal species vary considerably in the extent to which they metabolize foreign compounds. Most species have a preferred route of conjugation but other routes are still available and utilized. Furthermore, the balance between different phase II pathways can

depend on the concentration of phase I product delivered (Fry 1987). No increase in the number of pyrene conjugates formed was observed when terrestrial isopods were fed a single high dose of pyrene compared to continuous exposure of pyrene for 14 d in the food, however, the relative levels of pyrene metabolites did change (Stroomberg et al. 1999). Thus, identification of a sulfate conjugate at the higher pyrene exposure concentration seems to suggest either an induction of phase I enzymes resulting in increased production of 1-hydroxypyrene, or saturation of the glucuronidation pathway at faster rates of 1-hydroxypyrene generation, leading to accumulation of unconjugated 1-hydroxypyrene thereby increasing sulphate and other conjugation pathways.

RELATIVE DISTRIBUTION OF PYRENE METABOLITES IN TISSUE AND GUT FLUID

Of the total pyrene in worm tissue roughly 73% was in the form of 1-hydroxypyrene glucuronide and only 2% as 1-hydroxypyrene indicating an efficient turnover of 1-hydroxypyrene in *N. diversicolor* tissue. Christensen et al. (Christensen et al. 2002a) reported up to 50% of total body burden as aqueous metabolites in *N. diversicolor* after 42 days of exposure to sediment-associated pyrene. The results for *N. diversicolor* presented in this paper agree with those seen for B[a]P and benz[a]anthracene in nereid polychaetes. In both *N. diversicolor* (75%) and *N. virens* (58%), most B[a]P was recovered as aqueous metabolite and in *N. virens* ≤10% of total body burden was recovered as benz[a]anthracene (Kane Driscoll and McElroy 1996; McElroy 1985). In another deposit-feeding marine polychaete, *Arenicola marina*, more than 80% of total body burden remained as pyrene in both a short (11 day) and a long term (52 day) experiment (Christensen et al. 2002a) and Kane Driscoll and McElroy

(1996) reported less than 20% of total body burden as polar and aqueous B[a]P metabolites in a screening experiment of seven species of marine polychaetes.

A similar mass balance made for gut fluid yielded 90% as 1-hydroxypyrene glucuronide and 9% as 1-hydroxypyrene. Though based on a triplicate measure of the pooled gut fluid sample, the mass balance for gut fluid presented here seems only to suggest that the relative distribution of pyrene-derived compounds in gut fluid resembles that of tissue. In a study of 9-OH-B[a]P, a highly lipophilic primary metabolite of B[a]P, in American lobster (*Homarus americanus*) Li and James (2000) found that 9-OH-B[a]P was readily conjugated to sulfate and glucose, and that despite its high lipophilicity, 9-OH-B[a]P was excreted from the lobster hemolymph and tissue much more rapidly than B[a]P. More studies on pharmacokinetics of hydroxylated PAH and their metabolic conjugates in individual animals are needed in order to identify if this holds true in tissue and gut fluid of deposit-feeding marine invertebrates.

Fecal elimination is assumed to be the major route of removal of contaminants in marine polychaetes (Forbes et al. 1996; Goerke and Ernst 1977; Kane Driscoll and McElroy 1997). Unlike other marine invertebrates such as mollusks and crustaceans, annelid worms do not have an easy separable digestive gland commonly used in biotransformation studies. Annelids have specialized tissue associated with the alimentary tract (chloragogen), whose function is comparable to the mussel digestive gland as well as the vertebrate liver. Assuming that chloragogen tissue is the site with the highest biotransformation capacity in marine polychaetes, elimination of aqueous metabolites through the gut would be expected. Elimination of metabolic products through the gut is supported by the identification of 1-hydroxypyrene and

1-hydroxypyrene glucuronide in both gut fluid and defecation water from *N. diversicolor* (Figure 2.4). Goerke and Ernst (1977) found that fecal matter from *Nereis virens* contained between 7.5 and 41% of the accumulated dose of PCB, of which 72-94% was polar metabolites. Polar and aqueous metabolites of PAHs eliminated in fecal matter accounted for up to 50% of accumulated dose in the two deposit-feeding polychaetes, *Capitella* sp. I. and *N. diversicolor* (Forbes et al. 1996; Kane Driscoll and McElroy 1997). Whether the 1-hydroxypyrene glucuronide identified in defecation water is eliminated directly to the aqueous phase or is desorbed from fecal matter after defecation remains to be investigated.

ORIGIN OF IDENTIFIED METABOLITES

1-hydroxypyrene might be of either biotic and abiotic origin. The pyrene-contaminated sediment used in the experiment was allowed to sit in the lab for a week prior to worm addition and the containers were exposed to day/night cycles of artificial light throughout the acclimation and experimental period. 1-hydroxypyrene has been identified as a product of photochemical oxidation although this process yields primarily 1,6- and 1,8-pyrenequinones (Sigman et al. 1998). However, sediment samples taken at the time of worm addition and at the end of the experiment showed no detectable traces of 1-hydroxypyrene (see Table 5.1) and it seems likely that abiotic processes did not contribute significantly to the measured 1-hydroxypyrene. It is also well known that PAHs can be degraded aerobically by microorganisms (Cerniglia 1992). Low-molecular weight PAH degrades readily in sediments whereas high-molecular weight PAH appear more resistant to microbial attack, even in aquatic ecosystems which are chronically

exposed to petrogenic hydrocarbons (Heitkamp and Cerniglla 1987). Microorganisms associated with *N. diversicolor* gut or body surfaces could be responsible for the observed biotransformation of pyrene. However, prokaryotic microorganisms in general utilize dioxygenase enzymes to incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols, and the proposed microbial catabolic pathway for pyrene does not produce 1-hydroxypyrene (Cerniglia 1992). Because 1-hydroxypyrene is the only measured phase I metabolite it seems likely that the measured pyrene-derived compounds are neither abiotic nor microbial in origin and are instead due to worm metabolism.

Using pyrene as a model compound for PAH metabolism in marine invertebrates has the clear advantage over, for example, B[a]P (McElroy et al. 2000) and fluoranthene (Forbes et al. 2001) by having only one phase I metabolite. Detection of 1-hydroxypyrene provides insight into accumulation of pyrene, integrated over all uptake routes, and modulated by bioavailability. 1-hydroxypyrene is only detected if pyrene is accumulated and metabolized by the animal. Pyrene usually co-occurs with the more toxic PAHs and the limited number of total phase I and II metabolites makes pyrene an attractive model compound to assess for both PAH exposure and species specific biotransformation capacity of marine invertebrates.

Limitations in the analytical procedure used to study biotransformation products should always be considered carefully even when dealing with simple biotransformation pathways like the one presented for pyrene in this chapter. It is possible that other polar metabolites that do not exhibit UV absorption at 339 nm or at the chosen excitation/emission fluorescence wavelengths were present in the samples of *N.*

diversicolor. Although the wavelength 254 nm is better for PAH measurements in complex mixtures, higher PAHs like pyrene have absorption maxima at higher wavelengths that can be utilized for more selective analysis (Krstulovic et al. 1976). Loss of aromaticity of one of the four rings with the formation of phenanthrene-like compounds would therefore not be detected by the present method. The software associated with the HPLC used in this study can extract multiple wavelength chromatograms from recorded DAD data and no extracted 254 nm chromatograms had additional peaks besides the three presented in Figure 2.1. Lack of additional peaks at 254 nm does not account for the possibility of pyrene-derived compounds falling outside the analytical window, i.e. co-eluting with tissue components in the first 5 minutes of the solvent gradient program. The presence of phenanthrene-like compounds originating from *N. diversicolor* metabolism of pyrene could be resolved by redoing the total metabolite experiment using radiolabeled pyrene. Stroomberg et al. (1999) reported a pyrene metabolite not showing UV absorbance at 339 nm in the hepatopancreas of a terrestrial isopod exposed to ^{14}C-pyrene. The metabolite never exceeded 5% by area of total radioactivity found in the sample and could not be conclusively identified as pyrene-derived by its DAD spectrum. Based on these arguments and the data presented in this paper it therefore seems likely that 1-hydroxypyrene and 1-hydroxypyrene glucuronide are the primary phase I and phase II metabolites of pyrene in *N. diversicolor*.

The liquid:liquid extraction scheme of radiolabeled PAH commonly applied in biotransformation studies yields little information on chemical speciation of the separated metabolites. Subsequent analysis of separated fractions, primarily using HPLC/UV, has been limited to phase I biotransformation products with commercially available standards

(e.g. Christensen et al. 2002a; Forbes et al. 2001; McElroy et al. 2000). The HPLC method presented in this paper is fast, less labor intensive than liquid:liquid extraction schemes, and incorporates both phase I and Phase II metabolites in the same chromatogram. Furthermore, it emphasizes the use of HPLC with fluorescent detection in studies of PAH biotransformation products. Fluorescence detection is usually 2 – 3 orders of magnitude more sensitive than UV detection, and by choosing excitation/emission wavelengths that are specific to the assumed PAH metabolites, low detection limits can be obtained (Santana Rodríguez and Padrón Sanz 2000). This study also shows that LC/MS is a powerful analytical tool for identification of phase II biotransformation products (Figure 2.5) even without standards and prior knowledge of the chemical speciation of metabolites. Modern ion-trap LC/MS^n allows multiple MS experiments to be performed across an HPLC peak affording more detailed structural data, and recent developments in ionization technology will expand the application of LC/MS to include the otherwise undetectable, non-polar, parent PAHs (Robb et al. 2000).

3. IDENTIFICATION OF 1-HYDROXYPYRENE GLUCURONIDE IN TISSUE OF MARINE POLYCHAETE *NEREIS DIVERSICOLOR* BY LIQUID CHROMATOGRAPHY/ION TRAP MULTIPLE MASS SPECTROMETRY

ABSTRACT

1-hydroxypyrene glucuronide is identified as the single major aqueous metabolite of the tetracyclic aromatic hydrocarbon pyrene, in tissue from a deposit feeding marine polychaete, *Nereis diversicolor*. Identification was performed using an iontrap mass spectrometer (LCQ Deca from ThermoFinnegan) fitted with a APCI probe and connected to a HPLC/DAD system. Besides 1-hydroxypyrene, the 339 nm UV trace of tissue samples from pyrene-exposed worms only showed one dominating peak that could be related to pyrene metabolism. Negative APCI-MS of this supposed 1-hydroxypyrene conjugate gave a characteristic signal at m/z 429 corresponding to the molecular ion of 1-hydroxypyrene glucuronide plus eluent adducts, $[M - H + 2H_2O]^-$. Fragmentation pathways were studied by isolating the abundant ion at m/z 429 in the ion trap and performing multiple mass spectrometric experiments (MS^n). MS^2 of m/z 429 gave a simple product ion spectrum with a dominant ion at m/z 393 corresponding to the molecular ion $[M - H]^-$ of 1-hydroxypyrene glucuronide. The product ion spectrum collected in the MS^3 experiment for the $[M - H]^-$ at m/z 393 gave ions at m/z 217, m/z 175 and m/z 113 corresponding to fragment ions of 1-hydroxypyrene (m/z 217) and glucuronide (m/z 175), and m/z 113 is a known fragment ion of m/z 175. Two low intensity peaks which could be related to pyrene metabolism by their DAD absorption

spectra were also present in the 339 nm UV chromatogram of tissue samples. The peaks could not be identified by their MS spectra in negative ion mode due to ion suppression by very abundant co-eluding impurities. The present method shows that LC/MS^n is a fast and useful analytical tool for identification of aqueous PAH biotransformation products in samples from relatively small marine invertebrates with limited sample preparation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a broad class of environmentally persistent organic compounds that are ubiquitous in both marine and terrestrial environments (Neff 1985). Marine invertebrates are known to accumulate PAHs from both particulate and dissolved phases (Meador et al. 1995) and the ability of marine polychaetes to metabolize PAHs has been documented for a few species (e.g. Forbes et al. 2001; McElroy et al. 2000). In marine invertebrates the ability to metabolize PAHs varies widely within and among phyla, though rates are much less than those observed in vertebrates (Livingstone 1994). Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P-450 enzyme suite which converts hydrophobic, lipid-soluble, parent PAH to water soluble metabolites in a two phase process. The enzymes of Phase I metabolism introduce a functional group (OH, COOH, NO_2 etc.) into the PAH, to which conjugase or other Phase II enzymes attach a large polar moiety (glucuronic acid, glutathione, sulfate, amino acid, etc.) so that the resulting conjugate can be readily excreted from the animal.

Traditionally, metabolite identification in marine invertebrates has been done using liquid:liquid extraction schemes of radiolabeled parent PAH (e.g. McElroy et al.

2000), identifying metabolites as activity measured in nonpolar, polar and aqueous solvent fractions. However, this approach does not yield any information about the chemical speciation of the aqueous phase II metabolites, and lack of commercially available standards makes identification using, e.g. HPLC, a complicated task. Furthermore, the PAHs often used in invertebrate biotransformation studies, benzo[a]pyrene (BaP) and fluoranthene, have complicated metabolite patterns with several phase I metabolic intermediates (Forbes et al. 2001; McElroy et al. 2000) thereby increasing the number of potential phase II biotransformation products.

Recent developments in liquid chromatography-ion trap mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ES) makes it possible to obtain considerable structural information on, and even identify biotransformation products (Coldham et al. 1998; Gu et al. 1999; Kostiainen et al. 1997) without the use of standards and with limited knowledge of metabolic capacity of the species under investigation. In the present study 1-hydroxypyrene glucuronide is identified using $LC/MS^3/APCI^-$, as the dominant aqueous metabolite of pyrene in tissue from the deposit feeding marine polychaete *Nereis diversicolor*.

Figure 1.1 shows the proposed biotransformation route for pyrene in *Nereis diversicolor* with three phase II biotransformation products commonly observed in marine invertebrates (Livingstone 1994). Measurement of pyrene metabolites, primarily 1-hydroxypyrene, in excretory products of animals and humans has gained considerable attention as a potential biomarker for PAH exposure and is widely used as a biomarker to study human PAH exposure (Levin 1995). 1-hydroxypyrene is the only observed phase I

metabolite of pyrene in *Nereis diversicolor* (Giessing et al. 2002) and also the only observed metabolic intermediate when pyrene is administered to pigs, rats, rabbits and a terrestrial isopod (Boyland and Sims 1964; Jacob et al. 1989; Keimig et al. 1983; Stroomberg et al. 1999). Thus, the limited number of potential phase II metabolites makes 1-hydroxypyrene an excellent marker in studies of species-specific PAH metabolic capabilities.

EXPERIMENTAL

Pyrene (98%), 1-hydroxypyrene (98%), ammonium acetate and HPLC grade solvents were purchased from Sigma-Aldrich (Copenhagen, Denmark). All water was Milli-Q quality (Millipore, MA, USA) and was filtered through a 0.22 μm filter prior to use.

POLYCHAETE COLLECTION AND SAMPLE PREPARATION

The marine deposit feeding polychaete, *Nereis diversicolor*, used in the experiment was collected at Store Havelse, Roskilde Fjord, Denmark in September of 2001. Detailed description on pyrene exposure setup and sample preparation is given by Giessing et al. (2002). In short, worms were exposed to sediment spiked with pyrene (50 μg g^{-1} sediment, dry weight) for 5 days prior to extraction. Tissue was homogenized in methanol using a Tissue Tearor (Biospec Products, Inc., OK, USA), centrifuged (500 g) for 3 minutes and supernatant was passed through a 0.45 μ syringe filter prior to injection on the LC/MS without any further sample preparation. A total of 10 worms were analyzed using the method presented here.

CHROMATOGRAPHY AND MS ANALYSIS

Due to the acidic characteristics of the supposed glucuronide conjugate of 1-hydroxypyrene, the samples were analyzed using $APCI^-$ on an Iontrap LCQ Deca from ThermoFinnigan (San Jose, CA, USA) connected in series to a HPLC diode array detector (DAD) system (TSP Spectra Systems). The system was controlled with Xcalibur software (vers. 1.2) and the following mass spectrometric conditions was used: capillary temperature 200˚C, APCI vaporizer temperature 450˚C, source voltage –6 kV, full scan (m/z = 80-600), negative ion mode, sheath gas flow 60 arbitrary units of nitrogen, setting the divert valve to the MS between 9-21 minutes and 33-35 minutes. The analytical column was a Supelcosil LC-PAH column, 250 x 4.6 mm, 5μm fitted with a C18 Supelcoguard guard column (Supelco, PA, USA). The eluent consisted of acetonitrile/10 mM ammonium acetate (pH = 5) and the gradient profile applied was 5/95 directly to 90/10 over 40 min, and then hold 90/10 for 10 min at a flow rate of 0.8 ml min^{-1}. UV detection was performed by measuring absorbance at 339 nm and collecting UV absorption spectra in the 250-400 nm range.

A 1-hydroxypyrene glucuronide standard was prepared using human UGT1A9 Supersomes from Gentest (Woburn, MA, USA). UGT1A9 is the most efficient catalyst of glucuronidation of 1-hydroxypyrene among the human isoforms of glucuronosyl-transferases (UGT) (Luukkanen et al. 2001). A 0.2 mL reaction mixture containing 0.05 mg UGT1A9 protein/mL, 1 mM uridine diphosphoglucuronic acid (UDPGA), 10 mM $MgCl_2$, 0.025 mg/mL alamethicin and 50 μM 1-hydroxypyrene in 50 mM Tris (pH = 7.5) was incubated at 37˚C for 20 minutes. The reaction was stopped by addition of 100 μL 94% acetronitrile/6% glacial acetic acid and centrifuged (10,000 g) for 3 minutes. 100 μL

of supernatant was injected directly on the LC/MS. Pyrene and 1-hydroxypyrene standards were prepared in methanol and data on standards will not be shown in the interest of conciseness.

RESULTS AND DISCUSSION

IDENTIFICATION OF 1-HYDROXYPYRENE GLUCUROINIDE

Figure 3.1 shows the extracted ion chromatogram (EIC) of m/z 392.5-393.5 and the 339 nm UV trace of a tissue sample from a worm exposed to sediment-associated pyrene. Both the 339 nm trace and the EIC show a distinct peak at 15.88 min and 15.91 min respectively that is not present in the chromatogram of an unexposed worm (data not shown). The large peak C at 15.88 min has a DAD spectrum like that of 1-hydroxypyrene, Figure 3.1B. The 339 nm trace shows two additional peaks at 43.86 and 34.73 minutes, marked A and B. The high absorption region in the first 7 minutes and the small peak at 11.27 minutes are due to endogenous compounds from polychaete tissue and are not related to pyrene metabolism. Pyrene and 1-hydroxypyrene are readily identified by comparing their retention times (43.86 min and 34.73 min respectively) and their DAD absorption spectra (Figure 3.1A and B) to those of standards. The large peak at 15.88 min gave a DAD spectrum (Figure 3.1C) like that of 1-hydroxypyrene (Figure 3.1B), and co-eluted with the prepared 1-hydroxypyrene glucuronide standard. Figure 3.2 (top) shows a full scan $APCI^-$/MS of the peak at 15.91 minutes. The dominating peak at m/z 429 corresponds to deprotonated 1-hydroxypyrene glucuronide plus eluent adducts, $[M - H + 2H_2O]^-$; m/z 393 is the 1-hydroxypyrene glucuronide ion $[M - H]^-$, and m/z 217 corresponds to deprotonated 1-hydroxypyrene $[M - H - glucuronide]^-$.

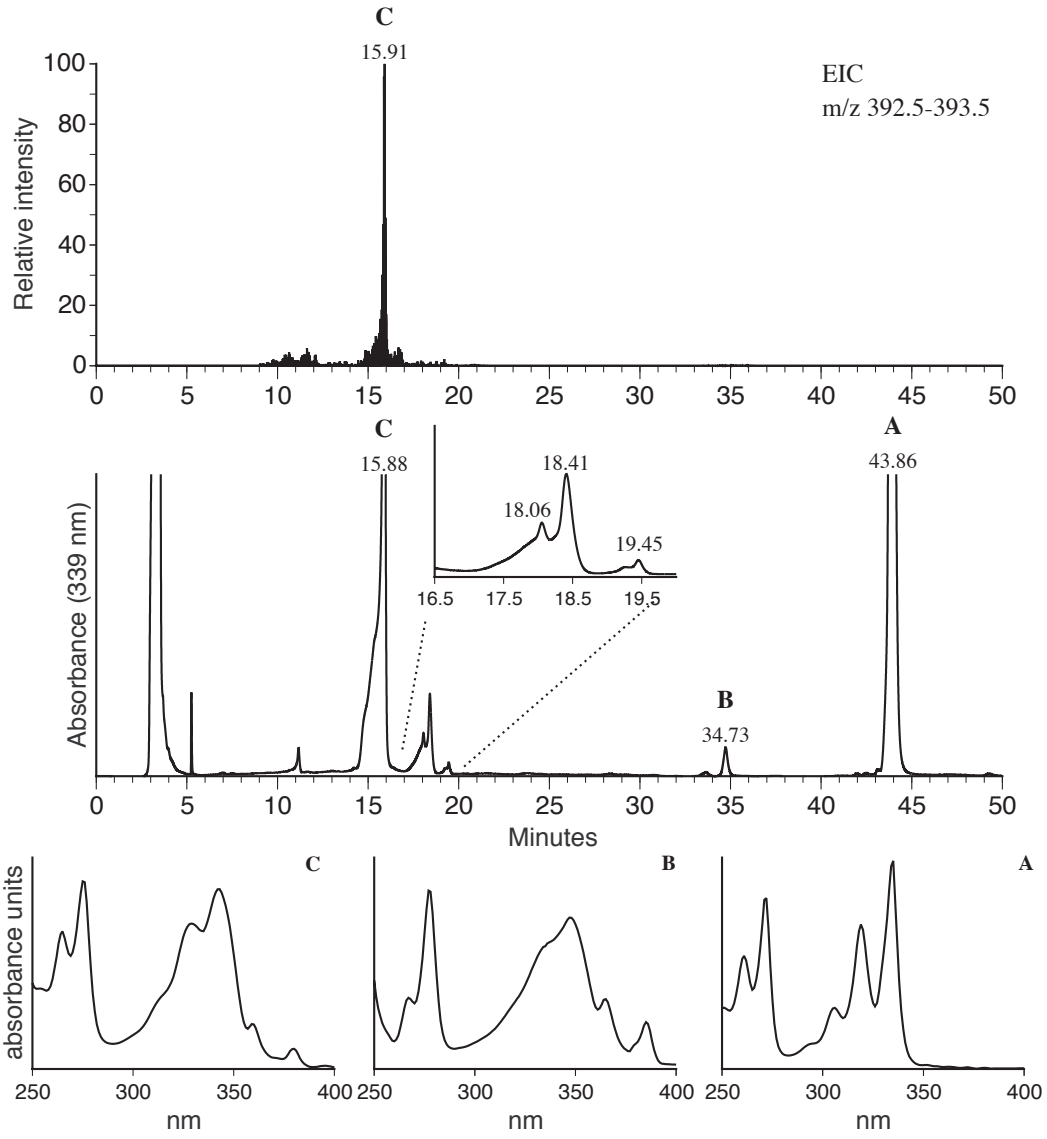

Figure 3.1 LC/MS of pyrene metabolites from *Nereis diversicolor*.

