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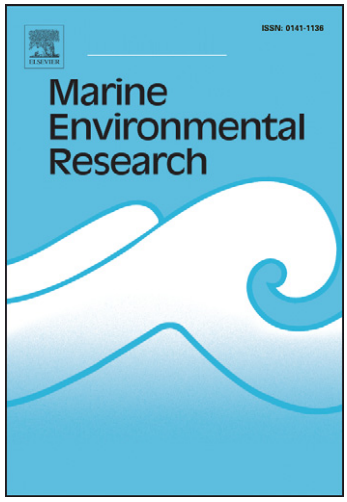

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1 **Title: Biotransformation of polycyclic aromatic hydrocarbons in marine**

2 **polychaetes**

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Abstract

Deposit-feeding polychaetes constitute the dominant macrofauna in marine environments that tend to be depositional centers for organic matter and contaminants. Polychaetes are known to accumulate polycyclic aromatic hydrocarbons (PAHs) from both particulate and dissolved phases but less is known about the mechanisms underlying elimination of accumulated PAHs. An important pathway of elimination is through biotransformation which results in increased aqueous solubility of the otherwise hydrophobic PAHs. Biotransformation in marine polychaetes proceeds in a two phased process similar to those well studied in vertebrates, phase I enzymes belonging to the Cytochrome P450 (CYP) enzyme family, along with a few phase II enzymes have been identified in marine polychaetes. In this review we aim at highlighting advances in the mechanistic understanding of PAH biotransformation in marine polychaetes by including data obtained using analytical chemistry and molecular techniques. In marine polychaetes induction of CYP enzyme activity after exposure to PAHs and the mechanism behind this is currently not well established. Conflicting results regarding the inducibility of CYP enzymes from polychaetes have led to the suggestion that induction in polychaetes is mediated through a different mechanistic pathway, which is corroborated by the apparent lack of an AhR homologous in marine polychaetes. Also, none of the currently identified *CYP* genes from marine polychaetes are isoforms of those regulated by the AhR in vertebrates. Relatively few studies of phase II enzymes in marine polychaetes are currently available and most of these studies have not measured the activity of specific phase II enzymes and identified phase II metabolites but used an extraction technique only allowing determination of the overall amount of phase II metabolites. Studies in insects and various marine invertebrates suggest that in invertebrates, enzymes in the important phase II enzyme family, UDP-glucuronosyl transferases primarily use glucoside as co-substrate as opposed to the vertebrate cosubstrate glucuronic acid. Recent studies in marine polychaetes have however identified

glucuronidation of PAHs indicating no mechanistic difference in co-substrate preference among UDP-glucuronosyl transferases between vertebrates and marine polychaetes but it might suggest a mechanistic difference between marine polychaetes and insects.

Keywords: CYP enzymes, metabolism, phase I and phase II enzymes, PAH, induction, elimination, trophic transfer.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) constitute one of several classes of organic pollutants present in the marine environment primarily as a consequence of human activities. PAHs are hydrocarbons composed of two or more fused aromatic (benzene) rings. Important sources of PAHs to the marine environment include atmospheric fallout, spillage and seepage of petroleum and oil products, and industrial and domestic sewage. Concern over the environmental impact of PAHs is due to their persistence (Neff, 1985), ability to bioaccumulate (Landrum et al., 1991; Weston, 1990), toxicity (Swartz et al., 1990) and potential carcinogenicity (DeWitt et al., 1992; Penning, 1993). Due to their low aqueous solubility and hydrophobic character, PAHs readily adsorb to organic and other particulate matter and therefore accumulate in marine sediments (Ferguson and Chandler, 1998). Deposit-feeders such as marine polychaetes ingest large amounts of bulk sediment and are thereby exposed gastrointestinally and via body surfaces to sorbed PAHs and PAHs desorbed into pore water. The common lugworm *Arenicola marina* for example ingest up to 20 times its own body weight of wet sediment per day (Cadée, 1976). Gut fluid hydrolysis and solubilization are of vital importance in determining bioavailability of food substrates (Mayer et al., 2001). Deposit-feeding invertebrates have evolved an enclosed extracellular digestive geometry which enables them to efficiently retain both digestive agents and digestive products and thus thrive on a nutritionally poor sedimentary diet (Andresen and Kristensen, 2002; Mayer et al., 2001) Contaminant exposure is increased by particle selectivity in food selection, and bioavailability is enhanced by the animals' attempt to solubilize food from ingested particles. Thus, for these organisms, ingestion is the primary route of uptake of particle-associated contaminants (Forbes et al., 1998; Penry and Weston, 1998; Weston, 1990; Weston and Mayer, 1998b). Good agreement between the proportion of contaminant solubilized in isolated gut fluid and the proportion of contaminant absorbed during gut passage suggests that digestive solubilization is the limiting factor

in determining bioavailability of sediment-bound contaminants in deposit feeding polychaetes (Weston and Mayer, 1998a); (Ahrens et al., 2001; Rust et al., 2004; Selck et al., 2003). Polychaetes are richly abundant in sediments and have been reported to constitute up to 50% of sediment macro fauna by number (Reish and Gerlinger, 1997). Hence, polychaetes are the dominant species in environments that tend to be depositional centers for organic matter and organic contaminants like PAHs (Jumars et al., 1990). Polychaetes are known to accumulate significant amounts of PAHs from their environment and steady-state body burdens are a function of biotransformation and elimination processes. Although much remains to be elucidated, polychaete biotransformation of PAHs appears to be similar in principle to the two-step process observed in vertebrates. Phase I enzymes primarily cytochrome P450 enzymes (CYP enzymes) catalyze introduction of a functional group into the PAH which slightly increases water solubility. Subsequently, phase II enzymes catalyze covalent attachment of a large polar group which extensively increases water solubility (James, 1987; Giessing et al., 2003a; Giessing and Lund, 2002; Giessing et al., 2003b; Jørgensen et al., 2005a; Li and James, 1993; van den Hurk and James, 2000) thereby enhancing the elimination of PAHs (Burchell and Coughtrie, 1989; Livingstone, 1998).

In organisms efficiently biotransforming PAHs, analysis of only parent compound might result in underestimation of total PAH exposure. Therefore, increased knowledge on PAH biotransformation in benthic invertebrates is important in order to improve the understanding of PAH mediated effects in the marine environment and thereby also how PAHs should be handled in risk assessment. Also, the potential for formation and trophic transfer of metabolites that are more toxic than the parent PAH makes information on biotransformation pathways and capacities relevant to investigate on a larger ecological scale. In this review we will summarize the current knowledge of PAH biotransformation in marine polychaetes and draw parallels to biotransformation in vertebrates

where the best understanding of the involved enzymes is present. The present knowledge on PAH biotransformation indicates that the enzymes involved, the metabolites formed, and the basic mechanisms are similar in vertebrates and invertebrates, including marine polychaetes. It is generally acknowledged that the overall biotransformation pathway is conserved, and important differences in biotransformation of PAHs between marine polychaetes and other species have not been observed. However, at the more detailed level, conflicting results are published, especially regarding the inducibility of polychaete CYP enzymes and the apparent lack of AhR mediated regulation of CYP enzymes involved in PAH biotransformation. Furthermore, the knowledge about phase II biotransformation of PAHs in marine polychaetes is very limited impeding general conclusions about the importance of the different phase II enzymes, substrate specificity and capacity. In this review, special attention is given to these questions, analysing studies in which specific metabolites, especially phase II metabolites, of PAH biotransformation are identified as well as studies where the capacity of specific biotransformation is investigated. Finally, molecular techniques allowing identification of specific CYP enzymes, their expression, regulatory capacity and activity towards PAHs are highlighted to increase the mechanistic understanding of the biotransformation of PAHs in polychaetes.

