Bioaccumulation of persistent organic pollutants (POPs) and biomarkers of pollution in Mediterranean deep-sea organisms

Bioacumulación de contaminantes orgánicos persistentes (COPs) y biomarcadores en organismos del mar Mediterráneo profundo

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Samuel Koenig

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Universidad de Barcelona

Facultad de Biología – Departamento de Fisiología

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Samuel Koenig

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Institut de Ciències del Mar (ICM)

Institut de Diagnosi Ambiental i Estudis de l`Aigua (IDAEA)

Consejo Superior de Investigaciones Científicas (CSIC)

Directora:	Co-directora:	Tutora:
Dra. Montserrat Solé	Dra. Pilar Fernández	Dra. Isabel Navarro
ICM-CSIC	IDAEA-CSIC	UB

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1. General introduction

1

General introduction

1.1 Contamination of the deep-sea

The deep sea (depth > 1000 m) is the largest ecosystem on Earth and has long been considered to be the last region to have remained untouched from anthropogenic disturbance. However, more recently, there has been growing concern on the impact of human activities on deep-sea ecosystems (Ramirez-Llodra et al., 2011). Man-made chemicals have been shown to occur in deep-sea areas remote from anthropogenic pollution sources and it has been postulated that the deep ocean may act as sink for highly persistent contaminants that enter the marine environment (Ballschmiter et al., 1997; Froescheis et al., 2000; Looser et al., 2000; Mormede and Davies, 2003). In the water column, many of these pollutants tend to bind to particles, facilitating their export from surface waters to the bottom of the ocean (Dachs et al., 2002; Wania and Daly, 2002). Benthic organisms living on the deep-sea floor are thus exposed to these contaminants and previous studies have shown that these compounds accumulate in deep-sea biota in the Mediterranean Sea (Porte et al., 2000; Storelli et al., 2007), the Atlantic (Kramer et al., 1984; Mormede and Davies, 2003) and the Pacific Ocean (Ramu et al., 2006; Takahashi et al., 2010). These pollutants can pose a threat to humans and wildlife due to their high toxicity and persistence in the environment (Darnerud, 2003; Vasseur and Cossu-Leguille, 2006). However, little is known on the extent of contamination of the deep-sea and the potential impact it may have on deep-sea ecosystems is even less understood.

1.2 Selected contaminants

1.2 1 Persistent organic pollutants (POPs)

POPs have been defined as organic substances that are resistant to chemical, biological and physical degradation, bioaccumulate through the food chain, are prone to long-range transport and can cause adverse effects to human health and the environment. After the call of the Governing Council of the United Nations Environment Program (UNEP) for global action to be taken on POPs in 1995, the treaty of the Stockholm Convention on POPs was signed in 2001, which aimed to eliminate or restrict the use and emissions of twelve initially selected compounds by 2004. The general and common physical-chemical characteristics of POPs include environmental persistence and semi-volatility, which favors their long-range transport. In addition, they are hydrophobic with high bioaccumulation and biomagnification potentials and present various toxic effects. Initially, only organochlorine compounds were included in the Stockholm Convention, but more recently other compounds such as brominated or fluorinated organic compounds have been added to the list of POPs.

The POPs selected in the present work are characteristic of different pollutant classes and origins, providing representative cases for the description of the processes involved in the observed bioaccumulation patterns. The selected contaminant groups include legacy as well as emergent POPs, to not only allow for comparisons with previous pollution studies, but also provide novel information on baseline levels of emergent contaminant classes in the Mediterranean deep-sea environment.

Pentachlorobenzene (PeCB) and hexachlorobenzene (HCB)

PeCB and HCB are both by-products that are generated during the fabrication of organochlorine solvents. HCB has also been used as fungicide for seed treatment, but its production was banned in the United States in 1966 and was included in the initial Stockholm Convention treaty in 2001. Both, HCB and PeCB have been shown to be toxic to aquatic organisms, causing long-term adverse effects in the environment (Malcolm et al., 2004). In addition, the International Agency for Research on Cancer (IARC) has considered HCB a possible human carcinogen, potentially causing soft-tissue and thyroid cancers (Grimalt et al., 1994) and it has also been listed as endocrine

disruptor by the EU (European Union Prioritization List for Endocrine Disrupting Compounds).

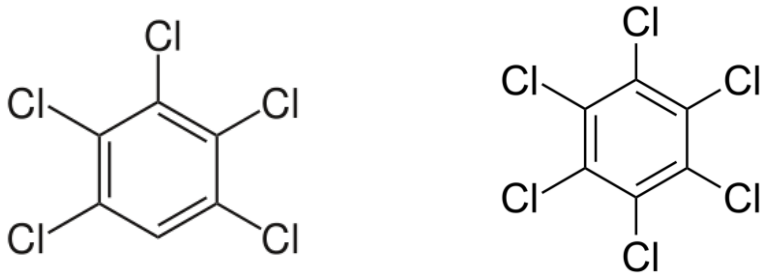


Figure 1.1 Chemical structure of PeCB and HCB.

Hexachlorocyclohexanes (HCHs)

HCHs comprise eight stereoisomers, although only four of these are chemically stable, namely α-, β-, γ- and δ-HCH. A technical mixture of these four isomers was heavily used as pesticide during the 1960s and 1970s, however, this mixture was progressively replaced by γ-HCH, also called lindane, as it is the only isomer with significant insecticide activity. The use of lindane has been banned in 2009 under the Stockholm convention, although a specific exemption still allows its application for lice and scabies treatment. In comparison to most other POPs, HCHs have a relatively high water solubility and a low vapor pressure and are thus predominantly found in the gaseous phase in the atmosphere or in the dissolved phase in water, unlike other more hydrophobic POPs, which are usually associated with the suspended particulate matter (Mackay et al., 1992). Lindane can have neurotoxic effects by interfering with the GABA neurotransmitter and has been classified as potential human carcinogen by the IARC. Moreover, the β-HCH isomer has been associated with thyroid disruption and negative effects on brain development (Álvarez-Pedrerol et al., 2008).

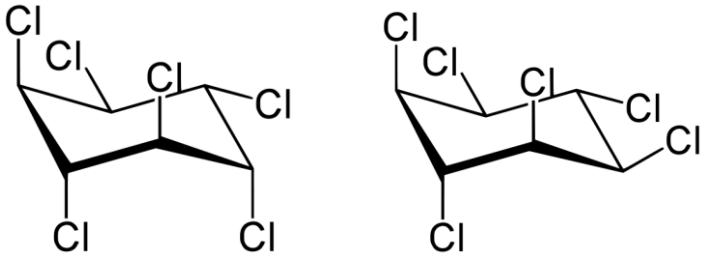


Figure 1.2 Molecular structure of α-, and γ-HCH.

Polychlorinated biphenyls (PCBs)

PCBs encompass a family of 209 congeners containing 2-10 chlorine atoms, which were predominantly used from the 1930s onwards in the manufacturing of electrical equipment such as transformers and capacitors because of their heat absorbing and electrical insulating properties. PCB production was banned in 1979 in the United States and included in the initial treaty of the Stockholm Convention in 2001. The number and position of the chlorine atoms in the molecule largely determine its behavior in the environment and toxicity. The water-solubility and vapor pressure decrease with increasing chlorination of the molecule, while the lipophilicity increases, resulting in higher persistence and bioconcentration/biomagnification of the highly-chlorinated congeners. Ortho-chlorinated PCBs (*e.g.* PCB 77, 169) have a similar planar structure as dioxins and furans and are thus considered highly toxic. In addition, the IARC concluded that PCBs are likely to be carcinogenic and some congeners are thought to have neurotoxic, thyroid-disrupting and estrogen-mimicking properties (Garcia-Reyero et al., 2007).

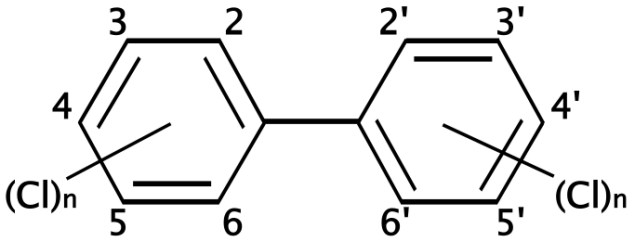


Figure 1.3 Generic chemical structure of PCBs (n=2-10)

Dichlorodiphenyltrichloroethane and metabolites (DDTs)

DDT was widely used as an agricultural insecticide and to control of disease vectors for malaria and typhus from the 1940s until the 1970s. However, due to its very high toxicity to humans and wildlife its application was progressively banned in most developed countries during the 1970s and 1980s. Moreover, its use was restricted to disease vector control under the Stockholm Convention on POPs, although in some countries such as India and North Korea it is still being applied as agricultural insecticide. The main compound used in the technical mixtures is the *p,p'*-DDT isomer, which progressively degrades in aquatic environments into even more persistent metabolites, primarily *p,p'*-DDE (Wolfe et al., 1977). Potential health effects caused by DDT and DDE exposure include genotoxicity and endocrine disruption. In addition, population declines of predatory bird species have been linked to the use of DDT, as *p,p'*-DDE has been shown to cause eggshell thinning of bird eggs, causing a reduction of their breeding success (Vasseur and Cossu-Leguille, 2006).

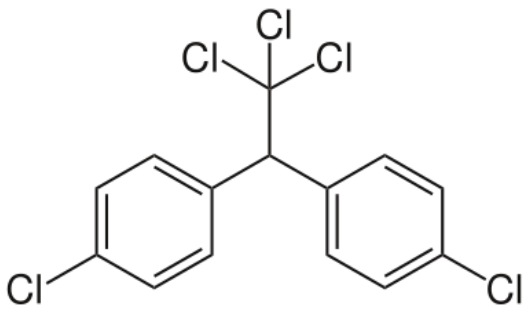


Figure 1.4 Chemical structure of *p,p'*-DDT

Polybrominated diphenyl ethers (PBDEs)

PBDEs consist of 209 congeners that have been used as flame retardants in a wide array of products such as plastics, foams, textiles and electronic devices. Commercial PBDE mixtures include the penta-, octa- and decaBDE mixtures, depending on the predominant congeners used in the different formulations. Unlike most other POPs

mentioned so far, PBDEs have only been in the environment since the 1970s and their production is still in the process of being phased out. Among the commercial mixtures used, the penta- and octa-BDE formulations have been progressively replaced by the less toxic decaBDE mixture, which however, has been shown to degrade to lower brominated and more toxic congeners in the environment (Ross et al., 2009). Concerns on the potential toxicity and detrimental effects on the environment include developmental neurotoxicity, endocrine-disruption due to thyroid-mimicking effects and immunotoxicity (Darnerud, 2003; Birnbaum and Staskal, 2004).

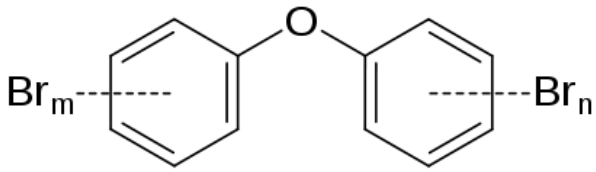


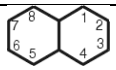
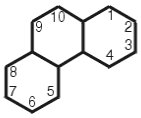
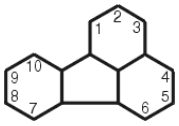
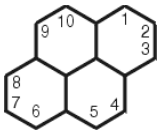
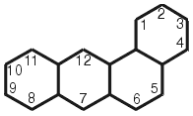
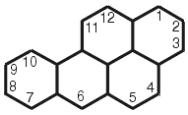
Figure 1.5 Generic chemical structure of PBDEs (n+m=2-10)

1.2.2 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are a unique class of organic pollutant that contain two or more fused aromatic rings, which originate from natural processes (biogenic and diagenic) as well as human activities, including petrogenic (derived from fossil fuel) and pyrogenic (derived from incomplete combustion processes) sources. As shown in previous studies, PAHs are also exported to the deep-sea by vertical transport of sinking particles and are thus of interest for this work (Bouloubassi et al., 2006). However, unlike the above-mentioned POPs, PAHs are readily metabolized and thus only accumulate at low levels within the muscle tissue of fish. In this context, the analysis of PAH metabolites in bile has been advocated to provide a better indicator for PAH contamination in fish (Escartin and Porte, 1999; Beyer et al., 2010). Although PAHs include a wide variety of structurally different substances, the US Environmental Protection Agency (EPA) has identified 16 compounds that are environmentally relevant due to their potential toxicity to mammals and aquatic organisms. Moreover, six of these EPA priority pollutants have been classified as probable human carcinogens by the IARC (Table 1.1). The carcinogenic potential of PAHs is related to the formation of DNA adducts that can cause DNA

damage and altered gene expression, potentially resulting in mutations and cancer development (Akcha et al., 2003). In particular, in some cases highly reactive hydroxylated metabolites are generated during the cytochrome P450-mediated phase I metabolism, which exhibit cytotoxic, mutagenic and carcinogenic characteristics.