Extracted ion chromatogram (EIC) m/z 392.5-393.5 and 339 nm UV chromatogram of a tissue extract from a single polychaete. Figure 3.1A and Figure 3.1B are readily identified as the UV spectra of pyrene and 1-hydroxypyrene respectively. The insert show an enlargement of the low intensity peaks in the 16-21 minute window, see text for details.

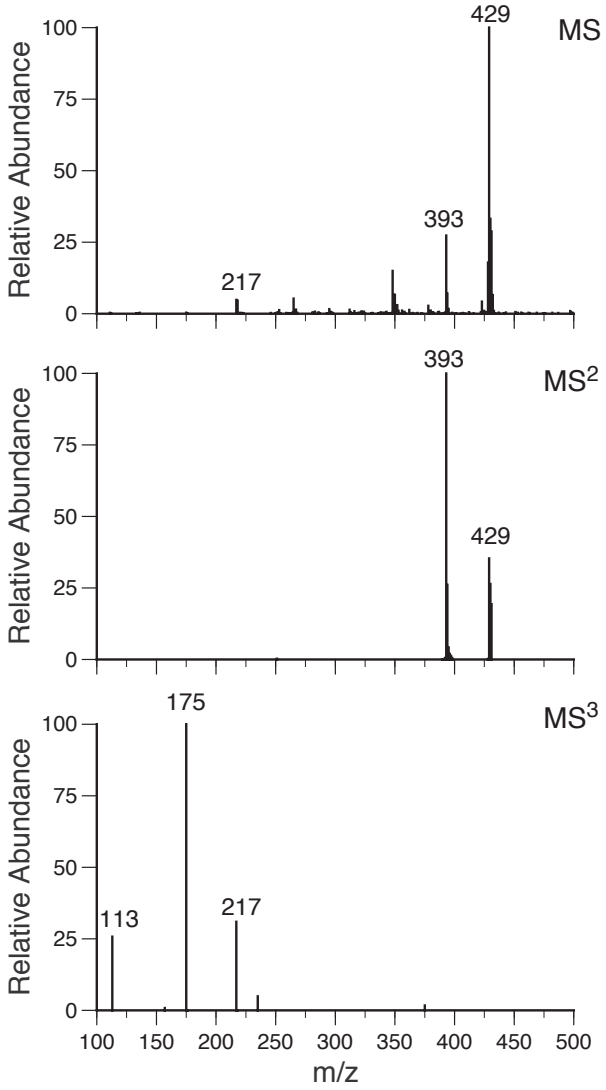

Figure 3.2 APCI$^-$ MS^3 analysis of peak C shown in Figure 3.1**.**

1-hydroxypyrene glucuronide is identified at m/z 393 as $[M - H]^-$. See text for details.

Ion-trap MS^n allows multiple MS experiments to be performed across an HPLC peak thereby affording more detailed structural information.

To understand fragmentation pathways and connect them back to the parent molecule, MS^2 and MS^3 experiments were conducted by isolating the dominant m/z value of each MS^n experiment in the ion trap and applying 30% (arbitrary units) of the available collision energy. MS^2 experiments of m/z 429 yields a simple product ion spectrum with a dominant ion at m/z 393, corresponding to the molecular ion $[M - H]^-$ with loss of eluent adducts (Figure 3.2, middle). The product ion spectrum collected in the MS^3 experiments (Figure 3.2 bottom) for the $[M - H]^-$ ion at m/z 393 yields a relatively simple spectrum with ions at m/z 217, 175 and 113, corresponding to fragment ions of 1-hydroxypyrene (m/z = 217) and glucuronide (m/z = 175); and m/z 113 is a known fragment of m/z 175 (Yang et al. 1999).

The formation of m/z of 175 (loss of 176 Da) and m/z 217 suggests that the $[M – H]^-$ ion at m/z 429 is a glucuronide conjugate, because a neutral loss of 176 Da is generally used as a specific method for detecting *O*-glucuronide conjugates in triple quadrupole mass spectrometry (Dalvie et al. 1997; Rudewicz and Straub 1986; Smith et al. 1996; Velic et al. 1995). Kostiainen et al. (1997) reported an accurate mass measurement of m/z 393.0986 for 1-hydroxypyrene glucuronide using negative-ion quadrupole low resolution electrospray mass spectrometry. In this study the measured mass of the 1-hydroxypyrene glucuronide $[M - H]^-$ ion was m/z 393. Accurate mass measurements using a low resolution instrument, like the LCQ-Deca, requires the sample ion to be completely separated from background ions which is not achieved for the real-world sample by the present chromatographic method. However, analysis of the prepared

glucuronic acid standard gave DAD and MS^3 spectra identical to those presented in Figure 3.1C and Figure 3.2.

Most enzymes responsible for biotransformation of xenobiotics in mammals have also been identified in invertebrates. Phase I enzymes, like cytochrome P450s, have been found or indicated in annelids, crustaceans, echinoderms and molluscs (Brown et al. 1998; Den Besten 1998; James and Boyle 1998; Lee 1998). In the case of phase II metabolism, glucose and sulphate conjugation is indicated to predominate over glucuronic acid conjugation in marine invertebrates (Livingstone 1998). However, as for mammals and fish, large species-specific differences in phase II metabolism are observed among invertebrates and identification of 1-hydroxypyrene glucuronide as the major phase II metabolite in *Nereis diversicolor* further emphasizes this commonly observed trend.

IDENTIFICATION OF LOW INTENSITY PEAKS

The 339 nm UV chromatogram also showed two peaks at 18.06 and 18.41 minutes (insert on Figure 3.1) of low intensity, and their DAD absorption and MS spectra are presented in Figure 3.3A and Figure 3.3B respectively. The small peak at 19.45 minutes was too weak to be related to pyrene metabolism by its DAD spectrum. Both peaks had a 1-hydroxypyrene DAD spectrum but did not give conclusive mass spectra in negative ion mode. Deconjugation with glucuronidase-arylsulfatase enzymes of identical tissue samples from *Nereis diversicolor* have been shown to hydrolyze effectively the 18.41 minute peak but not the 18.06 minute peak to 1-hydroxypyrene, indicating the presence of a sulfate conjugate (see Chapter 4). Failure to observe $[M - H]^-$ at m/z 297 of

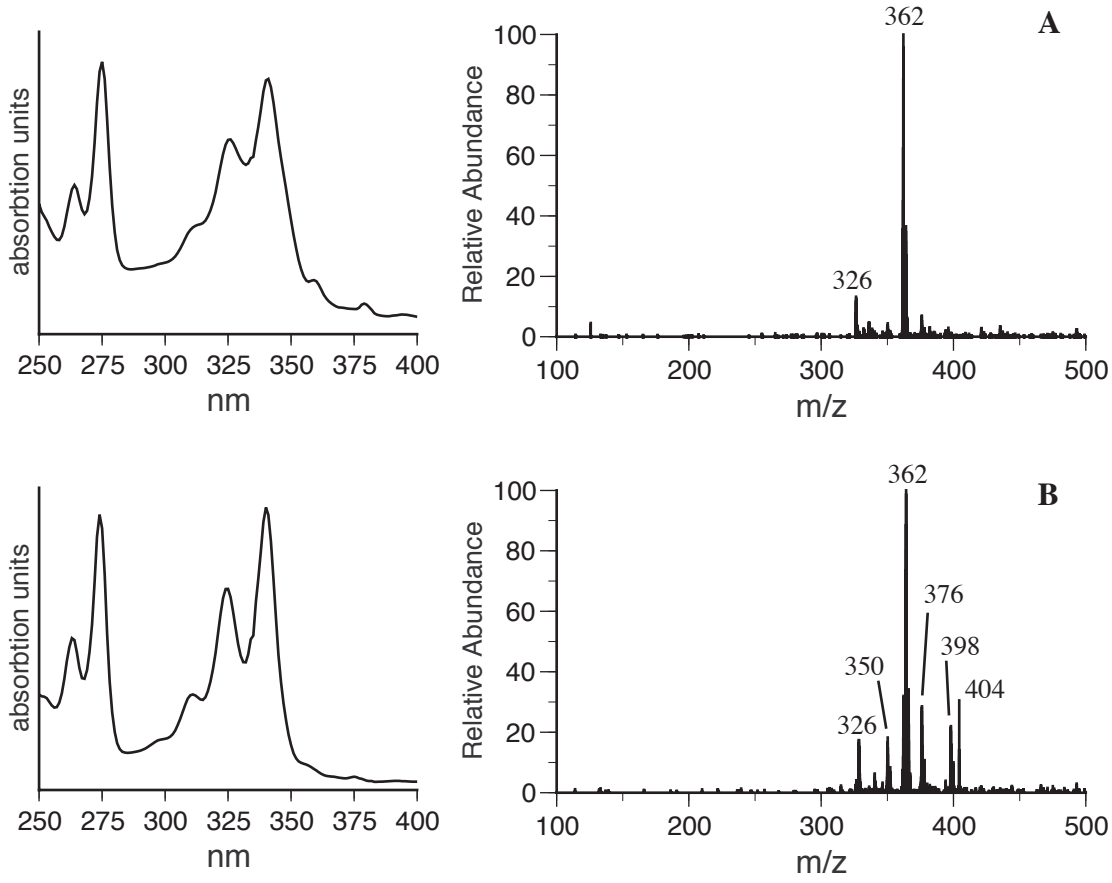

Figure 3.3 DAD absorption and negative ion spectra (MS) of the two small peaks presented in Figure 3.1.

The peaks at **A** 18.06 min and **B** 18.41 min could not be identified by their MS spectra due to ion suppression by very abundant co-eluding worm tissue impurities. See text for details.

a sulfate conjugate in Figure 3.3 is probably a case of ion suppression by very abundant co-eluding impurities. MS spectra of tissue extract from unexposed worms showed intense ions of m/z 362 and m/z 326 in negative ion mode (data not shown), indicating coelution of unidentified worm tissue compounds with the 1-hydroxypyrene conjugates.

The present study shows that LC/MS^n is a useful analytical tool for identification of phase II biotransformation products in tissue samples from relatively small marine invertebrates. Biotransformation experiments commonly use samples of tissue with the highest biotransformation capacity like digestive gland (hepatopancreas) or liver. Unlike other marine invertebrates like mollusks and crustaceans, annelid worms do not have an easy separable digestive gland. Annelids have specialized tissue (chloragogen), which function is comparable to the mussel digestive gland as well as the vertebrate liver, associated with the alimentary tract. Thus, due to the size and morphology of *Nereis diversicolor*, it is not possible to separate the 'reactive' tissue for analysis to increase analyte concentration. Instead, whole worm homogenate is used thereby increasing background ion signal considerably. Identification of the two low intensity peaks in negative ion mode will require development of a sample clean up procedure to eliminate influence of co-eluding background ions. However, the intensity of the peaks in the present study were always low and they are probably insignificant on a mass-balance scale.

Recent developments in LC/MS ionization technology will expand the application of LC/MS to include the otherwise undetectable non-polar parent PAHs (Robb et al. 2000). The present method is fast, provides detailed information on chemical speciation of pyrene metabolites, and is less labor intensive than the liquid:liquid extraction schemes

commonly applied in PAH biotransformation studies. Furthermore, choosing pyrene as a model compound for PAH metabolism reduces metabolite complexity so that information on species-specific PAH metabolic capabilities, much needed in risk assessment models, can be obtained.

4. SYNCHRONOUS FLUORESCENCE SPECTROMETRY OF 1-HYDROXYPYRENE: A RAPID SCREENING METHOD FOR IDENTIFICATION OF PAH EXPOSURE IN TISSUE FROM MARINE POLYCHAETES

ABSTRACT

The uptake of polycyclic aromatic hydrocarbons (PAHs) by marine deposit-feeding invertebrates can be determined by screening for PAH-derived metabolites. We identified conjugated 1-hydroxypyrene as the major metabolite in tissue of four species of deposit feeding polychaetes, *Nereis diversicolor*, *Nereis virens*, *Arenicola marina*, and *Capitella* sp.I. Synchronous fluorescence spectroscopy (SFS) provides a fast and simple method for both qualitative and quantitative analysis of 1-hydroxypyrene in all four species. We used HPLC with fluorescence detection combined with enzymatic hydrolysis of conjugated metabolites to validate the SFS assay and to investigate species-specific metabolite conjugation patterns. We observed good correlation between 1-hydroxypyrene concentrations determined by the two methods. A tentative aqueous metabolite identification scheme indicates that Nereid polychaetes predominately make use of glucuronide conjugation whereas *Capitella* sp. I. and *Arenicola marina* appear to utilize predominantly sulfate and/or glucoside conjugation. The usefulness of 1-hydroxypyrene as a biomarker for PAH exposure in deposit-feeding invertebrates is discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the marine environment (Neff 1985). PAHs are mainly formed as products from the combustion of fossil fuels but are also natural components of unaltered fossil fuels. The most important inputs of PAH to the marine environment are atmospheric fallout, spillage and seepage of petroleum and oil products, and industrial and domestic sewage. Concern over the fate and effect of PAHs is due to their persistence, ability to bioaccumulate, and acute toxicity (Cerniglia 1991; DeWitt et al. 1992; Landrum et al. 1991; Swartz et al. 1990; Weston 1990). Because some members of the PAH group found in the environment are also procarcinogens (Menzie et al. 1992), there is additional concern about trophic transfer of PAH residues and biotransformation products with ultimate exposure to humans.

PAHs are primarily bound to sediment particles and are readily accumulated by marine deposit-feeding invertebrates (Meador et al. 1995). Marine polychaetes have known PAH metabolic capabilities (Forbes et al. 1996; Giessing et al. 2002; McElroy et al. 2000), though the ability to metabolize PAH varies widely within and among phyla and rates are much lower than those observed in vertebrates (Livingstone 1994). Metabolism of PAHs in marine invertebrates is apparently related to the cytochrome P-450 enzyme suite which converts hydrophobic, lipid-soluble, parent PAH to water soluble metabolites, but the mechanisms by which the involved enzymes are regulated is still poorly understood (Hahn 1998).

Routine monitoring of PAHs in the marine environment usually involves the determination of parent PAHs in sediment samples. Benthic invertebrates are often used

to assess the toxicity, bioavailability, and the potential trophic transfer of PAH from sediments (Chapman and Wang 2001; Di Toro et al. 1991; Næs et al. 1999). However, chemical analysis of invertebrates provides little useful information for compounds that are rapidly metabolized by some species and information on species-specific PAH metabolic capacity is therefore critical in evaluating bioaccumulation data. Effects of PAHs are mainly caused by their metabolites rather than by parent compounds (James and Kleinow 1993; Livingstone 1993). Thus development of simple methods for detection and quantification of PAH metabolites in marine invertebrates may serve as useful screening tools for preliminary stages of environmental risk assessment of PAH-contaminated sediment.

Although the PAH pyrene is not known for its toxicity, it is one of the most dominant PAHs in the marine environment, and it usually co-occurs with other PAHs that are metabolized into toxic products. Furthermore, 1-hydroxypyrene is the only metabolic intermediate of pyrene observed in invertebrates such as the marine deposit-feeding polychaete *Nereis diversicolor* (Giessing et al. 2002) and the terrestrial isopod *Porcellio scaber* (Stroomberg et al. 1999). Measurement of 1-hydroxypyrene in excretory products of animals and humans has gained considerable attention as a potential biomarker for PAH exposure and is widely used as a biomarker to study human PAH exposure (Levin 1995; Schaller et al. 1993).

Biomarker studies using 1-hydroxypyrene have previously dealt with liquid matrices such as urine and fish bile (e.g. Lin et al. 1994; Schaller et al. 1993). This approach cannot be utilized when dealing with small tissue samples from marine polychaetes and a more elaborate liquid:liquid partitioning scheme is usually applied (e.g.

Christensen et al. 2002a; McElroy et al. 2000). Even though conventional fluorescence spectroscopy has excellent sensitivity in determining trace PAH, its application to mixtures of fluorescent compounds is limited (Santana Rodríguez and Padrón Sanz 2000). This limitation is especially evident when the compounds have high structural similarity, as do pyrene and 1-hydroxypyrene. Such spectral interferences can sometimes be resolved by using synchronous fluorescence spectroscopy (SFS). Scanning both the excitation and emission monochromator with a constant wavelength difference, rather than scanning the excitation monochromator while keeping the emission constant, results in an important simplification of the fluorescence spectra and in the ideal case only one spectral element remains (Vo-Dinh 1982). By reducing spectral interference, individual components of moderately complex mixtures can be identified with limited sample preparation and chromatographic separation.

We report here a simple fluorometric method that allows rapid detection and quantification of 1-hydroxypyrene in small samples of tissue extracts from *Nereis diversicolor* exposed to sediment-associated pyrene. We have used this method for qualitative analysis of pyrene metabolism in three other species of marine deposit-feeding polychaetes, *Arenicola marina*, *Capitella* sp. I., and *Nereis virens*. High pressure liquid chromatography (HLPC) with fluorescence (HPLC/F) and UV/vis detection (HPLC/UV) was used as a complementary, quantitative method for confirmation of the SFS results of *Nereis diversicolor* and to investigate species-specific metabolite conjugation patterns.

MATERIALS AND METHODS

Pyrene (98%), 1-hydroxypyrene (98%), and Na_2SO_4 (>99%) were purchased from Aldrich Chemical Company (Steinheim, Germany). HPLC grade acetonitrile, hexane, chloroform and methanol (all Chromosolv® grade) were acquired from Reidel-deHaën AG (Seelze, Germany). HPLC-grade water was obtained from Merck (Darmstadt, Germany). Glucuronidase-arylsulfatase (from *Helix pomatia*, EC 3.2.1.31 and EC 3.1.6.1., 30 and 60 U/mL respectively) was supplied by ICN Biochemicals (Aurora, OH, USA). All chemicals were used as received.

TEST ANIMALS, EXPERIMENTAL SETUP, AND SEDIMENT SPIKING

Individual *Nereis diversicolor* were collected on a sand flat in the Damariscotta River, South Bristol, Maine, USA in June of 1999 and July 2000 and at Store Havelse, Roskilde Fjord, Denmark in October and November of 1999. Gravid females were avoided. Three additional species of marine polychaetes, *Arenicola marina*, *Capitella* sp. I., and *Nereis virens*, were also tested for pyrene metabolism. *Arenicola marina* was collected at Store Havelse, Denmark at the same time as *Nereis diversicolor*. *Capitella* sp. I was from a mature culture (20 year old) obtained from SUNY, Stony Brook (originally identified to sibling species by J. P Grassle, Rutgers Univ. New Jersey, USA). Cultured *Nereis virens* was purchased from a commercial worm supplier in Roskilde, Denmark. All animals were kept in culture in mm-sieved sediment and with 0.45 μm filtered seawater for a minimum of 7 days prior to use.

The following procedure was followed in all sediment contaminations. Sediment was collected at the same time as worms, sieved (1 mm) and frozen (-18˚C) prior to use. Thawed sediment was contaminated with pyrene by adding the desired amount of pyrene dissolved in a minimum of acetone to a slurry of sediment and 0.45 μm filtered seawater. Seawater used in the experiments was always filtered unless otherwise stated. The slurry was placed on a stand with a power drill, fitted with a mud slinger, and was mixed continuously for a minimum of 24 hours. The slurry was then allowed to settle, the water was decanted, and the sediment was allowed to sit at 5˚C for one week prior to use in microcosms.

ACCUMULATION OF PYRENE IN *NEREIS DIVERSICOLOR*

We investigated accumulation of pyrene and induction of pyrene metabolism over time in *Nereis diversicolor* by exposing worms to 136 μg pyrene g^{-1} sediment (dw) for a total of 9 days. Six separate plastic containers, each with five worms and 500 mL pyrene-contaminated sediment, were placed in a tank in a flow-through system with running seawater. One container was sacrificed daily on day 1 through 5 and one was sacrificed on day 9. Two separate containers containing five worms each were kept in a separate system as non-exposed references to be sampled at the beginning and end of the experiment. Pyrene and 1-hydroxypyrene were identified and quantified using SFS and traditional fluorescence spectroscopy.

Concentration-dependent pyrene metabolism in *Nereis diversicolor* was investigated by exposing worms to sediments containing 1, 20, 50 and 100 μg pyrene g^{-1} (dw). Five worms in each treatment were exposed in a 3 L plastic container with 500 mL

sediment and 1 L 0.45μm filtered sea water for 5 days. A separate container with 5 worms was kept in clean sediment for reference. Each container was fitted with an airstone and bubbled continuously through the experiment. Fresh filtered seawater was added daily. Tissue samples were extracted as described below and analyzed for pyrene and 1-hydroxypyrene using HPLC/UV and SFS.

SAMPLE TREATMENT

Accumulation experiment: Worms that were collected on days 1-5 and 9 were allowed to defecate in individual petri dishes with seawater for a minimum of 12 hours, a time sufficient to purge their guts of sediment prior to extraction (Kane Driscoll and McElroy 1996). The worms were weighed and transferred to test tubes and 2 mL 50:50 methanol/water was added. The worms were then homogenized using a Tissue Tearor (Biospec Products, Inc. Bartlesville, OK, USA) at full speed for 1 minute and subsequently adding 2 mL chloroform. Tissue homogenates were extracted in chloroform to avoid aqueous-pyrene derived compounds that might interfere with 1-hydroxypyrene quantification. The samples were sonicated for 10 min and centrifuged at 2500 rpm for 10 min to precipitate any debris and the chloroform phase was removed. This procedure was repeated twice to a total chloroform volume of 6 mL. The chloroform phase was then dried over anhydrous sodium sulfate, blown down under a gentle stream of nitrogen gas and redissolved in 1 mL methanol.

Concentration series experiment: Tissue extraction was performed by homogenizing whole worms in 2 mL methanol using a Tissue Tearor at full speed for 1 minute and subsequently sonicating samples for 10 minutes. Samples were then

centrifuged at 2500 rpm for 10 minutes to precipitate any debris. 500 μL of supernatant was filtered through a 0.45 μm syringe filter and transferred directly to brown HLPC vials without any further sample preparation.