2. Phase I Biotransformation in marine polychates

The knowledge on CYP enzyme function and regulation in polychaete species primarily originates from biochemical studies, e.g. PAH metabolite formation via enzyme activity. Most studies primarily established that monooxygenase activities are present in polychaetes and estimated the biotransformation of PAHs by measuring production of metabolites (Forbes et al., 2001; Fries and Lee, 1984; Lee, 1981; Lee and Singer, 1980; Lee, 1998; Lee et al., 1979; McElroy, 1990; McElroy et al., 2000; McElroy, 1985b; Rust et al., 2004; Selck et al., 2003). For example, several studies

have investigated biotransformation of PAHs in *Capitella* sp. I. Selck et al. (2003) found that *Capitella* sp. I biotransformed fluoranthene accumulated from porewater and sediment. Approximately 18% of fluoranthene extracted from whole-body tissue were polar (phase I) metabolites (Selck et al., 2003). Also, Bach et al. (2005) found 20% of total PAH to be polar metabolites in *Capitella* sp. I after 15 days exposure to 30 µg fluoranthene/g sediment, however, in the closely related sibling species *Capitella* sp. S only 3% of total fluoranthene were present as polar (phase I) metabolites, indicating large species-specific difference in biotransformation capacity.

Several experiments have established that also *Nereis virens* is capable of biotransforming PAHs such as benzo(a)pyrene (B(a)P) and benz(a)anthracene (B(a)A) via enzyme catalysed reactions (Lee, 1981). The major metabolite produced in *N. virens* exposed to B(a)P was 3-hydroxybenzo(a)pyrene, indicating a CYP catalysed hydroxylation (Fries and Lee 1984; Lee 1998). In *N. virens* CYP enzyme activity has been found in the microsome fraction of gut tissue, whereas mitochondrial CYP enzyme activity was not identified (Lee and Singer, 1980). Several other studies reported CYP enzyme activity in microsomal fractions (McElroy, 1985b**;** Jørgensen et al., 2005a; McElroy, 1990). Few studies investigated CYP enzyme activity in specific tissues, e.g. gut tissue (Jørgensen et al., 2005), whereas most studies used whole-worm homogenates (McElroy, 1985a). This could possibly lead to an under-estimation of CYP enzyme activity as the activity in somatic tissue is lower than in gut including intestinal (chloragogenic) tissue. Rust et al. (2004) investigated the biotransformation of B(a)P in six marine polychaete species. Biotransformation capacity was determined as % B(a)P biotransformed after 7 days exposure to B(a)P-contaminated sediment. The investigated polychaete species showed a wide range of biotransformation efficiency ranging from 92% in *Spio setosa*, 85% in *Nereis succinea*, 72% in *Nereis virens*, 27% in *Nephys incise*, 14% in

Cirriformia grandis and 6% in *Clymenella torquata* (Rust et al., 2004). Also, McElroy et al (2000) investigated the biotransformation of B(a)P in four polychaete species (*Nereis succinea*, *Pectinaria gouldii*, *Haploscolopolous* sp. and *Capitella* sp. I) exposed to sediment associated ^{3}H-B(a)P for four days. The fractions of biotransformed B(a)P were determined to 96%, 7%, 38% and 42%, respectively (McElroy et al., 2000). This large species-specific difference in biotransformation efficiency has not been explained. However, differences in PAH elimination strategies (biotransformation versus flushing of un-metabolised PAH) have been indicated and might reflect some of the difference in biotransformation efficiency (Christensen et al., 2002b). Also, differences in CYP enzyme inducibility (discussed below) might explain some of the difference in biotransformation efficiency as phase I is often the limiting step in the overall biotransformation (Jørgensen et al., 2005a). Penry and Weston (1998) investigated biotransformation of B(a)P and phenanthrene to more water-soluble metabolites in *Abarenicola pacifica*. The extent of B(a)P biotransformation was limited (less than 10 %) whereas the biotransformation of phenanthrene was more extensive with 20-70% of the ^{14}C associated with water-soluble metabolites after 48 h of exposure, suggesting a CYP enzyme mediated pathway (Penry and Weston, 1998). Furthermore, phenanthrene metabolite production was significantly higher in worms acclimated to low (0.08%) organic carbon sediment compared to high (0.45%) organic carbon sediment, likely due to a combination of reduced bioavailability and reduced feeding rate (Penry and Weston, 1998). The importance of physiological acclimation, which includes changes in digestive processes and thereby contaminant solubilization, on biotransformation is currently not understood. The CYP enzyme mediated B(a)P hydroxylase activity in whole body homogenates of *Nereis diversicolor* and *Platynereis dumerilii* B(a)P was 15.8 ± 0.2 and 8.1 ± 1.6 pmol min^{-1} mg^{-1} protein, respectively (Sole and Livingstone, 2005). This is lower than *N. virens* gut tissue pyrene hydroxylase activity (V_{max}), which was determined to be 0.36 nmol min^{-1} mg^{-1} protein (Jørgensen et al., 2005a). This difference

could in part be explained by the use of whole body and gut tissue homogenates, respectively. Also, B(a)P exposure might have a toxic effect on *N. diversicolor* and *P. dumerilii* whereas pyrene is considered to be much less toxic. Finally, different assays and analytical techniques were used to determine the enzyme activities in the two studies. Also three different species, with possibly slightly different "editions" of the same CYP enzymes were used.

Identification, both qualitatively and quantitatively, of the metabolites formed is essential for a deeper mechanistic understanding of the PAH biotransformation process in polychaete species. However, some PAHs such as the intensively studied compounds B(a)P and fluoranthene are biotransformed to several different phase I metabolites which each potentially are conjugated to different endogenous phase II substrates making identification of all metabolites a difficult task. For example, biotransformation of B(a)P was examined in detail in *N. succinea*, where three of the B(a)P metabolites were identified by HPLC: 7,8-diol, 1,6 or 3,6-diol and 7-hydroxy B(a)P (McElroy et al., 2000). In another study, biotransformation of fluoranthene was examined in *Capitella* sp. I and more than 30 different peaks were found after HPLC analysis (Forbes et al., 2001). The metabolites were more hydrophilic than fluoranthene. Of the 30 peaks only two peaks were tentatively identified as 3- and 8-hydroxyfluoranthene (Forbes et al., 2001). By investigating biotransformation of a single PAH with a simple metabolic pattern it is possible to obtain novel information about the specific enzymes involved in phase II biotransformation and their relative importance. The four-ringed PAH pyrene has a compact molecular structure that restricts oxidative attack, resulting in formation of only one phase I metabolite, 1-hydroxypyrene, which is commercially available and has been identified in eukaryotes. Consequently, the number of phase II metabolites is low making quantitative metabolic analysis more simple. Therefore, pyrene has been used as a model compound in several studies in humans, fish and terrestrial invertebrates

(Stroomberg et al., 2003). Few studies have investigated biotransformation of pyrene in marine polychaetes using HPLC and LC/MS analysis. In the marine polychaetes *Nereis diversicolor* and *Nereis virens*, 1-hydroxypyrene was the only identified phase I metabolite (Giessing and Lund, 2002; Giessing et al., 2003a; Jørgensen et al., 2005a).