Table 1.1 Chemical structure of the six PAHs classified as probable human carcinogens by the IARC

Substance	Structure
Naphthalene	
Phenantrene	
Fluoranthene	
Pyrene	
Benz[a]anthracene	
Benzo[a]pyrene	

1.2.3 Alkylphenols (APs)

Alkylphenol ethoxylates (APEs) represent a class of nonionic surfactants that are extensively used in domestic and industrial products, such as detergents, pesticide formulations, fuel, lubricants, among others. Among the different APE compounds, nonylphenol (NPE) and octylphenol ethoxylates (OPE) are the most commonly used surfactants. Environmental sources include municipal wastewater and sewage treatment plant discharges or direct emissions into the environment, where they degrade to more persistent and toxic shorter-chain alkylphenol (AP) compounds such as nonylphenol (NP) and octylphenol (OP) (Ying et al., 2002). Since the 1990s, there has been growing concern on the toxicity of these compounds due to their ability to interfere with the estrogen-receptor, potentially causing endocrine disruption (Nimrod and Benson, 1996). Thus far, there are no reports on the presence of APs in deep-sea biota. However, due to their environmental persistence, they could potentially be subject to vertical transport to deeper waters and thus pose a risk for deep-sea ecosystems, particularly with regard to their endocrine disruptive potencies. Similarly to PAHs, APs are also readily metabolized in the liver and the assessment of their biliary metabolites provides a good indicator of recent AP exposure in fish (Beyer et al., 2011).

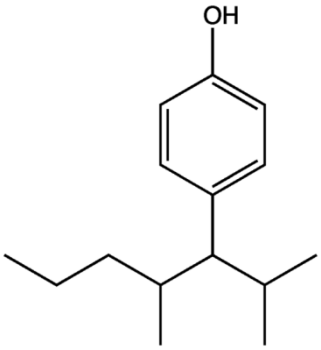


Figure 1.6 Chemical structure of nonylphenol

1.2.4 Mercury

Mercury (Hg) is a trace element present in the Earth's crust, which can be released from natural (*e.g.* volcanic activity, forest fires) and anthropogenic sources (*e.g.* coal burning, mining, steel production) (Díez, 2009). In water bodies, mercury is readily transformed by chemical and biological (*i.e.* bacterially mediated) pathways to form organomercury compounds such as methylmercury (MeHg). However, the origin and cycling of MeHg in the oceans and the mechanisms of its formation are still under debate. For instance, deep waters and sediments have been suggested as potential source of methylated mercury compounds (Kraepiel et al., 2003; Ogrinc et al., 2007). In contrast, other studies have suggested the formation of methylmercury to occur at intermediate depths within the water column by planktonic organisms (Cossa et al., 2009; Heimbürger et al., 2010). Methylmercury, the most toxic mercury species, tends to bind to sulfhydryl groups of proteins and readily biomagnifies in the food chain (Mason et al., 2006). Adverse effects of MeHg include neurotoxicity, genotoxicity and endocrine disruption on a wide range of organisms (Scheuhammer et al., 2007).

Although mercury is not considered a POP, it is of great concern for human health due to the high toxicity of MeHg, which is the dominant chemical form of Hg present in fish (Harris et al., 2003). In particular, elevated levels of Hg have been reported for deep-sea fish from different parts of the world's oceans, with particularly high levels detected in Mediterranean species. Thus, Hg accumulation is of particular interest for this work, especially considering the fact that it includes species that are commercially exploited for human consumption such as the red-shrimp *Aristeus antennatus*.

1.3 Biomarkers

1.3.1 Definition

Biomarkers have been generally defined as measures of changes in biological parameters resulting from exposure to an environmental stressor (NRC, 1987). Biomarker responses are usually determined at the sub-organismal level as molecular, cellular, genetic, immunological and physiological measures, used as a means to provide early detection of exposure and adverse effects of pollutants on organisms

(Peakall, 1992; van der Oost et al., 2003). While the impacts of marine pollution at the higher levels of biological organization (*i.e.* population/community/ecosystem) are the ultimate concern, they are generally too complex and far removed from the causative events to be useful as ecotoxicological tools to detect and predict effects of environmental stress (Clements, 2000; Moore et al., 2004). Distress signals measured at lower levels of biological organization (*e.g.* molecular, biochemical responses) are thought to precede disturbances at higher biological levels (Figure 1.7) and biomarkers could thus potentially serve as 'early warning' prognostic indicators of pollution effects (Schlenk, 1999; Clements, 2000; van der Oost et al., 2003; Moore et al., 2004).

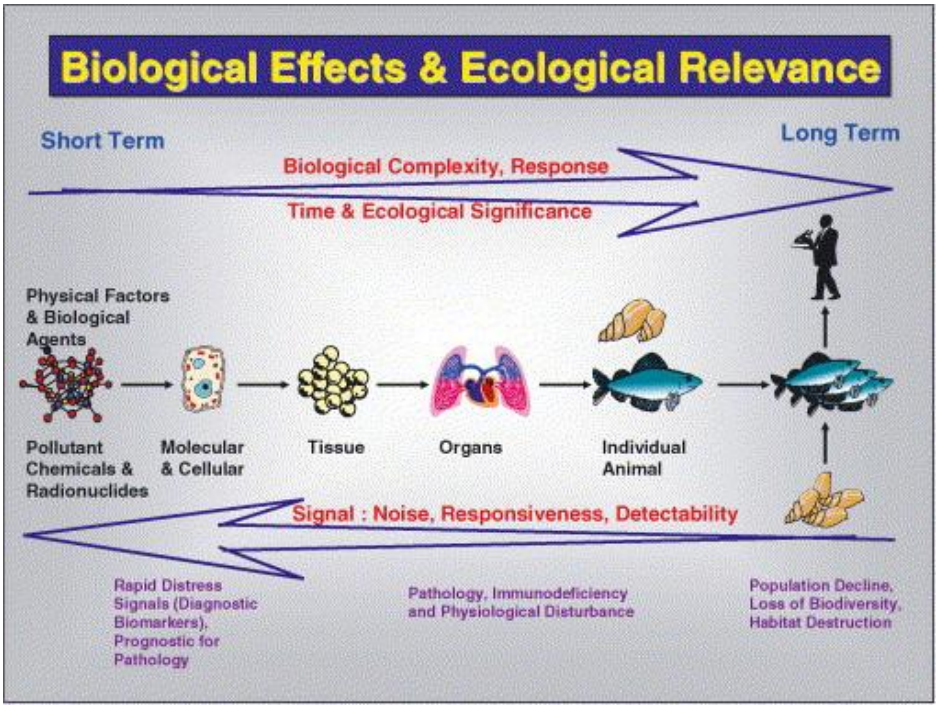


Figure 1.7 Signal delectability and ecological relevance of biological effects at different levels of biological organization (from Moore et al., 2004, Fig.1, p.250).

However, a single biomarker response cannot unequivocally provide a measure of environmental degradation and the use of a suite of biomarkers has thus been advocated (Handy et al., 2003; Galloway et al., 2004). Moreover, biomarkers are susceptible to natural variability due to abiotic (*e.g.* temperature, salinity, dissolved oxygen) and biotic factors (*e.g.* gender, age, size, reproductive stage) (Whyte et al., 2000; van der Oost et al., 2003; Martínez-Álvarez et al., 2005) and contaminant-induced stress signals can

therefore sometimes be masked by the intrinsic variability of biomarker activities (Sheehan and Power, 1999). For the practical application of biomarkers, the careful experimental design of field studies, data normalization and the characterization of potential confounding abiotic and biotic factors can help minimizing their variability (Flammarion and Garric, 1999; Handy et al., 2003; Sanchez et al., 2008).

Among the most commonly studied biomarkers are enzymes involved in the detoxification of xenobiotics and their metabolites, such as biotransformation and antioxidant enzymes, which are typically assessed in the liver where most of the metabolic processes take place (van der Oost et al., 2003). The present study has also focused mainly on these two biomarker categories, as outlined below.

1.3.2 Xenobiotic metabolism

The biotransformation of xenobiotics usually involves two types of enzymatic reactions, namely the phase I and phase II metabolism processes:

The phase I usually consists of an alteration of the original foreign molecule such as oxidation, reduction or hydrolysis, where a functional group (*e.g.* -OH, -COOH) is introduced to increase its water-solubility. For most xenobiotic compounds, the phase I metabolism is catalyzed by the mixed function oxidase (MFO) system, which includes the cytochrome P450-dependent (CYP450) monooxygenases. CYP450 constitute a superfamily of monooxygenase enzymes that are involved in the biotransformation of a variety of endogenous and exogenous compounds, found in eukaryotic as well as prokaryotic organisms (Nelson, 2003; Nelson and Strobel, 1987; Rewitz et al., 2006; Snyder, 2000). Among these enzymes, the content and activity of CYP1A is the most studied biomarker in aquatic organisms, used as a proxy of exposure to aryl hydrocarbon receptor (AhR) agonists including PAHs, coplanar PCBs, dioxins and other drugs (Goksøyr and Förlin, 1992; Whyte et al., 2000; van der Oost et al., 2003). In the aquatic environment, the induction of the enzyme 7-ethoxyresorufin-*O*-deethylase (EROD) has been widely used as a highly sensitive marker of exposure to CYP1A-inducing contaminants in laboratory and field studies (Whyte et al., 2000). Other enzymes involved in the phase I metabolism are carboxylesterases (CbEs), which catalyze the hydrolysis of a wide range of ester-containing chemicals (Satoh and Hosokawa, 2006; Wheelock et al., 2008).

The phase II metabolism involves the addition of a larger endogenous polar molecule (*e.g.* glucoronid, glutathione, sulphate) to the phase I reaction product to further increase its water-solubility and facilitate its excretion. The conjugation of phase I metabolites with glutathione is catalyzed by the glutathione-*S*-transferases (GST), a superfamily of multifunctional enzymes (Nimmo, 1987).

1.3.3 Antioxidant enzymes

All aerobic life can result in the production of potentially harmful reactive oxygen species (ROS) as a result of incomplete oxygen reduction during aerobic processes. In addition, numerous environmental contaminants have been shown to increase the intracellular generation of ROS, potentially resulting in oxidative stress (Winston and Di Giulio, 1991). However, in a healthy cell, the production of ROS is counteracted by the antioxidant defense system, which includes antioxidant enzymes like superoxide dismutase (SOD), catalase, (CAT) and glutathione peroxidase (GPX), as well as free radical scavenging molecules such as vitamin C and carotenoids (Winston and Di Giulio, 1991; van der Oost et al., 2003; Valavanidis et al., 2006). Thus, the induction of these antioxidant responses can be used as proxies for increased contaminant-induced ROS production.

1.3.4 Application of biomarkers

The above-mentioned biomarkers can be measured at different levels of intracellular responses, such as gene transcript expression, protein expression and functional enzyme activity. The induction of transcript expression as response to environmental stress is usually rapid and precedes changes in protein expression and activity, as shown for instance for the CYP1A induction in the marine fish *Stenotomus chrysops* (Hahn and Stegeman, 1994; Regoli et al., 2011). The more recently developed molecular techniques are thus often used to support or substitute biochemical analyses. However, a number of studies have shown that the transcript expression of a gene and the catalytic activity of its product do not always vary coherently (Tom et al., 2003; Kammann et al., 2008; Nahrgang et al., 2009; Trisciani et al., 2011), as in some cases the same pollutant that inhibits the activity of a protein may simultaneously induce the transcription of the protein-encoding gene (Nikinmaa and Rytkönen, 2011). Thus, the combination of biomarkers at different response levels may provide a more robust monitoring approach in field study scenarios, where complex contaminants mixtures tend to occur.

1.4 Framework and study sites

The present thesis was carried out in the framework of two multidisciplinary deep-sea research projects, namely the Spanish national project PROMETEO and the European-funded project BIOFUN, both forming part of the European Community's Seventh Framework Program HERMIONE. The PROMETEO project represents an integrated study on environmental factors and their influence on biological responses in the area of the Blanes canyon (NW Mediterranean), focusing on seasonal sampling and high depth resolution on a relatively small spatial scale (Figure 1.8). The BIOFUN project aimed to investigate the link between biodiversity patterns and ecosystem functioning in relation to environmental conditions on a large geographic scale (Figure 1.8). Both projects also aimed to address the issue of anthropogenic impacts on deep-sea ecosystems, including studies on the exploitation of fisheries resources, accumulation of litter on the seafloor as well as chemical contamination of deep-sea biota, with the latter being addressed in this thesis.