Tissue samples from *Arenicola marina*, *Capitella* sp. I., and *Nereis virens* were prepared as described above. Due to the large size of *Arenicola marina* and *Nereis virens* their gut tissue was substituted for whole worm homogenate. Unlike other marine invertebrates such as mollusks and crustaceans, annelid worms do not have an easily separable digestive gland. Annelids have specialized tissue (chloragogen) associated with the alimentary tract, the function of which is comparable to the mussel digestive gland. Thus, due to the size and morphology of *Nereis diversicolor* and *Capitella* sp. I., it is not possible to separate the 'reactive' tissue for analysis.

ENZYMATIC HYDROLYSIS

The presence of glucuronic acid and sulphate conjugates of 1-hydroxypyrene in tissue samples was studied by enzymatic hydrolysis of tissue samples. Glucuronidase-arylsulfatase enzymes effectively hydrolyse the conjugates to give free 1-hydroxypyrene. Tissue methanol extracts were diluted 1:100 with 0.22 μm filtered deionized water (DI) and incubated for 2 hours at 37˚C with 10 μL of glucuronidase-arylsulfatase solution. After addition of 500 μL ice-cold 100% ethanol the samples were mixed and centrifuged at 3000 rpm for 5 minutes to precipitate denatured protein. The supernatant was transferred to brown HPLC vials analyzed and using HLPC/F.

SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

Accumulation of pyrene in *Nereis diversicolor* was followed using SFS with a constant wavelength difference of 34 nm between excitation and emission wavelengths channels. SFS was performed on a Hitachi F-4500 spectrofluorometer using a 1 cm methyl acrylate cuvette (VWR, Bridgeport, NJ, USA), with 5 nm slit widths on the emission and excitation channels, a PMT voltage of 700 V and a response time of 0.1 seconds. Calibration curves were made by standard additions of pyrene and 1-hydroxypyrene in hexane to a tissue sample of an unexposed worm. Quantification was done by measuring peak heights and not area because this approach was less influenced by spectral overlap. Calibration curves had regression coefficients $r^2 > 0.98$ for both pyrene and 1-hydroxypyrene. Identification of 1-hydroxypyrene in tissue samples of *Nereis virens, Capitella* sp. I. and *Arenicola marina* using SFS was done as described above.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

Quantification of pyrene and 1-hydroxypyrene in tissue samples from *Nereis diversicolor* in the concentration series experiment was done by HPLC/UV. The method consisted of an acetonitrile/water gradient scheme using a reverse phase Supelcosil LC-PAH column, 15cm x 4.6 mm, 5μm (Supelco, Bellefonte, PA, USA). The acetonitrile/water (v/v) gradient profile used for quantification of pyrene and 1-hydroxypyrene was 50:50 for 5 minutes, 100:0 over 20 minutes, and 100:0 for 8 minutes at a flow rate of 0.85 mL min^{-1}. Column temperature was kept at 28˚C. The system consisted of two Gilson 306 solvent pumps, a Gilson 831 Temperature Regulator,

a Gilson 234 Autoinjector, an ERC 3415 degasser, and a Gilson 205 Fraction Collector. The injection volume was 100 μL on a 500 μL stainless steel injection loop. Detection was performed with a Gilson 119 UV/vis detector by measuring absorbance at 339 nm. Quantification was done by measuring peak area using external calibration curves of pyrene and 1-hydroxypyrene standards in acetonitrile. Calibration curves had regression coefficients $r^2 > 0.98$ for both pyrene and 1-hydroxypyrene.

Total metabolite pattern was analysed on a Hitachi D-7000 HPLC using a method optimized for pyrene metabolites originally provided by Dr. Gerard Stroomberg (AquaSence, Amsterdam, NL). The method is described in detail in Giessing and Lund (2002); in brief a Vydac C18 reverse phase column 25cm x 4.6 mm, 5μm (Supelco, Bellefonte, PA, USA) was used with an acetonitrile/10 mM ammonium acetate (pH = 5) (v/v) gradient. The gradient profile was 5/95 directly to 90/10 over 40 min, and then hold 90/10 for 10 min at a flow rate of 0.8 mL min^{-1}. Column temperature was kept at 28˚C. Detection was performed by measuring absorbance at 339 nm and fluorescence $\lambda_{EX/EM}$ = 346/384 nm for metabolites/conjugates and $\lambda_{EX/EM}$ = 333/384 nm for parent compound. Injection volume was 100 μL. Peaks were identified with a Hitachi L-7450 Diode Array Detector (DAD).

RESULTS AND DISCUSSION

PYRENE ACCUMULATION AND METABOLISM IN *NEREIS DIVERSICOLOR*

Figure 4.1 shows classical fluorescence (Em 430 nm) and SFS ($\Delta\lambda$ = 34 nm) spectra of pyrene and 1-hydroxypyrene standards, and tissue extracts from a *Nereis diversicolor* exposed for 5 days to 136 μg pyrene g^{-1} sediment (dw). SFS simplifies the fluorescence spectra of a pyrene and 1-hydroxypyrene mixture (Figure 4.1A) and pyrene and 1-hydroxypyrene are readily identified at 339 nm and 355 nm respectively (Figure 4.1B). The synchronous fluorescence spectra of exposed *Nereis diversicolor* (Figure 4.1C) showed two peaks at 350 nm and 338 nm corresponding to spectrum of a 1-hydroxypyrene and pyrene standard. Spectra of unexposed worms showed no peaks other than the broad protein band at ~280 nm with varying intensity (Mayer et al. 1999). The small blue shift observed in the spectrum can in part be explained by the complex nature of the sample matrix and the presence of conjugated 1-hydroxypyrene and non-covalent complexes between 1-hydroxypyrene and endogenous tissue components. Ariese et al. (1993a) reported that the spectrum of glucuronide-conjugated 1-hydroxypyrene was blue-shifted 5 nm and was 2–fold more intense than that of free 1-hydroxypyrene. Generally, an increase in degree of conjugation (i.e. extent of aromatic structure) leads to a shift of absorption and fluorescence spectra to longer wavelengths and to an increase in fluorescence intensity. Two major metabolites, 1-hydroxypyrene and a pyrene-1-glucuronide conjugate, have been identified in the tissue extracts of *Nereis diversicolor* exposed to sediment-associated pyrene (see Chapter 2 and 3, Giessing and Lund 2002), with pyrene-1-glucuronide constituting >70% of total pyrene-derived

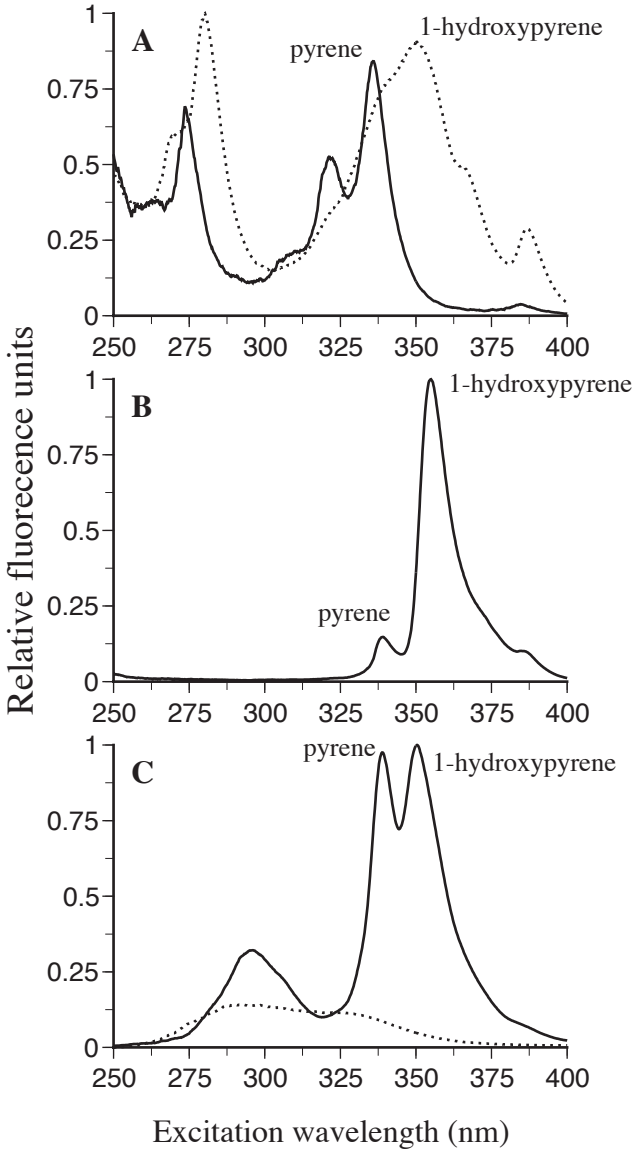

Figure 4.1 Fluorescence spectra of pyrene and 1-hydroxypyrene.

A: Traditional fluorescence spectrum of PAH standards scanning the excitation channel while measuring emission at 430nm. **B**: Synchronous fluorescence spectrum (SFS, $\Delta\lambda$ = 34 nm) of a mixture of pyrene and 1-hydroxypyrene. **C**: SFS spectrum ($\Delta\lambda$ = 34 nm) of tissue extract from a *Nereis diversicolor* exposed to pyrene contaminated sediment and an unexposed worm (dotted line).

compounds measured in tissue (See Chapter 2, Giessing et al. 2002). The peak labeled 1-hydroxypyrene in Figure 4.1C is the signal from all compounds containing the 1-hydroxypyrene fluorophore and thus the combined fluorescence signal from 1-hydroxypyrene, pyrene-1-glucuronide and possibly other minor 1-hydroxypyrene conjugates (e.g. sulfates and glucosides).

The worms reached quasi-steady state tissue concentrations of both pyrene and 1-hydroxypyrene after approximately 3 days of exposure (Figure 4.2). Subsequently, an exposure period of 5 days was chosen in later experiments for all species studied. Similar quasi-steady state tissue concentration within 2-3 days of exposure to PAH has previously been reported for deposit feeding marine polychaetes (Forbes et al. 1996; Kure 1997).Worms in the exposed treatment were noticeably affected by the pyrene exposure. The majority of the worms burrowed in when added to the microcosms but some remained on the sediment surface or resurfaced several hours after addition. However, all worms burrowed in on the day of addition and all but four were collected alive from the containers on sampling days. The four that escaped were collected alive from the overflow tank and thus no mortality was observed.

The worms showed increasing accumulation of pyrene with increasing exposure concentration up to 50 μg g^{-1} sediment followed by lower accumulation at 100 μg pyrene g^{-1} sediment (Figure 4.3). Worm tissue concentrations at 100 μg pyrene g^{-1} sediment exposure concentration were not significantly different from the 50 μg g^{-1} concentration (Student t test, $P = 0.18$). However, the trend is confirmed by adding the data for day 5 from Figure 4.2 to the data in Figure 4.3 (data points with open symbols).

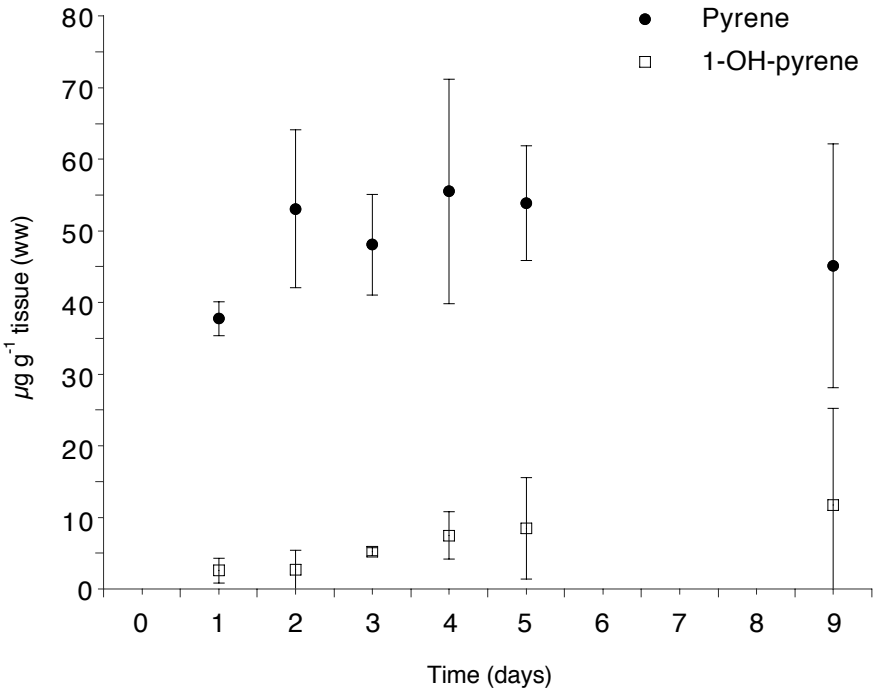

Figure 4.2 Accumulation of pyrene and formation of 1-hydroxypyrene in *Nereis diversicolor* exposed to sediment-associated pyrene.

Concentrations are measured by SFS. Data are presented as average ± standard deviation, n = 5 except for day 1 and day 3, n = 3.

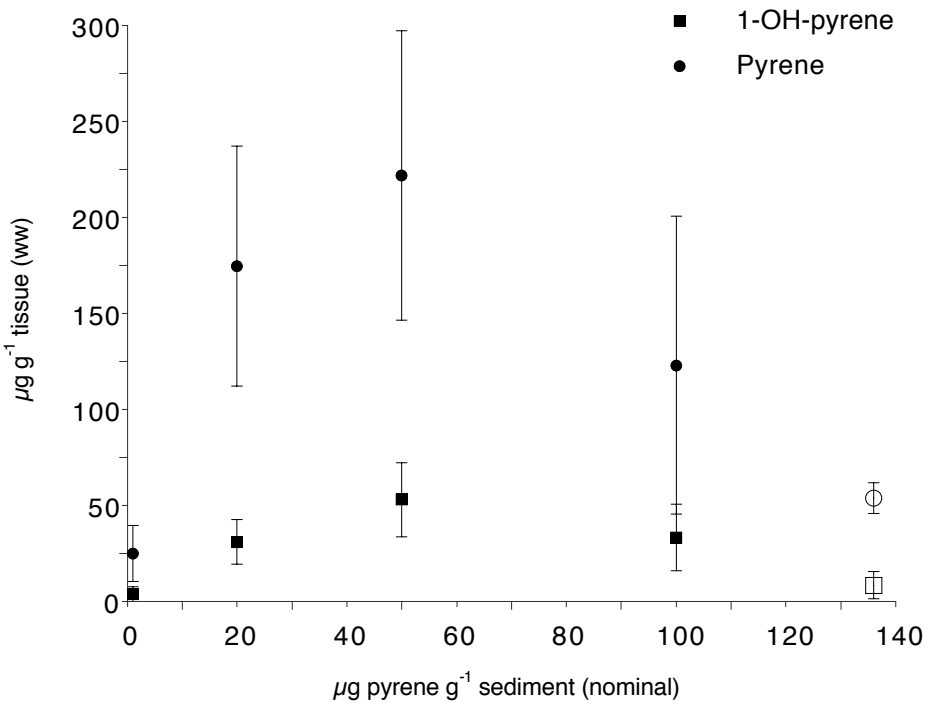

Figure 4.3 Concentrations of pyrene and 1-hydroxypyrene in *Nereis diversicolor* exposed to 1, 20, 50 and 100 μg pyrene g^{-1} sediment (dw).

Data are presented as average ± standard deviation. The open data points at 136 μg pyrene g^{-1} sediment are the averages ± standard deviations of day 5 from the data presented in Figure 4.2.

The apparent decrease in pyrene accumulation above exposure concentrations of 50 μg pyrene g^{-1} sediment is possibly due to changes in *Nereis diversicolor* feeding behavior, such as a decrease in feeding rate. When exposed to 16 μg g^{-1} fluoranthene the feeding rate of *Arenicola marina* showed an exponential decline with increasing exposure concentration and was reduced to 4% of that of control worms (Kure 1997). Data on changes in feeding rate in *Nereis diversicolor* under pyrene exposure are lacking, but the bell shape of the accumulation curve seems to suggest that there is a threshold concentration below which *Nereis diversicolor* metabolic capacity can keep pyrene tissue concentrations below acute toxic levels. However, this conclusion is somewhat speculative and the influence of pyrene on the feeding behavior of *Nereis diversicolor* warrants further investigation.

Both pyrene accumulation and formation of 1-hydroxypyrene as a function of exposure concentration were significantly different from zero (ANOVA, $P = 0.0014$ and $P = 0.02$ respectively) and there was a highly significant positive correlation between the measured pyrene and 1-hydroxypyrene concentrations ($p < 0.01$). Tissue concentrations of 1-hydroxypyrene from exposures 20, 50 and 100 μg pyrene g^{-1} sediment treatments in the concentration series experiment (Figure 4.3) were also quantified using SFS. There was a positive correlation (though not significant, $p = 0.0814$) between 1-hydroxypyrene concentrations measured by the two methods with SFS concentrations being on average 1.7 times higher than HPLC – all but one of the data points are above the 1:1 line on Figure 4.4.

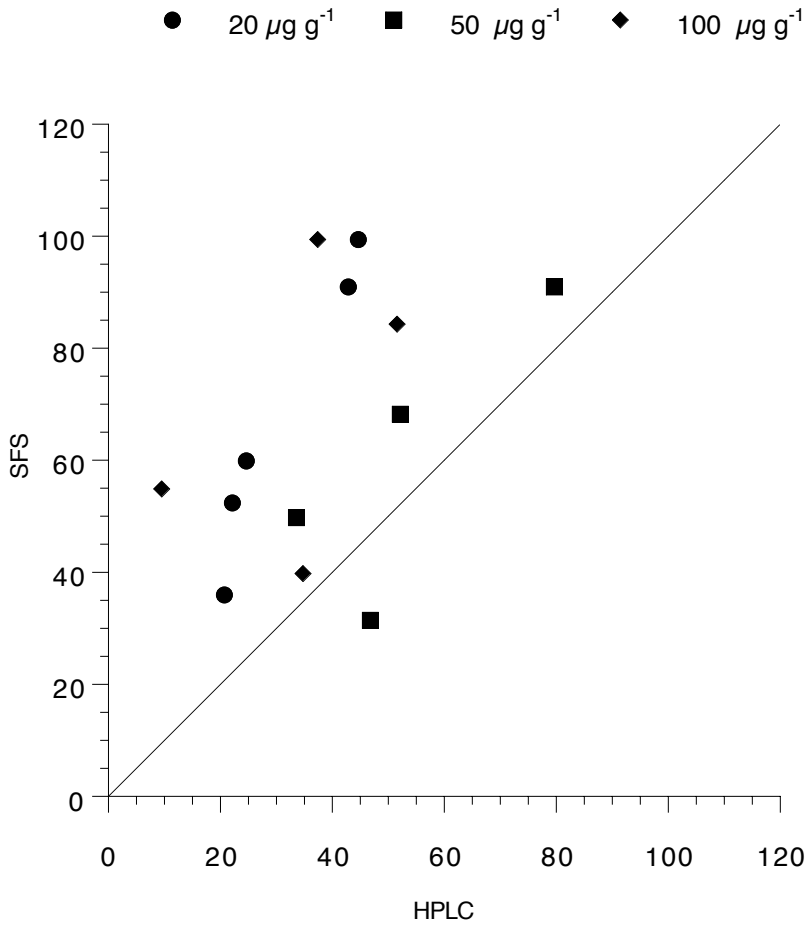

Figure 4.4 Correlation between 1-hydroxypyrene concentrations (μg g^{-1} tissue wet weight) measured by HPLC/UV and SFS.

The straight line indicates the 1:1 ratio and the pyrene exposure concentration is indicated by the legend on the figure. There was a good correlation though not significant ($p = 0.0814$) between concentrations measured by the two methods.

As mentioned earlier the SFS signal measured is the combined signal of all compounds with the 1-hydroxypyrene fluorophore. Ariese et al. (1993a) reported a 2.2-fold smaller fluorescence yield of free 1-hydroxypyrene compared to 1-hydroxypyrene glucuronide. Thus SFS-determined 1-hydroxypyrene concentrations (i.e. $\sum$ of all 1-hydroxypyrene containing species) would be expected to be higher than HPLC.

The inequivalency can also in part be explained due to the unique fluorescent response of the pyrene-derived compounds exhibited at different solvent mixtures in the chromatographic program. SFS quantification was performed in methanol whereas HPLC/F quantification used an acetonitrile/water gradient. Lin et al. (1996) observed a similar difference when comparing fixed wavelength fluorescence and HPLC/F, using a linear acetic acid-water and methanol gradient program, for determining benzo[a]pyrene (BaP) exposure in fish bile. In another fish bile study, high correlation between BaP-type metabolites measured by HPLC/F and pyrene-type metabolites measured by SFS indicated the suitability of using the latter as a surrogate for exposure to BaP-type PAHs (Lin et al. 1994). Future studies on pyrene metabolism in marine invertebrates using the SFS method should employ enzymatic hydrolysis of the samples to liberate conjugated 1-hydroxypyrene prior to quantification.

PYRENE METABOLISM IN 3 ADDITIONAL SPECIES OF MARINE POLYCHAETES

Figure 4.5 shows SFS spectra of methanol extracts of tissue from *Nereis diversicolor*, *Nereis virens, Capitella* sp. I. and *Arenicola marina* exposed to sediment-associated pyrene. *Nereis diversicolor* and *Capitella* sp. I. showed two distinct spectral elements, pyrene and 1-hydroxypyrene-type metabolites, in the 340-350 nm region.