Investigations of CYP enzyme inducibility in marine polychaetes have resulted in conflicting suggestions regarding inducibility of CYP enzymes responsible for PAH biotransformation. In some studies, CYP enzymes were found to be inducible (Forbes et al., 1996; Jørgensen et al., 2005a; Lee, 1981; Lee and Singer, 1980; Lee et al., 1979) whereas others reported constitutive expression of CYP enzymes (Driscoll and McElroy, 1996; Driscoll and McElroy, 1997; McElroy, 1985a; McElroy, 1990; McElroy, 1985b). A three fold increase in CYP enzyme activity was detected 48h after feeding *N. virens* clams that had been maintained in water containing 10 µg/l B(a)A for six days (Lee and Singer, 1980). In the small marine polychaete, *Capitella capitata* CYP enzyme activity could be detected only after exposure to B(a)A or crude oil for periods of 3 to 6 weeks (Lee and Singer, 1980; Lee et al., 1979). In another experiment, both control and fluoranthene pre-exposed *Capitella* sp. I biotransformed fluoranthene. Pre-exposed worms did so more efficiently, indicating induction of CYP enzymes (Forbes et al., 1996). Driscoll & McElroy (1996) investigated three polychaete species, *Leitoscoloplos fragilis*, *Nereis diversicolor* and *Scolecolepides viridis* and found species differences not only in their ability to biotransform B(a)P, but also in the inducibility of metabolic activity. The worms were exposed to sediment-associated B(a)P with or without 3-MC (16 µg/g wet weight sediment) for nine days The ability of *L. fragilis* to biotransform B(a)P was limited and not inducible, whereas *N. diversicolor* biotransformed B(a)P extensively, but the activity was not induced by exposure to 3-methylcholanthrene (3-MC) which is a potent inducer of PAH metabolism in vertebrates. In contrast, *S. viridis* biotransformed B(a)P and

3-MC slightly induced B(a)P biotransformation (Driscoll and McElroy, 1996). Contrary to these results, McElroy (1985a) observed no increase in B(a)P hydroxylase activity in *N. virens* after pre-exposure. Also, McElroy (1990) found no induction of B(a)P hydroxylase activity in *N. virens* after pre-exposure to 3-MC. Worms were injected with a solution of 1 mg/l 3-MC in corn oil at a dose of 20 mg/kg wet weight and B(a)P hydroxylase activity was determined after 96 h (McElroy, 1990). Also, no induction of B(a)P hydroxylase activity was found in *N. diversicolor* and *S. viridis* after 3-MC pre-exposure (Driscoll and McElroy, 1996). However, the same authors suggested in a later publication that 3-MC might not be a particularly good inducer of polychaete CYPs (Driscoll and McElroy, 1997). In accordance with Lee and Singer (1980) and Lee (1981) we found approximately 3 fold induction of pyrene hydroxylase activity in *N. virens* gut tissue after pre-exposure to 10 µg pyrene/g sediment for 3 and 7 days (Jørgensen et al. 2005a). Also, additional experiments using microsomes pooled from *N. virens* gut tissue demonstrated an approximately 3-fold induction of pyrene hydroxylase activity after 5 days exposure to 10 µg pyrene/g sediment (Unpublished results Jørgensen and Giessing). Comparison of the different studies on CYP enzyme inducibility in marine polychaetes is difficult as large species-specific differences have been reported. Also, the use of different PAHs, inducers, concentrations and exposure duration seems to complicate direct comparison and thereby allow for general conclusions about inducibility of CYP enzymes in polychaetes.

It was previously suggested that the mechanism of CYP enzyme induction in invertebrates including polychaetes must be different from that in vertebrates (Lee, 1998). Though the exact mechanism of PAH mediated CYP enzyme induction is not yet understood in any marine invertebrate species, the few studies at the molecular level on *CYP* gene expression (see section 6. Molecular mechanisms underlying biotransformation in marine polychaetes) indicate that the

mechanism resembles that of vertebrate *CYP* gene expression except for a lower regulatory capacity. In polychaetes, up to 3 fold upregulation of *CYP* gene expression has been determined (Rewitz et al., 2004); (Li et al., 2004)) compared to 10-100 fold upregulation in vertebrates (Hahn et al., 1998; Livingstone, 1998). In vertebrates, phase I biotransformation of PAHs is primarily mediated by CYP1A enzymes, with expression regulated via the aryl hydrocarbon-receptor (AhR). Currently, no AhR homologues have been identified from marine polychaete species and it has previously been suggested that the AhR is absent in some invertebrate groups (Hahn, 1998; Hahn and Stegeman, 1992). AhR homologues have been characterized from invertebrate species including the nematode *Caenorhabditis elegans* and the soft shell clam *Mya arenaria*, but they do not have binding affinity for the prototypical AhR ligands 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and β-naphtoflavone (BNF) distinguishing them from vertebrate AhRs (Butler et al., 2001; Mimura and Fujii-Kuriyama, 2003). In polychaetes, no CYP1 enzyme homologues have currently been identified and other CYP enzymes than the CYP1 subfamily seem to be involved in PAH biotransformation, e.g. CYP enzymes with highest homology to CYP4 enzymes (Rewitz et al., 2004); (Li et al., 2004); (Jørgensen et al., 2005b). These CYP enzymes are likely to be transcriptionally regulated by a different receptor than AhR. In vertebrates, CYP4 enzymes are transcriptionally regulated by the peroxisome proliferator activated receptor (PPAR) belonging to the nuclear receptor (NR) family (Waxman, 1999). However, no NRs have currently been identified in marine polychaete species. The present knowledge on inducibility of polychaete CYP enzymes indicates that the levels of inductions are lower compared to vertebrates, but that at least some CYP enzymes are inducible. Future work will hopefully identify and mechanistically elucidate receptors mediating the transcription regulation of *CYP* genes involved in PAH biotransformation in marine polychaetes. Identification of *CYP* genes involved in PAH biotransformation and receptors mediating their regulation is possible by homology identification between different species

followed by PCR and sequencing. However, a future sequencing project using a marine polychaete model-species is the best initiative to elucidate how phase I biotransformation of PAHs is regulated and which specific CYP enzymes are involved.

3. Phase II biotransformation in marine polychaetes

Most previous studies of polychaete PAH biotransformation did not identify phase II metabolites or characterize the involved phase II enzymes. Instead extraction methods separating compounds into fractions of different water solubility were used (Christensen et al., 2002b). However, in a few studies, phase II enzymes involved in PAH biotransformation in marine polychaete species as well as the ensuing metabolites were characterized (Giessing et al., 2003a; Giessing et al., 2003b; Jørgensen et al., 2005a). These studies demonstrated that glucuronosyl transferases and sulfotransferases dominate phase II PAH biotransformation in polychaetes. However, the few studies conducted to date exclusively used pyrene as model PAH thereby the potential importance of some phase II enzymes like glutathione-s-transferases (GST) escape the analysis, because an epoxide is not formed during pyrene biotransformation.