PROMETEO

Integrated study of deep canyons and slopes of the Western Mediterranean Sea

- Blanes canyon and adjacent slope
- Seasonal sampling cruises
- 900-1500 m depth

BIOFUN

BIOdiversity and ecosystem FUNctioning in contrasting southern European deep-sea environments: from viruses to megafauna

- Western, Central and Eastern
- Transmediterranean cruise (June 2009)
- 1200-3000 m depth

The Mediterranean Sea is divided into two major basins separated by the relatively shallow Sicilian Strait (400 m) into the western and central-eastern basin. The average depth of the sea-bed is approximately 1500 m, with a maximum depth of > 5000 m off the southern coast of Greece. Deep-sea communities within the Mediterranean are relatively isolated from those from the Atlantic because of the shallow sill of Gibraltar (300 m) that separates both oceans. The main hydrological features of the

Mediterranean deep-sea are high and constant temperatures at all depths below 200 m (12.5-14.5 ºC), high salinity (38.0-39.5 PSU) and high oxygen levels (4.5-5 mL/L) (Sardà et al., 2004a; Danovaro et al., 2010). Moreover, compared to the Atlantic Ocean, the deep Mediterranean waters are considered oligotrophic due to low primary production in surface waters and decreasing nutrient concentrations from west to east, with the eastern basin being one of the most nutrient-deprived areas of the world. Despite its oligotrophic nature and low biomass values, the Mediterranean Sea harbors a high biodiversity and number of endemic species. Furthermore, a number of habitats that potentially represent biodiversity *hot spots* can be found in the Mediterranean deep basin, including open continental slope, submarine canyons, seamounts, deep-water coral systems and hydrothermal vents (Danovaro et al., 2010). However, Mediterranean deep-sea fish and crustaceans dwelling below 1000 m depth generally exhibit highly conservative ecological strategies, characterized by low fecundity and low metabolic rates, making them especially vulnerable (Sardà et al., 2009).

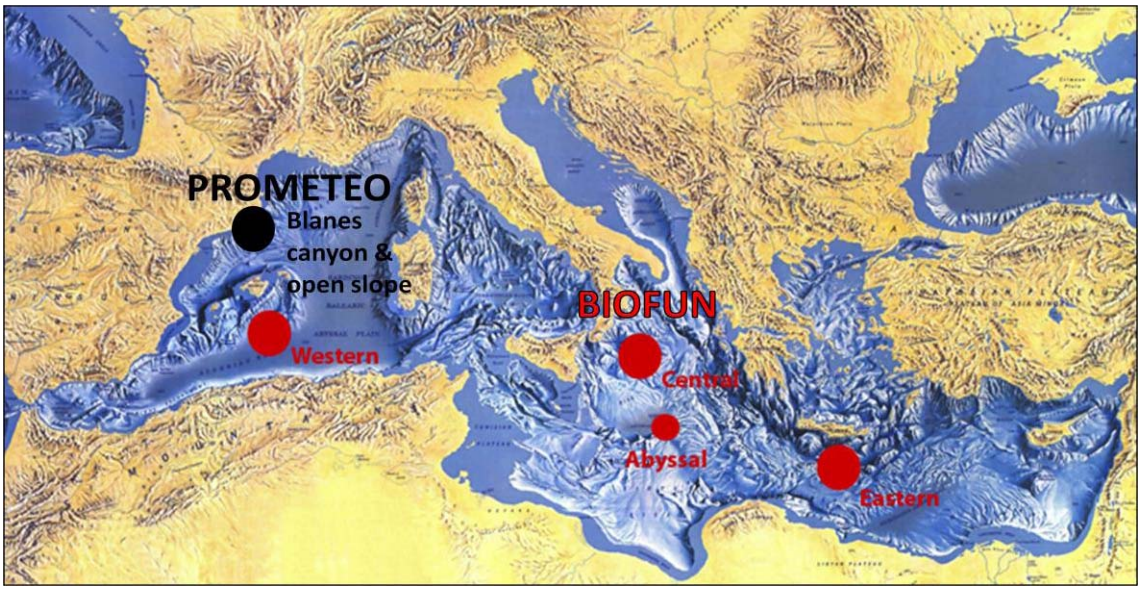


Figure 1.8 Sampling locations of the PROMETEO (black) and BIOFUN (red) projects

The present study focused mainly on samples from the PROMETEO study site (*i.e.* Blanes canyon and adjacent open slope) located on the Catalan slope in the northwestern Mediterranean. The NW Mediterranean continental shelf is characterized by the presence of a number of large submarine canyons. The general hydrodynamic circulation pattern consists of the cross-slope Northern current, which flows southwards

from the Gulf of Lions across the continental shelf. Moreover, the high evaporation of surface waters, which is typical of the Mediterranean, and wind-induced cooling during winter-time periodically causes the formation of large masses of open ocean dense water, resulting in major episodic events such as dense shelf water cascading (DSWC) (Canals et al., 2006; Palanques et al., 2006; Company et al., 2008). During these events, which tend to occur every 6 to 10 years, cold shelf water masses cascade down the continental slope transporting large amounts of sediment and organic matter to the deep-sea environment (Canals et al., 2006; Palanques et al., 2006). These important hydrodynamic processes have been shown to influence the population structure of deep-sea organisms such as the recruitment enhancement of the red-shrimp *Aristeus antennatus* following cascading events (Company et al., 2008), but also to increase the transport of particle-bound organic contaminants from surface waters to the deep-sea floor (Salvadó et al., 2012c). As a significant portion of these water masses is channelled through submarine canyons (Canals et al., 2006), canyon environments might be subject to higher contaminant input than the adjacent open slope areas and are thus of particular interest when studying anthropogenic impact in deep sea environments.

The Blanes canyon is one of the largest submarine canyons on the NW Mediterranean continental margin (Canals et al., 2004a). It has a NW-SE orientation and the upper canyon is located at less than 4 km from the coastline at a depth of 60 m, progressively widening with increasing depth (Zúñiga et al., 2009). The topography of the canyon exhibits a V-shaped cross-section in the upper region, an indication of high erosion, and a U-shape cross-section in the lower region as a result of higher sediment deposition (Figure 1.9) (Sardà et al., 2004a). The canyon walls present distinct bathymetric characteristics, limited by a relatively smooth eastern and a steep western wall. Studies on hydrodynamic processes have observed a higher downward particle flux within the canyon axis compared to the adjacent open slope areas, where the upper canyon receives the highest downward particle flux, associated with the continental input of sediments via the Tordera River (Zúñiga et al., 2009; López-Fernández et al., 2012). The NW Mediterranean submarine canyons are biodiversity and endemism hot spots and function as essential habitats to megafaunal deep-sea communities (Gili et al., 1999; Gili et al., 2000; Sardà et al., 2004a), highlighting the importance to study the potential impact of anthropogenic disturbances on these key environments.

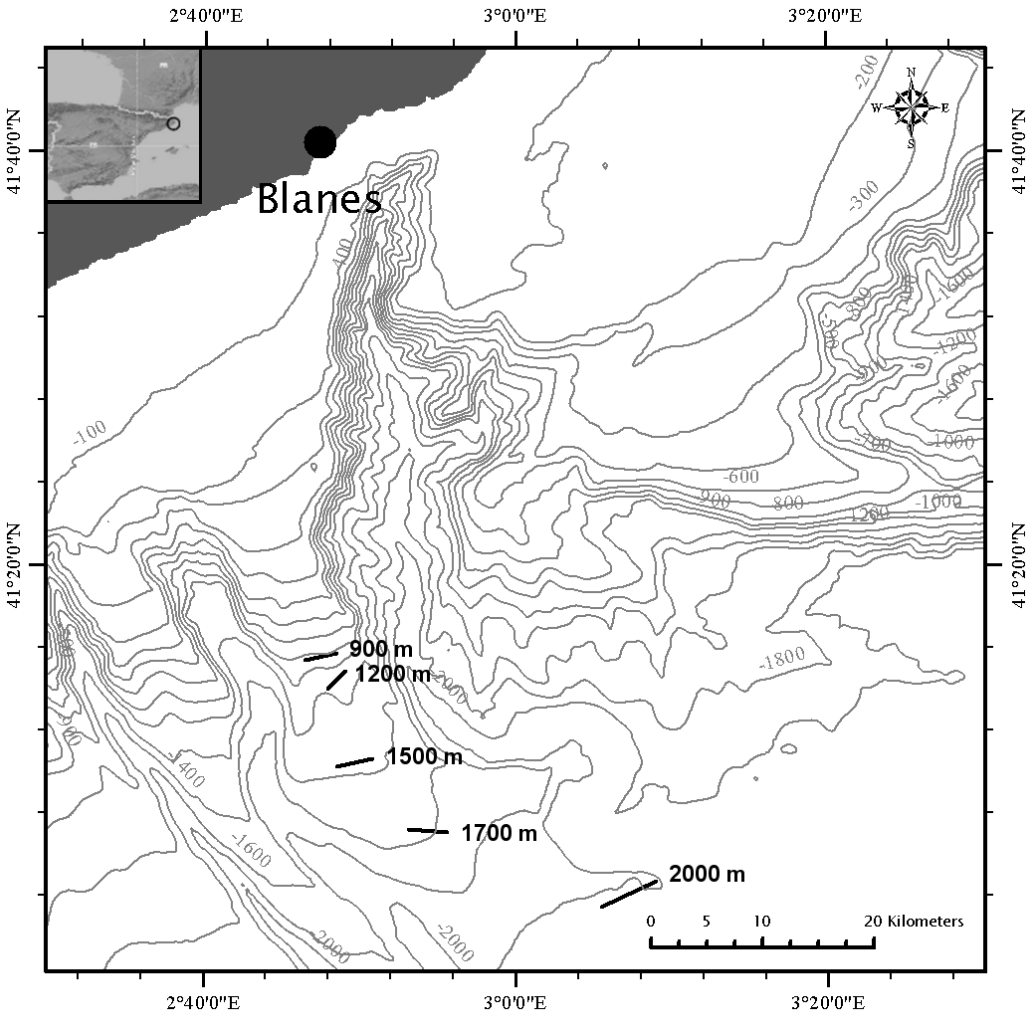


Figure 1.9 Map of Blanes canyon, NW Mediterranean by J.A. García, using ESRI® ArcMap™ 9.3 and bathymetric data from Canals et al. (2004b).

In addition, one study included samples from the BIOFUN sampling station in the Western Mediterranean (WM), located southwards off the Balearic Islands (see Figure 1.8). Due to this its remoteness from the coastal mainland, the WM site is presumably less impacted by anthropogenic activities compared to the area of the Blanes canyon.

1.5 Selected species

The deep-sea species sampled in the present work include twelve fish and one crustacean species. A detailed description of the four predominantly used species is

provided below, while the characteristics of all other selected species is summarized in Table 1.2.

Alepocephalus rostratus (Alepocephalidae, Alepocephaliformes)

A. rostratus is found in the western Mediterranean, as well as some parts of the eastern Atlantic. It represents one of the dominant species of the western Mediterranean deep-sea basin with a relatively wide depth distribution, ranging from 300 m to 2300 m, with a maximum abundance at mid-slope depths between 1000-1500 m. It also exhibits a trend of increasing size and age with depth up to 1500 m, reaching maximum length of 45 cm and age of 23 years (Morales-Nin et al., 1996). This species is considered a non-migratory macroplankton feeder, preying mainly on gelatinous macroplankton (Cartes et al., 2002). Moreover, mature individuals have been found all year round, although a peak in spawning activity is known to occur during summer and autumn (Morales-Nin et al., 1996; Fernandez-Arcaya et al., 2012).

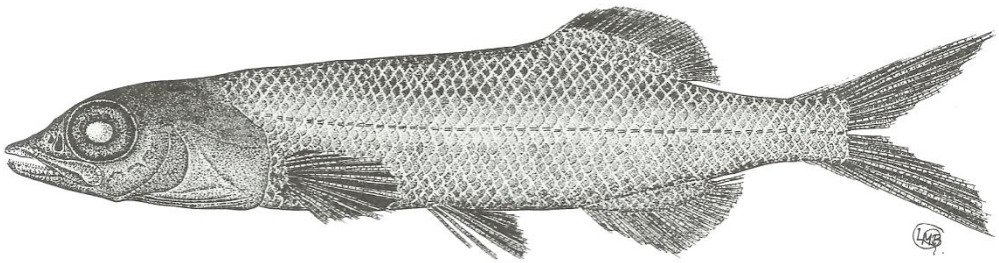


Figure 1.10 *Alepocephalus rostratus* (from Mercader et al., 2001)

Coelorinchus mediterraneus (Macrouridae, Gadiformes)

C. mediterraneus is found throughout the Mediterranean and occurs at depths ranging from 1200 m to 2200 m depth, exhibiting a peak in abundance at 1500 m. It can reach sizes of 9.8 cm PAL[i] and live up to 10 years (Massutí et al., 1995; Fernandez-Arcaya et al., 2012). Furthermore, it has been shown to preferably feed on benthic prey (*e.g.* polychaetes, amphipods), but also benthopelagic organisms (*e.g.* copepods) (Carrassón and Matallanas, 2002). Mature individuals are found throughout the year except during spring, indicating a continuous reproductive cycle (Fernandez-Arcaya et al., 2012).