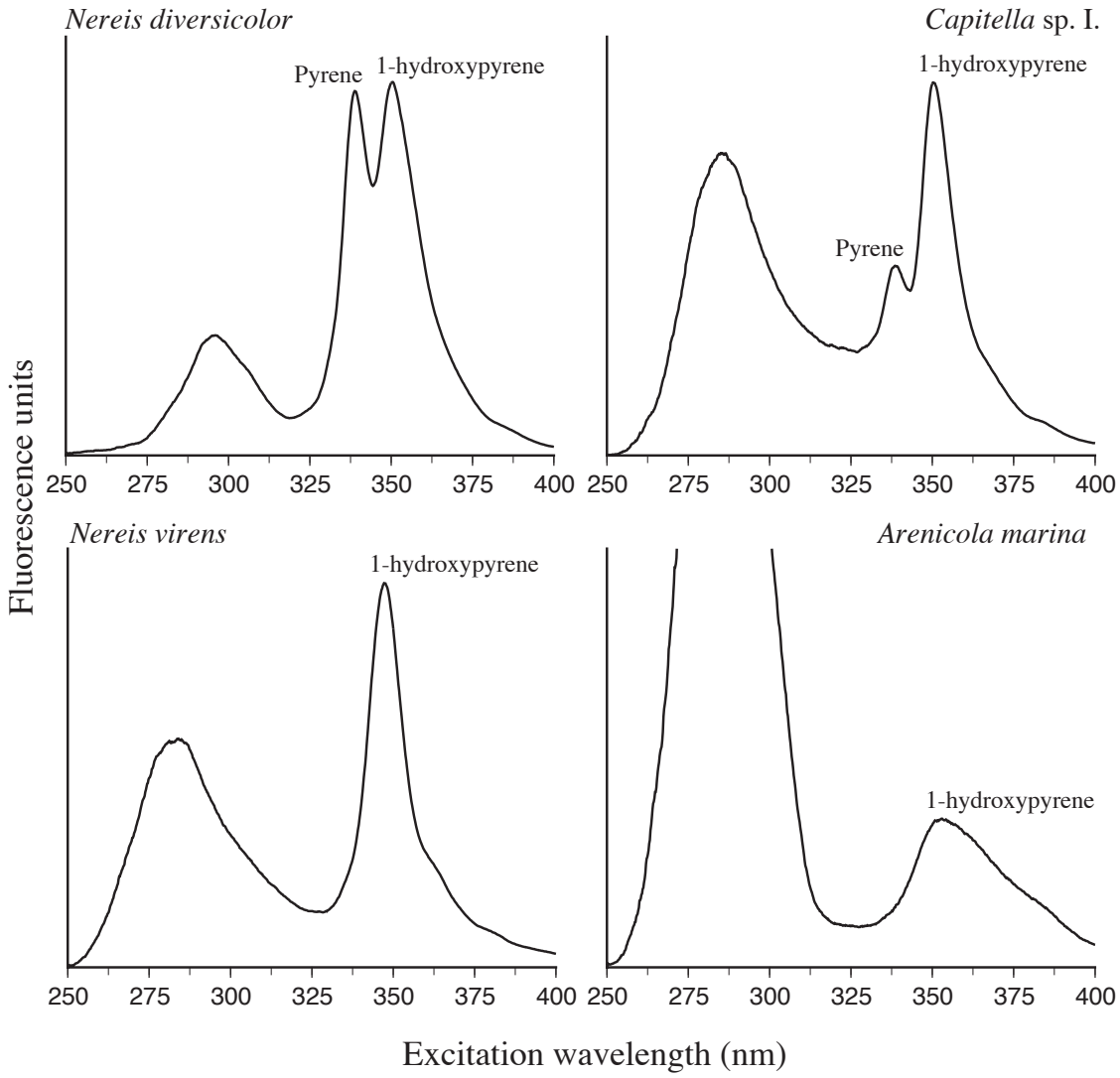

Figure 4.5 Synchronous fluorescence spectroscopy ($\Delta\lambda$ = 34 nm) of tissue extracts from 4 species of marine polychaetes.

Two distinct spectral elements, pyrene and 1-hydroxypyrene-type metabolites, are readily identified in all four species.

SFS spectra of *Nereis virens* and *Arenicola marina* only showed one peak that can be related to pyrene metabolism i.e. the 1-hydroxypyrene peak at ≈350 nm. Lack of a pyrene peak is due to rapid turnover of pyrene to 1-hydroxypyrene at the lower exposure concentration (5 μg g^{-1} as opposed to 136 and 50 μg pyrene g^{-1} sediment) and to quenching by the complex sample matrix. Both worms had intense protein peaks around 280 nm.

Identification of 1-hydroxypyrene as the only metabolic intermediate of pyrene in all 4 species of worms is corroborated by the HPLC/F chromatograms (Figure 4.6). Lower chromatograms marked *a* are diluted samples identical to those presented in Figure 4.5 and upper chromatograms marked *b* are the same sample after enzymatic hydrolysis with glucuronidase-arylsulfatase. In all 4 worms, treatment with glucuronidase-arylsulfatase caused a significant increase in 1-hydroxypyrene signal at 32.5 min. The chromatogram of *Nereis diversicolor* before hydrolysis shows three peaks at 19.0 min, 21.7 min and 22.2 min. The peak at 19.0 min is 1-hydroxypyrene glucuronide (see Chapter 3, Giessing and Lund 2002) and it is, together with the small peak at 22.2 min, hydrolysed after treatment with glucuronidase-arylsulfatase. The peak at 22.2 min is therefore likely a sulfate conjugate. The peak at 21.7 min is not affected by glucuronidase-arylsulfatase and remains to be identified. Glucoside and sulphate conjugations are thought to predominate over glucuronic acid conjugation in marine invertebrates (Livingstone 1998). Using this commonly observed trend the nonhydrolysable peak at 21.7 min is suggested to be a glucoside conjugate.

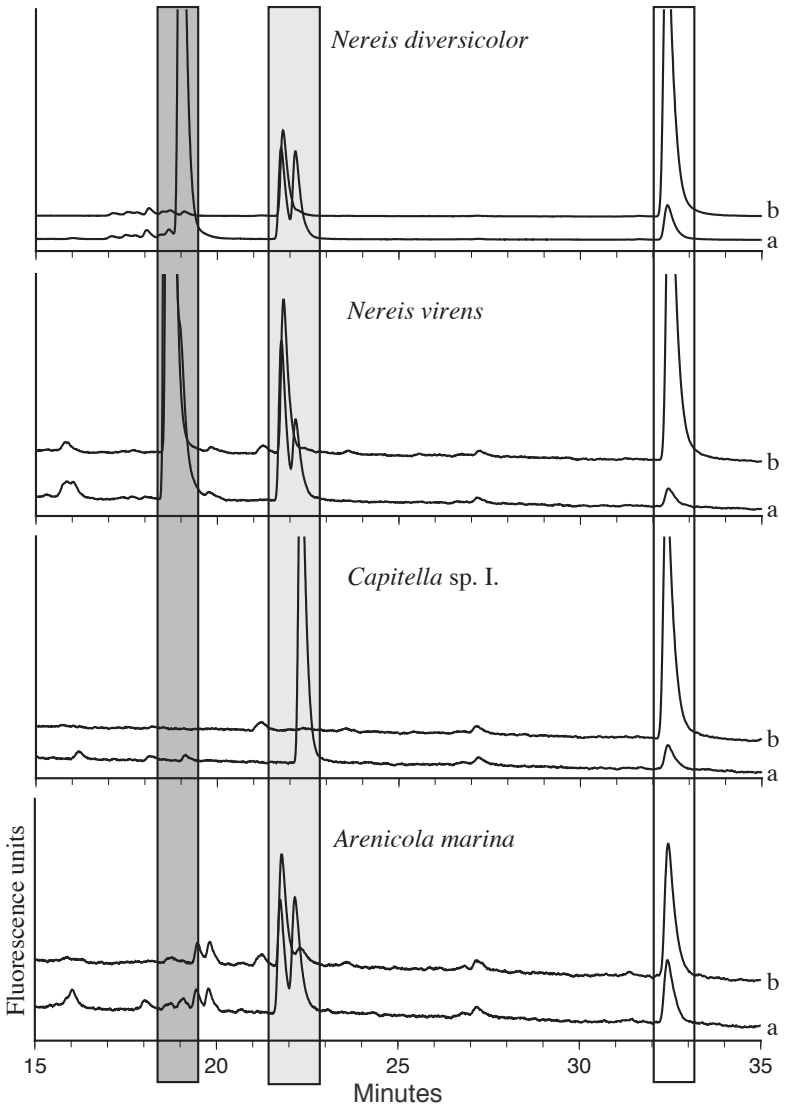

Figure 4.6 HPLC/F chromatograms of tissue samples presented in Figure 4.5. Lower chromatogram (marked a) for each species is the sample prior to enzymatic hydrolysis and the upper (marked b) shows the effect of enzymatic hydrolysis. 1-hydroxypyrene appears at 32 min (white band) in tissue from all four species and the peak increases in size after treatment with glucuronidase-arylsulfatase. The traces (a and b) are offset and abscissa is limited to metabolite time window for visualization purposes. The darker gray band to the left is 1-hydroxypyrene glucuronide and the light gray band in the middle contains 1-hydroxypyrene sulfate and 1-hydroxypyrene glucoside peaks.

By applying the identification scheme to all 4 species (visualized by the bands on Figure 4.6) distinct differences in pyrene metabolism, especially in conjugated metabolites, are observed among the 4 species.

Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P450 enzyme suite, which converts hydrophobic, parent PAH to water soluble metabolites in a two phase process. The enzymes of Phase I metabolism introduce a functional group (OH, COOH, NO_2 etc.) into the PAH, to which Phase II enzymes attach a large polar moiety (glucuronic acid, glutathione, sulfate, amino acid, etc.) to facilitate excretion. *Nereis diversicolor*, *Nereis virens*, and *Capitella* sp. I. all have known cytochrome P450-like PAH metabolic capabilities (Forbes et al. 2001; Giessing and Lund 2002; McElroy 1985). However, identification of 1-hydroxypyrene is the first direct identification of cytochrome P450-like PAH metabolism in *Arenicola marina*. Previous studies have failed to detect pyrene metabolites (Christensen et al. 2002a) or a PAH metabolizing system in *Arenicola marina* (Payne and May 1979), though arenicolids are known to excrete aqueous metabolites of pyrene and other PAHs (Christensen et al. 2002a; Kure 1997; Weston 1990).

The tentative phase II metabolite identification presented above suggests that nereid polychaetes predominantly make use of glucuronide conjugation whereas *Capitella* sp. I. and *Arenicola marina* appears to utilize predominantly sulfate and/or glucoside conjugation respectively. An interesting question becomes whether an advantage is gained by using either of these strategies. Deposit-feeding polychaetes like *Capitella* sp. I. often dominate oil-contaminated sediments (Grassle and Grassle 1974; Grassle and Grassle 1976; Pearson and Rosenberg 1978) whereas *Arenicola marina* is

known to be more sensitive to PAH contamination (Kure 1997). The gut of a deposit-feeder is assumed to be the primary route of both uptake and elimination of organic contaminants in deposit-feeding polychaetes (Forbes et al. 1998; Mayer et al. 1996) and elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion (Giessing et al. 2002). Assuming that our tentative identification of phase II metabolites in *Arenicola marina* is correct, hydrolysis of 1-hydroxypyrene glucoside by glucosidase enzymes present in gut fluid of most deposit-feeders (Mayer et al. 1997) would release 1-hydroxypyrene which would potentially reabsorbed into the animal thereby increasing toxicity. A similar process, called entero-hepatic circulation, has been observed in vertebrate as well as invertebrate species (James 1987).

Synchronous fluorescence spectroscopy of whole worm homogenates is a straightforward, fast, and simple method for the detection of pyrene metabolites and assessment of PAH exposure in marine, deposit-feeding polychaetes. The complete analysis takes less than 5 min. Even though the present study suggests that HPLC/UV/F surpasses SFS as a quantitative analytical technique for pyrene and its metabolites, SFS can provide useful and rapid method and could be a good screening tool for PAH exposure in marine invertebrates. Detection of 1-hydroxypyrene can indicate accumulation of pyrene, integrated over all uptake routes, and taking into account bioavailability. Activities of enzymes such as cytochrome P450 and 7-ethoxyresorufin-O-deethylase (EROD) can be used as biochemical markers of environmental contamination. However, these enzymes can be induced by multiple substrates (e.g. PAHs, dioxins and

PCBs) and no detection of pyrene metabolites at zero exposure makes the SFS method more specific to PAH exposure.

The use of 1-hydroxypyrene as a biomarker for total PAH exposure has successfully been applied to fish bile. Field studies have indicated that the sensitivity of the SFS method is sufficient for field monitoring close to industrialized and urbanized areas (Ariese et al. 1993c; Krahn et al. 1987; Van der Oost et al. 1994). Furthermore, studies have shown that the total PAH metabolite profile in fish bile is roughly constant at different locations with comparable PAH sources, and that 1-hydroxypyrene, easily quantified by SFS, can be used as a relative measure for the total uptake of PAHs (Ariese et al. 1993b; Krahn et al. 1987; Lin et al. 1994). Future experiments will reveal if a similar correlation between total PAH and 1-hydroxypyrene can be made in marine invertebrates in both laboratory and field experiments.

5. OXIDATIVE COUPLING DURING GUT PASSAGE IN MARINE DEPOSIT-FEEDING INVERTEBRATES

ABSTRACT

We tested for oxidative coupling of organic contaminants by enzymes in gut fluid from marine deposit-feeding invertebrates. Hydroxylated metabolites, which can arise from deposit-feeding invertebrates accumulation of polycyclic aromatic hydrocarbons (PAHs), can participate in oxidative coupling reactions with the formation of stable covalent bonds. Gut fluid from 4 species of marine deposit-feeding invertebrates (3 polychaetes and 1 holothuroid) catalyzed oxidative coupling of pyrenol (1-hydroxypyrene) in an apparent oxidative enzymatic reaction. Unaltered *Nereis virens* gut fluid had high total oxyradical scavenging capacity indicating the presence of fast-acting antioxidants. An antioxidant enzyme capable of oxidative coupling, a heme peroxidase, was tentatively identified in gut fluid from *Nereis virens* by dye decolorization assays and enzyme inhibitor studies. The enzyme was not isolated and purified. Of total extractable pyrenol in fecal matter from polychaetes exposed to sediment associated pyrene, 70% was released after acid hydrolysis, indicating the presence of ester bonds between pyrenol and sedimentary organic matter. Consistent with these findings, *Nereis virens* gut fluid also catalysed formation of dityrosine, a marker of oxidative damage in proteins. Oxidative coupling of PAHs will reduce subsequent bioavailability, toxicity, and transport of these compounds in marine environments. Furthermore, oxidative coupling of PAHs represents a new sink for organic contaminants

in marine sediments and suggests a biological mechanism for the formation of aquatic humic material in general.

INTRODUCTION

Deposit-feeding invertebrates dominate metazoan life in marine sediments, both in terms of abundance and biomass (Lopez and Levinton 1987; Pearson and Rosenberg 1978). These animals are major controllers of sediment ecosystems and their vertical and horizontal mixing of sediment particles is an important driving force behind chemical reactions and transport of organic matter in marine sediments (Aller and Aller 1998; Aller et al. 2001; Levinton 1995). Furthermore, most bioturbators maintain contact with the overlying water by ventilating seawater through their burrow system (irrigation), thereby increasing transport of ions and gases (e.g. NH_4^+ and O_2) across the sediment water interface (Kristensen 1985).

Marine sediments consists of only a few percent organic matter and even organic-rich sediments may be 95% mineral phase. Because most sedimentary organic matter is refractory, deposit-feeders survive on a remarkably poor food source (Jumars and Wheatcroft 1989; Lopez and Levinton 1987). Deposit-feeders meet their nutritional requirements by ingesting large amounts of bulk sediment. Species like the common lugworm (*Arenicola marina*, Polychaeta) ingest up to 20 times their own body weight of wet sediment per day (Cadée 1976). Deposit-feeding metazoans also have evolved an enclosed extracellular digestive geometry which lets them efficiently retain both digestive agents and digestive products and thus thrive on a nutritionally poor sedimentary diet (Mayer et al. 2001a).

Deposit feeders guts have a unique chemistry compared to that of ambient sediment. Gut fluids from these animals contain a wide range of digestive agents (e.g. enzymes and surfactants) and high concentrations of dissolved organic matter (DOM) rich in proteinaceous materials (Mayer et al. 1997). They also contain high concentrations of dissolved transition metals, especially manganese and iron, along with high concentrations of potential metal ligands (Chen et al. 2000). Metals like iron, manganese and copper can, at very low concentrations, redox cycle and catalyze the generation of various highly reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$).

The formation of ROS is a normal consequence of essential biochemical reactions in aerobic organisms (Dhainaut and Scaps 2001; Halliwell and Gutterridge 1999). ROS are produced in biological tissues from the univalent reduction of oxygen to water and from activities of several enzymatic systems. If ROS are not rapidly detoxified by cellular antioxidants, they may cause significant damage such as peroxidation of membrane lipids, oxidative damage to proteins and nucleic acids, and may disturb vital cellular functions and energy homeostasis (Gutteridge and Halliwell 1990; Hyslop et al. 1988). Production of ROS can be increased by intake of natural and pollutant pro-oxidant xenobiotics and trace metals and by changing oxygen tension in the environment (Livingstone et al. 1990; Winston and Digiulio 1991). Marine invertebrates living in intertidal environments are exposed to varying concentration of both O_2 and H_2O_2 (Abele-Oeschger et al. 1994). Due to the instability and reactivity of ROS and their potential to damage cells and tissue, both antioxidant enzymes (e.g. superoxide dismutase, glutathione peroxidases and catalases) and low molecular mass molecules (e.g.

glutathione, vitamins E and C, and carotenoids) are made to protect against their adverse effects (Larson 1997).

Oxidative coupling of products from microbial degradation of organic residues can catalyze the formation of terrestrial humic substances (Bollag et al. 1998). Oxidative coupling is mediated by both enzymes and abiotic catalysts that are present in soils. Enzymatic processes are mediated by oxidoreductive enzymes, like peroxidases and polyphenol oxidases, while oxides of Al, Fe and Mn and clay minerals can serve as abiotic catalysts (Filip and Preusse 1985; Naidja et al. 2000; Shindo and Huang 1984). Peroxidases are powerful antioxidant enzymes that oxidizes another substrate, such as a substituted phenol, in the reduction of a peroxide (e.g. H_2O_2). The enzymatic oxidation produces free radicals or reactive quinones that covalently couple or polymerize to form humic polymers (Bollag 1983). Because degradation products of many pollutants e.g. polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), also have phenolic characteristics they may be incorporated into organic matter during humification processes (Richnow et al. 1994; Richnow et al. 1995). Oxidative coupling of pollutants to natural macromolecular organic matter is believed to decrease bioavailability, toxicity and transport in natural environments (Bollag 1992) and has been suggested to be a major sink for organic pollutants in soils (Bollag 1983). Oxidative coupling of phenols by inorganic species such as MnO_2 has also been recognized as important in formation of aquatic humic substances (Larson and Hufnal 1980).

The gut of a marine deposit-feeder is assumed to the primary route of both uptake and elimination of organic contaminants (Forbes et al. 1996; Mayer et al. 1996). We have recently identified pyrenol (1-hydroxypyrene), a phenolic metabolite of the tetra-cyclic

PAH pyrene, in gut fluid of deposit-feeding polychaete *Nereis diversicolor* (see Chapter 2, Giessing et al. 2002). Although pyrenol is an intermediate of pyrene metabolism, its presence in gut fluid provides a phenolic substrate capable of participating in oxidative coupling reactions. Oxidative coupling of PAH metabolites during gut passage has important implications for the subsequent fate of these chemicals and introduces a novel fate for organic contaminants in marine sediments.

This study investigated oxidative coupling reactions of PAHs in gut fluid of marine deposit-feeding invertebrates. The combined effect of digestive chemistry and ingestion of sediment rich in redox cycling metals makes the gut of deposit-feeders a reaction zone with oxidative coupling potential. High activities of cytosolic antioxidant enzymes have been reported in gut tissue (chloragog) of deposit-feeding marine polychaetes (Buchner et al. 1996). Furthermore, the aforementioned identification of pyrenol in gut fluid makes it a suitable model substrate for studying oxidative coupling reactions. Pyrenol has known oxidative coupling potential (Richnow et al. 1997) and both pyrenol and pyrene have the analytical sensitivity needed for work below aqueous solubility. However, due the possibility of competing reactions in gut fluid and the low aqueous solubility of PAHs, identification of oxidative coupling products (e.g. a pyrenol dimer) is a complicated task. Thus, for product studies a more abundant naturally occurring compound with higher aqueous solubility was chosen.

Tyrosine, an essential amino acid with phenolic functionality, was chosen for product identification experiments. A typical feature of oxidative stress in the physiological pH range is the formation of tyrosyl radicals by ROS. Protein-bound dityrosine, a major product of tyrosine modification in proteins, is formed through a

carbon-carbon bond between two tyrosine radicals (Jacob et al. 1996; Pennathur et al. 1999). Tyrosine coupling is an irreversible process which may lead to molecular cross-links in proteins thereby initiating dysfunction and aggregation. Identification of dityrosine formation catalyzed by gut fluid will provide strong evidence for oxidative coupling reactions of naturally occurring compounds during gut passage in marine deposit-feeding invertebrates.

Oxidative coupling reactions were studied using gut fluid from the omnivorous marine polychaete *Nereis virens* and 3 other marine deposit feeding invertebrates (2 polychaetes and 1 echinoderm). Fecal matter from a congener species, *Nereis diversicolor,* exposed to sediment-associated pyrene (see Chapter 2) was used to test for the possible influence of oxidative coupling on fate of pyrene in marine sediments. Because deposit-feeder gut fluid is a complex soup of proteins, lipids and metals, involvement of peroxidase-like enzymes in oxidative coupling reactions was studied by measuring the antioxidant capacity of unaltered gut fluid and by using dye decolorization assays and inhibitor studies.

MATERIALS AND METHODS

Horseradish Peroxidase (EC 1.11.1.7), Remazol Brilliant Blue R (RBBR), α-keto-γ-methiolbutyric acid (KMBA) and NaN_3 were obtained from Sigma (St. Louis, MO, USA). Pyrene (98%), pyrenol (98%), reduced glutathione (98%), 2,2'-azobis-amidinopropane (ABAP) and $Na_2S_2O_5$ was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Hydrogen peroxide (30%) and NaCN were purchased from Fisher Scientific (Fairlawn, NJ, USA). L-(+)-cysteine, Na_2HPO_4 (>99%), NaOH and HCl

were obtained from J. T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol, hexane, dichloromethane, and acetone, all ChromAR® HPLC grade, were purchased from Mallinckrodt (Paris, KT, USA). 100% Ethanol was obtained from Quantum Chemical Corporation (Tuscola, IL, USA). All chemicals were used as received.

PAH stock solutions were prepared by dissolving pyrene and pyrenol in methanol and adding 1 mL methanol stock to 1 L 0.02M Na_2HPO_4 buffer, with pH adjusted to 7 with HCl, to give final concentrations of 129 μg L^{-1} and 134 μg L^{-1} respectively. Stock concentrations were based on an aqueous pyrene solubility of 0.64 μM (Meyerhoffer and McGown 1991). To our knowledge there exist no data on aqueous solubility of pyrenol. Because addition of O-containing functional groups generally increases the aqueous solubility of aromatic compounds we assumed that pyrenol was more soluble than pyrene.