In insects and other invertebrates glucosidation is considered a more important phase II conjugation pathway than glucuronidation that is most important in vertebrates. Livingstone (1998) suggested that glucuronidation might be restricted to vertebrate species and that glucosidation might be the corresponding pathway in invertebrates. However, Giessing et al. (2003a) demonstrated formation of glucuronide conjugates in *N. diversicolor*, and Jørgensen et al. (2005a) observed glucuronide conjugation in the closely related nereid *N. virens*. In other marine invertebrates like mussels and crustaceans, glucosidation appears to be the primary reaction of glucuronosyl transferase enzymes.

Accordingly, co-substrate preference in glucuronosyl transferase reactions is likely to be species specific.

Another important phase II enzyme, sulfotransferase, has been identified in several marine polychaetes. Sulphate metabolites are generally less abundant than other phase II metabolites in the investigated marine polychaetes (Fillmann et al., 2004; Giessing et al., 2003a; Giessing and Lund, 2002; Jørgensen et al., 2005a). However, in two species of marine clams *Mya arenaria* and *Protothaca staminea* exposed to pyrene and 1-hydroxypyrene for 10 days, pyrene-1-sulphate was identified as the major phase II metabolite (Simpson et al., 2002). In the marine polychaetes *N. virens* and *N. diversicolor* pyrene-1-glucuronide was the most prominent phase II conjugate present in tissue, even though pyrene-1-sulfate and pyrene-1-glucoside were also found in both species (Giessing et al., 2003a; Jørgensen et al., 2005a). This leads to the proposed biotransformation pathway for pyrene shown in **Figure 1**. In two other marine polychaetes, *Capitella capitata* and *A. marina*, pyrene-1-sulfate and pyrene-1-glucoside were the most prominent metabolites identified, respectively (Giessing et al., 2003b). The results from these four marine polychaete species emphasize the extensive inter-specific differences in phase II conjugation pathways even among closely related species (**Table 1**). This difference among species together with the limited amount of studies presently prohibit a more general conclusion on the relative importance of different phase II conjugation pathway in polychaetes.

The biotransformation of PAHs in marine polychaetes generally proceeds efficiently even though there are large differences in biotransformation capacity and inducibility among polychaete species. *N. virens* is considered an efficient biotransformer, and we found that more than 80% of the pyrene derived compounds extracted from gut tissue were present as phase II metabolites after 5 days

exposure (Jørgensen et al., 2005a). In *N. diversicolor*, approximately 75% of total pyrene extracted from whole worms after five days of exposure were present as pyrene-1-glucuronide (Giessing et al., 2003a). In another study with *N. diversicolor* exposed to B(a)P, 75% was recovered as aqueous metabolites (Driscoll and McElroy, 1996). In *Capitella* sp. I approximately 45% of the fluoranthene extracted from whole worm homogenate were aqueous metabolites after 10 days exposure (Selck et al., 2003). In agreement, Bach et al. (2005) found 38% aqueous metabolites of fluoranthene after 15 days exposure in *Capitella* sp. I. In *Capitella* sp. S, only 7% aqueous metabolites of fluoranthene were found after 15 days exposure (Bach et al., 2005), indicating large species specific differences in biotransformation capacity between the two sibling species. Furthermore, 89% of the fluoranthene present in *Capitella* sp. S after 15 days exposure was parent fluoranthene, indicating that this species is a poor biotransformator of fluoranthene (Bach et al., 2005). Also, Christensen et al. (2002a) found 50% of total pyrene in *N. diversicolor* as aqueous metabolites after 42 days exposure, whereas in *A. marina*, less than 20% was present as aqueous pyrene metabolites after 52 days exposure, indicating a large difference in biotransformation efficiency and/or excretion pathways between these two polychaete species. Comparisons of produced PAH metabolites in marine polychaetes (**Table 2**) show significant species-specific differences in biotransformation efficiency. In all studies, low percentage of phase I metabolites are found in polychaetes which corresponds well with the general notion that phase I biotransformation is the rate limiting step in the overall biotransformation pathway. Large differences between species are seen in the percentage of PAH present as parent and phase II metabolites. This emphasizes the importance of increasing the knowledge of the phase II enzymes. To our knowledge only one study has investigated the enzyme activity and inducibility of phase II enzymes in marine polychaetes. Glucuronosyl transferase (with glucuronic acid and glucoside as substrate, respectively) and sulfotransferase enzyme activity were investigated. Neither enzyme was induced by exposure to sediment associated

pyrene (1 μg/g sed. and 10 μg/g sed.) or B(a)A (1 μg/g sed. and 10 μg/g sed.) (Jørgensen et al., 2005a). The kinetic parameters of these enzymatic reactions were also investigated. Glucuronidation had high apparent V_{max} and relatively low K_m, glucosidation had relatively low apparent V_{max} and high K_m and sulfation had relatively low apparent V_{max} and low K_m (Jørgensen et al., 2005a). As phase II biotransformation in marine polychaetes are much less investigated compared to phase I, the first step in a further understanding of these enzymes is to include determination of phase II enzyme activity, production of specific phase II metabolites as well as determination of phase II enzyme capacity and inducibility in future studies. This will increase the general knowledge about phase II biotransformation enzymes in marine polychaetes and make it possible to determine if these enzymes resemble their vertebrate counterparts as the few studies to date indicate.

4. Excretion

In polychaete species the major route of excretion of xenobiotics is assumed to be via the gut. The gut of a polychaete is lined with a specialized tissue, chloragogen tissue, which has a function that resembles that of the vertebrate liver. In accordance, Giessing et al. (2003a) found conjugates of pyrene in both gut fluid and defecaetion water from *N. diversicolor*, indicating that pyrene metabolites after phase I and II biotransformation are eliminated via the gut in this organism. The few studies on elimination of PAHs and PAH metabolites indicate that there are large species specific differences between different polychaetes in how they eliminate PAHs (Christensen et al., 2002a; Driscoll and McElroy, 1997). In an experiment comparing elimination rates of B(a)P between three different species of polychaetes, elimination of both parent compound and metabolites was faster in *N. diversicolor* and *M. viridis* which efficiently biotransform B(a)P compared to *L. fragilis* (Driscoll and McElroy, 1997). However, elimination might also be affected