[i] Pre-anal length (PAL)

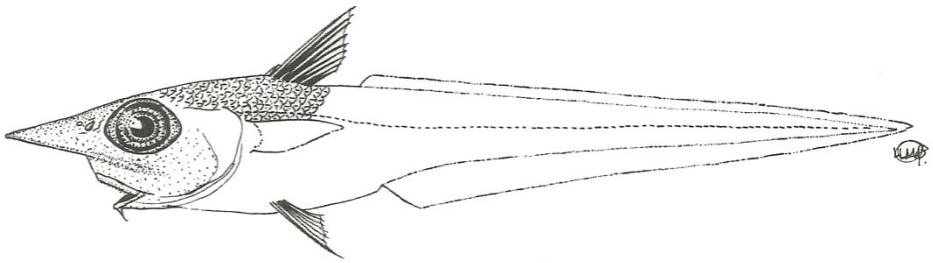


Figure 1.11 *Coelorinchus mediterraneus* (from Mercader et al., 2001)

Lepidion lepidion (Moridae, Gadiformes)

L. lepidion is also an endemic Mediterranean species with a wide depth distribution ranging from 500 m to 2300 m depth. Its highest abundance is found at 1200 m depth, where it also represents one of the dominant species and reaches maximum size of 33 cm and age of 10 years (Morales-Nin, 1990; Rotllant et al., 2002; Fernandez-Arcaya et al., 2012). Although it has previously been shown to reproduce almost continuously, except during summer (Rotllant et al., 2002), no mature individuals were caught during the seasonal sampling conducted in the framework of the PROMETEO project, indicating that this species may not reproduce every year (Fernandez-Arcaya et al., 2012). Studies on the diet of *L. lepidion* have shown that it presents a diverse feeding strategy, actively preying on supra- and epibenthic organisms (Carrassón et al., 1997)

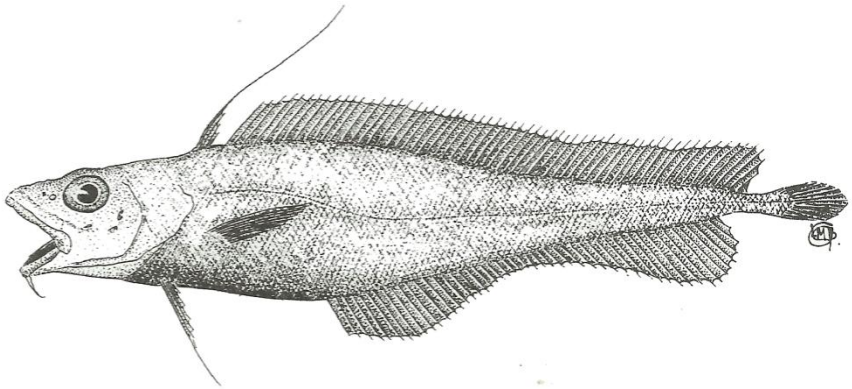


Figure 1.12 *Lepidion lepidion* (from Mercader et al., 2001)

Aristeus antennatus (Decapod)

A. Antennatus is a eurybathic shrimp species that can be found throughout the Mediterranean Sea and along the NW African Atlantic coast, constituting one of the most valuable commercial fishery resources in the Mediterranean (Company et al., 2008). It has a known depth range from 80 m down to 3300 m depth, exhibiting an abundance peak at 700 m depth, although a second, smaller peak has also been observed at 1500 m (Sardà et al., 2004b). Mature individuals aggregate between late winter and early summer at their shallower depth range to reproduce and a peak in reproductive activity occurs from May until September (Demestre, 1995; Sardà et al., 2003). Male shrimp can grow up to sizes of 30 mm (CL[i]), while females have been shown to reach CL of 60 mm, both reaching ages of probably no more than 5 years (Company et al., 2008). The diet of *A. antennatus* mainly consists of infauna such as polychaetes, bivalves and small crustaceans (Cartes et al., 2002).

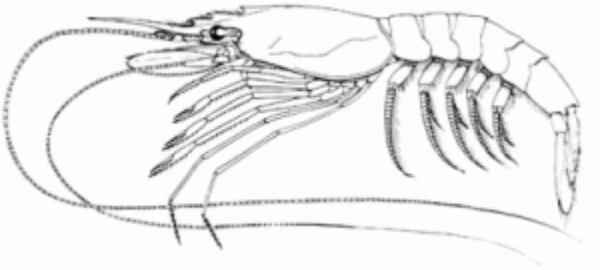


Figure 1.13 *Aristeus antennatus*

Other fish species included in the present work are *Bathypterois mediterraneus* (Ipnopidae, Aulopiformes), *Cataetyx laticeps* (Bythitidae, Ophiidiformes), *Coryphaenoides guentheri* (Macrouridae, Gadiformes), *Coryphaenoides mediterraneus* (Macrouridae, Gadiformes), *Lampanyctus crocodilus* (Myctophidae, Myctophiformes), *Nezumia aequalis* (Macrouridae, Gadiformes), *Nezumia sclerorhynchus* (Macrouridae, Gadiformes), *Mora moro* (Moridae, Gadiformes) and *Trachyrynchus scabrus* (Macrouridae, Gadiformes), as summarized in Table 1.2.

[i] Carapace length (CL)

Table 1.2 Biological characteristics of 12 deep-sea fish species and the crustacean *A. antennatus.*

Species	Depth range	DMA	L∞	$\delta^{15}N$	Diet
Alepocephalus rostratus	500-2300 m	1300 m	45.0 cm	9.86	Macroplankton (non-migratory)[a]
Bathypterois mediterraneus	800-3300 m	1750 m	18.0 cm	11.39	Benthopelagic plankton (non-migratory)[b]
Cataetyx laticeps	1750-3000 m	2800 m	48.5 cm	12.75	Epibenthic prey[c]
Coelorinchus mediterraneus	1200-2200 m	1500 m	9.8 cm[i]	12.60*	Infauna[a]
Coryphaenoides guentheri	1400-3000 m	1750 m	6.5 cm[i]	11.00	Benthic feeder[d]
Coryphaenoides mediterraneus	1500-3000 m	2700 m	10.5 cm[i]	10.97	Benthopelagic and benthic feeder[d]
Lampanyctus crocodilus	350-3000 m	500 m	22.5 cm	8.09	Macroplankton (migratory)[a]
Lepidion lepidion	500-2300 m	1200 m	33.5 cm	11.20	Benthopelagic and benthic feeder[e]
Mora moro	350-1300 m	1100 m	45.0 cm	11.78	Nekto-suprabenthos (Active predator)[e]
Nezumia aequalis	300-1500 m	600 m	6.5 cm[i]	13.78*	Nekto-suprabenthos[a,f]
Nezumia sclerorhynchus	600-1500 m	1200 m	4.7 cm[i]	12.22	Nekto-suprabenthos[f]
Trachyrhynchus scabrus	350-1500 m	900 m	21.2 cm[i]	10.25	Infauna[a]
Aristeus antennatus	80-3300 m	700 m	6.0 cm[ii]	9.61	Infauna[a]

Depth range from published data and *DeepMed Research Group Database* (ICM-CSIC)
DMA: depth of maximum abundance extracted from the *DeepMed Research Group Database* (ICM-CSIC)
L∞ based on published data and *DeepMed Research Group Database* (ICM-CSIC)
[i] pre-anal length, [ii] carapace size
$\delta^{15}N$: nitrogen stable isotope values from Tecchio et al. (In Prep.), except values indicated by * from Polunin et al. (2001)

[a] (Cartes et al., 2002); [b] (Carrassón and Matallanas, 2001); [c](Mauchline and Gordon, 1984); [d] (Carrassón and Matallanas, 2002); [e] (Carrassón et al., 1997); [f] (Marques and Almeida, 1998)

2. Aims and objectives

2

Aims and objectives

The overall objective of the present thesis was to investigate the bioaccumulation and potential effects of POPs on a range of Mediterranean deep-sea organisms. Specific aims that were addressed are:

(1) to determine levels of legacy and emergent POPs, as well as other relevant contaminant classes, in Mediterranean deep-sea organisms

(2) to assess interspecies variation in the accumulation of organic contaminants in relation to habitat, feeding strategies and metabolic capacities

(3) to determine pollution biomarkers and characterize the natural variability of those that are to be implemented as proxies for adverse effects of pollutant exposure in biota and to validate the use of gene expression biomarkers in deep-sea species

(4) to determine differences between sampling sites in contaminant bioaccumulation and biomarker responses

(5) to identify potential candidate species for future monitoring studies

The results of the present research project are presented as a compendium of the following scientific publications:

1. Samuel Koenig, David Huertas and Pilar Fernández. Legacy and emergent persistent organic pollutants (POPs) in NW Mediterranean deep-sea organisms. (Submitted to *Chemosphere*)

2. Samuel Koenig, Montserrat Solé, Cristal Fernández-Gómez and Sergi Díez. New insights into mercury bioaccumulation in deep-sea organisms from the NW Mediterranean and their human health implications. (Submitted to *Science of the Total Environment*)

3. Samuel Koenig and Montserrat Solé, 2012. Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms. *Marine Environmental Research*, 79, 122-131

4. Samuel Koenig, Pilar Fernández, and Montserrat Solé, 2012. Differences in cytochrome P450 enzyme activities between fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). *Aquatic Toxicology* 108, 11-17.

5. Samuel Koenig, Pilar Fernández, Joan B. Company, David Huertas and Montserrat Solé. Are deep-sea organisms dwelling within a submarine canyon more at risk from anthropogenic contamination than those from the adjacent open slope? A case study of Blanes canyon (NW Mediterranean). Accepted in *Progress in Oceanography* Special Issue: Mediterranean Deep Canyons.

6. Samuel Koenig, Cinta Porte, Montserrat Solé and Joachim Sturve. Biliary PAH and alkylphenol metabolites, biomarker enzyme activities and gene expression levels in the deep-sea fish *Alepocephalus rostratus*. (To be submitted to *Environmental Science and Technology*).

3. Impact factor of published articles

Las abajo firmantes, Montserrat Solé Rovira y Pilar Fernández Ramón, como directoras de la tesis doctoral con título: *"Bioaccumulation of persistent organic pollutants (POPs) and biomarkers of pollution in Mediterranean deep-sea organisms"* presentada por Samuel Koenig, certifican que los trabajos presentados y que se relacionan a continuación, han sido publicados o se encuentran en proceso de publicación en las revistas científicas que se detallan. Todas ellas están incluidas en el ISI y su índice de impacto, según el Journal Citation Reports 2011, es el indicado en cada caso. Así mismo, declaran que el doctorando ha participado muy activamente en la ejecución y elaboración de los trabajos que se presentan, tal y como se describe en cada uno de ellos:

1. **Samuel Koenig**, David Huertas and Pilar Fernández. Legacy and emergent persistent organic pollutants (POPs) in NW Mediterranean deep-sea organisms. Sometido a la revista Chemosphere. Índice de impacto: 3,613, 1er cuartil en el área de medio ambiente.

El trabajo presentado constituye un estudio original sobre los niveles de contaminantes orgánicos persistentes (POPs) en organismos abisales de la región del Cañón de Blanes, situada en el Mediterráneo Nord-occidental. El estudio incluyó la determinación tanto de aquellos contaminantes más conocidos, como los policlorobifenilos (PCBs) o el DDT y derivados, como de nuevas familias de compuestos, los polibromodifenil éteres (PBDEs), que recientemente han sido incluidos en la lista del Convenio de Estocolmo sobre POPs. La contribución del doctorando incluyó desde la toma de muestra de peces y crustáceos en las diferentes campañas realizadas a bordo del buque *García del Cid*, hasta el análisis de los niveles de contaminantes orgánicos en dichas muestras, así como la interpretación de los resultados obtenidos y elaboración y redacción del manuscrito. La contribución del resto de coautores se centró en la supervisión y diseño de los trabajos realizados, así como la discusión y corrección del manuscrito (Pilar Fernández) y el apoyo logístico y ayuda en la toma de muestra y análisis de los contaminantes en el laboratorio (David Huertas).

2. **Samuel Koenig**, Montserrat Solé, Cristal Fernández-Gómez and Sergi Díez. New insights into mercury bioaccumulation in deep-sea organisms from the NW Mediterranean and their human health implications. Sometido en Science of the Total Environment. Índice de impacto 3,286, 1er cuartil en el área de medio ambiente

El estudio que se describe en este trabajo surgió del interés mutuo de dos grupos de investigación pertenecientes al Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA) y al Instituto de Ciencias del Mar (ICM). Fruto de esta colaboración se ha podido determinar el nivel de contaminación por mercurio en organismos de la zona del Cañón de Blanes, incluidas algunas especies de gran valor comercial, obteniendo unos resultados científicos novedosos y de gran relevancia para la salud humana. La contribución del doctorando incluyó el diseño y enfoque del trabajo, además del estudio, interpretación de los resultados obtenidos y redacción del manuscrito. La contribución del resto de coautores se centró en el análisis mercurio en las muestras estudiadas (Cristal Fernández-Gómez) y en la discusión y corrección del trabajo presentado (M. Solé y S. Díez).

3. **Samuel Koenig** and Montserrat Solé, 2012. Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms. Marine Environmental Research, 79, 122-131. Índice de impacto 2,276, 1er cuartil en el área de "Marine and freshwater biology".