TEST ANIMALS

Animal species used in the experiments were *Nereis virens*, *Arenicola brasiliensis*, and *Arenicola marina* (all Annelida: Polychaeta) and *Parastichopus californicus* (Echinodermata: Holothuroidea). *Nereis virens* was purchased from a commercial worm supplier in Egdecomb, Maine in July of 2000 and October of 2001. Digestive fluid from *Nereis virens* was carefully extracted as described in Mayer et al. (2001b). Animals were held in seawater and dissected within 24 hours after collection. Dissection and digestive fluid removal were performed under air, to avoid contamination by seawater, by making a small insertion in the outer body wall to expose the gut, inserting a pipette through the gut wall, and withdrawing digestive fluid. Fluid from 80

individuals was pooled and centrifuged for 5 min at 420 g (IEC Centra MP4R centrifuge), filtered (0.22 μm) and stored at -80˚C prior to use. Gut fluid used in the experiments was always filtered through a 0.22 μm syringe filter unless otherwise stated. Gut fluid from the 3 other species extracted in a similar manner was taken from our stock of digestive fluids stored in 1 mL aliquots at -80˚C. *Arenicola marina* originated from sand flats at Lubec, Maine (courtesy of I. Voparil), *Arenicola brasiliensis* was from San Francisco, California (courtesy of D. Weston) and *Parastichopus californicus* was from Puget Sound, Washington (courtesy of L. Self).

GUT FLUID ASSAYS

An oxidative coupling assay using pyrenol and pyrene, as phenolic and non-phenolic coupling substrates respectively, was developed using horseradish peroxidase (HRP) and synchronous fluorescence spectroscopy (SFS). The fluorescence intensity of aqueous reaction mixtures containing HRP and pyrenol or pyrene were recorded before and after 5 consecutive additions of 1 mmol H_2O_2. The fluorescence intensity of pyrenol at $\lambda_{EX/EM}$ = 350/384 nm fell immediately below detection upon addition of H_2O_2whereas pyrene fluorescence ($\lambda_{EX/EM}$ = 338/372 nm) was lowered by only 2% after each addition of H_2O_2 (data not shown). The significant loss of pyrenol fluorescence indicates effective binding of pyrenol to itself or to other reactants in the sample matrix. The fluorescence signal was not affected by addition of either DI instead of H_2O_2 or H_2O_2 without HRP (data not shown).

The gut fluid assay was identical to the HRP assay described above except that 30 μl of 0.22 μm filtered gut fluid was substituted for HRP and added to 1.5 mL PAH stock

solution. To reduce quenching of the pyrenol fluorescence signal gut fluid was diluted 100-fold and all 3 experiments were performed with an Instech cuvette stirrer (Instech Laboratories, Inc., PA, USA), during data collection. 15 μL 3% H_2O_2 was added and the SFS signal ($\Delta\lambda$ = 34nm) was recorded over time. Although the method worked well for qualitative studies, the background fluorescence signal from gut fluid precluded quantification. Instead a chromatographic method was developed.

GUT FLUID ASSAYS WITH CHROMATOGRAPHIC ANALYSIS

First experiment: Oxidative coupling potential of gut fluid (July 2000) was tested by adding 100 μL gut fluid to 1 mL of PAH stock in 1.5 mL Eppendorf tubes and initiating the reaction by addition of 50 μL 0.1 M H_2O_2. The control was a sample with 50 μL DI added instead of H_2O_2. All treatments were done in triplicate. The samples were placed in an incubator oven mounted on an orbital shaker at 37˚C for 1 hour. After incubation, 0.5 mL of the sample was transferred to a clean Eppendorf tube and 0.5 mL of ice-cold 100% ethanol added to precipitate protein. The samples were mixed and centrifuged at 420 g at 0˚C for 5 minutes and 0.5 mL of the supernatant was transferred directly to brown HPLC vials without any further preparation or extraction. Gut fluids from three additional deposit feeding invertebrates (*Arenicola brasiliensis*, *Arenicola marina*, and *Parastichipus californicus*) were incubated and analyzed in triplicate as described above. Analysis of dissolved pyrene and pyrenol was done using high pressure liquid chromatography with fluorescence detection (HPLC/F, see below).

Second experiment: To test for oxidative capacity of unmodified *Nereis virens* gut fluid (October 2001), incubations without H_2O_2 were followed over time. We added 1 mL

gut fluid to10 mL PAH stock in glass, and left it in the dark at room temperature. The reaction mixture was subsampled in duplicate (2 x 100 μL) and treated as in the first experiment. As a control 250 μM sodium metabisulfite ($Na_2S_2O_5$) was added to inhibit the hypothesized oxidative process.

Recovery of pyrene by the protein precipitation method in the time-series experiment was 77% ± 8 (mean ± std, n = 3) of samples analyzed immediately after gut fluid was added to the PAH stock. An extraction test showed that recovery of pyrene with the centrifugation step omitted was slightly higher 85% ± 7 (t-test, $p > 0.05$, n = 3) indicating some co-precipitation of pyrene with the protein. However, pyrene recovery remained constant through out the experimental period both with and without centrifugation and therefore centrifugation was applied as a clean-up procedure prior to HPLC analysis.

REMAZOL BRILLIANT BLUE DECOLORIZATION ASSAY

H_2O_2-dependent decolorization of certain dyes can indicate oxidoreductive enzyme activity – e.g. Remazol Brilliant Blue R (RBBR) has been used for peroxidases in fungi (e.g. Glenn and Gold 1983; Pasti and Crawford 1991; Vyas and Molitoris 1995). We tested small volumes of gut fluid by incubating a RBBR solution and activating with H_2O_2. Initial experiments showed that RBBR decolorization depends on initial concentration of H_2O_2 (10-100 μM) and the pH (6-8) of the reaction mixture. Highest reaction potential was observed at pH 8 with an initial H_2O_2 concentration of 100 μM (data not shown), which was used in subsequent RBBR experiments. Reaction mixtures consisted of 2 mL 1 mM RBBR in 0.1M phosphate buffer. To all reaction mixtures was

added 20 μL gut fluid and the initial absorbance at 595 nm was recorded. Controls consisted of RBBR with only gut fluid or H_2O_2 added. Influence of known inhibitors of oxidative enzymes (Dunford 1999; Saunders et al. 1964) were tested by adding NaN_3, NaCN, $Na_2S_2O_5$, and cysteine as aqueous solutions to the reaction mixture after addition of gut fluid and prior to the addition of H_2O_2. Decolorization of RBBR was studied in polystyrene cuvettes (VWR, NJ, USA) using a Hitachi 3010 spectrophotometer with a slit width of 2 nm, and a scan speed of 600 nm/min. Decolorization was visualized by recording UV absorption spectra of reaction mixtures in the 400-700 nm range and decolorization was quantified using a molar absorption coefficient of RBBR (ε_{595} = 6050 M^{-1} cm^{-1}) (Vyas and Molitoris 1995). Inhibition was calculated as $(A_i/A_0 - 1) \times 100$ where A_i is the absorption at different concentrations of inhibitor and A_0 is the absorption prior to addition of inhibitor.

TOTAL OXYRADICAL SCAVENGING CAPACITY ASSAY

One approach in assessing antioxidant capacity of a biological fluid is to measure its capability to absorb ROS by determining its total oxyradical scavenging capacity (TOSC) (Winston et al. 1998). Peroxyl radicals generated by thermal homolysis of 2,2'-azobis-amidinopropane (ABAP) cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene. In the presence of an antioxidant (in this case gut fluid) the oxidation of KMBA to ethylene is quantitatively reduced. Ethylene formation is then monitored by gas chromatography with flame ionization detection (GC-FID) of head space gas in the reaction vessel. Reaction mixtures consisted of 0.20 mM KMBA and 50 μL diluted gut fluid mixed in a 10 mL test tube to a final volume of 1 mL. Gut fluid

dilutions are indicated in the legend of Figure 5.7. The test tube was sealed with a rubber septum and 50 μL of 0.4 M ABAP was injected through the septum. The reaction mixture was allowed to equilibrate at 35˚C in a thermostated waterbath prior to addition of ABAP. A head space sample was drawn immediately after addition of ABAP using a 1 mL syringe and a Hamilton GC needle and analysed on a Shimadzu GC-14A/FID using a 30 m MEGABORE DB-1 column (J&W Scientific). Sample volume was 300 μL. All reactions were carried out at 35˚C because at this temperature peroxyl radicals are generated at a constant rate from ABAP (1.66×10^{-8} M s^{-1}). Head space samples were then drawn every 10 minutes for 60 minutes. Reduced glutathione was used as a reference antioxidant at concentrations indicated in the legend of Figure 5.7.

Based on the difference of ethylene formation between control (absence of peroxyl radical scavengers i.e. gut fluid) and samples containing gut fluid, TOSC values were quantified according to the equation: $\mathrm{TOSC} = 100 - (\int \mathrm{SA} / \int \mathrm{CA} \times 100)$ where $\int \mathrm{SA}$ and $\int \mathrm{CA}$ are the numerically integrated areas under the kinetic curves, of sample (SA) and control (CA) reactions respectively. Areas were calculated using Kaleidagraph™ (Synergy Software).

TYROSINE EXPERIMENT

As dityrosine is not commercially available it was synthesized by oxidation of L-tyrosine with HRP modified after Malencik et al. (1996). To a 5 mM L-tyrosine solution was added 10 mg HRP (116 e.u./mg) and 1.25 mmol H_2O_2 (0.5 mol H_2O_2/mol tyrosine) and briefly swirled. The reaction was stopped after 20 min by addition of 175 μL ß-mercaptoethanol to the reaction mixture. The golden brown solution was lyophilized and

then dissolved in HPLC buffer. HRP was separated by centrifugation in Microsep centrifugation tubes (Pall Life Sciences, MI, USA, 30.000 MW) at 5000 g for 30 min before injection on HPLC. Analysis of synthesized oxidation products was performed by RP/HPLC as described below.

Formation of L-tyrosine oxidation products by *Nereis virens* gut fluid was studied by incubating 3 mL 0.5 mM L-tyrosine in 0.1 M Na_2HPO_4 buffer (pH 8) with 150 μL gut fluid. The reaction mixture was left in the dark for 3 h. The reaction mixture was then centrifuged twice through Ultrafree-CL centrifugal filters (Millipore, Bedford MA, USA), with 30.000 MW and 10.000 MW cutoff respectively, for 30 min at 5000 g. The final filtrate was diluted 1:10 with HPLC buffer and analyzed using HPLC/F as described for the di-tyrosine standard.

SAMPLE PREPARATION AND TOTAL AMINO ACID ANALYSIS

Fecal matter from *Nereis diversicolor* was collected in a previous experiment (Giessing et al. 2002). 2 g of freeze dried fecal matter was extracted (Voparil and Mayer 2000) with acetone:hexane (1:1 by volume) for 20 min at 115˚C in a microwave (CEM MSP 1000). 200 mg of the microwave-extracted fecal matter residue was subsequently hydrolyzed with 5 mL 6N HCl at 150˚C for 22h in an acid digestion bomb (Parr Instrument Co., Moline IL, USA). The acid hydrolysate was adjusted to pH = 7 with NaOH and extracted 3 times with dichloromethane (DCM). The DCM phase was then dried over anhydrous sodium sulfate, blown down under a gentle stream of nitrogen, redissolved in 60/40 acetonitrile/water and analyzed using HPLC/F. Sediment spiked

with pyrene used for worm exposure was used as control sediment and treated as described for fecal matter above.

Total amino acid (TAA) contents of gut fluids were analysed according to Mayer et al. (1995). 10 μL of gut fluid (100 μL for *Parastichopus californicus*) was first hydrolysed in 6N HCl at 110˚C for 22 hours, and the hydrolyzed samples were derivatized with orthophthaldialdehyde (OPA) followed by fluorometric detection.

SPECTROSCOPY AND CHROMATOGRAPHY

In the HRP assay pyrenol and pyrene were analyzed by synchronous fluorescence spectroscopy (SFS). Using SFS with $\Delta\lambda$=34nm, pyrene and pyrenol spectra are reduced to single peaks at $\lambda_{EX/EM}$ of 338/372 nm and 350/384 nm respectively. We used methyl acrylate cuvettes in a Hitachi F-4500 spectrofluorometer, with operating conditions of 2.5/5 nm slit widths (emission /excitation), and a response time of 0.1 sec.

In gut fluid experiments, pyrene and pyrenol were analyzed on a Hitachi D-7000 HPLC system by injection of aqueous supernatants on a Supelcosil LC-PAH column, 25cm x 4.6 mm, 5μm (Supelco, Bellefonte, PA, USA). The acetonitrile/water (v/v) gradient profile used was 60/40 for 5 minutes and directly increase to 100/0 over 30 min, and then hold 100/0 for 10 min at a flow rate of 1.5 mL min^{-1}. Fluorescence detection was performed at $\lambda_{EX/EM}$ = 346/384 nm for pyrenol and $\lambda_{EX/EM}$ = 333/384 nm for pyrene. Injection volume was 250 μL and column temperature was kept at 30˚C.

HPLC analysis of tyrosine oxidation products was done on a Beckman Ultrasphere ODS column (250 x 4.6 mm, 5μm particles). Oxidation products were eluted isocratically at 1.8 mL/min with a mobile phase of 0.1M Na_2HPO_4 - 0.1 mM EDTA,

adjusted to pH 5.9 with acetic acid. L-tyrosine oxidation products were detected by fluorescence detection with $\lambda_{EX/EM}$ = 280/410 nm.

RESULTS

GUT FLUID ASSAYS

Nereis virens gut fluid catalyzed a significant reduction in pyrenol fluorescence intensity at $\lambda_{EX/EM}$ = 350/384 nm when activated with 1 μmol H_2O_2 (Figure 5.1). The observed pyrenol fluorescence intensity decreased immediately after H_2O_2 addition to pyrenol and gut fluid mixtures. Pyrenol - gut fluid and pyrenol - H_2O_2 showed no similar decrease in fluorescence. An identical experiment with pyrene showed no decrease of pyrene fluorescence in any of the 3 treatments (data not shown).

We define oxidative coupling potential as the decrease in recovery of PAH from an aqueous solution after incubation with invertebrate gut fluid activated with H_2O_2. Incubations with *Nereis virens* gut fluid activated with H_2O_2 showed a pronounced reduction in the amount of dissolved pyrenol to less than 0.5 % of the concentration measured in the control experiment (Figure 5.2). Gut fluid did not show any significant oxidative coupling potential when incubated with pyrene. Pyrene recoveries were 63% ± 2 and 66% ± 1 for H_2O_2 activated and control, respectively, which were not significantly different (t-test, $p > 0.05$). Recoveries of pyrenol and pyrene in all gut fluid treatments were all statistical significantly lower than recoveries, 94% ± 8 and 86% ± 5 respectively, from an aqueous standard (t-test, $p < 0.05$). Sorption of pyrene and pyrenol into the reaction vessel polymer could account for < 1% of the loss during the 1h incubation

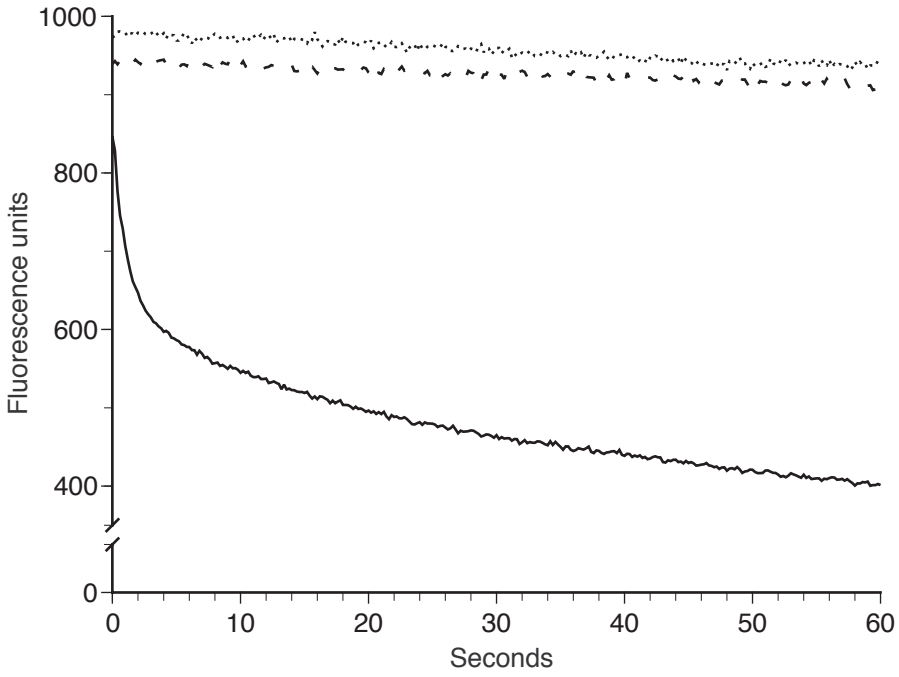

Figure 5.1 Fluorescence time scan of an aqueous pyrenol and *Nereis virens* gut fluid. Pyrenol stock solution was mixed with either gut fluid (dashed), 10 μl 0.1M H_2O_2 (dotted line), or both added (solid line). Fluorescence was recorded at $\lambda_{EM/EM}$ 350/384nm with continuous stirring of the cuvette. Gut fluid was diluted 1:100 to reduce pyrenol quenching

period. Thus lower than expected recoveries are most likely due to uptake of PAH by gut fluid DOM. Pyrenol recovery in the DI control treatments in experiment one was 75% ± 5. Based on a time series experiment using the same gut fluid, roughly 80% of pyrenol loss during the 1 h incubation period in the control treatment (i.e. DI addition) can be explained by oxidative coupling reactions without addition of H_2O_2 (data not shown).

Gut fluid from all of the 4 species tested showed significant pyrenol oxidative coupling potential when incubated with H_2O_2 (Figure 5.3). Pyrenol recoveries were lower than 0.5% in the polychaete species while gut fluid from *Parastichopus californicus* showed recovery that was 48% of the control incubation. Recovery of pyrenol from controls (i.e. DI additions) varied between 53-93% among the four species. TAA content of the gut fluid from the 4 species was 0.6 mg mL^{-1}, 52.5 mg mL^{-1}, 21.7 mg mL^{-1}, 41.3 mg mL^{-1} for *Parastichopus californicus*, *Arenicola marina*, *Nereis virens*, and *Arenicola brasiliensis* respectively. There was a moderate but not statistically significant negative correlation (Pearson product-moment correlation, $r = -0.85$, $P = 0.15$, $n = 4$) between amount of TAA in the gut fluid and average recovery of pyrenol. Thus, with the caveat of non-H_2O_2-activated pyrenol loss in control treatments explained above, variation in pyrenol recovery in DI controls can to some extent be explained by sorption to and subsequent co-precipitation with protein during sample preparation. *Nereis virens* gut fluid lowered the dissolved pyrenol concentration without addition of H_2O_2 (Figure 5.4) to less than 10% after 2 hours. Adding the oxidative inhibitor $Na_2S_2O_5$ reduced both the apparent rate and the final magnitude of removal. Pyrene concentration declined insignificantly. 70% of total extractable pyrenol in fecal matter from a congener species *Nereis diversicolor* exposed to sediment-associated pyrene was in the acid hydrolysis

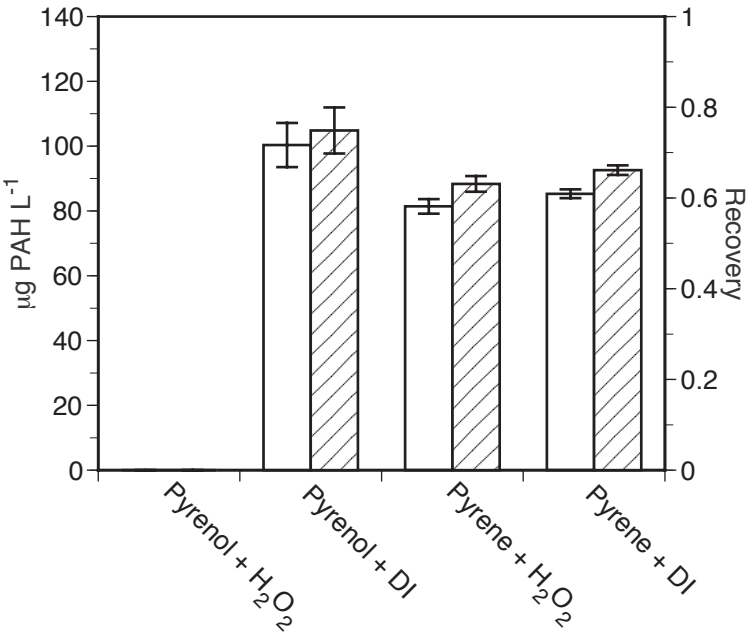

Figure 5.2 Oxidative coupling of pyrenol by *Nereis virens* gut fluid.

Residual aqueous PAH in 10:1 mixtures of PAH stock and gut fluid after incubation with 5 μmol H_2O_2. White bars are in μg/L and dashed bars are recovery. Data are presented as average ± SD (n = 3).

pool (Table 5.1), and constituted 0.88% of total extractable PAH (pyrene + pyrenol) from fecal matter. Pyrene extractability on the other hand, did not increase after acid hydrolysis in either fecal matter or control sediment.

RBBR DECOLORTIZATION ASSAY

Gut fluid from *Nereis virens* could effectively decolorize RBBR when H_2O_2 was added to the reaction mixture (Figure 5.5A). In absence of gut fluid, addition of H_2O_2 had no influence of the RBBR decolorization reaction (Figure 5.5B, dotted line). The visible spectra of the reaction mixture at 1h intervals and 3 consecutive additions of 100 mmol H_2O_2 (Figure 5.5A, I-III) showed that RBBR underwent degradation and that the decrease in absorbance was not due to physical removal when compared to spectra of different RBBR concentrations (Figure 5.5B, 1-4). Inactivation of a heme-containing enzyme may be brought about by the addition of a substance which can attach to the sixth co-ordination position of the metal atom in the heme group (e.g. NaCN and NaN_3). Inactivation of an oxidative enzyme can also result from addition of a reducing agent (e.g. $Na_2S_2O_5$), which reduces the active metal of the prosthetic group or by addition of low molecular weight non-enzymatic antioxidants (e.g. cysteine) which lower the amount of peroxide that can reduced by the enzyme. NaCN, $Na_2S_2O_5$, and cysteine could all inhibit the decolorization of RBBR by up to 35% (Figure 5.6, A-C). In the case of NaN_3 (Figure 5.6D) the influence was less conclusive and repeated experiments with varying azide concentrations did not improve results (data not shown). Studies of mechanism-based inactivation agents on HRP have established that sodium azide is oxidized to a free radical that does not add to the iron or the porphyrin nitrogens of the heme group in a

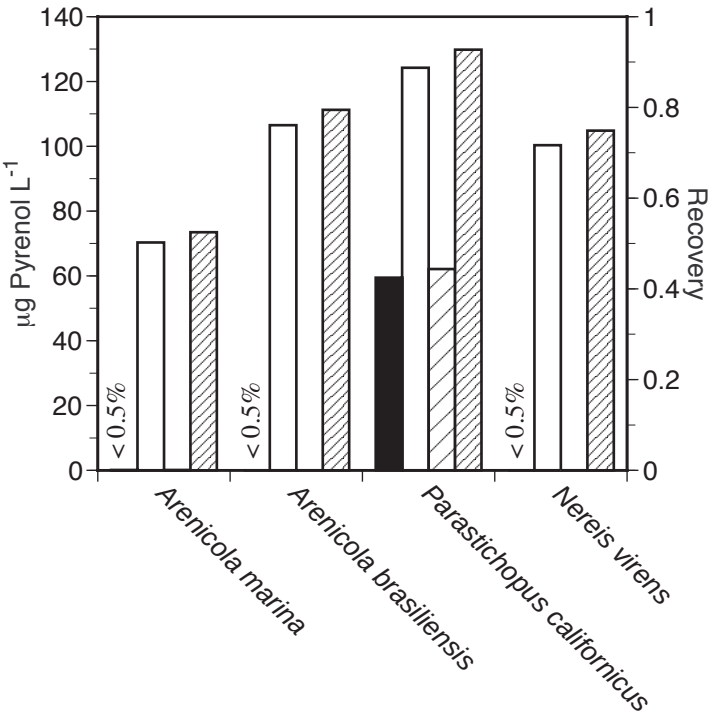

Figure 5.3 Oxidative coupling by gut fluid from 4 species of deposit-feeding invertebrates.