by gut retention time and could be decreased by presence in the gut of de-conjugating enzymes such as β-glucuronidase, leading to re-absorption in a process analogous to vertebrate enterohepatic circulation (Mulder et al., 1990). This has not been investigated in polychaetes. However, Mayer et al (1995) found that gut-fluid from several deposit-feeding invertebrates including the polychaetes *A. marina* and *N. virens* contains glucosidase activity and possibly also esterase enzymes capable of de-conjugation. Preliminary data indicate that sulfate conjugates are present in higher concentrations in the water phase of microcosms with *N. virens* or *N. diversicolor* compared to glucuronide and glucoside conjugates (Unpublished results Jørgensen and Giessing) despite that the most prominent conjugate in both *N. virens* and *N. diversicolor* tissues are pyrene-1-glucuronide (Giessing et al., 2003a; Jørgensen et al., 2005a). This could indicate either that glucuronide conjugates might be de-conjugated by βglucuronidase in these polychaete species or that sulfate conjugates are excreted faster than glucuronide conjugates. (Christensen et al., 2002b) observed differences in removal paths of accumulated pyrene between *N. diversicolor* and *A. marina*. The major pathway for removal of pyrene from *N. diversicolor* was release of water-soluble metabolites whereas the major pathway of removal from *A. marina* was flushing of un-metabolised pyrene (Christensen et al., 2002b). Unfortunately, the water-soluble metabolites were not identified in this experiment. Also, previous investigations have indicated that the rate of elimination of B(a)P metabolites in *Marenzellaria viridis* and *N. diversicolor* appeared to be slower than elimination of the parent compound (Driscoll and McElroy, 1997). This result agrees with the general notion that PAH metabolites are eliminated quite inefficiently by aquatic invertebrates (James, 1989).

PAHs are primarily eliminated from marine polychaetes in the form of conjugates, and the environmental fate of these conjugates is presently unknown. Since the eukaryotic PAH metabolism does not introduce ring opening, the mineralisation to CO_2 of PAH metabolites excreted from

eukaryotic organisms must be conducted by bacteria (Cerniglia and Heitkamp, 1989). It has been suggested that after excretion to the environment, conjugated PAHs are readily hydrolysed releasing the phase I metabolites (Giessing and Johnsen, 2005). Recently, Giessing and Johnsen (2005) showed that marine pyrene degrading bacteria did not degrade pyrene metabolites excreted from *N. diversicolor* whereas pyrene was indeed degraded. Furthermore, none of six isolated pyrene degrading bacterial strains could utilise 1-hydroxypyrene as their sole carbon and energy source. In addition, 1-hydroxypyrene reduced the respiration rates of all six strains suggesting a direct toxic effect of 1-hydroxypyrene and supports the negligible degradation of pyrene metabolites excreted from *N. diversicolor* (Giessing and Johnsen, 2005). Since bacteria conceivably are unable to degrade phase I metabolites of some PAHs, the biogeochemical fate of these metabolites is currently unknown and remains to be elucidated.

5. Trophic transfer

PAHs themselves are relatively inert molecules and it is generally accepted that except for nonpolar narcosis due to incorporation into the phospholipid bilayer of membranes, toxic effects of PAHs are caused by their metabolites rather than by the parent compounds (Livingstone, 1993). Biotransformation enzymes thus play a dual role of ridding the organism of parent PAH through modification and eventual elimination, but also of creating toxic metabolic intermediates. In vertebrates, the initial CYP catalyzed oxidations of PAHs are either mono-hydroxylations or epoxidations, epoxides being hydrolyzed to vicinal trans-dioles catalyzed by epoxide hydrolase (EH) or thiolyzed to glutathione conjugates catalysed by GST. However, in PAHs containing a “bay region” like e.g. B(a)P, epoxides formed in PAH “bay region” are not hydrolyzed by EH due to steric hindrance. Such PAHs have been found to be carcinogenic and mutagenic in mammalian species (Penning, 1993); (Chen et al., 1996). In marine polychaetes, metabolites of PAHs such as

B(a)P have been found to cause DNA damage. In *Capitella* sp. I, DNA damage was also detected after exposure to sediment-associated fluoranthene (Bach et al., 2005; Palmqvist et al., 2003). The potential for production of carcinogenic and mutagenic metabolites in marine polychaetes leads to concern about trophic transfer of PAH residues and biotransformation products as polychaetes may play important roles in the transfer of contaminants from sediments to biota, because of their association with sediments and their significance as food source for bottom feeding fish and other epifaunal organisms (Clements et al., 1994; McElroy and Sisson, 1989). As the biotransformation capacity of fish and other vertebrate organisms is higher than that of invertebrates, transferred PAHs and metabolites might be further biotransformed, either by further CYP catalysed oxidation that might lead to formation of DNA reactive metabolites or by biotransformation enzymes resulting in increased hydrophilicity and elimination (James, 1989; Palmqvist et al., 2006).

Trophic transfer involving marine polychaetes has been investigated in few experiments. Rice et al. (2000) investigated growth, *CYP1A* expression and DNA adduct formation in juveniles of the flatfish *Pleuronectes vetulus* fed the polychaete *Armandia brevis* exposed to harbour sediment or sediment contaminated with B(a)P, Arochlor 1254 or dichlorodiphenyldichloroethylene (DDE). The growth of *P. vetulus* fed exposed worms was slower than that of those fed non-exposed worms. Also, fish fed exposed worms all showed increased expression of CYP1A immunostaining with a polyclonal antibody. However, hepatic PAH-DNA adducts were found only in fish exposed to B(a)P-exposed polychaetes (Rice et al., 2000). Palmqvist et al. (2007) investigated the trophic transfer to *N. virens* of ^{14}C-labelled fluoranthene from two *Capitella* sibling species differing in their ability to biotransform. *N. virens* fed *Capitella* sp. I (effective biotransformator) accumulated higher levels of fluoranthene derived compounds than did *Capitella* sp. S (poor biotransformator). There was however, no indications of DNA damage in *N. virens* fed either of the two fluoranthene

exposed *Capitella* species (Palmqvist et al., 2006). Also, McElroy and Sisson (1989) demonstrated transfer of metabolites of ^{14}C-labelled B(a)P formed by *N. virens* via the diet to winter flounder *Pseudopleuronectes americanus*, resulting in the presence of phase I and phase II metabolites in liver and intestine. The current data indicates that transfer of PAH metabolites between trophic levels might occur and have effects on the predator organism. Especially the potential of some PAH metabolites to cause DNA damage is reason for concern. However, a recent study on trophic transfer of PAHs found that relative high concentrations of PAHs are found in organisms at low trophic levels and that PAHs undergo trophic dilution in the marine food web resulting in relatively low PAH concentrations in organisms at high trophic levels (Wan et al., 2007). This is most likely due to low assimilation efficiency and high biotransformation capacity in organisms at high trophic levels (Wan et al., 2007). Since only few studies are available, the extent of trophic transfer of PAHs and PAH metabolites involving marine polychaetes is largely unknown.