El trabajo describe y discute la variación estacional de biomarcadores que se relacionan con exposición a contaminantes y que pueden estar influenciados por parámetros físicos del medio. Debido a las características de estabilidad física del medio abisal, este estudio nos brinda la oportunidad de analizar las variaciones naturales y avanzar en el conocimiento de la influencia de otros factores estrictamente biológicos. En este trabajo se pudo contar con la ayuda de dos estudiantes de Master bajo la estrecha supervisión de M. Solé y del doctorando. El número de datos obtenidos y la complejidad de los resultados han requerido un tratamiento estadístico complejo que el doctorando ha trabajado intensamente. La redacción y elaboración del manuscrito han sido en gran parte mérito del doctorando bajo la supervisión de M. Solé.

4. **Samuel Koenig**, Pilar Fernández, and Montserrat Solé, 2012. Differences in cytochrome P450 enzyme activities between fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). Aquatic Toxicology 108, 11-17. Índice de impacto 3,761, 1er cuartil en el área de medio ambiente y toxicología

El estudio que se presenta en este trabajo surgió de una iniciativa del propio doctorando a partir del análisis de los resultados químicos obtenidos que mostraban diferencias significativas en las capacidades metabólicas de los dos grupos de organismos estudiados (peces y crustáceos). Estas diferencias quedan reflejadas en la obtención de una distribución de los diferentes congéneres de PCBs analizados característica para cada tipo de organismo. Tanto el análisis químico como de biomarcadores y gran parte de la discusión y elaboración del trabajo han sido realizados por el doctorando con la ayuda de P. Fernández y M. Solé.

5. **Samuel Koenig**, Pilar Fernández, Joan B. Company, David Huertas and Montserrat Solé. Are deep-sea organisms dwelling within a submarine canyon more at risk from anthropogenic contamination than those from the adjacent open slope? A case study of Blanes canyon (NW Mediterranean). Aceptado en la revista Progress in Oceanography Special Issue: Mediterranean Deep Canyons. Índice de impacto 3,142, 1er cuartil en el área de oceanografía.

El estudio que se describe en este trabajo constituye uno de los objetivos principales del proyecto en el que se enmarca esta tesis doctoral: determinar la influencia de los cañones submarinos y los procesos oceanográficos asociados a ellos, en el transporte y acumulación de contaminantes orgánicos persistentes en las zonas de aguas profundas. La contribución del doctorando incluyó la toma de muestra y análisis de todos los organismos estudiados. El rigor científico y la calidad de los datos obtenidos han permitido realizar un estudio fiable de la hipótesis de partida. Aunque los resultados no son muy concluyentes, el doctorando ha realizado una interpretación y discusión de los datos obtenidos de un gran interés científico. El resto de coautores han participado en la discusión y corrección del manuscrito, excepto en el caso de D. Huertas que también

participó activamente en la toma de muestra y análisis de los contaminantes orgánicos estudiados.

6. **Samuel Koenig**, Cinta Porte, Montserrat Solé and Joachim Sturve. Biliary PAH and alkylphenol (AP) metabolites, biomarker enzyme activities and gene expression levels in the deep-sea fish *Alepocephalus rostratus*. En preparación. Este trabajo está previsto enviarlo a la revista Environmental Science and Technology de índice de impacto 5.228, 1er cuartil en el área de ciencias ambientales.

Este trabajo integra tres escalas de respuesta biológica y sus relaciones. Analiza la expresión génica, la actividad enzimática y la formación de metabolitos como consecuencia de una exposición a contaminantes en el medio natural. Para la consecución de este trabajo se ha contado con la inestimable ayuda de dos equipos de investigación muy competentes. La determinación de metabolitos en bilis se realizó en el laboratorio de la Dra. C. Porte (IDAEA), mientras que los estudios de expresión génica se llevaron a cabo durante dos estancias cortas que el doctorando realizó en el laboratorio del Dr. J. Sturve (Universidad de Gotemburgo, Suecia). Las determinaciones de actividades enzimáticas se realizaron en el ICM-CSIC. La integración de los tres equipos ha sido un éxito, en parte gracias a la capacidad de trabajo del doctorando, así como su buen criterio a la hora de discutir los resultados obtenidos. M. Solé participó también en la coordinación de los grupos y discusión de los resultados.

Barcelona, 5 de Julio de 2012

Fdo.: Montserrat Solé Rovira

Fdo.: Mª Pilar Fernández Ramón

4. Summary of results and general discussion

4

Summary of results and general discussion

Despite the growing concern on the anthropogenic impact on deep-sea ecosystems, information on the occurrence and potential effects of POPs in deep-sea organisms is scarce. Therefore, the present thesis aimed to provide an integrated study on the bioaccumulation and adverse effects of POPs on Mediterranean deep-sea biota, combining chemical and biochemical analyses. The levels of different contaminant classes, including legacy and emergent POPs, namely PCBs, DDTs, HCHs and PBDEs as well as other contaminant groups such PAHs and APs were determined in different deep-sea species to gain further insight into bioaccumulation dynamics of these different compounds and to identify potential contaminant types that could be of particular concern for the Mediterranean deep-sea. Furthermore, enzymatic and molecular biomarker techniques were applied as proxies for biological effects in these organisms resulting from POP exposure. In particular, the natural variability of biomarkers was further characterized to help establish baseline levels of biomarker data, which will facilitate their application and subsequent interpretation in future deep-sea monitoring studies. In addition, the selection of potential candidate species for future studies as well as sampling constraints is further discussed.

4.1 Contaminant levels

4.1.1 Persistent organic pollutants (POPs) in muscle tissue

Organochlorine (OC) (*i.e.* PCBs, DDTs, HCHs, PeCB and HCB) and brominated contaminants (*i.e.* PBDEs) were measured in muscle tissue of three deep-sea fish species, *Alepocephalus rostratus, Coelorinchus mediterraneus* and *Lepidion lepidion* and the red-shrimp *Aristeus antennatus* (paper 1). Detected concentrations for the legacy POPs were within the range of previous findings from the NW Mediterranean deep-sea (Porte et al., 2000) and exhibited the commonly observed bioaccumulation pattern of PCBs ≈ DDTs >> HCHs ≥ HCB. Although PCB and DDT levels are thought to have decreased in marine sediments over the last decade (Gómez-Gutiérrez et al., 2007; Salvadó et al., 2012a), this trend was not reflected in deep-sea biota and concentrations are similar to those detected over a decade ago (Porte et al., 2000). The levels of PBDEs were approximately one order of magnitude lower than the OC compounds, which is accordance with other studies that have simultaneously investigated OC and PBDE levels in Atlantic and Pacific deep-sea fish (Webster et al., 2009; Takahashi et al., 2010). In addition, this trend is also consistent with sedimentary data from the NW Mediterranean deep-sea (Salvadó et al., 2012a; Salvadó et al., 2012b), reflecting the relatively recent emissions of PBDEs in the environment as compared to the legacy POPs such as PCBs and DDTs.

The congener profiles of the analyzed POPs revealed the predominant presence of high-molecular-weight (HMW) compounds. These compounds are more hydrophobic than low-molecular-weight congeners, which results in higher bioaccumulation potential and enhanced particle-bound transport from surface waters to deep-sea sediments (Dachs et al., 2002; Scheringer et al., 2004). Furthermore, the compound distribution of DDTs in fish is dominated by the degradation product DDE, reflecting the lack of recent input of the parent compounds, in agreement with their ban as agricultural pesticide in the 1970s. Similarly, consistent with the gradual substitution of technical HCH mixtures containing all four isomers by lindane (γ-HCH), the ΣHCHs profile detected in fish was mainly composed of γ-HCH.

The PBDE profiles in fish muscle tissue were also similar to the general accumulation patterns observed in other studies where BDE 28, 47, 99, 100 and 154 were the most

important congeners. However, it is difficult to elucidate from which one of the technical BDE mixtures (*i.e.* penta-, octa- and decaBDE) these compounds originate, since many HMW BDEs have been shown to degrade to lower brominated compounds in the environment. For instance, the pentaBDE commercial formulation contains high levels of BDE 47 and 99, however, these compounds can also be generated during the debromination of higher brominated congeners such as BDE 209 (Salvadó et al., 2012b). Furthermore, the bioavailability of certain BDE compounds may also influence their accumulation rates in biota. This is especially important for BDE 209. This compound is often present at high concentrations in the environment, however its bioavailability is relatively low (Eljarrat et al., 2004) and thus bioaccumulation profiles do not necessarily reflect the sedimentary contamination pattern.

The present study has shown that not only the well-known legacy POPs, which have been in the environment for decades, may be of concern for the deep-sea, but also more recent contaminant types such as PBDEs. In particular, considering that future projections predict that PBDE levels will surpass PCB levels in marine organisms within the next decade, future studies should also focus on the impact that these novel contaminants may have on the deep-sea environment.

4.1.2 Total mercury (THg) levels in muscle tissue

Another pollutant class analyzed in this study was total mercury (THg). Although toxic effects associated to this element are related specifically to the presence of methylmercury (MeHg), in the present work we analyzed THg levels because MeHg represents the major chemical form of mercury stored in fish muscle tissues (80-90 % of THg) (Harris et al., 2003) and a similar relationship between THg and MeHg has been demonstrated for the red-shrimp *A. antennatus* (Minganti et al., 1996). Overall, the measured Hg levels described in paper 2 were higher than those reported for other deep-sea species from the Atlantic Ocean (Cronin et al., 1998; Mormede and Davies, 2001), with all, except one, species exceeding the European limit for safe consumption of 0.5 µg/g w.w. The mercury "anomaly" of the Mediterranean Sea has been the subject of extensive studies for the last decades and has resulted in international campaigns such as the UNEP MED POL program, which aims to assess the impact of mercury

contamination on the Mediterranean marine environment (Aston and Fowler, 1985; UNEP/FAO/WHO, 1987; Cossa and Coquery, 2005). Moreover, the two commercially exploited species, the fish *Mora moro* and the highly valuable red-shrimp *Aristeus antennatus*, clearly exceeded guideline values and their consumption may pose a risk for human health. In this context, the fact that the exploitation of deep-sea waters is becoming increasingly important as a fisheries resource stresses the need to pay particular attention to the potential risk for humans resulting from the consumption of deep-sea organisms.

4.1.3 PAH and alkylphenol (AP) metabolites in bile

Furthermore, other pollutant classes that were analyzed in this thesis include PAHs and APs. However, once taken up by an organism, these contaminants are generally more readily metabolized than the previously mentioned pollutants and they therefore tend to accumulate at lower rates in muscle tissues. Thus, in this work, the levels of their metabolites excreted via the bile were assessed in the fish species *A. rostratus* as indicator of recent exposure to these compounds (paper 6). Among the five OH-PAHs analyzed (*i.e.* 1-naphthol, 2-naphthol, 9-fluorenol, 9-phenantrol and 1-pyrenol), 1-naphthol contributed approximately 90 % to the ΣOH-PAHs, indicating a petrogenic origin of PAH contamination. This finding is in contrast to previous results of biliary PAH metabolites in *A. rostratus* from the NW Mediterranean, where mostly 1-pyrenol was detected (Escartin and Porte, 1999), indicating a different source of PAHs. It is however noteworthy that the concentration of ΣOH-PAHs was similar in both studies.

NP and OP were detected in all bile samples, including fish from up to a depth of 2000 m at a relatively remote site in the Western Mediterranean. This finding further highlights the ubiquitous distribution of these contaminants in the environment and shows that they are transported to the deep-sea where they are taken up by organisms. The concentrations observed in *A. rostratus* were similar to those found in two coastal fish species sampled at depths between 100 and 500 m on the Northern Iberian shelf in the Atlantic Ocean (Fernandes et al., 2008), but lower than previous results reported for *Mullus barbatus* from the NW Mediterranean coast in the vicinity of the Rhone outfall (Martin-Skilton et al., 2006). To our knowledge, the present study is the first one to

determine the presence of AP metabolites in deep-sea fish, since the few field studies investigating their presence in the marine environment focused on more shallower-dwelling species. These findings indicate the importance to further investigate the presence of these relatively novel contaminant class in deep-sea environments and their potential detrimental effects on biota as they are known endocrine disruptors (Nimrod and Benson, 1996).