Residual aqueous pyrenol in 10:1 mixtures of PAH stock and gut fluid from 4 deposit feeding invertebrates incubated with 5 μmol H_2O_2. Black (H_2O_2 activated) and white (DI control) bars indicate pyrenol in μg/L and dashed bars indicate pyrenol recovery. Data are presented as average ± SD (n = 3).

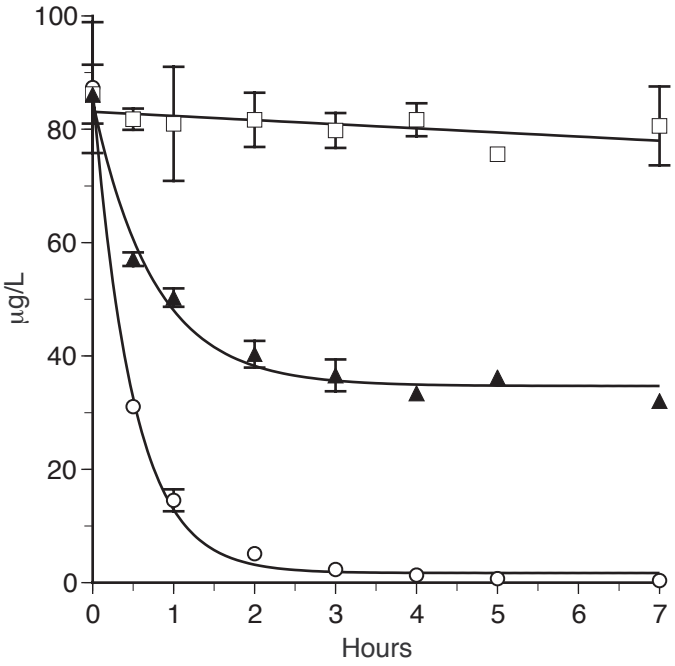

Figure 5.4 Oxidative coupling of pyrenol by *Nereis virens* gut fluid without H_2O_2. Residual aqueous pyrenol in 10:1 mixtures of PAH stock and Nereis virens gut fluid in the absence of external H_2O_2.The concentration of dissolved pyrenol (open circles) is reduced to less than 10% of initial aqueous concentration within 2 hours of incubation. Adding an oxidative enzyme inhibitor (250 μM $Na_2S_2O_5$) to the assay (filled triangles) increases pyrenol recovery whereas pyrene concentration (open squares) remains constant over the experimental period. Error bars (± 1 SD) are given when their size exceeds that of the symbol used.

Table 5.1 PAHs in fecal matter from pyrene exposed polychaetes.

Sequential microwave assisted solvent extraction and acid hydrolysis of fecal matter from pyrene exposed *Nereis diversicolor*. Data are presented as average ± standard deviation μg g^{-1} sediment (dw) of triplicate samples with the relative amounts (percent) given in parenthesis.

	Microwave extraction		**Acid hydrolysis**	
	Control	Fecal pellets	Control	Fecal pellets
Pyrene	18.6 ± 1.6 (92)	7.5 ± 0.6 (95)	1.6 ± 0.24 (8)	0.4 ± 0.2 (5)
Pyrenol	nd.	0.03 ± 0.01 (30)	nd.	0.07 ± 0.007 (70)

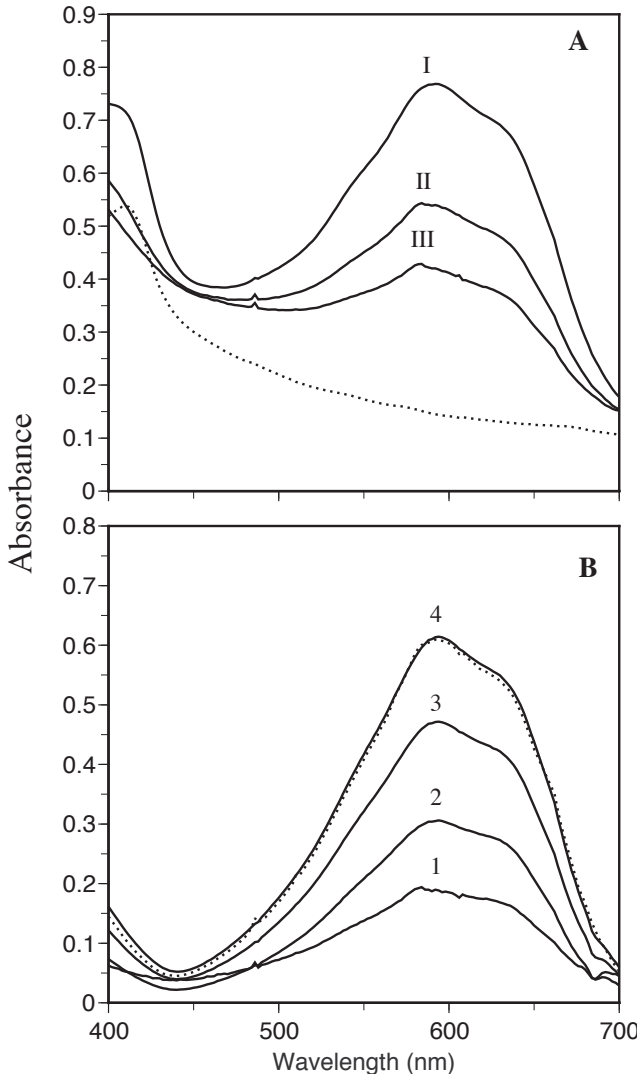

Figure 5.5 Remazol brilliant blue decolorization by *Nereis virens* gut fluid.

A Visible spectra of RBBR at various stages of the decolorization catalyzed by gut fluid from *Nereis virens* before (I) and after 2 additions of 100 mmol H_2O_2 (II and III) with 1 h interval and **B** at (1) 25 μM, (2) 50 μM, (3) 75 μM, and (4) 100 μM concentrations of native RBBR. The dotted line in **A** is the visible spectra of gut fluid in buffer and in **B** the visible spectra after 2 additions of 100 mmol H_2O_2 to RBBR.

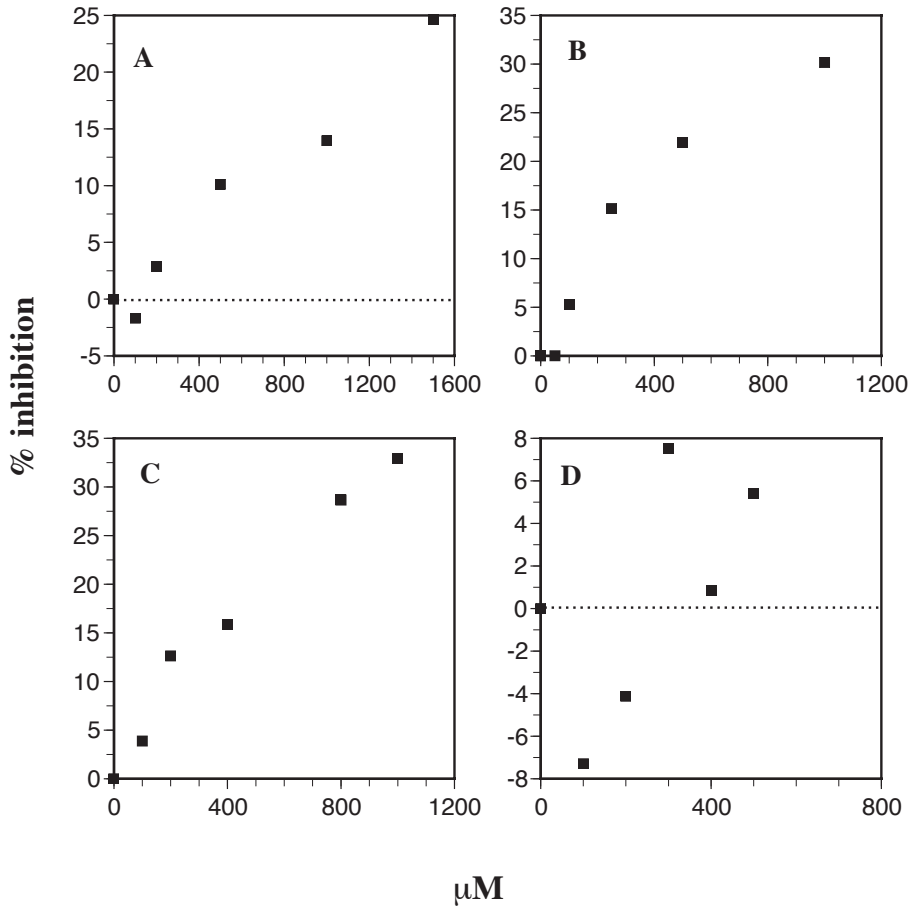

Figure 5.6 Influence of various concentrations of enzyme inhibitors on RBBR decolorization reaction.

(**A**) Sodium cyanide, (**B**) sodium metabifulfite, (**C**) cysteine, and (**D**) sodium azide, (pH = 8).

peroxidase (DePillis et al. 1990 and references therein) thereby inhibiting enzyme activity.

Enzyme activity can also be reduced by heat inactivation. Boiling gut fluid, but allowing it to cool to room temperature prior to use, had no effect on the RBBR decolorization (data not shown).

TOTAL OXYRADICAL SCAVENGING CAPACITY

Gut fluid from *Nereis virens* strongly reduced ethylene production from KMBA, even at a 1:1000 dilution (Figure 5.7). Ethylene concentration was significantly reduced with decreasing dilution of gut fluid and increasing glutathione concentration. At lower dilutions of gut fluid (1:50) a distinct induction period of 60 min was observed. Calculating the area under the curves for all dilutions of *Nereis virens* gut fluid and control experiments and using a gut fluid protein concentration of 21.7 mg mL^{-1} gave a TOSC value of 1.82 ± 0.14 per μg TAA (n = 4).

TYROSINE EXPERIMENT

L-tyrosine was oxidized to dimers and trimers when incubated with gut fluid without the addition of H_2O_2 (Figure 5.8). Three fluorescent peaks in gut fluid-incubated samples were identified by HPLC/F (Figure 5.8A). HPLC/F of the dityrosine standard prepared with HRP gave three peaks with identical retention times (Figure 5.8B), which are identified as dityrosine, trityrosine and pulcherosine based on the identification made by Jacob et al. (1996). Dityrosine in gut fluid sample was identified by addition of a small aliquot of manually collected dityrosine from the HRP-prepared standard (Figure 5.8C).

A large peak at 8.7 min was not identified. It may be a second dimer, isodityrosine, of tyrosine oxidation with the two tyrosine units linked by a biphenyl ether bridge (the two rightmost tyrosine units in pulcherosine on Figure 5.8) which has been identified in plant cell-wall glycoprotein (Fry 1982).

DISCUSSION

The observed oxidative coupling potential of polychaete gut fluid and the tentative identification of a heme-containing oxidoreductive enzyme indicates that metabolites of PAHs can undergo enzyme catalyzed oxidative coupling during gut passage in a marine deposit-feeder. Evidence for an enzymatically catalyzed process is further corroborated by the high antioxidant capacity of unaltered gut fluid and covalently bound pyrenol in fecal matter. Binding of contaminants to humic matter will decrease the amount of material to interact with biota, both micro- and macrofauna, thus reducing toxicity of the compounds and rendering them resistant to further biotransformation (i.e. microbial degradation) and thus represent a new sink for organic contaminants in marine sediments. Furthermore, identification of dityrosine as an oxidative coupling product (Figure 5.8) suggests that gut passage could be important in creating humic-like polymers through the formation of stable covalent bonds thereby, forming recalcitrant organic matter in marine sediments.

Due to the complex nature of gut fluid, the resolution of the employed method is currently too low to include product studies of other compounds with oxidative coupling potential present in marine sediments (e.g. brown algae phlorotannins). Future product

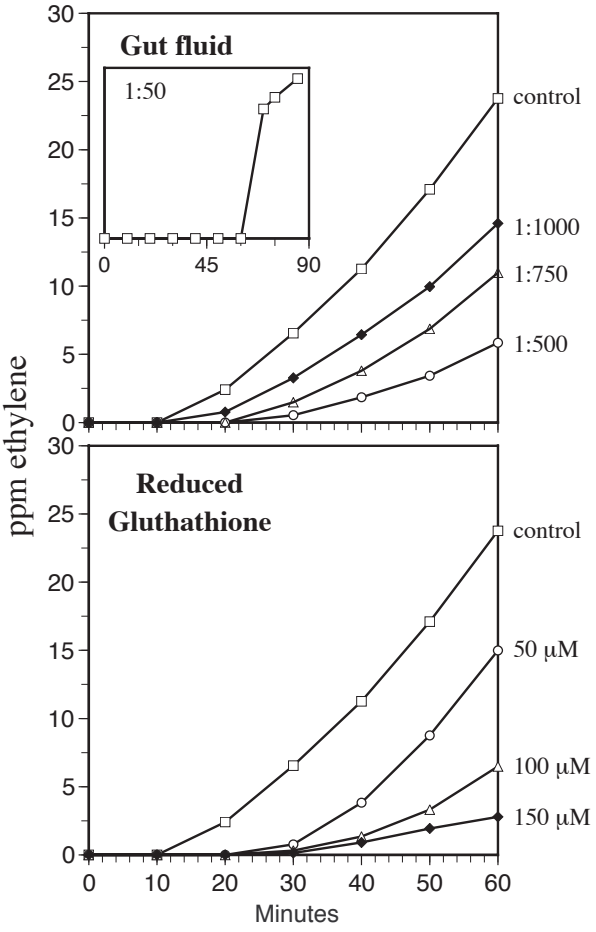

Figure 5.7 Total oxyradical scavenging capacity of gut fluid.

Time course of ethylene production from oxidation of KMBA by peroxyl radicals in the presence of different dilutions of *Nereis virens* gut fluid (top panel) and reduced gluthathione (bottom panel).

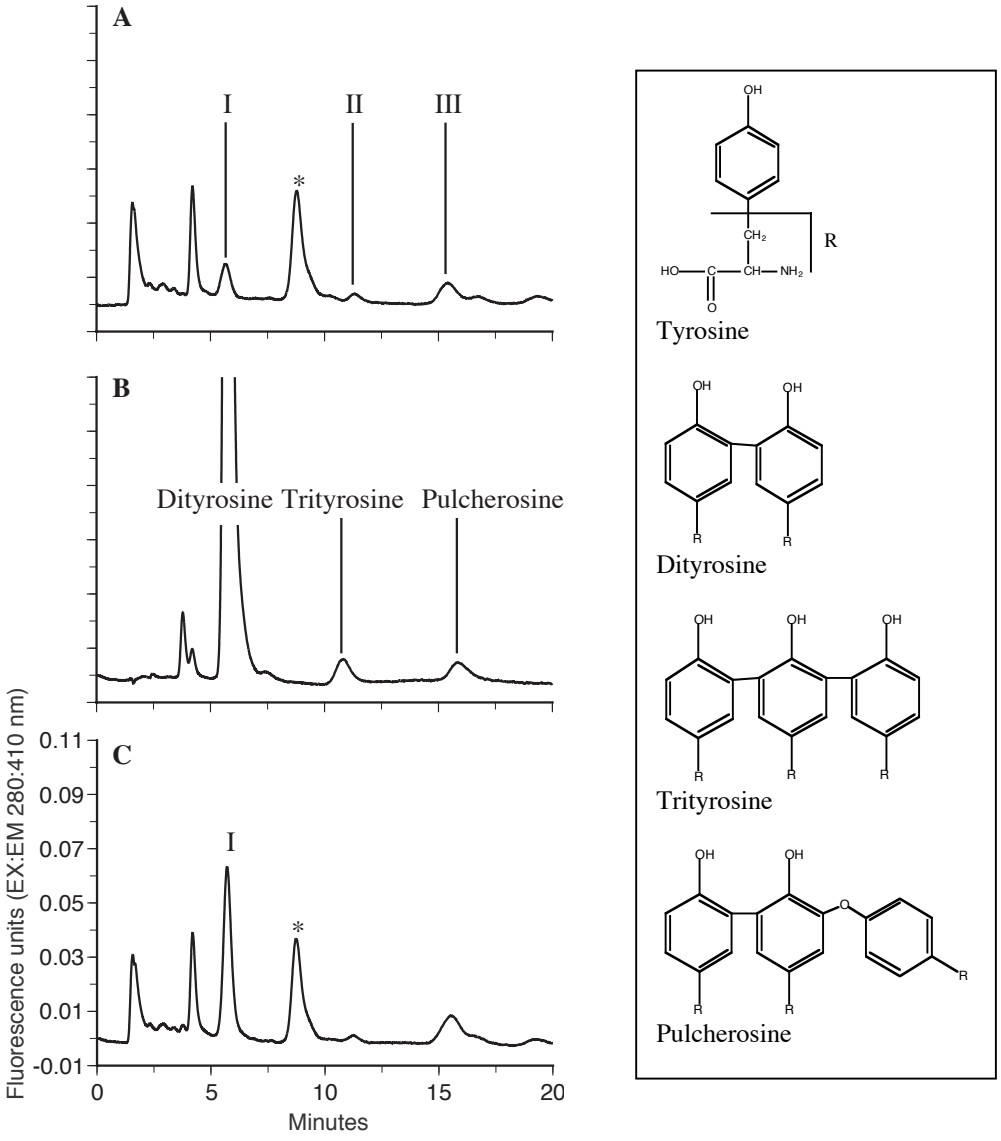

Figure 5.8 Tyrosine oxidation catalysed by *Nereis virens* gut fluid.

Oxidation products, peaks I-III, are identified as dityrosine, trityrosine, and pulcherosine based on a HRP-prepared standard and on the identification made by Jacob et al. (1996). Peak marked with an asterisk is unidentified. See text for details.

studies awaits isolation of the proposed peroxidase. However, due to its chemical stability and its widespread use as a marker for oxidative stress in human disease, dityrosine in marine sediments might prove a valuable marker for the influence of marine macrofauna on preservation of organic matter in the marine sediments.

OXIDATIVE COUPLING

Oxidative coupling potential is here operationally defined as the decrease in recovery of pyrenol from aqueous solution after incubation with invertebrate gut fluid activated with H_2O_2. However, even without H_2O_2 activation pyrenol recovery is less than 10% after 2 hours of incubation with *Nereis virens* gut fluid (Figure 5.4). Reactions of this nature, either polymerization or coupling reactions, will affect fluorescence characteristics of pyrenol, resulting in loss of the original peak at $\lambda_{EX/EM}$ = 350/384 nm. Generally an increase in degree of conjugation (i.e. extent of aromatic structure) leads to a shift of absorption and fluorescence spectra to longer wavelengths and to an increase in fluorescence yield. However, fluorescence characteristics of aromatic hydrocarbons containing more than one substituents are difficult to predict and effects of, for example, polymerization or oxidative coupling to DOC cannot simply be extrapolated from the individual substituents. To our knowledge there exist no data on the effect of polymerization on fluorescence characteristics of pyrenol in the literature.

Richnow et al. (1997) demonstrated efficient binding of 4 aromatic alcohols, including pyrenol, to soil humic substances using a HRP assay. The authors observed that cross-linking between naphthols was not significant in the presence of humic substances. Binding of other environmentally relevant chemicals (i.e. aliphatic and aromatic

hydrocarbons, fatty acids, and aliphatic alcohols) to humic acid in a HRP/H_2O_2 assay was also tested, but only aromatic alcohols were found to be reactive. Thus formation of pyrenol polymerization products are not likely with the high DOC commonly observed in gut fluid.

It is possible that non-covalent processes influenced the fate of pyrene and pyrenol in our experiments and hence loss of pyrenol fluorescence signal. The lower than expected recovery of pyrene and pyrenol (Figure 5.4 and Figure 5.5) might in part be due to structural changes in organic matrix brought on by an enzyme-catalyzed oxidative reaction. Formation of aryl and alkyl ethers catalyzed by HRP is known to increase conformational stability and molecular size of humic substances (Cozzolino and Piccolo 2002). Oxidative coupling of dissolved organic matter (DOM) present in gut fluid may result in increased non-covalent binding of pyrenol and pyrene, decreasing recoveries. The lower recoveries could also in part be explained by sorption of pyrenol and pyrene on solid metal oxides. Gut fluids from deposit feeding invertebrates contain high concentrations of dissolved transition metals (Chen et al. 2000) which might be oxidized to particulate metal oxides by H_2O_2. In general, only at fractions of organic matter (f_{OC}) lower than 0.002 does sorption to mineral surfaces contribute to total sorption of a hydrophobic compound (Schwarzenbach et al. 1993). Thus with the DOC concentrations commonly observed in gut fluid, sorption of pyrenol and pyrene to metal oxides are considered insignificant for the low recoveries.

Of the four deposit feeding marine invertebrates tested for oxidative coupling potential of pyrenol, only gut fluid from the sea cucumber, *Parastichopus californicus*, left significant amounts of dissolved pyrenol after the one hour incubation period (Figure

5.3). Echinoderms (*Parastichopus californicus*) and polychaetes (*A. marina*, *A. brasiliensis*, and *Nereis virens*) differ in terms of digestive strength (hydrolytic enzyme activity); digestive fluids from echinoderms tend to be rather weak and those from polychaetes tend to be relatively strong (Mayer et al. 1997). Total amino acid content of gut fluid from the 4 species used in this experiment follow the same trend and can in part explain the variation in pyrenol recoveries in control treatments observed among the 4 species. Activities of deposit feeder digestive enzymes (i.e protease, lipase and esterase) generally correlate well with one another (Mayer et al. 1997) and if the enzymes that cause oxidative coupling of pyrenol are similarly correlated, it would seem reasonable to assume that their activity would follow a similar cross-phyletic pattern.

