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Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet *Chelon labrosus*

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ABSTRACT

Thicklip grey mullets *Chelon labrosus* inhabit coastal and estuarine areas where they can be chronically exposed to commonly released pollutants such as polycyclic aromatic hydrocarbons (PAHs) and perfluorinated compounds. These pollutants can also originate from accidental spills, such as the Prestige oil spill in 2002, which resulted in the release of a heavy fuel oil that affected coastal ecosystems in the Bay of Biscay. Peroxisome proliferation (PP), induced biotransformation metabolism, immunosuppression and endocrine disruption are some of the possible biological effects caused by such chemicals. With the aim of studying the effects of organic toxic chemicals on such biological processes at the transcriptional and at the cell/tissue level, juvenile mullets were exposed to the typical mammalian peroxisome proliferator perfluorooctane sulfonate (PFOS), and to fresh (F) and weathered (WF) Prestige-like heavy fuel oil for 2 and 16 days. First, fragments of genes relevant to biotransformation, immune/inflammatory and endocrine disruption processes were cloned using degenerate primers. Fuel oil elicited a significant PP response as proved by the transcriptional upregulation of *palmitoyl-CoA oxidase* (*aox1*), *peroxisome proliferator activated receptor α* (*pparα*) and *retinoic X receptor*, by the AOX1 activity induction and by the increased peroxisomal volume density. PFOS only elicited a significant induction of AOX1 activity at day 2 and of PPARα mRNA expression at day 16. All treatments significantly increased catalase mRNA expression at day 16 in liver and at day 2 in gill. *Cyp1a* transcription (liver and gill) and EROD activity were induced in fuel oil treated organisms. In the case of phase II metabolism only hepatic glutathione S-transferase mRNA was overexpressed in mullets exposed to WF for 16 days. Functionally, this response was reflected in a significant accumulation of bile PAH metabolites. WF treated fish accumulated mainly high molecular weight metabolites while F exposure resulted in accumulation of mainly low molecular ones. Fuel oil significantly regulated immune response related *complement component C3* and *hepcidin* transcription followed by a significant regulation of inflammatory response related apolipoprotein-A1 and fatty acid binding protein mRNAs at day 16. These responses were accompanied by a significant hepatic inflammatory response with lymphocyte accumulations (IRLA) and accumulation of melanomacrophage centers (MMC). PFOS did not elicit any transcriptional response in the studied biotransformation and immune related genes, although histologically significant effects were recorded in IRLA and MMC. A significant reduction of lysosomal membrane stability was observed in all exposed animals. No endocrine disruption effects were observed in liver while brain aromatase mRNA was overexpressed after all treatments at day 2 and

Abbreviations: AhR, aryl hydrocarbon receptor; AOX1, palmitoyl-CoA oxidase; ApoA1, apolipoprotein-A1; B(*a*)P, benzo(*a*)pyrene; BKME, bleached kraft pulp and paper mill effluents; CAT, catalase; CC3, complement component C3; CYP19A, aromatase; CYP1A, cytochrome P450 1A; DECR, Δ^2,Δ^4-dienoyl-CoA reductase-2; EROD, 7-ethoxyresorufin O-deethylase; ERα, estrogen receptor α; F, fresh heavy fuel oil; FABP, fatty acid binding protein; FF, fixed wavelength fluorescence; GST, glutathione S-transferase; IRLA, inflammatory response with lymphocyte accumulations; LP, labilization period; MFP1, multifunctional protein; MMC, melanomacrophage centres; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PMP70, peroxisomal membrane protein of 70 kDa; PP, peroxisome proliferation; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response elements; ROS, reactive oxygen species; RXR, retinoid X receptor; THIO, 3-ketoacyl-CoA thiolase; TNFα, tumor necrosis factor α; UGT, UDP-glucuronosyltransferase; VTG, vitellogenin; V_{vp}, peroxisomal volume density; WF, weathered heavy fuel oil.

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estrogen receptor α was downregulated under WF exposure at day 16. These results show new molecular and cellular biomarkers of exposure to organic chemicals and demonstrate that in mullets PP could be regulated through molecular mechanisms similar to those in rodents, although the typical mammalian peroxisome proliferator PFOS and heavy fuel oil follow divergent mechanisms of action.

1. Introduction

Littoral areas are important pollutant recipients via tanker spills, urban run-offs, industrial and sewage effluents or atmospheric deposition. In November 2002 the tanker Prestige sunk offshore Galicia spilling more than 60,000 tons of heavy fuel oil. In subsequent months, this heavy fuel oil enriched in high molecular weight polycyclic aromatic hydrocarbons (PAHs) and metals such as Se, Mo, Ni and V arrived at beaches and cliffs all along the North coast of the Iberian Peninsula (Martínez-Gómez et al., 2006).

In order to study the biological effects of PAHs, different exposure and effect biomarkers can be used. Among them, peroxisome proliferation (PP) in aquatic organisms has been proposed as a promising biomarker of exposure to organic compounds such as PAHs (Cancio and Cajaraville, 2000; Cajaraville et al., 2003). In this way, PP has been demonstrated in different fish species exposed to crude and lubricant oils, PAHs, polychlorinated biphenyls (PCBs) and phthalate ester plasticizers (Cancio and Cajaraville 2000; Cajaraville et al., 2003). PP is characterized by increased hepatic peroxisomal volume density which in rodents is accompanied by the transcriptional upregulation of enzymes involved in lipid homeostasis, such as those participating in peroxisomal β-oxidation: palmitoyl-CoA oxidase (AOX1), multifunctional protein (MFP1) and 3-ketoacyl-CoA thiolase (THIO) (Qi et al., 2000). In vertebrate responsive species, PP is under the regulation of the peroxisome proliferator activated receptor α (PPARα), a transcription factor that belongs to the nuclear receptor superfamily (Isseman and Green, 1990) and that acts in conjunction with its heterodimerization partner retinoid X receptor, RXR (Van den Heuvel et al., 2006; Raingeard et al., 2009). Considering that piscine genes coding for peroxisomal β-oxidation enzymes contain putative PPARα/RXR binding motifs in their promoter regions (Bilbao et al., 2009), induction of peroxisomal genes in aquatic organisms might be regulated transciptionally, similar to rodents. However, so far most of the studies reporting PP in aquatic species have only measured the process in terms of increased peroxisomal volume density or increased AOX1 activity (reviewed in Cancio and Cajaraville, 2000; Cajaraville et al., 2003).