4.1.4 General contamination aspects

The fact that all contaminant classes analyzed in the present study were detected in the selected deep-sea organisms further suggests that the deep-sea may actually act as a sink for persistent anthropogenic contaminants, originating from land-based or coastal emission sources. Results showed that Mediterranean deep-sea organisms are potentially exposed to a wide range of contaminants, some of them potentially posing a risk to deep-sea ecosystem as well as human health. However, the fact that these contaminants are eventually exported to the deep-sea does not necessarily mean that concentrations are higher in deeper-dwelling species, although this has been suggested by other studies (Froescheis et al., 2000; Looser et al., 2000). At least for POPs, PAHs and APs, the detected levels appeared to be lower than those found in coastal Mediterranean species, which are presumably closer to the emission sources. For instance, PCB and DDT levels found in this study were generally lower than results reported for shallower species from the same region (*e.g. Mullus barbatus*). However, caution is advised when drawing conclusions on the fate of these chemicals based on direct comparisons of contaminant levels between surface and deep-sea species because of the potential differences in these organisms' biological and/or ecological characteristics, which are known to influence the bioaccumulation of contaminants (*e.g.* feeding strategy, trophic level, longevity, metabolic capacities). Considering that the above-mentioned contaminants are presumably exported from surface waters to the deep-sea via vertical particle-associated transport, which was also reflected in the bioaccumulation profiles observed in the present work (*i.e.* dominance of highly hydrophobic compounds), a potential explanation for the lower levels in deep-sea biota could be that a significant fraction of these pollutants entering the marine environment is degraded and/or retained within the upper layers of the water column. However,

despite the idea that only a portion of the organic contaminants present in the water column eventually sink to the seafloor, the present findings further suggest that anthropogenic pollutants that are of general environmental concern due to their high persistence and hydrophobicity, may also pose a risk for deep-sea ecosystems.

In contrast to the other contaminants included in this thesis, which mainly originate from land-based or coastal sources and transported to the deep-sea via particle-bound transport, Hg shows a different environmental behavior. In this sense, a clear increase of Hg accumulation with habitat depth was observed in twelve fish species. This trend is in accordance with previous studies conducted in the Atlantic (Monteiro et al., 1996) and Pacific Ocean (Choy et al., 2009), indicating that the increase of Hg with depth is a general phenomenon occurring throughout the world's oceans. Although the formation and cycling of MeHg (the main Hg form in biota) in the water column are still not fully understood, existing theories point to other sources of MeHg than surface waters. For instance, while Kraepiel et al. (2003) concluded that MeHg found in pelagic tuna fish originated from deep waters or sediments, more recent studies suggested that MeHg formation occurs within the water column at intermediate depths (100-600 m) (Cossa et al., 2009; Heimbürger et al., 2010). Thus, Hg could be of major concern for deep-sea biota and human health due to the increasing trend of deep-sea fisheries to substitute declining fish stocks.

Furthermore, the presence of emergent contaminant groups such as PBDEs should be further monitored in deep-sea environments, in particular with regard to the Mediterranean region where information is still very scarce. In this sense, the recent increasing emissions of decaBDE (*i.e.* BDE 209) could be of particular concern for deep-sea biota because of its fate in the aquatic environment. Although BDE 209 is relatively persistent in sediments, it can degrade into more toxic and bioavailable congeners via photodegradation and/or biotic debromination processes (Ross et al., 2009). In this context, a recent study by Salvadó et al. (2012b) conducted within the NW Mediterranean basin showed that the proportion of BDE 209 tends to progressively decrease with increasing depth as it degrades to lower brominated congeners during sediment transport and resuspension. Thus, deep-sea biota could be exposed to BDE mixtures dominated by lower brominated, more toxic congeners compared to, for

instance, the less-degraded decaBDE mixture occurring in areas closer to the original emission sources.

4.1.5. Interspecies differences

Differences in POP bioaccumulation among species are probably related to variations in feeding strategies and metabolic capacities. Overall, the most striking differences in accumulation patterns were observed between fish and the red-shrimp *A. antennatus*. For instance, as outlined in paper 4, *A. antennatus* exhibited different proportions of CYP1A-inducing PCB congeners (*e.g.* PCB 118, 138, 169) and lower proportions of CYP2B-inducers (*e.g.* PCB 153, 180, 194) than fish, coinciding with the respective absence of CYP1A-like and high CYP2B-like enzyme activities measured in the hepatopancreas. The fact that most of the 209 existing PCB congeners are thought to be CYP2B-inducers probably explains the lower PCB levels in *A. antennatus* in relation to the other POPs analyzed in paper 1 (*e.g.* DDTs). However, the DDT and PBDE accumulation profiles also presented distinct patterns in fish and crustacea. While the most dominant DDT compound found in fish is *p,p'*-DDE, *A. antennatus* primarily accumulated the o*,p'*-DDE metabolite. Furthermore, the PBDE profile also indicated distinctive metabolization capacities between *A. antennatus* and the fish species, as suggested by the very high proportions of BDE 153 and 209 and the high BDE 99/100 and BDE 153/154 ratios present in shrimp samples (paper 1). However, BDE profiles also reflected a potential influence of feeding strategies on the accumulation pattern of certain congeners, as indicated by the differences between the infaunal feeders (*i.e. C. mediterraneus, A. antennatus*) and the more pelagic-feeding species (*i.e. A. rostratus, L. lepidion*).

Similar to POPs, the analysis of THg in twelve deep-sea fish species and the crustacean *A. antennatus* (paper 2) revealed differences among species. In this case, interspecific variations were mainly explained by the variations in trophic levels, which is in accordance with the general idea that Hg accumulation increases through the food web (Mason et al., 2006; Senn et al., 2010), due to its tendency to bind to sulfhydryl groups of proteins and its low elimination rates (Harris et al., 2003; Amlund et al., 2007). Although former studies have also observed a significant relationship between trophic

levels and POP concentrations in biota, these compounds are generally highly lipophilic and differences in lipid content of muscle tissue among species may sometimes be of more importance with regard to their bioaccumulation rate than their trophic position.

4.2 Biomarkers

Biomarkers were used as proxies to evaluate potential adverse effects resulting from contaminant exposure in different deep-sea species. The enzymatic responses that were analyzed include cytochrome P450 enzymes (CYPs) such as 7-ethoxyresorufin-*O*-deethylase (EROD) or pentoxyresorufin-*O*-deethylase (PROD), carboxylesterase (CbE) and glutathione-*S*-transferase (GST) as well as antioxidant responses such as catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and superoxide dismutase (SOD). As mentioned before, these responses are susceptible to natural variability and their seasonal fluctuations were evaluated. In addition, a range of biomarker genes (*i.e.* CYP1A, CAT, GR, SOD and Vtg) were identified in *Alepocephalus rostratus* and their expression was applied in a field study.

4.2.1 Natural variability of enzymatic activities

A suite of enzymatic biomarkers were used as proxies to detect potential adverse effects resulting from POP exposure. However, many biomarker responses are susceptible to variability due to natural biotic (*e.g.* reproduction, size) and abiotic factors (*e.g.* temperature, salinity, food availability). Thus, a characterization of the intrinsic seasonal variability of enzymatic biomarkers is crucial and it was conducted for the fish species *A. rostratus* and *L. lepidion*, as well as the red-shrimp *A. antennatus*..

The seasonal variability of six enzyme activities (*i.e.* EROD, GST, CbE, GPX, CAT, GR) in *A. rostratus* and *L. lepidion*, as well as the red-shrimp *A. antennatus* was assessed in paper 3. As the physical-chemical properties of the water (*i.e.* temperature, salinity) did not fluctuate during the study period, their influence on biomarker variability was thought to be negligible and, thus the observed natural variations were mostly linked to the reproductive cycle and food input from surface waters. In

particular, as shown for *A. rostratus*, female fish are more susceptible to seasonal variations as their sexual maturation appears to influence many biomarker responses, especially those involved in the metabolism of xenobiotics and endogenous hormones (*e.g.* EROD, GST, CbE). These results indicated that despite the deep-sea being regarded as a relatively stable environment, it is important to first characterize potential factors that may influence the variability of biomarkers before using them as markers of POP exposure. In particular, the size, sex and gonad maturity stage of specimens should be recorded when conducting monitoring studies.

4.2.2 Validation of the use of gene expression as biomarker

The sampling of deep-sea organisms is very different from the sampling conditions in other field studies. Long hauling and trawl recovery times and the fact that most organisms arrive dead onboard after net retrieval cannot be avoided in deep-sea sampling. Thus, the first step was to ensure that the RNA samples extracted from the fish liver satisfied the quality criteria required for qPCR analysis (Biorad Experion™: RQI > 7). All samples met the established quality standards, demonstrating that deep-sea sampling techniques are suitable to conduct field studies using molecular biomarker assays.

In order to quantify the expression of specific biomarker genes in *A. rostratus* samples, initial "gene fishing" experiments were necessary to identify potential biomarker genes as no mRNA sequences were available for this or any closely related species. To this end, several available sequences from other fish species were aligned and analyzed for conserved regions among them. Based on these conserved regions in the alignment, consensus primers were designed and a PCR was performed followed by a gel electrophoresis to see if a potential gene amplification was achieved. Obtained PCR products were sequenced and checked for homology to existing gene sequences from other fish species. A suite of 9 biomarker genes, namely cytochrome P450 1A (CYP1A), catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), vitellogenin (Vtg), acetylcholinesterase (AChE) and metallothionein (MT) were tested and out of theses, 5 genes were successfully sequenced (*i.e.* CYP1A, CAT, GR, SOD and Vtg). Moreover, several housekeeping candidate genes were evaluated, namely β-

actin, elongation factor (EF1a), ubiquitin, α-tubulin, ribosomal RNA 12S and ribosomal RNA 16S. All reference genes were successfully identified and sequenced, of which three were quantified in samples (*i.e.* β-actin, EF1a, rRNA 12S) to normalize quantitative PCR cycle threshold (Ct) data. After the initial identification of gene sequences, specific qPCR primer pairs were designed and optimized to reach efficiencies of 100 % ± 5%.

The application of the qPCR analysis for the site comparison of biomarker gene expression in *A. rostratus* (paper 6 and section 4.3) has shown that this technique may provide an important complementary tool to study the effect of pollution exposure in deep-sea fish. In fact, the sensibility of this assay seemed to be greater than that obtained by the application of traditional enzymatic biomarkers, further advocating its use in future deep-sea studies.

4.3 Field studies

One of the main objectives of the multidisciplinary deep-sea projects (*i.e.* PROMETEO and BIOFUN) was to evaluate potential differences in anthropogenic impact on deep-sea organisms between sampling sites. Two studies were conducted that aimed to achieve this objective by combining chemical and biomarker data. The first of these two studies (paper 5) was conducted within the framework of the PROMETEO project and focused on the spatial variability of pollution levels at a relatively small spatial scale, contrasting contaminant levels and biomarker responses between specimens sampled from two sites in the NW Mediterranean. A second study (paper 6), that was conducted within the framework of the BIOFUN project, investigated differences between samples from the Catalan slope (CS), off the Spanish mainland coast, and the Western Mediterranean (WM) site, located in the open ocean off the Balearic Islands. The first site comparison was conducted on the NW Mediterranean shelf, where the impact of anthropogenic contaminants on deep-sea biota was contrasted between the Blanes canyon (BC) and adjacent open slope (OS). As canyon environments are thought to receive higher input of particle-associated pollutants, organisms dwelling within BC might be exposed to higher contaminant levels. Results have shown that within the upper canyon at 900 m depth, a pollution gradient occurs between the two sites, which

was reflected in higher bioaccumulated POPs and higher xenobiotic metabolism and antioxidant responses in the fish *L. lepidion* and the red-shrimp *A. antennatus*. However, these differences between sites were not detectable at greater depths (*i.e.* 1500 m), suggesting that the higher pollution input is potentially restricted to the head of the canyon. Overall, the study showed that deep-sea biota living within the upper region of BC may be more at risk of experiencing adverse effects due to pollution exposure.

The second site comparison was conducted on a larger geographical scale (paper 6). Similarly to the former study conducted in the BC area, chemical and biomarker responses varied concomitantly, indicating higher pollution exposure in *A. rostratus* from the WM than on the CS. Higher AP metabolites in bile coincided with higher biomarker gene expressions in samples from WM, in particular, a 35-fold induction in Vtg expression in male fish. Thus, these findings not only indicated the presence of a pollution gradient between sites but also that endocrine-disrupting substances reach the deep-sea floor and potentially exert negative effects on biota. Furthermore, the higher levels of pollution exposure in the WM compared to the CS is somewhat surprising due to the remoteness of the WM site from industrial activities and major pollution sources, showing that the contamination pattern of the deep-sea is not necessarily predictable from the location of major land-based or coastal emission sources and that the origin and transport of contaminants in these remote areas are still poorly understood.

It is worth emphasizing that differences between sites in both studies were observed based on chemical analyses and biomarker results, supporting the combination of both techniques as a robust approach to assess the impact of organic contaminants on deep-sea organisms.

4.4 Candidate sentinel species

The identification of potential sentinel species is crucial if the impact of anthropogenic contamination on the deep-sea is to be appropriately assessed in future monitoring studies. In the present work, several candidate species were selected and successfully used to investigate the bioaccumulation and effects of POPs. However, results have also

shown that the selection of one single species to monitor the impact of pollutants in the deep-sea does probably not provide an unequivocal representation of the degree of environmental degradation. In particular, sampling constraints and confounding biological parameters of the species can sometimes impede their use for the assessment of potential pollution gradients. In this sense, the adequate choice of an appropriate species may also vary depending on the question that the investigation aims to address. For instance, for the large-scale site comparison between CS and WM, *A. rostratus* appeared to be a good indicator species. However, in paper 5, a pollution gradient between OS and BC was only detected within the upper canyon region at 900 m depth, but *A. rostratus* was not present in trawls at this depth within the canyon, thus impeding its use to contrast pollution levels between the two sites.