6. Molecular mechanisms underlying biotransformation in marine polychaetes

To increase the mechanistic understanding of the specific CYP enzymes that mediate the first step in PAH biotransformation, identification of *CYP* genes from marine polychaetes has been initiated. To date only few *CYP* genes have been fully sequenced and investigated (**Table 3**). In *N. virens*, two *CYP* genes were identified and sequenced (Jørgensen et al., 2005b); they were named CYP4BB1 (GenBank accession number **AY453407**) and CYP342A1 (GenBank accession number **AY453408**) by the Cytochrome P450 Nomenclature Committee. *N. virens* CYP342A1 shares less than 40% amino acid identity with other CYP enzymes and was therefore assigned to a new family, but has the highest homology with CYP enzymes belonging to the CYP4F family. The other identified *N. virens* gene CYP4BB1 was assigned to a new subfamily, but shared highest similarity to CYP4F. Furthermore, two *CYP* genes from *Capitella* sp. I have been identified CYP4AT1

(GenBank accession number **AY574044**) and CYP331A1 (GenBank accession number **AY574043**) which shared highest homology to the CYP4F and CYP45 subfamily, respectively (Li et al., 2004). In a recent review on CYP enzymes, the phylogenetic relationship of the identified marine polycheate CYPs is established by comparison with CYPs from other marine invertebrates (Rewitz et al., 2006). The two CYP genes (CYP4BB1 and CYP342A1) from *N. virens* cluster together with CYP4AT1 from *Capitella* sp.I and *Mytilus galloprovincialis* (mussel) in the 4 clan whereas CYP331A1 also from *Capitella* sp.I, CYP30 from *Mercenaria mercenaria* (clam) and CYP45 from Homarus americanus (lobster) cluster in the 3 clan (Rewitz et al., 2006).

Compared to vertebrates, the invertebrate CYP4 family is more diverse comprising numerous isoforms even within a single species. In vertebrates, CYP4 enzymes are primarily involved in fatty acid metabolism, but some enzymes are also recognised for their involvement in metabolism of exogenous compounds (Kikuta et al., 1999). It has been suggested that CYP4 enzymes in vertebrates function at the interface between metabolism of endogenous and exogenous substrates (Fischer et al., 1998). In insects the CYP4 family has been suggested to be involved in toxin metabolism (Dunkov et al., 1996; Scott et al., 1994). This is supported by the greater number and broader sequence diversity of CYP4 genes in insects which indicate that the corresponding enzymes could have a similar role as vertebrate CYP2 enzymes, that are involved in drug metabolism (Dunkov et al., 1996). This hypothesis is supported by Danielson et al. (1998) who related strong and highly specific upregulations of CYP4 mRNA expression in *Drosophila mettleri* after barbiturate exposure to a pattern of xenobiotic responsiveness more similar to vertebrate drug metabolising enzymes than to putative vertebrate CYP4 homologs (Danielson et al., 1998). The suggested function of insect CYP4 enzymes in biotransformation of exogenous compounds is based on the general thought that CYPs involved in xenobiotic biotransformation are often

transcriptionally inducible by substrates upon which they act (Whitlock, 1986). Therefore most investigations have used mRNA expression studies to determine inductions after exposure to xenobiotics and thereafter suggested involvement in xenobiotic biotransformation (Carino et al., 1994; Danielson et al., 1998; Snyder, 1998b; Snyder et al., 1995; Tares et al., 2000). Accordingly, identified *CYP* genes from marine polychaetes have primarily been investigated with regard to possible functions by mRNA expression studies. In Rewitz et al (2004), northern blot analysis showed induction of *CYP4(2) (CYP342A1)* by crude oil, B(a)A and clofibrate. In contrast the *CYP4(1) (CYP4BB1)* gene expression was not induced by any of the treatments, including clofibrate, which is a known inducer of CYP4F in vertebrate species (Simpson, 1997). Based on the transcriptional upregulations by xenobiotics found with the *N. virens* CYPs, it was suggested that the *CYP* genes might be involved in xenobiotic biotransformation (Rewitz et al., 2004). Also, CYP4AT1 and CYP331A1 from *Capitella* sp. I was investigated by real-time PCR after exposure to sediment associated PAHs (Li et al., 2004). CYP4AT1 was not induced except after exposure to one concentration of 3-MC whereas CYP331A1 was induced by B(a)P and fluoranthene (Li et al., 2004). However, the authors suggested that the two *CYPs* are relatively constitutively expressed. The induction levels found in marine invertebrates appear to be lower compared to insects and vertebrates, which could indicate that the regulation of the CYP enzyme expression is less sophisticated in marine polychaetes compared to vertebrates. However, upregulation of transcription does not necessarily result in an increased amount of produced enzyme, therefore, experiments at the enzyme level are also necessary.

In vivo experiments employing total CYP enzyme activity in polychaetes can be used to investigate induction of the total enzyme level and the relationship between inducers and substrate. However, it is seldom possible to separate activities contributed by different CYP isoforms, limiting the value of

this approach. Activity of specific CYP enzymes can be investigated using heterologous expression followed by determination of activity of the specific CYP isoform, which is necessary to demonstrate that an exogenous compound that upregulated mRNA levels is in fact substrate for the enzyme. Therefore, heterologous expression of *CYP* genes is a valuable tool for investigating if the substrate in question is metabolised by the specific CYP enzyme. This type of biotransformation study with specific CYP enzymes is needed to directly demonstrate catalytic activity and involvement in PAH biotransformation. The activity of CYP4BB1 and CYP342A1 from *N. virens* was determined with pyrene as a substrate and both enzymes catalysed the production of 1-hydroxypyrene (Jørgensen et al., 2005b). However, differences in CYP family, substrate and expression system make it difficult to directly compare catalytic activities from other invertebrate species. An alternative to measuring specific CYP enzyme activity could be Western blotting with antibodies or DNA probes. However, due to low sequence similarity between vertebrate and invertebrate CYP enzymes, antibodies raised against vertebrate CYPs do not seem to bind specifically when used on marine invertebrates (Brown et al., 1998; Snyder, 2000). Specific antibodies raised against specific *CYP* genes identified in marine polychaetes would provide a invaluable tool in elucidating the mechanism of regulation and function of polychaete CYPs. Few studies using specific CYP antibodies have been conducted in marine invertebrates (Snyder and Mulder, 2001), including anti-CYP2L from spiny lobster *Panilirus argus* and anti-CYP45 from lobster *Homarus americanus* (Snyder and Mulder, 2001).

7. Conclusion and perspectives

The data currently available on PAH biotransformation in marine polychaetes indicates that the mechanism resembles that of mammalian biotransformation with few exceptions. There are

contradicting results on inducibility of CYP enzymes in marine polychaetes with some studies finding CYP enzyme activity to be inducible by 2-3 fold whereas other studies find that CYP enzymes are constitutively expressed. However, large differences in the different enzyme assays complicate direct comparison of the results. Also, these assays measure total CYP enzyme activity, thereby blurring the possible induction of one or few CYP isoforms. Recent investigations on the molecular level determining *CYP* gene sequences and -expression after exposure to xenobiotics including PAHs indicate that *CYP* gene expression in marine polychaetes can not be categorised as either inducible or constitutive. It appears that some CYP isoforms are inducible whereas others are constitutively expressed, as expected from vertebrate studies. Currently, the most obvious difference appears to be that inductions of CYP1A enzymes involved in PAH biotransformation in vertebrates is mediated through the Ah receptor. In marine polychaetes and most other invertebrates neither the CYP1A isoform nor AhR homologues have been identified. Future studies should include experiments in which molecular techniques are used to identify *CYP* genes and receptors from marine polychaetes that are involved in PAH biotransformation.