Sequential microwave-assisted organic solvent extraction, followed by acid hydrolysis, of fecal matter from *Nereis diversicolor* exposed to sediment associated pyrene gave 70% of total extractable pyrenol in the acid hydrolysis pool (Table 5.1). Though pyrenol constituted less than 1% of total extractable PAH (i.e. [pyrene] + [pyrenol]) in fecal matter, the significant increase in pyrenol extractability (133%) after acid hydrolysis suggests that pyrenol was bound through ester bonds in fecal matter. Richnow et al. (1997) found 0.26% - 0.61% of total degraded PAHs, as ester-bound pyrene, phenanthrene and anthracene metabolites in humic substances isolated from a biodegradation experiment with artificially contaminated soil. They also found 0.03% of total degraded pyrene as ether-linked pyrenols.

A smaller but discernible increase in pyrene extractability, both from fecal matter and control sediment, was observed after acid hydrolysis. Organic matter on sediments occur in somewhat isolated and porous patches of organic chains with non-aqueous

cavities for hydrophobic sorbates like pyrene to 'dissolve' in. Depending on the provenance of the organic matter, acid hydrolysis might perturb these patches, making more pyrene susceptible to organic solvent extraction.

Pyrenol is the only hydroxylated metabolite of pyrene in *Nereis diversicolor* (Giessing et al. 2002). Rapid intracellular turnover of pyrenol to a glucuronic acid conjugate limits the amount of pyrenol leaking into the gut thereby reducing the amount of pyrenol available to participate in oxidative coupling reactions. PAHs with more hydroxylated metabolites than pyrene (e.g. benzo[a]pyrene and fluoranthene) might undergo more significant oxidative coupling during gut passage, making this reaction a significant biogeochemical sink for PAH in marine sediments.

OXIDOREDUCTIVE ENZYMES AND OXYRADICAL SCAVENGING CAPACITY OF GUT FLUID

As previously mentioned decolorization of Remazol Brilliant Blue R has been extensively used to identify oxidoreductive enzyme activity, especially in fungi. The dependence of the gut fluid-catalysed decolorization of RBBR on H_2O_2 (Figure 5.5) and inhibition of the reaction with known inhibitors of oxidative enzymes (Figure 5.6), indicates that the observed oxidative coupling reaction is in part an enzymatic process. Furthermore, inhibition by cyanide suggests catalytic metal centers such as a heme prosthetic groups. Enzyme activities may be diminished by mechanical breakdown of the enzyme molecule, for example by heating. Peroxidases are perhaps the most thermo-stable of all enzymes (Saunders et al. 1964) and the activity of heat-inactivated enzyme is often regained upon cooling to room temperature (Lu and Whitaker 1974). Similarly, we found heat inactivation, followed by cooling, had no impact on oxidative coupling

activity. The combination of these results suggest that the enzyme responsible is a heme peroxidase.

Peroxidases are powerful antioxidants (Dunford 1999). In the TOSC assay at 1:50 dilution of gut fluid a distinct induction period, in which ethylene formation was totally inhibited or retarded relative to controls, was observed (insert on Figure 5.7 top panel) indicating the presence of fast acting antioxidants (Winston et al. 1998). Furthermore, other naturally occurring antioxidants, e.g. low molecular weight compounds such as vitamin C, might be present in the reaction mixture competing with antioxidant enzymes for peroxyl radicals generated by ABAP. Though direct evidence for vitamin C acting as an extracellular antioxidant *in vivo* is limited, in humans for example, vitamin C is secreted into gastric juice at higher concentrations than those observed in plasma (Halliwell and Gutterridge 1999).

A high TOSC value would be expected if the oxidative coupling reactions are due to an antioxidant enzyme. *Nereis virens* gut fluid gave TOSC value of 1.82 ± 0.14 per μg TAA. Previous studies have shown that protein derived amino acids (i.e. high molecular weight forms) constitute about half of TAA in *N. virens* gut fluid (Mayer et al. 1997) thus doubling the TOCS value when normalized to protein. Regoli and Winston (1998) reported TOSC values of 0.39 ± 0.02 μg^{-1} protein to 1.47 ± 0.08 μg^{-1} protein in rat liver and basal stalk of sea anemones (*Bunodosoma cavernata*), respectively, using the same method. The same study showed TOSC values that were 60-90% higher in digestive gland of mussel (*Mytilus edulis*) and pyloric caeca of starfish (*Leptasterias epichlora*) than in rat liver cytosolic fractions. Enzymatic oxidative coupling of pyrenol is further supported by the relatively high TOSC value of gut fluid in combination with the

indication of fast acting antioxidants. Furthermore, the high TOSC value of gut fluid indicates that deposit-feeder guts are places of significant ROS production, possibly resulting from ingestion of large amounts of sediment rich in redox-cycling transitions metals.

It is also possible that dissolved metals and particulate metal oxides contribute significantly to the oxidative coupling potential of gut fluid. Among metal oxides, hydroxides, and oxyhydroxides, manganese oxides are the most powerful catalysts in transformation of phenolic compounds to humic substances (Shindo and Huang 1982; Shindo and Huang 1984). Larson and Hufnal (1980) reported that dissolved metal cations, clays, and insoluble oxides promoted the polymerization of polyphenolic compounds and that stream water and sediment appears to catalyze the same reaction. Their experiments also showed that phenols without adjacent hydroxyl groups were nearly inert to auto-oxidation or to MnO_2-promoted polymerization at pH 7.6. It is not possible to discern the relative importance of enzymatic versus metal catalyzed oxidative coupling of pyrenol our simple test system. Oxidative coupling in vivo is most likely promoted by both enzymatic and abiotic reactions, with potential synergistic effects.

Oxidoreductive enzymes like peroxidases occur in protozoa, mollusca, annelida, echinodermata, and vertebrata (Dunford 1999; Saunders et al. 1964). Peroxidases defend against ROS formed as a normal consequence of essential biochemical reactions like mitochondrial and microsomal electron transport systems and phagocytosis (Dhainaut and Scaps 2001; Halliwell and Gutterridge 1999). High levels of halogenated aromatic compounds have been found in marine invertebrates (Gribble 1994; King 1986; Woodin et al. 1987) and are believed to be produced as secondary metabolites through the action

of haloperoxidases (Chen et al. 1991; Fielman and Targett 1995; Woodin et al. 1987). Little is known about the presence and/or function of extrasomatic peroxidases in aquatic invertebrates. Defretin (1941) reported the presence of peroxidase in the mucus of the parapodial glands of *Nereids*. Based on the distribution of the enzyme, Defretin argued that the observed activity was due to a peroxidase but did not isolate and identify the enzyme. On a more general level, vital cellular antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase have been identified in aquatic animals with marked quantitative differences among animals in levels and response of the enzymes (reviewed in Felton 1995; Winston 1991). Though Halliwell and Gutterridge (1990) concluded that extracellular antioxidant enzymes contribute little to the antioxidant status of human extracellular fluids, marine deposit-feeder guts might represent an environment where extracellular, indeed extrasomatic, antioxidant enzymes are important factors in protecting against ROS produced as a consequence of the unique digestive environment.

Neuhauser & Hertenstein (1978) suggested that soil invertebrates use peroxidase enzymes as a defense mechanism against invasion of microbes. They also observed that peroxidase activity was present in tissue of most invertebrates (slugs, earthworms, millipedes) that feed largely on material rich in lignins and/or tannins. The origin of the peroxidase or other oxidoreductive enzymes in gut fluid of marine deposit-feeding invertebrates remains unclear. This type of enzyme could be actively secreted by the animal as protection against ROS, as a defense mechanism against pathogenic microorganisms, or as a digestive agent. Deposit-feeding macrofauna consume and digest sedimentary bacteria and though gut fluid from marine deposit-feeding polychaetes have

bacteriolytic activities (Hymel and Plante 2000; Plante et al. 1996), bacterial biomass is considered to constitute only a minor fraction (<10%) of the bulk carbon requirements of deposit-feeders (Cammen 1980b; Cammen 1989). Marine bacteria in general secrete extracellular enzymes and ingested bacteria that remain viable may contribute to invertebrate digestion, though no microbial extracellular enzymes have been identified as oxidoreductases (Harris 1993). Peroxidase activity in gut fluid could simply be a consequence of lysis of prey or digestive tract epithelial cells. Activities of cytosolic antioxidant enzymes have been reported to be up to 57 times higher in gut tissue than in somatic tissue of *Arenicola marina* (Buchner et al. 1996) and deposit-feeding polychaetes are thought to retain gut fluid by antiperistaltic pumping (Mayer et al. 1997) with the potential to build up enzyme activities.

OXIDATIVE COUPLING OF TYROSINE

Enzyme-catalyzed oxidative coupling in animal guts not only suggests a mechanism for incorporation of PAHs into humic matter but also suggests a biological mechanism for the formation of aquatic humic material in general. Oxidative coupling of free tyrosine is not precluded, but dityrosine is readily formed in by oxidative coupling of tyrosine residues in polypeptides (e.g. Audette et al. 2000; Michon et al. 1997). Formation of tyrosyl radicals can also initiate lipid peroxidation (Savenkova et al. 1994). Deposit-feeders typically process large amounts of sediment on a daily basis (Cadée 1976; Lopez and Levinton 1987), which might catalyze significant oxidative coupling of organic compounds.

Formation models of marine humic matter, both dissolved and sedimentary, may be categorized into two groups: the small molecule polymerization model and the biopolymer degradation and polymerization model (Ishiwatari 1992). Of the former category the melanoidin or 'browning reaction' model has been proposed as one possible pathway for formation of humic substances (Yamamoto and Ishiwatari 1989a; Yamamoto and Ishiwatari 1989b). Melanoidens are produced by condensation reactions between amino acids and carbohydrates (Maillard reaction). However, the reaction between dissolved free carbohydrates and amino acids at the low concentrations found in most marine systems is considered highly unlikely in natural systems (Ishiwatari 1992). Another small molecule polymerization model, the polyunsaturated lipid-condensation model, assumes that polyunsaturated fatty acids or lipids containing polyunsaturated carbon chains polymerize into humic substances. The degradation and polymerization model assumes that biopolymers are first degraded to small molecules which then re-polymerize to produce humic substances. Macromolecular material thus represents the remains of biopolymers after the labile components (i.e. readily hydrolysable lipids, polysaccharides and proteins) have been degraded. Neither of the two model categories incorporates a direct biologically mediated pathway for the formation of aquatic humic matter. Deposit-feeder guts represent a reaction zone whose biogeochemical conditions are considerably different from what is observed in the surrounding sediment. High concentrations of DOC, redox-cycling metals, and an apparent high oxidoreductive enzyme activity in gut fluids would provide a favorable environment for oxidative coupling of organic matter to occur. Oxidative coupling during gut passage may represent a significant biological pathway for formation of marine humic matter.

6. MICROBIAL DEGRADATION OF PAH METABOLITES FROM A MARINE DEPOSIT-FEEDING POLYCHAETE. PRELIMINARY RESULTS

ABSTRACT

We compared the mineralization of [4,5,9,10-^{14}C]pyrene and its eukaryotic [4,5,9,10-^{14}C]pyrene metabolites. Metabolites were obtained by exposing the deposit-feeding marine polychaete, *Nereis diversicolor*, to sediment-associated ^{14}C-pyrene and using a tissue homogenate as inoculum in microbial degradation experiments. Neither metabolites nor pyrene were substantially mineralized by microbial populations in sediment from a pristine Danish Fjord. A lag phase of 40 d was observed and only 2.6% and 1.7% of total label had evolved as $^{14}CO_2$ after 175 d from metabolite and pyrene treatments respectively. Pre-exposure to unlabelled pyrene for 60 days significantly increased mineralization and supported 80% and 15% mineralization of the ^{14}C-label added in pyrene and metabolite treatments respectively. No lag period was observed and mineralization was reduced by amendment of crude oil. Mineralization of both pyrene and metabolites in a naturally aged oil contaminated sediment was less than 0.6 % of total label after 50 days exposure. A micro titer plate method indicated that 1-hydroxypyrene, the single eukaryotic metabolic intermediate of pyrene, is not utilized as a sole source of carbon and energy by strains of known pyrene degraders. Microbial degradation of pyrene is not enhanced by production of aqueous and polar metabolites by marine invertebrates. The evidence strongly suggests that enhanced degradation of larger PAHs

in marine sediments is primarily due to bioturbation and irrigation processes of infaunal invertebrates.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of aquatic and terrestrial environments (Hites et al. 1980; Neff 1985). PAHs are hydrocarbons composed of two or more fused aromatic (benzene) rings. PAHs enter environments from a variety of anthropogenic sources such as industrial effluents and seepage, spillage of petrochemical products, and incomplete combustion of fuel and other organic materials and from natural sources such as forest fires and volcanic eruptions (Johnson et al. 1985; Neff 1985). Due to their low aqueous solubility and hydrophobic character, PAHs are readily adsorbed to particulate organic matter and solid surfaces when released into the marine environment (Karickhoff and Morris 1985). Thus marine sediments act as a sink, accumulating PAHs from multiple sources.

It is well known that PAHs can be degraded by microbial populations indigenous to marine sediments (Cerniglia and Heitkamp 1985; MacGillivray and Shiaris 1994). Low-molecular weight PAHs (≤ three fused benzene rings) degrade readily in sediments whereas high-molecular weight PAHs (≥ four fused benzene rings) appear more resistant to microbial attack (Cerniglia 1991), even in aquatic ecosystems which are chronically exposed to petrogenic hydrocarbons (Heitkamp and Cerniglla 1987). Low molecular weight PAHs are known to undergo microbial degradation under anaerobic conditions (Grbic-Galic et al. 1991) but only a few studies have demonstrated degradation of larger PAHs under anaerobic conditions (Coates et al. 1996; Coates et al. 1997; Zhang and

Young 1997). There are also only a few reports of anaerobic degradation of complex mixtures of PAHs and the capacity to degrade PAHs is most likely the result of previous long-term exposure to high concentrations (Coates et al. 1997). Limited information exists on microbial utilization of the larger PAHs as sole sources of energy and carbon; their degradation of larger PAHs probably results from cometabolic reactions that occur very slowly in a natural environment (reviewed in Kanaly and Harayama 2000).

Lack of accumulation of oxygenated metabolic intermediates in sediments containing larger PAHs supports the hypothesis of Herbes and Schwall (1978) that the rate limiting steps in catabolic aerobic degradation of PAHs is the initial ring-oxygenation and that intermediate intracellular metabolites are further oxidized almost as rapidly as they are produced. Aerobic degradation rates of PAHs in marine sediments are typically slow and microbial degradation is primarily thought to be limited by the concentration of PAHs solubilized in the aqueous phase (Bosma et al. 1997; Harms and Bosma 1997). Furthermore, molecular oxygen is essential to catalyze the initial oxygenation by microorganisms (Cerniglia and Heitkamp 1985). Microorganisms play an important role in the recycling of elements such as carbon, nitrogen and phosphorous and this activity quickly depletes oxygen. The rate of oxygen consumption thus generally exceeds the rate at which oxygen can be replenished (Fenchel et al. 1998). Deposit-feeding invertebrates, such as polychaete worms, are known to stimulate microbial degradation rates of both natural organic material in sediments (e.g. Aller et al. 2001) as well as sediment-associated contaminants (e.g. Holmer et al. 1997). Reworking of sediment by deposit-feeding macrofauna extends aerobic degradation by distributing oxygen further into the sediment subsurface layers (Fenchel 1996). Repetitive oscillating

redox conditions have been shown to stimulate organic matter degradation (Aller 1994) and PAH degradation potentials of burrow sediment are generally higher than degradation potentials for non-burrow sediment (Chung and King 1999).

Marine invertebrates are known to accumulate and metabolize PAHs (e.g. Forbes et al. 2001; Giessing et al. 2002; McElroy et al. 2000). Insofar as low bioavailability controls microbial degradation of sediment associated PAHs, increased bioavailability due to animal metabolic processes would therefore increase degradation, either by enhancing diffusion and/or by providing the PAHs in a more readily degradable form. Furthermore, animal activities will increase oxygen penetration in upper sediment layers and flush burrows of parent compound and metabolites and maintain a concentration gradient favoring desorption of PAHs into burrow wall sediment and burrow water.

The present study was carried out to evaluate if production of polar and aqueous metabolites by a marine invertebrate increases the microbial degradation potential of ^{14}C-labeled PAH in pristine and oil contaminated sediments. Pyrene, a tetracyclic PAH, was chosen as a suitable model PAH because of its simple metabolic pattern. 1-hydroxypyrene is the only intermediate metabolite of pyrene commonly observed in both invertebrate and vertebrate species (e.g. Giessing et al. 2002; Jacob et al. 1989; Levin 1995; Stroomberg et al. 1999) thereby limiting the potential number of aqueous metabolites. In the deposit-feeding marine polychaete *Nereis diversicolor*, 1-hydroxypyrene glucuronide was identified as the dominant aqueous metabolite which constituted 73% of total identifiable pyrene-derived compounds in tissue and gut fluid of the worm (Giessing et al. 2002). An experiment providing 1-hydroxypyrene as a sole source of energy and carbon to individual bacterial strains was also performed using a

new microtiter plate method (Johnsen et al. 2002) for detection of bacterial growth on crystalline PAHs. Increased degradation rates initiated by animal metabolism will be especially interesting for the larger PAHs which are otherwise recalcitrant in marine sediments. Sediment from a site polluted with a heavy bunker oil (Pécseli et al. 2002) was used to study microbial degradation potential of PAH metabolites in a naturally aged oil contaminated sediment.

MATERIALS AND METHODS

MINERALIZATION EXPERIMENTS

Because ^{14}C-labeled pyrene metabolites are not commercially available, labeled metabolites were obtained by exposing *Nereis diversicolor* (25 individuals) to sediment spiked with 0.05 μCi ^{14}C-pyrene g^{-1} sediment (dw) equivalent of 0.2 μg ^{14}C-pyrene g^{-1} sediment for 5 days. [4,5,9,10-^{14}C]pyrene (>98%) was obtained from Amersham (Nycomed Amersham, Buckinghamshire, England). Worms and sediment were collected at Store Havelse, Roskilde Fjord, Denmark in April of 2002. Previous studies have shown that *Nereis diversicolor* produces predominantly 1-hydroxypyrene glucuronide (73%) and small amounts of 1-hydroxypyrene (2%) when exposed to sediment-associated pyrene (Giessing et al. 2002). After the exposure period, worms were homogenized in 10 mL methanol using a Tissue-Tearor™ (Biospec Products, Inc. Bartlesville, OK, USA). The sample was centrifuged for 10 min at 5000 rpm (Jouan GR412 centrifuge) and the supernatant containing metabolites and unmetabolized pyrene was decanted and saved for use in mineralization experiments. Unexposed worms were homogenized in the same manner and the supernatant was used to prepare a ^{14}C-pyrene stock solution. Both stock

solutions (referred to as metabolite and pyrene stocks hereafter) had an activity of roughly 200000 dpm/mL. The relative distribution of aqueous (1-hydroxypyrene glucuronide) versus polar (1-hydroxypyrene) and parent (pyrene) ^{14}C-label in the metabolite stock solution was determined by liquid:liquid extraction. 100 μL of metabolite stock was dissolved in 1.9 mL H_2O and extracted with three times 2 mL dichloromethane (DCM). Aqueous metabolites constituted 75% of the activity of the two phases (see below for ^{14}C analysis).

All mineralization experiments were carried out in replicates or triplicates in 250 mL Erlenmeyer flasks fitted with KOH traps suspended from silicone stoppers. 100 μL of either metabolite or pyrene stock solution was added to each flask and the methanol was allowed to evaporate. 10 g of wet sieved (< 1mm) sediment was subsequently added to each flask along with 100 mL of autoclaved seawater (15‰). All equipment and seawater used was autoclaved prior to use. The flasks were fitted with $^{14}CO_2$-traps containing 5 mL 0.1 M KOH, sealed, and placed on an orbital shaker table at 100 rpm and left in the dark at 12˚C. To a set of samples were added 100 μL metabolite stock solution and 30% w/v NaCl and used as a killed control. Measurements of $^{14}CO_2$ were carried out at regular time intervals by removing 3 mL of the trap KOH, discarding the remainder, and replenishing with 5 mL of fresh 0.1 M KOH. For ^{14}C analysis, all samples were added 15 mL of scintillation fluid (EcoScint A, National Dianogstics, Hessle Hull, UK), left in the dark overnight, and analyzed by liquid scintillation counting in a Beckman 1409 liquid scintillation counter. Counts of the killed controls were not different from triplicate counts of 3 mL neat KOH samples on any of the sample days and the killed control samples were used for background correction.

To study the effect of pyrene pre-exposure and oil contamination two additional degradation experiments were setup. Both experiments were done in replicates. The pre-exposure experiment was comparable to the experiment described above except one set of samples were added 10 g of sediment pre-exposed to 20 μg pyrene g^{-1} sediment for 60 days, and a second set of samples were added 10 g of pre-exposed sediment containing 1% (w/w) North Sea crude oil (provided by the Statoil Oil Refinery, Kalundborg, Denmark).

Naturally aged oil contaminated sediment was collected in Grønsund in September, 2002 and sieved (< 1mm) to remove mussel shell fragments and plant debris prior use. Three treatments and one killed control were set up all containing 10 g (wet weight) of sediment. Treatments were amended with ^{14}C-pyrene stock, ^{14}C tissue homogenate, and ^{14}C aqueous metabolites only. Aqueous metabolites were separated by liquid:liquid extraction and the samples were stored and treated as described above.

MICRO TITER PLATE ASSAY

The microtiter plate method for detection of bacterial growth on crystalline PAHs utilizes the reduction of a water-soluble respiration indicator, WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} (Roche Molecular Biochemicals, Mannheim, Germany), in combination with easily degradable carbon sources, to a colored formazan. The method is described in detail in Johnsen et al. (2002). Briefly, PAH-mineralizing strains are grown on PAHs crystals in microtiter plates for 7 to 10 days. The tetrazolium dye WST-1 is then added along with easily degradable carbon (i.e. pyruvate, succinate, and glucose) to boost flow of electrons and

thus the increase the reduction of WST-1 to the colored formazan, and plates are incubated for 60 min. The change in absorbance, Δ(A450 nm - A630 nm), is read before and after the WST incubation period. The cells, and hence the intensity of the color, proliferate only in wells amended with a substrate they can metabolize. Thus, the intensity of the color is a measure of the metabolic potential of the cells in each well.