Furthermore, the characterization of the natural variability of biomarkers in paper 3 has shown that all selected species experience seasonal variations in enzyme activities. With regard to *A. rostratus*, results revealed that the use of male fish may significantly reduce the potential fluctuations of these enzymatic responses. Similarly, in paper 5, male *A. rostratus* also exhibited a clearer gene expression response pattern, indicating that within a given species the selection of fish of different sex can influence their applicability as indicator species.

In addition, the present work has shown that the bioaccumulation and impact of pollutants can differ greatly between fish and crustacea. *A. antennatus* exhibited clearly distinctive bioaccumulation patterns for most POPs, which were, at least partially, related to their differential capacities to metabolize these compounds. Thus, the use of crustacean species may provide further insight into contamination processes that would not be elucidated by the analysis of exclusively fish species. It is therefore important to include crustacean species in deep-sea monitoring studies to complement the data obtained by the analysis of fish species. The fact that *A. antennatus* represents one of the most abundant crustacean species in Mediterranean Sea, has a very wide depth distribution and is one of the most valuable fisheries resources, makes it a good sentinel species for future studies.

5. Conclusions

5

Conclusions

- Legacy and emergent POP classes, as well as PAHs, APs and THg, are present in deep-sea biota from the NW Mediterranean Sea. In general, concentrations were lower than those reported in other marine organisms from areas close to the emission points. However, these results indicate the potential of these pollutants to be transported to the deep-sea and to negatively affect the organisms inhabiting these areas.

- Hg contamination constitutes an exception of this general pattern as THg accumulation in deep-sea fish increases with habitat depth and detected concentrations were above the EU consumption limits, potentially posing a significant risk to human health.

- General accumulation patterns indicate that organisms dwelling within the Blanes submarine canyon may be particularly at risk of experiencing pollution-induced adverse effects due to the presence of higher POP inputs to the canyon compared to the adjacent open slope. These higher inputs could be the consequence of enhanced vertical transport of hydrophobic, particle-associated

contaminants related to the hydrodynamic processes that affect particle flux and sediment resuspension in this area.

- Chemical and biomarker data varied consistently, suggesting that the combined use of these techniques may provide an adequate tool to assess the contamination impact on deep-sea organisms. In particular, the analysis of biomarker gene expression responses is a promising ecotoxicological tool for future deep-sea monitoring studies.

- Despite the lack of seasonal fluctuations of environmental factors such as temperature and salinity in the Mediterranean deep-sea, enzymatic biomarker activities of organisms are subject to significant variability as a result of natural confounding factors such as sex, size, reproductive activity and food availability.

- Chemical and biomarker studies indicate differences in contaminant bioaccumulation and biomarker responses among species. In particular, marked differences were observed between fish and crustacea, with the red shrimp *A. Antennatus* exhibiting significant differences in metabolic activities, which are reflected in its POP bioaccumulation patterns compared with fish. Thus, the additional use of crustacean species is advocated to complement biomonitoring data obtained from fish. Furthermore, the selection of appropriate sentinel species for deep-sea monitoring studies may depend on the aims of the study due to sampling constraints.

6. Resumen

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Resumen

6.1 Introducción

A pesar de su situación remota respecto a las fuentes potenciales de contaminación antropogénica, estudios recientes han mostrado que las zonas profundas del mar se encuentran bajo el impacto de compuestos tóxicos que provienen de actividades humanas. En el medio acuático, muchos de estos compuestos tienden a asociarse a las partículas en suspensión, lo cual facilita su transporte desde las aguas superficiales al mar profundo. En base a esto, algunos estudios previos han sugerido que el fondo marino podría actuar como un sumidero para los contaminantes más persistentes. En este contexto, los denominados contaminantes orgánicos persistentes (COPs o POPs según terminología inglesa) constituyen un problema especialmente importante debido a su persistencia, toxicidad, capacidad de bioacumulación en los seres vivos y distribución global (Scheringer et al., 2009). De hecho, varias familias de COPs han sido detectadas en organismos de gran profundidad del mar Mediterráneo, el océano Atlántico y el Pacífico (Porte et al., 2000; Mormede and Davies, 2003; Takahashi et al., 2010). No obstante, el conocimiento sobre la extensión de la contaminación en las zonas profundas del mar y su impacto potencial sobre estos ecosistemas es todavía muy escaso.

6.1.1 Contaminantes seleccionados

Bajo la denominación de contaminantes orgánicos persistentes (COPs) se engloban varias familias de contaminantes orgánicos de origen antropogénico que presentan una elevada resistencia a la degradación, tanto biótica como abiótica, tienen tendencia a bioacumularse y biomagnificarse a través de la cadena trófica y se encuentran distribuidos de forma global en el planeta, debido fundamentalmente a su transporte a larga distancia por vía atmosférica. Además, todos ellos poseen propiedades tóxicas con efectos adversos probados en todos los organismos vivos, incluido el hombre. Todas estas características dieron lugar a diversos tratados internacionales que culminaron con la firma en 2001 del Convenio de Estocolmo sobre COPs, auspiciado por el Programa de Naciones Unidas para el Medio Ambiente (PNUMA), en el que los países firmantes se comprometían a reducir o eliminar su emisión o uso y a promover la investigación de su impacto en el medio ambiente y salud humana. Originalmente la lista de COPs regulados por el Convenio de Estocolmo incluía doce compuestos o familias de compuestos organoclorados (OCs), entre los que destacaban los bifenilos policlorados (PCBs), el hexaclorobenceno (HCB), el diclodifeniltricloroetano (DDT) y las dioxinas y furanos, entre otros. Posteriormente la lista se ha ampliado incluyendo nuevas familias de contaminantes considerados emergentes como los polibromodifenil éteres (PBDEs). En la presente tesis se han analizado una selección de contaminantes orgánicos persistentes representativos de los diferentes tipos de COPs, tanto aquellos incluidos en la lista inicial del Convenio de Estocolmo básicamente OCs como los considerados contaminantes emergentes, cuyas características se detallan a continuación.

Compuestos Organoclorados. Entre los OCs estudiados se encuentran el penta- (PeCB) y el hexaclorobenzeno (HCB), ambos subproductos de la fabricación de disolventes organoclorados, aunque el HCB también se utilizó como fungicida para el tratamiento de semillas. El HCB se considera como posible carcinógeno y disruptor endocrino, con una elevada toxicidad hacía los organismos acuáticos. Otra familia de OCs incluida en este estudio son los cuatro isómeros del hexaclorociclohexano (HCHs), α-, β-, γ- y δ-HCH, usados extensivamente como insecticidas durante los años sesenta y setenta. De los cuatro isómeros, solo el γ-HCH, también conocido como lindano, presenta una actividad insecticida significativa, por lo que la mezcla técnica que contiene los cuatro isómeros fue sustituida progresivamente por el lindano.

Los PCBs representan otra clase de COPs con numerosas aplicaciones industriales, sobre todo en productos electrónicos como transformadores por su capacidad de absorción del calor. Los PCBs se caracterizan por su elevada estabilidad química y su lipofilicidad, que aumenta con el grado de cloración de la molécula, por lo que los PCBs más clorados poseen una gran tendencia a la bioacumulación en los seres vivos. Entre los 209 congéneres existentes, aquellos que contienen átomos de cloro en posición orto (*e.g.* PCB 77, 169) poseen una estructura molecular plana similar a la de las dioxinas y, por consiguiente, se consideran compuestos muy tóxicos. Finalmente entre los OCs analizados se incluye también el DDT, insecticida muy utilizado hasta los años setenta en la agricultura industrial y para el control de vectores de patologías como la malaria. El compuesto aplicado mayoritariamente es el *p,p'*-DDT, que se degrada progresivamente en el medio acuático dando lugar al *p,p'*-DDE, todavía más persistente y tóxico que el compuesto original. Tanto el DDT como sus productos de degradación pueden provocar efectos genotóxicos y de disrupción endocrina, identificándose como el factor principal responsable de la disminución de algunas poblaciones de aves rapaces.

Entre los COPs emergentes seleccionados destaca la familia de los PBDEs, utilizados como retardantes de llama en una gran variedad de productos como plásticos, textiles y aparatos electrónicos. A diferencia de los otros COPs mencionados hasta ahora, los PBDEs son de origen relativamente reciente, emitidos al medio ambiente a partir de los años setenta. El principal problema ambiental asociado a la presencia de estos compuestos en el medio radica en su capacidad de provocar efectos neurotóxicos y de disrupción endocrina, debido a su similitud estructural con las hormonas de la glándula tiroides.

Además de los COPs mencionados, también se incluyeron en este estudio otros tipos de contaminantes como los hidrocarburos aromáticos policíclicos (PAHs, según terminología inglesa), una familia de compuestos orgánicos formados por dos o más anillos aromáticos de origen natural y antropogénico, relacionado fundamentalmente con la combustión incompleta de la materia orgánica. Los PAHs incluyen un gran número de compuestos, de los cuales 16 han sido identificados por la Agencia de Protección Ambiental de EEUU (US EPA) como contaminantes ambientales prioritarios, debido a su elevada toxicidad en mamíferos y organismos acuáticos. De

hecho estos compuestos son conocidos por su actividad mutagénica, carcinogénica y teratogénica.

Otro grupo de contaminantes orgánicos incluidos en el estudio son los alquilfenoles (APs), utilizados principalmente como surfactantes en productos domésticos e industriales, como los detergentes, pesticidas y lubricantes, entre otros. El interés ambiental de los APs radica en sus efectos de disrupción del sistema endocrino, debido a su capacidad de interferir con el receptor de estrógeno.

Finalmente, otra substancia contaminante incluida en el presente trabajo fue el mercurio (Hg). Este elemento ha sido objeto de numerosos tratados internacionales, entre los que destacan OSPAR (Mar del Norte), HELCOM (Mar Báltico) y AMAP (Ártico), todos ellos con el objetivo de reducir el consumo y los aportes de Hg al medio ambiente. Recientemente, un estudio preparado por el Programa de Naciones Unidas para el Medio Ambiente (UNEP) concluyó que este metal ha tenido un impacto en el medio ambiente y la salud humana a escala global. La problemática ambiental del Hg está asociada a los efectos nocivos sobre la salud humana que puede provocar su presencia en los organismos acuáticos en forma de metilmercurio (MeHg). Aunque el origen y la formación de MeHg en el océano es todavía tema de investigación, se han detectado niveles altos de Hg en peces marinos, especialmente en zonas profundas del Mediterráneo. Por lo tanto, la acumulación de este contaminante es especialmente relevante en el contexto de este estudio considerando que se incluyen especies de interés comercial para el consumo humano como por ejemplo, la gamba roja *Aristeus antennatus*.

6.1.2 Biomarcadores

Los biomarcadores se definen como medidas de la alteración de procesos biológicos causadas por contaminantes ambientales. Estas alteraciones se pueden evaluar a nivel celular, inmunológico y fisiológico, como indicadores de exposición a contaminantes y sus efectos adversos en organismos. Entre los biomarcadores más utilizados en estudios de contaminación se encuentran las enzimas responsables del metabolismo de xenobióticos y las antioxidantes. La biotransformación de xenobióticos tiene lugar generalmente mediante dos tipos de reacciones enzimáticas consecutivas, que incluyen

las reacciones metabólicas de fase I y II. Para la mayoría de los xenobióticos el metabolismo de fase I tiene lugar a través de las enzimas del citocromo P450 (CYP), entre las cuales destaca la actividad de CYP1A, medida como etoxiresorufina-O-deetilasa (EROD), como el biomarcador más utilizado en organismos acuáticos como indicador de exposición a contaminantes inductores del receptor de hidrocarburos de arilo como los PAHs, dioxinas y algunos PCBs. Destacan también las carboxilesterasas, enzimas de fase I que catalizan la hidrólisis de una gran variedad de compuestos que contienen un enlace tipo ester. El metabolismo de fase II supone la conjugación del metabolito generado en fase I con una molécula polar (*e.g.* glutatión, sulfato) para aumentar su solubilidad y, por consiguiente, facilitar su excreción por enzimas como las glutatión-*S*-transferasas (GST).

Otro tipo de biomarcadores aplicados en estudios de contaminación son las enzimas antioxidantes. Aunque las reacciones de oxidación son cruciales para la vida aeróbica, también generan especies reactivas de oxígeno (ROS) que pueden causar estrés oxidativo y dañar las estructuras celulares. La exposición a algunos contaminantes también puede dar lugar a la formación de ROS y causar estrés oxidativo en los organismos. El papel de las especies antioxidantes es reducir o prevenir la oxidación de otras moléculas y, por lo tanto, constituyen un mecanismo de defensa importante contra el estrés oxidativo. Entre las enzimas antioxidantes destacan la glutatión peroxidasa (GPX), glutatión reductasa (GR), catalasa (CAT) y superóxido dismutasa (SOD).