The CYP enzyme mediated phase I biotransformation of PAHs in marine polychaetes can result in the formation of metabolites that are potentially carcinogenic. This causes concern for trophic transfer of PAH parent compounds, but also of metabolites formed in polychaetes, which are important prey items for fish. However, it is not possible to make any general conclusions regarding extend of the trophic transfer of PAH metabolites from polychaetes based on the few available studies. Experiments in which the specific phase I and II PAH metabolites from polychaete biotransformation are identified is limited, but the few available data suggest that the conjugation of PAH phase I metabolites appears to proceed in a manner that resembles vertebrate phase II biotransformation. The primary phase II conjugation pathway in marine polychaetes appears to

differ even among closely related species. However, the knowledge about marine polychaete biotransformation of PAHs is based on studies of very few species. Future studies should include investigations using additional species of marine polychaetes. Furthermore, elimination of the conjugated PAHs from polychaetes is currently not well investigated and the presence and activity of de-conjugating enzymes in gut tissue needs to be further elucidated in order to determine whether de-conjugation is an important factor in elimination of PAH metabolites. Finally, the environmental fate of PAH metabolites eliminated from polychaetes is largely unknown. However, a recent study indicates that pyrene degrading bacteria are not capable of degrading pyrene metabolites eliminated from *N. diversicolor* and the study indicated that the phase I metabolite was toxic to the pyrene-degrading bacteria. Future studies should include determination of excretion rates of produced phase II metabolites as well as determine the relative importance of the phase II enzymes in different polychaete species. Integration of molecular techniques and analytical chemical determination of produced metabolites will improve our understanding of the entire biotransformation pathway in the marine polychaetes thereby establishing whether it is distinct from vertebrate biotransformation. In conclusion, current data indicate that biotransformation of PAHs in marine polychaetes resemble that of vertebrates in a mechanistic perspective (Figure 2), except for lower response level regarding activity of biotransformation enzymes, inducibility and enzyme capacity as well as the apparent lack of a AhR mediated CYP enzyme regulation.

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Figure captions

Figure 1. Proposed biotransformation pathway of pyrene in *Nereis virens*. CYP450: Cytochrome P450 enzymes, ST: Sulfotransferase enzymes, UDPGT: urinidinediphosphosphateglycuronosyl transferase enzymes. From Jørgensen et al 2005a.

Figure 2. Generalised overview of processes that participate in the biotransformation and elimination of accumulated PAHs. The PAH will be biotransformed by phase I enzymes present in the organism and also, the PAH can bind to a receptor that is activated resulting in an increased expression of *CYP* genes. The CYP enzymes are the most important phase I enzymes and the induction will result in a higher efficiency of the phase I biotransformation. This is convenient since the phase I biotransformation is generally believed to be the rate-limiting step in the overall elimination process. The PAH phase I metabolite is then further biotransformed by phase II enzymes to PAH phase II metabolite which can either be eliminated from the organism or it can be de-conjugated to the phase I metabolite by enzymes (enterohepatic circulation).

1 **Tables**

2 Table 1. Identified phase II metabolites involved in PAH biotransformation in marine polychaetes.
3 The substrate was 1-hydroxypyrene in all experiments. Bold indicates that only the mentioned
4 phase II metabolite was identified and * indicates marine polychaete species.

5

Species	Substrate	Phase II metabolites identified	Reference
Capitella capitata *	1-hydroxypyrene	**Sulfate**	Giessing et al. 2003b
Arenicola marina *	1-hydroxypyrene	**Glucoside**	Giessing et al. 2003b
Nereis diversicolor *	1-hydroxypyrene	Glucoside, sulfate, glucuronide	Giessing et al. 2003a
Nereis virens *	1-hydroxypyrene	Glucoside, sulfate, glucuronide	Jørgensen et al. 2005
Carcinus maenas	1-hydroxypyrene	Glucoside, sulfate, unknown	Fillmann et al. 2004
Mya arenaria	1-hydroxypyrene	Sulfate, pyrenediol-hydrogensulfate	Simpson et al. 2002
Protothaca staminea	1-hydroxypyrene	Sulfate, pyrenediol-hydrogensulfate	Simpson et al. 2002
Mytilus galloprovincialis	B(a)P metabolites	Sulfate, glucuronide	Michel et al. 1995
Porcellio scaber	1-hydroxypyrene	Glucoside, sulfate	DeKnecht et al. 2001
Oniscus asellus	1-hydroxypyrene	Glucoside, sulfate	DeKnecht et al. 2001
Homarus americanus	9-hydroxy-B(a)P	Glucoside, sulfate	Li & James 2000

6

Table 2

Comparison of produced metabolites of PAHs in marine polychaetes.

Species	Parent PAH	Phase I	Phase II	Unextractable	Exposure	PAH and Conc.	Reference
Nereis virens	17 %	4 %	79 %	-	5 days	10 µg/g pyrene	Jørgensen et al. 2005a
Nereis virens	24 %	10 %	32 %	34 %	6 days	8 µg/g B(a)A	McElroy 1990
Capitella sp. I	37 %	20 %	38 %	4 %	10 days	30 µg/g Fluoranthene	Bach et al. 2005
Capitella sp. S	89 %	3 %	7 %	1 %	10 days	30 µg/g Fluoranthene	Bach et al. 2005
Nereis diversicolor	25 %	2 %	73 %	-	5 days	25 µg/g pyrene	Giessing et al. 2003a
Nereis diversicolor	5 %	5 %	78 %	12 %	9 days	20 ng/g B(a)P	Driscoll & McElroy 1996
Scolecolepides viridis	40 %	8 %	38 %	14 %	9 days	20 ng/g B(a)P	Driscoll & McElroy 1996
Leitoscoloplos fragilis	90 %	2 %	1 %	6 %	9 days	20 ng/g B(a)P	Driscoll & McElroy 1996
Arenicola marina	86 %	4 %	10 %	-	8 days	0.4 µg/g pyrene	Christensen et al. 2002a
Nereis diversicolor	56 %	7 %	37 %	-	10 days	0.4 µg/g pyrene	Christensen et al. 2002a