Toxicity of some xenobiotics depends among other factors, on the rate at which they are biotransformed (Ferreira et al., 2006). Biotransformation metabolism is generally subdivided in two processes: phase I metabolism consists in principally oxidative reactions where cytochrome P450 (CYP) monooxygenases play the central role as catalysts, resulting in hydroxylation of parent compounds. Among different CYP families, in vertebrates organic contaminants such as PAHs or PCBs are specifically oxidized by cytochrome P450 1A encoded by the *cyp1a* genes. These genes in turn are regulated by the substrates that their product enzymes help to metabolize, so their expression is upregulated in the presence of PCBs or PAHs under regulation of the aryl hydrocarbon receptor, AhR (Hahn and Stegeman, 1994; Jönsson et al., 2006), and thus enhanced *cyp1a* gene transcription or induced enzyme activity is used as a biomarker of exposure to these compounds (Hahn and Stegeman, 1994; Van der Oost et al., 2003). Phase II metabolism involves the conjugation of the hydroxylated compound with an endogenous substrate, thus facilitating the excretion of lipophilic chemicals by the addition of a polar group (Van der Oost et al., 2003). Addition of glutathione or UDP-glucuronic acid catalyzed by glutathione S-transferases (GST) and UDP-glucuronosyltransferases (UGT), respectively, are two important conjugation reactions for the further excretion of lipophilic chemicals via bile (Van der Oost et al., 2003). Significant effects of PAHs and halogenated xenobiotics on fish GST and UGT enzyme activities have been reported in field and laboratory studies (Van der Oost et al., 2003; Schreiber et al., 2006; Martin-Skilton et al., 2008; Simonato et al., 2008). The level of biliary PAH metabolites constitutes a sensitive biomarker to assess recent exposure to PAHs (Van der Oost et al., 2003). Interestingly, human *cyp1a1* contains 2 peroxisome proliferation response elements (PPRE) in its promoter region and several peroxisome proliferators are able to enhance *cyp1a1* transcription by a mechanism that still remains to be elucidated (Sérée et al., 2004). Similarly, Barbier et al. (2003) identified *ugt* as a PPARα and PPARγ target gene in mice while *gst* of the flatfish *Pleuronectes platessa* contains functional PPREs in its promoter region (Leaver and George, 1996; Boukouvala et al., 2004).

Induction of biotransformation metabolism and PP produce highly reactive oxygen species (ROS) as by-products (Jifa et al., 2006) leading to oxidative stress, which could result in mutagenesis or carcinogenesis. In this way, the antioxidant defense system may be induced in cells as response to ROS. Thus, measurement of components of the antioxidant defense system such as peroxisomal catalase (CAT) may be helpful to determine organism exposure to lipophilic organic compounds (Livingstone, 2001; Liu et al., 2007).

PAHs have also been described to alter vertebrate immunocompetence having an effect on fish resistance to pathogens (Reynaud and Deschaux, 2006). AhR agonists such as the model carcinogenic PAH benzo(*a*)pyrene or dioxins have been shown to be pro-inflammatory and cause immunosuppression (Carlson et al., 2002; Reynaud and Deschaux, 2006; Volz et al., 2006). Additionally, high throughput gene transcription studies have revealed that exposure to xenobiotics highly regulates genes involved in the piscine immune/inflammatory response (Williams et al., 2008).

Here, a laboratory experiment was designed where thicklip grey mullets *Chelon labrosus* were exposed to fresh heavy fuel oil (F), trying to mimic recently spilled oil and weathered heavy fuel oil (WF) in order to simulate the oil composition after long periods in water. Another group of mullets was exposed to perfluorooctane sulfonate (PFOS) as a typical mammalian peroxisome proliferator and hypolipidemic agent (Sohlenius et al., 1993; Seacat et al., 2003; Guruge et al., 2006). Fluorinated alkyl substances constitute a diverse class of chemicals that occur in a wide range of products. For example, PFOS is used as a surfactant and surface protector in carpets, leather and paper as well as in chemicals such as floor-polishes or fire-fighting foams (Giesy and Kannan, 2002). Perfluorinated compounds are resistant to hydrolysis, photolysis, biodegradation and metabolism in the environment, resulting in a high degree of environmental persistence and bioaccumulation (Giesy and Kannan, 2002; Hoff et al., 2005). Information regarding toxic effects caused by fluorinated compounds in aquatic organisms is scarce but they have been reported to alter plasma concentrations of both steroidal androgens and estrogens and to induce AOX1 activity in liver of fathead minnows *Pimephales promelas* (Oakes et al., 2004). The perfluorinated compound perflurooctanoic acid (PFOA), for instance, has been described as a weak PPARα agonist in fish (Leaver et al., 2005), while gene transcription profiling has suggested that PFOA might increase fatty acid β-oxidation in European flounder (Williams et al., 2008). Finally, recent transcriptomic studies have shown perfluorinated compounds to behave as weak