Growth on 1-hydroxypyrene and pyrene (Sigma-Aldrich, Vallensbæk, Denmark) was determined in triplicate in microtiter plates. 1-hydroxypyrene was dissolved in ethanol (2.5 mg/mL) and pyrene was dissolved in hexane (5.0 mg/mL). 40 µl 1-hydroxypyrene solution or 20 µL pyrene solution was added to each well. Controls were wells with the solvents alone. Inocula were grown in phosphate minimal medium (PMM), washed twice in PMM and diluted to an optical density of 0,020-0,030. 200 μL PMM and 10 μL inoculum were added to each well. The potential respiration was assayed after 10 days incubation by adding 50 µL electron donor solution (17 mM glucose. 17mM succinate, 17 mM pyruvate, 40 mM tris, pH 6.5) and 10 µL WST-1 to each well. The plates were incubated for 60 min at room temperature on a shaker table (300 rpm) and absorbance was measured with a microplate autoreader (Bio-Tek Instruments EL311) after WST-1 addition and again after the 60 min incubation period.

Mycobacterium sp. strains VM455, VM445, VM451, and LB501T were obtained from D. Springael (pers. com. and Bastiaens et al. 2000). *Mycobacterium* sp. strain GJ-3p was obtained from G. Zaitsev (pers. com) and *Gordona* sp. strain BP9 was obtained from B. Mahro (Kästner et al. 1998). All strains were stored in 40% (vol/vol) glycerol at –80˚C. Each inoculum was grown to the late exponential phase at room temperature in PMM medium supplemented with glucose (1 g L^{-1}) as the sole carbon source.

RESULTS AND DISCUSSION

MINERALIZATION EXPERIMENTS

Mineralization of ^{14}C-label to $^{14}CO_2$ was limited when the pristine sediment was incubated with either metabolite- or pyrene-stock solutions (Figure 6.1). An initial lag phase of 40 d was observed in both treatments. Though total $^{14}CO_2$ production at the end of the experimental period was higher in the metabolite treatment, only 2.5% and 1.6% of total added ^{14}C-label had evolved as $^{14}CO_2$ after 175 d in metabolite and pyrene treatments respectively. It was expected that the increased aqueous solubility of pyrene metabolites would increase microbial degradation compared to pyrene. However, the concentration of ^{14}C-label added to each flask, approx. 3 ng pyrene-derived compounds g^{-1} sediment, is too dilute to induce significant growth of microbial populations on pyrene or pyrene-derived compounds alone. The sediment used, from a pristine site in Roskilde Fjord, Denmark, contains less than 0.2% organic matter (Christensen et al. 2002b) and microbial populations able to utilize pyrene as an energy and carbon source might have been sparse. Thus, the combination of dilute substrates and low density of pyrene-degraders gave a minute but significant increase in degradation rate of eukaryotic pyrene metabolites by native microbial populations.

Pre-exposure to pyrene significantly induced microbial degradation of ^{14}C-pyrene in the same sediment as above (Figure 6.2). At the end of the 95 d experimental period, 80% of the total added ^{14}C-pyrene had evolved as $^{14}CO_2$ and no lag phase was observed (Figure 2A filled squares). A similar rapid evolution of $^{14}CO_2$ was observed in the metabolite treatment (Figure 2B, filled circles) though the magnitude was much less than

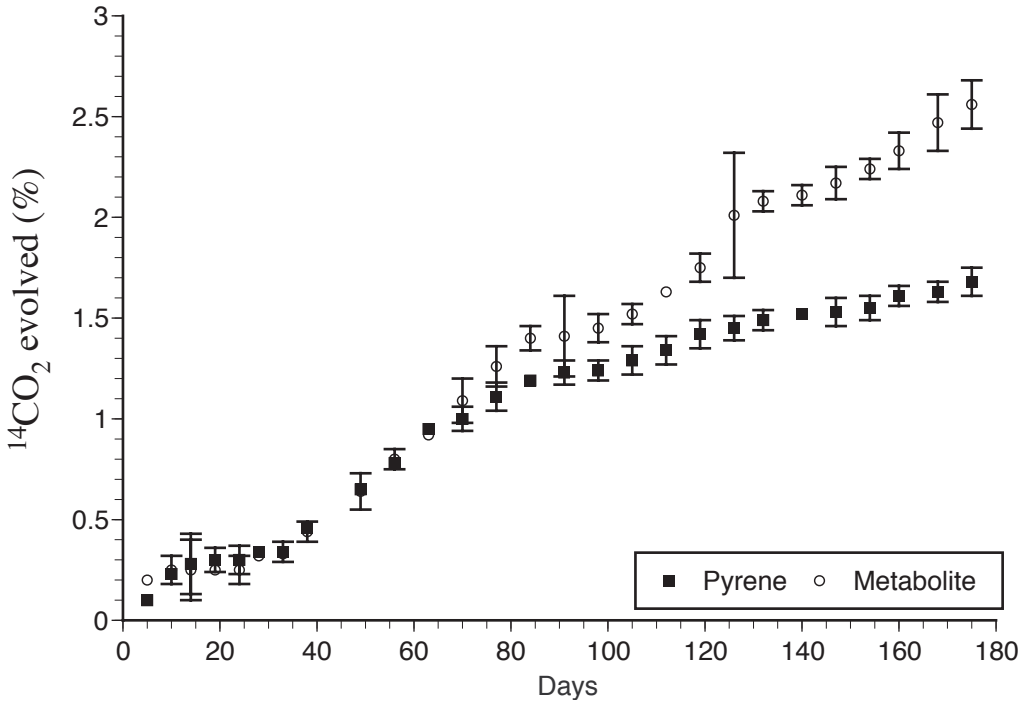

Figure 6.1 Microbial degradation of ^{14}C-pyrene and ^{14}C-pyrene metabolites in a pristine sediment.

Cumulative percent $^{14}CO_2$ evolved in sediment with ^{14}C-pyrene (squares) or with polychaete ^{14}C-pyrene metabolites added (circles). Average of triplicates ± 1 standard deviation: standard deviations are omitted when smaller than symbol used.

observed for ^{14}C-pyrene. Only 15% of the ^{14}C-label added had evolved as $^{14}CO_2$ after 95 d of incubation.

The *Nereis diversicolor* tissue homogenate used in this experiment contained 75% aqueous metabolites. The remaining 25% is a mixture of ^{14}C-pyrene and ^{14}C-1-hydroxypyrene. Previous experiments have shown that *Nereis diversicolor* rapidly converts 1-hydroxypyrene to a glucuronide conjugate and that only a few percent of total PAHs in worm tissue is in the form of 1-hydroxypyrene (Giessing et al. 2002). Assuming an identical ratio of pyrene-derived label in the tissue homogenate used in this experiment it seems reasonable to assume that the $^{14}CO_2$ evolved in the metabolite treatment is primarily due to microbial degradation of unmetabolized ^{14}C-pyrene. However, the relative mineralization did not evolve above 25% in the metabolite treatment during the experimental period, so degradation of ^{14}C-1-hydroxypyrene cannot conclusively be excluded.

To explore if the microbial degradation of 1-hydroxypyrene compounds could be boosted by adding a more bioavailable substrate, 1% (w/w) North Sea crude oil was added to the pyrene pre-exposed sediment. Crude oil is a complex mixture of hydrocarbons with some of the components, such as alkanes and low-molecular aromatics, being readily degradable by sedimentary microbial populations. Adding crude oil to the sediment had no positive effect on the degradation of worm metabolites (Figure 2B open circles). In fact a decrease in $^{14}CO_2$ evolution in both pyrene and metabolites treatments was observed (Figure 6.2 A and B). This decrease in $^{14}CO_2$ evolution indicated that some of the crude oil components may lower pyrene degradation, possibly by utilization of the more labile carbon sources of the crude oil or by utilization of the labile

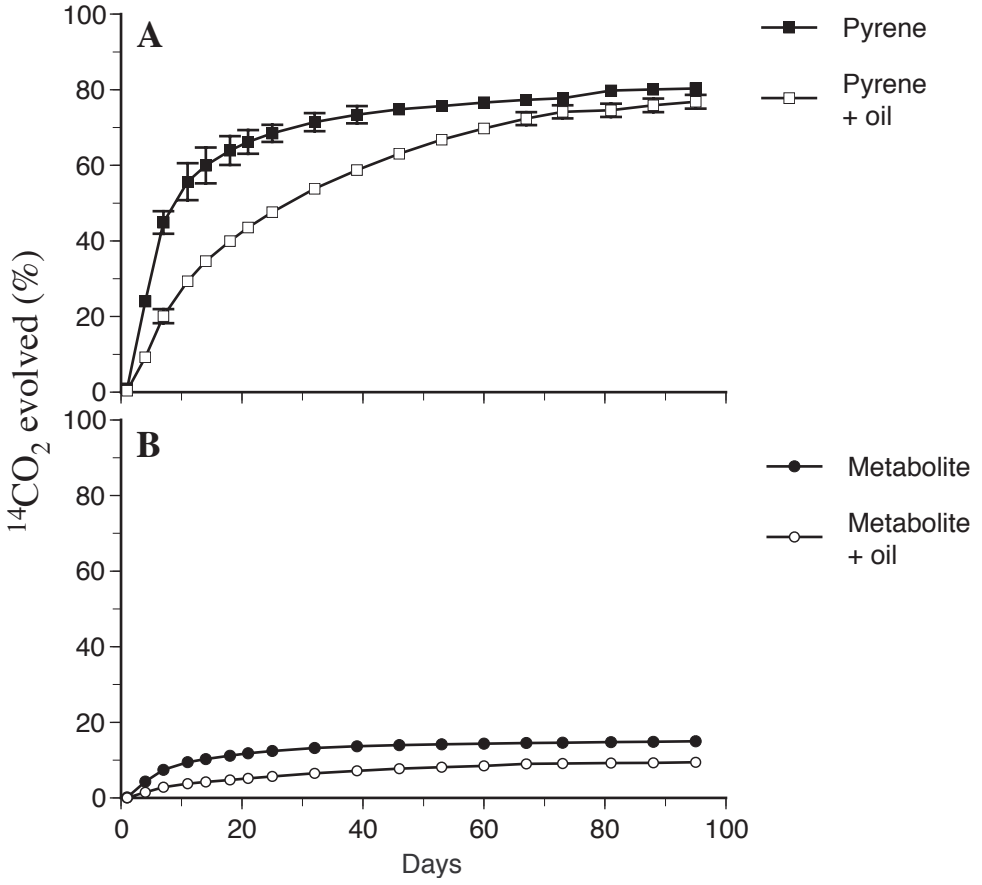

Figure 6.2 Microbial degradation of ^{14}C-pyrene and ^{14}C-pyrene metabolites in a pristine sediment pre-exposed to pyrene.

Cumulative percent mineralization of (**A**) ^{14}C-pyrene or with (**B**) polychaete ^{14}C-pyrene metabolites added in sediment pre-exposed to 20 μg pyrene g^{-1} sediment for 60 d. Average of duplicates ± 1 standard deviation: standard deviations are omitted when smaller than symbol used. Open symbols represents addition of 1% (w/w) North Sea crude oil to the pre-exposed sediment.

carbon source by rapidly growing indigenous microorganisms instead of the pyrene degraders. In either case, addition of crude oil further suggests that neither sedimentary pyrene degraders nor other indigenous microorganisms are capable of degrading pyrene metabolites. Future experiments will reveal if microbial degradation of 1-hydroxypyrene can proceed through a co-metabolic process.

Microbial degradation of pyrene and metabolites was not significant when incubated with naturally aged oil contaminated sediment (Figure 6.3). Less than 0.6 % of added label had evolved as $^{14}CO_2$ after 50 days of exposure in either of the three treatments. In the same period of time, $^{14}CO_2$ production in the pristine sediment was 0.65% and 0.64% for pyrene and metabolite treatments respectively. The oil spilled in the tanker accident was a heavy bunker oil with relatively high concentrations of both low and high molecular weight PAHs. The total PAHs concentration in sediment from polluted areas was up to 1 mg kg^{-1} (dw) 8 months after the spill (Pécseli et al. 2002). Inability of naturally occurring microbial populations in aged oil contaminated sediment to degrade pyrene metabolites suggests, that mineralization of pyrene metabolites does not proceed through a co-metabolic process.

MICRO TITER PLATE ASSAY

Strains of known pyrene degraders were unable to grow when given 1-hydroxypyrene as a sole source of energy and carbon (Figure 6.4). This further supports the negligible degradation of aqueous pyrene metabolites in both pristine (Figure 6.1) and contaminated sediments (Figure 6.2 and Figure 6.3).

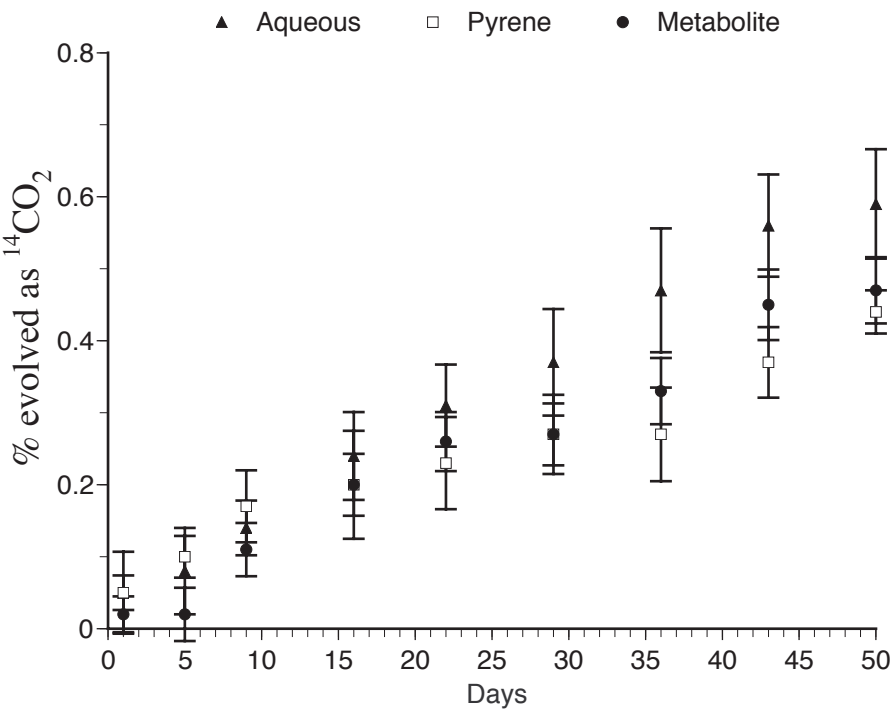

Figure 6.3 Microbial degradation of ^{14}C-pyrene and ^{14}C-pyrene metabolites in a naturally aged oil contaminated sediment.

Cumulative percent mineralization by oil contaminated sediment from Grønsund, Denmark. ^{14}C-pyrene (squares), 4,5,9,10-^{14}C-pyrene metabolites (circles), and ^{14}C-pyrene aqueous metabolites only. Average of replicates ± 1 standard deviation: standard deviations are omitted when smaller than symbol used.

The microtiter plate method is based on bacterial respiratory reduction of the tetrazolium salt WST-1 to a colored formazan by active cells.

All six strains tested showed significant reduction of WST-1 in the presence of suitable electron donors (e-donors) when initially grown on pyrene for 10 d (Figure 6.4, gray bars). Conversely, no significant reduction of WST-1 was observed when the strains were initially grown on 1-hydroxypyrene (Figure 6.4, black bars). The values for ethanol and hexane controls (Figure 6.4, light gray and white bars) with e-donors but without PAHs were higher than the 1-hydroxypyrene treatments for all six strains tested. The high level of survival and activity of *Mycobacterium* cells starved for 10 days may have been due to cryptic growth on the energy rich mycolic-acid wax surrounding the cells. Furthermore, the response lower than controls suggests that 1-hydroxypyrene is toxic at the concentration used. Even though the amount of crystalline pyrene and 1-hydroxypyrene added to each well was the same, greater aqueous solubility of 1-hydroxypyrene might account for the apparent increase in toxicity. To our knowledge there exist no data on aqueous solubility of 1-hydroxypyrene but, in general, addition of O-containing functional groups generally increases the aqueous solubility of aromatic compounds. Thus it is fair to assume that the aqueous solubility of 1-hydroxypyrene is at least equal or greater than the aqueous solubility of pyrene.

Little is known about microbial degradation of eukaryote metabolites such as 1-hydroxypyrene. Hwang et al. (2001) reported that 1-hydroxypyrene was resistant to degradation by microbial populations in surface water samples from a freshwater reservoir. Their study also showed that photo-oxidation was required for heterotrophic bacteria to be able to utilize 1-hydroxypyrene as a growth substrate.

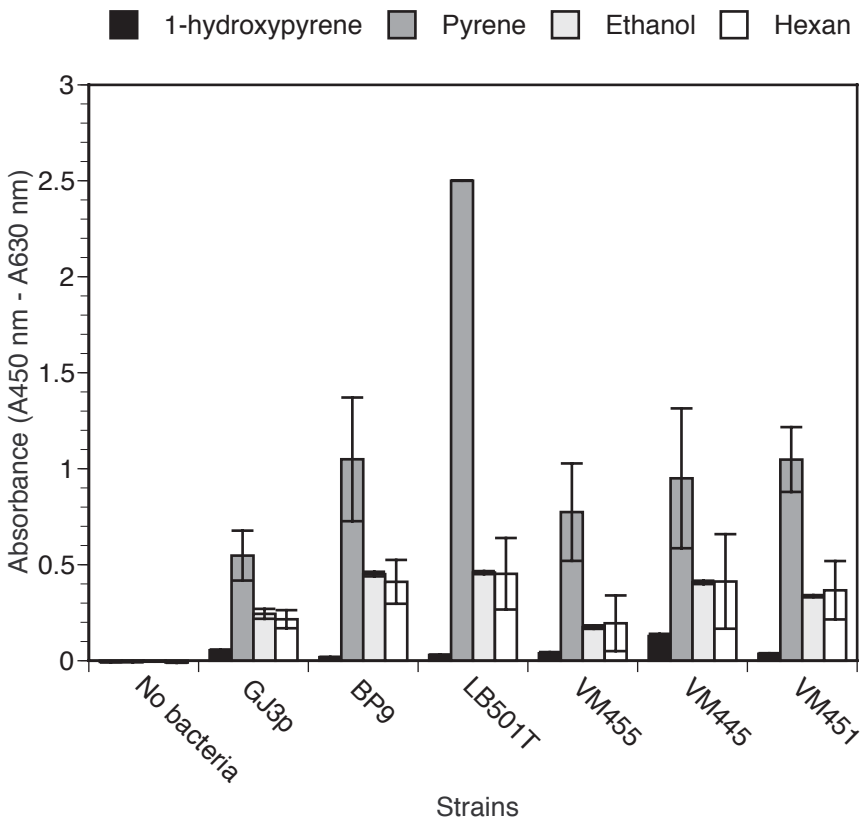

Figure 6.4 Respiratory reduction of WST-1 by strains of PAH degrading bacteria. Strains were grown on pyrene and 1-hydroxypyrene as a sole carbon and energy source in microtiter plates for 10 days. The plates were incubated with WST-1 for 60 min. Controls included wells with no PAH but with solvent used for PAH delivery added. Error bars represent ± 1 standard deviation.

In a recent microcosm experiment cumulative budgets of ^{14}C-pyrene showed a significant increase in water-soluble metabolites after 42 d in microcosms amended with *Nereis diversicolor* (Christensen et al. 2002b). The increase in water-soluble metabolites was not followed by an increase in microbial degradation expressed as total $^{14}CO_2$ production. After 42 d of exposure, total $^{14}CO_2$ production was significantly higher in the absence of *Nereis diversicolor* (Christensen et al. 2002b). The authors argued that removal of pyrene in proximity of the animals due to feeding activity, ventilation and diffusion from interstitial water may explain lower $^{14}CO_2$ production and suggested that any stimulation in microbial metabolism of ^{14}C-pyrene due to *Nereis diversicolor* was offset by diminishing concentrations of ^{14}C-pyrene in burrow wall sediments over time. In light of the data presented here, an alternative explanation for the absence of microbial degradation stimulated by *Nereis diversicolor* observed by Christensen et al. (2002b) can be offered. 1-hydroxypyrene and 1-hydroxypyrene glucuronide are poor carbon and energy substrates for indigenous as well as sedimentary microorganisms pre-exposed to pyrene. Thus buildup of aqueous metabolites and lack of increasing microbial degradation due to production of more soluble pyrene-derived compounds would be expected.

Although the chemical identity of the compounds was unknown, a build-up of water-soluble benz[a]anthracene metabolites has been detected in sediments with a natural mixed microbial community and degradation of both parent PAHs and intermediate products in the sediment was stopped or greatly slowed after 2 months by an unknown mechanism (Hinga and Pilson 1987). PAHs are relatively inert molecules and it is generally accepted that toxic and carcinogenic effects of PAHs are caused by their

metabolites rather than parent compounds (Livingstone 1993). Contrary to this, a related polychaete species - *Nereis virens* - has been shown to stimulate microbial degradation of benz[a]anthracene possibly be releasing more bioavailable metabolites of the compound (McElroy et al. 1990).

Eukaryotic metabolism of pyrene produces primarily 1-hydroxypyrene and 1-hydroxypyrene conjugates whereas eukaryotic metabolism of, for example benz[a]anthracene, fluoranthene and benzo[a]pyrene produces a suite of mono- and di-hydroxylated compounds (Forbes et al. 2001; McElroy 1990; McElroy et al. 2000). Prokaryotic organisms metabolise PAHs by initial dioxygenase attack incorporating both atoms of the oxygen molecule which leads to the formation of dihydrodiols that are in the *cis*-confuguration (Sutherland et al. 1995). Eukaryotic organisms oxidize PAHs via a cytochrome P450 monooxygenase to form an arene oxide by incorporating one oxygen atom into the PAHs, the other being reduced to water. The arene oxide is either further metabolised enzymatically to form a dihydrodiol that is in *trans*-configutration or undergoes non-enzymatic isomerization to form a phenol (Livingstone 1994). It is possible that eukaryotic di-hydroxylated PAHs metabolites may increase microbial degradation potentials as opposed to mono-hydroxylated and thus explain differential microbial degradation potential observed for the different PAHs. Differential microbial degradation of mono- and di-hydroxylated metabolites would lead to build up of potentially toxic mono-hydroxylated compounds with negative effects on both microbial and invertebrate populations.

The evidence presented here strongly suggests that formation of polar and aqueous metabolites by deposit-feeding marine invertebrates has limited influence on the

microbial degradation potential of sediment-associated pyrene. Whether or not this observation is a generally applicable to the larger PAHs remains to be investigated and future studies will reveal if stimulated degradation of larger PAHs in marine sediments is solely due to bioturbation and irrigation processes of infaunal invertebrates.

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