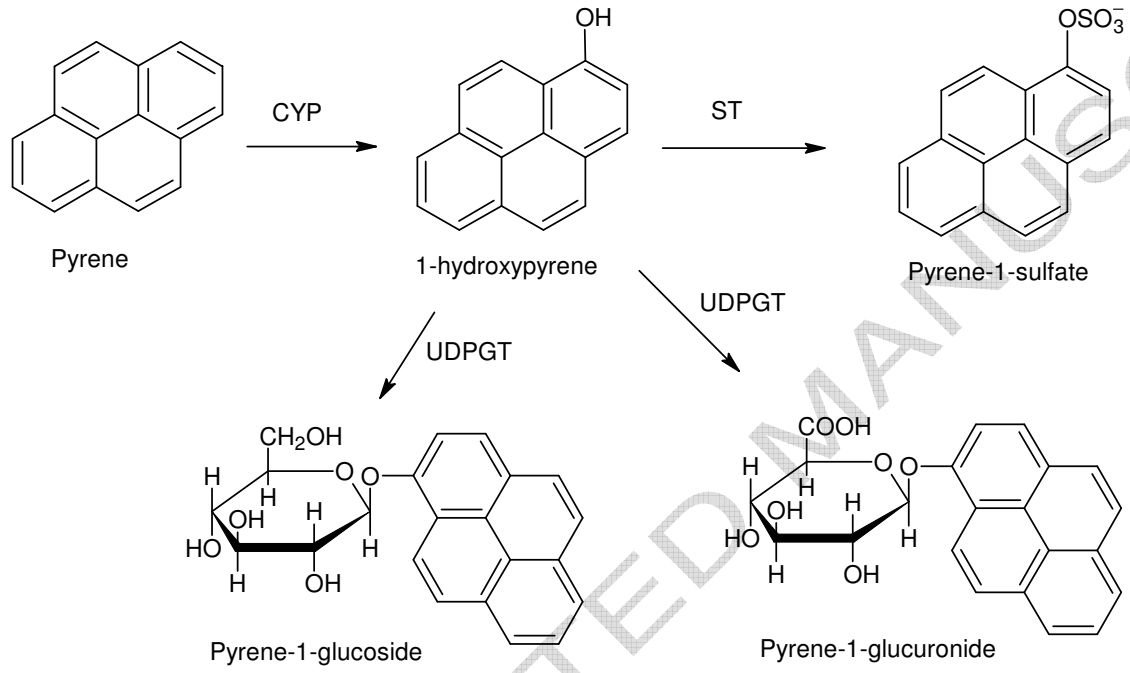
Table 3

Overview of *CYP* genes identified in marine invertebrate species.

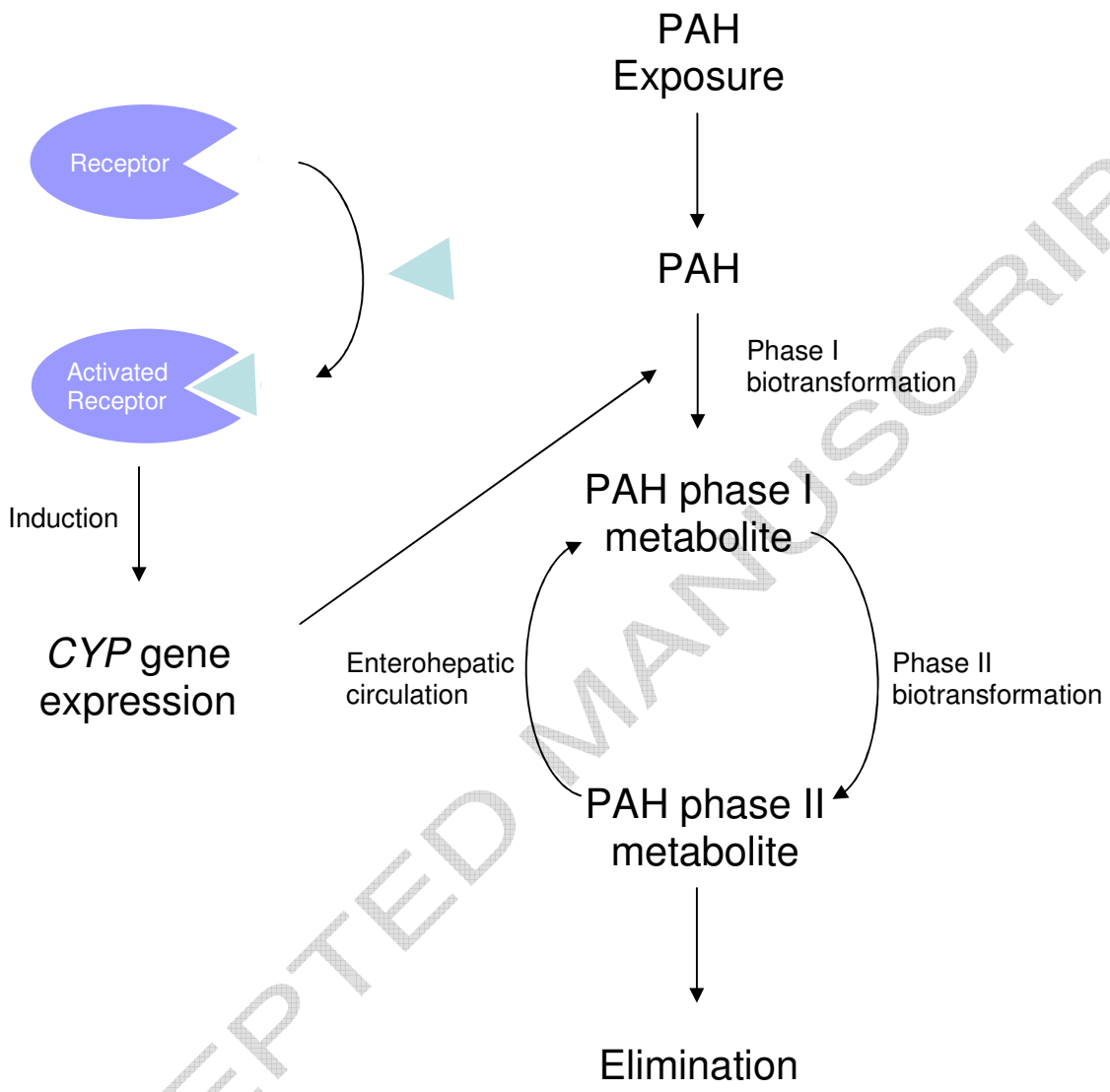
Species		CYP gene		Tissue	Reference
Nereis virens	polychaete	*CYP4BB1*	complete sequence	gut tissue	Jørgensen et al. (2005b)
Nereis virens	polychaete	*CYP342A1*	complete sequence	gut tissue	Jørgensen et al. (2005b)
Capitella Sp. I	polychaete	*CYP331A1*	complete sequence	whole worm	Li et al. (2004)
Capitella Sp. I	polychaete	*CYP4AT1*	complete sequence	whole worm	Li et al. (2004)
Haliotis rufescens	Abalone	*CYP4C17*	partial sequence	digestive gland	Snyder (1998a)
Lytechinus anamesis	sea urchin	*CYP4C19*	partial sequence	pyloric caeca	Snyder (1998a)
Lytechinus anamesis	sea urchin	*CYP4C20*	partial sequence	pyloric caeca	Snyder (1998a)
Mytilus galloprovincialis	Mussel	*CYP4Y1*	partial sequence	digestive gland	Snyder (1998a)
Mercenaria mercenaria	clam	*CYP30*	complete sequence	gonads	Brown et al. (1998)
Carcinus maenas	crab	*CYP330A1*	complete sequence	hepatopancreas	Rewitz et al. (2003)
Carcinus maenas	crab	*CYP4C39*	complete sequence	hepatopancreas	Rewitz et al. (2003)
Panilirus argus	spiny lobster	*CYP2L1*	complete sequence	hepatopancreas	James et al. (1996)
Panilirus argus	spiny lobster	*CYP2L2*	complete sequence	hepatopancreas	Boyle et al. (1998)
Penaeus setiferus	shrimp	*CYP4C16*	partial sequence	hepatopancreas	Snyder (1998a)
Homarus americanus	lobster	*CYP4C18*	partial sequence	hepatopancreas	Snyder (1998a)
Homarus americanus	lobster	*CYP45*	complete sequence	hepatopancreas	Snyder (1998b)

Figures

Figure 1



1 Figure 2



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