Las respuestas de estos biomarcadores se pueden evaluar a diferentes niveles de procesos intracelulares tales como la expresión génica, la expresión de la proteína y la actividad funcional de la proteína.

6.1.3 Marco y zona de estudio

Esta tesis se ha llevado a cabo en el marco de dos proyectos de investigación multidisciplinarios, el proyecto del Plan Nacional español PROMETEO y el proyecto europeo BIOFUN, ambos incluidos dentro del proyecto HERMIONE del 7º Programa Marco de la Unión Europea (FP7). Tanto PROMETEO como BIOFUN tienen como objetivo estudiar la influencia de los factores ambientales en el funcionamiento de los ecosistemas del mar Mediterráneo profundo a diferentes escalas geográficas. El

proyecto PROMETEO se centró en la zona del cañón de Blanes en el Mediterráneo noroccidental, mientras que en el proyecto BIOFUN se incluían las tres cuencas del mar Mediterráneo.

Las zonas profundas del Mediterráneo se caracterizan por unas condiciones físicas muy estables por debajo de los 200 m de profundidad, con temperaturas, salinidad y niveles de oxígeno altos. Comparado con el océano Atlántico, el Mediterráneo se considera un mar oligotrófico debido a su baja concentración de nutrientes y producción primaria. La región noroeste (NO) del Mediterráneo es más productiva que el resto de las cuencas como consecuencia de unas condiciones climáticas especiales caracterizadas por borrascas frecuentes. En esta zona, el enfriamiento del agua superficial durante los meses de otoño e invierno junto con un aumento de la salinidad, consecuencia de las tasas de evaporación elevadas características del Mediterráneo, da lugar a la formación de grandes masas de agua densa que se hunden. Este proceso implica un transporte rápido de aguas superficiales hacia las zonas profundas. Además, durante los inviernos muy fríos se producen corrientes estacionales que dan lugar a las llamadas cascadas de agua densa de plataforma (del inglés Dense Shelf Water Cascading; DSWC), capaces de arrastrar enormes cantidades de agua y sedimentos hacia las grandes profundidades del Mar Mediterráneo. Esta agua superficial densa se transporta a miles de metros de profundidad a través del talud, canalizada en gran parte por los numerosos cañones submarinos característicos de la plataforma continental del NO Mediterráneo. Además, junto a las grandes masas de aguas superficiales y sedimentos, los eventos DSWC suponen también un transporte importante de materia orgánica y contaminantes antropogénicos previamente acumulados en los sedimentos de la plataforma hacia el fondo del mar, donde acabarían acumulándose en los cañones submarinos. Entre los numerosos cañones del Mediterráneo noroccidental destaca por su tamaño el cañón de Blanes. Su situación cercana a la costa catalana favorece el aporte y acumulación de contaminantes, sobre todo de aquellos como los COPs, susceptibles de persistir en el medio marino durante largos periodos de tiempo.

6.2 Objetivos del estudio

El objetivo principal de esta tesis es investigar la bioacumulación COPs en organismos que habitan las zonas profundas del Mar Mediterráneo y los posibles efectos adversos asociados a su presencia. En particular, este trabajo pretende:

(1) determinar los niveles de COPs y otros contaminantes relevantes en organismos del mar Mediterráneo profundo

(2) investigar diferencias en la bioacumulación de COPs entre especies en función de su hábitat, dieta y capacidades metabólicas

(3) determinar diversos biomarcadores y caracterizar la variabilidad natural de aquellos aplicados en este estudio como indicadores de efectos adversos relacionados con la contaminación, así como validar el uso de la expresión génica como biomarcador en organismos de gran profundidad

(4) determinar las variaciones en la bioacumulación y efectos de COPs entre diferentes zonas de estudio del Mediterráneo Noroccidental (cañón de Blanes *vs* plataforma continental adyacente y costa catalana *vs* mar Balear)

(5) identificar posibles especies centinela adecuadas para futuros estudios de monitoreo.

6.3 Resultados y discusión general

6.3.1 Niveles de contaminación

En el presente estudio se incluyeron diferentes familias de COPs, tanto aquellos compuestos organoclorados (OCs) cuya producción y uso está prohibido en la mayoría de los países hace décadas (*i.e.* PCBs, DDTs, HCHs, CBs) como las incluidas recientemente en la lista del Convenio de Estocolmo (*i.e.* PBDEs). Las concentraciones de estos compuestos se midieron en musculatura de tres especies de peces, *Alepocephalus rostratus, Coelorinchus mediterraneus* y *Lepidion lepidion* y en la gamba roja *Aristeus antennatus* (artículo 1). Los niveles de OCs detectados fueron similares a los descritos en estudios previos realizados en la misma zona (Porte et al., 2000), con una abundancia relativa de los diferentes compuestos según la secuencia PCBs ≈ DDTs >> HCHs ≥ HCB. Los PBDEs se detectaron a niveles un orden de magnitud más bajos que los PCBs y DDTs, de acuerdo con su origen más reciente en el medio ambiente. En general, los compuestos de peso molecular más elevado dominaban los perfiles de congéneres de cada grupo de compuestos, probablemente debido a su mayor afinidad con el material particulado en suspensión que favorece su transporte vertical desde las aguas superficiales al mar profundo.

En cuanto a los estudios centrados en la presencia de metabolitos de PAHs en bilis, los resultados obtenidos indicaron concentraciones en *Alepocephalus rostratus* similares a los niveles detectados en la misma especie hace más que una década (Escartin and Porte, 1999), aunque el perfil de hidroxi-PAHs observado en este estudio es claramente diferente al descrito por Escartín y Porte, con un marcado carácter petrogénico caracterizado por el dominio del 1-naftol, que representa el 90 % de ΣOH-PAHs. También se detectaron en las muestras de bilis los metabolitos de los dos alquilfenoles estudiados, el octil y el nonilfenol, hasta una profundidad de 2000 m, lo que pone en evidencia que estos compuestos son transportados hasta las zonas profundas del mar.

El contenido de mercurio total (THg) en musculatura se usa generalmente como indicador de contaminación por metilmercurio (MeHg), ya que el 80-90 % de THg corresponde a MeHg, tanto en musculatura de peces (Harris et al., 2003) como en *A. antennatus* (Minganti et al., 1996). De las 13 especies de profundidad analizadas (artículo 2), todas menos una presentaron niveles por encima del límite de consumo de 0.5 μg/g peso fresco permitido por la UE. Además, se observó una relación significativa

entre la bioaccumulación de THg y la profundidad del hábitat de cada especie, con niveles de THg más altos en los peces que se encuentran a mayor profundidad. Aunque el origen y la formación de MeHg en el océano todavía no está totalmente establecido, Kraepiel et al. (2003) sugirieron que el MeHg acumulado en depredadores pelágicos como el atún proviene de los sedimentos del fondo marino, lo que explicaría el aumento de THg con la profundidad observado en este estudio. También conviene destacar las concentraciones altas de THg encontradas en las dos especies comerciales, la mora común *Mora moro* y la gamba roja *Aristeus antennatus,* que podrían suponer un riesgo importante para la salud humana.

6.3.2 Diferencias entre especies

En este estudio se han observado diferencias significativas en los perfiles de bioacumulación de contaminantes entre las distintas especies de organismos analizadas. En particular, las diferencias más destacadas se observaron entre los peces y la gamba roja *A. antennatus*. En el caso concreto de los PCBs, dichas diferencias se han relacionado con las actividades metabólicas características de cada grupo filogenético, mientras que en el caso de los DDTs y los PBDEs podrían deberse a diferencias en las capacidades metabólicas y/o en las estrategias de alimentación entre *A. antennatus* y las especies de peces estudiados.

Finalmente, también se observaron diferencias importantes en las concentraciones de THg entre especies, que en este caso correlacionaban con el nivel trófico de cada una de ellas. No obstante, también se ha observado que la profundidad del hábitat de cada especie, definida como la profundidad de abundancia máxima de la población, constituye otro factor importante en la acumulación de THg en estos organismos, de tal manera que los peces que se encuentran a más profundidad muestran tendencia a acumular más THg de lo que les correspondería por nivel trófico y masa corporal.

6.3.3. Biomarcadores

La mayoría de las actividades metabólicas utilizadas como biomarcadores poseen una marcada variabilidad ya que sus respuestas están afectadas por diversos factores, tanto

bióticos (*e.g.* reproducción, tamaño, sexo) como abióticos (*e.g.* temperatura, salinidad), factores que a su vez muestran variaciones estacionales. Por lo tanto, es necesario caracterizar la variabilidad natural de los biomarcadores enzimáticos antes de su aplicación en estudios de monitoreo de la contaminación. En la presente tesis se llevó a cabo la determinación de la estacionalidad de seis biomarcadores hepáticos (EROD, GST, CbE, GPX, CAT y GR –artículo 3-) en las dos especies de peces *A. rostratus* y *L. lepidion*, y en la gamba *A. antennatus.* En condiciones de temperatura y salinidad estables, típicas de las zonas profundas del Mediterráneo, se pudieron evaluar los factores determinantes de las fluctuaciones estacionales detectadas en las tres especies, que estaban relacionados con el ciclo reproductor y la abundancia de alimentos.

Además de la caracterización de la variabilidad natural de las respuestas enzimáticas, se ha validado por primera vez el uso de la expresión génica como biomarcador en una especie de profundidad, *A. rostratus*. En este sentido, se pudieron identificar cinco genes biomarcadores (CYP1A, CAT, GR, SOD y Vtg) y tres genes de referencia (β-actin, EF1a, rRNA 12S) cuya aplicación como biomarcadores supondría una herramienta complementaria importante en la investigación de los efectos de la contaminación en la fauna del mar profundo.

6.3.4 Diferencias entre zonas de estudio

Uno de los objetivos más importantes de esta tesis fue la evaluación de posibles diferencias del impacto antropogénico entre las distintas zonas seleccionadas mediante la aplicación combinada de análisis químicos y bioquímicos. En este sentido, se llevaron a cabo dos estudios de campo, uno realizado en el marco del proyecto PROMETEO centrado en la zona del cañón de Blanes y otro correspondiente al proyecto BIOFUN en el que se compararon dos zonas del mar Mediterráneo occidental.

El primer estudio investigó las diferencias en los niveles de contaminación entre organismos que habitan dentro del cañón de Blanes y aquellos pertenecientes a la plataforma continental adyacente. Los resultados del análisis químico y de biomarcadores indicaron la existencia de un gradiente de contaminación entre el interior y el exterior del cañón a 900 m, pero no a 1500 m, lo que sugiere que los organismos

que habitan la zona de la cabecera del cañón de Blanes presentan un riesgo mayor de sufrir efectos adversos asociados a la exposición a COPs.

Por otro lado, en el segundo estudio se investigaron los niveles de contaminación y posibles efectos negativos en peces de la especie *A. rostratus* capturados en dos zonas del Mediterráneo noroccidental: el margen de la costa catalana (CS) y en mar abierto cerca de las Islas Baleares (WM). En este caso se analizaron los niveles de metabolitos de PAHs y alquifenoles (AP) en bilis, junto con la expresión de cinco genes biomarcadores, detectándose diferencias significativas con ambos análisis entre las dos zonas seleccionadas. En particular, niveles elevados de APs en bilis coincidieron con una inducción 35 veces más alta del gen de la vitelogenina en peces macho, un indicador de disrupción endocrina.

6.3.5 Especies centinelas

La identificación de especies centinela adecuadas para la determinación del impacto relacionado con la presencia de contaminantes antropogénicos es crucial para futuros estudios de monitoreo. Sin embargo, hay que tener en cuenta varios factores a la hora de elegir una especie adecuada, como por ejemplo su distribución batimétrica y presencia en las zonas de estudio. En la presente tesis se evaluó la idoneidad de diferentes especies para su utilización como especie centinela a partir del análisis de biomarcadores (artículo 3). Los resultados indicaron una variabilidad estacional en las respuestas enzimáticas medidas en las tres especies analizadas, aunque en el caso de *A. rostratus* se observó que el uso de peces machos reducía la incertidumbre generada por las fluctuaciones naturales. Así mismo, las grandes diferencias detectadas en la bioacumulación de contaminantes y los efectos asociados a ésta entre peces y la gamba roja *A. antennatus* destacan la importancia de considerar no solo especies de peces sino también de crustáceos como posibles organismos centinela, ya que su uso puede proporcionar información complementaria a la obtenida a partir del análisis exclusivo de peces.

6.4 Conclusiones

- Se han detectado diversas familias de COPs y otros contaminantes relevantes, como PAHs, APs y THg, en organismos de zonas profundas del Mediterráneo noroccidental. Exceptuando el caso del THg, las concentraciones encontradas fueron en general más bajas que las descritas en organismos marinos de zonas próximas a las fuentes de contaminación. A pesar de ello, los resultados indican que estos compuestos pueden ser transportados hasta las regiones profundas del mar con el consiguiente riesgo para los organismos que las habitan.

- Se ha observado una relación entre la acumulación de THg en peces y la profundidad del hábitat de la especie, de tal manera que las concentraciones más altas se han detectado en los peces que viven a mayor profundidad. La contaminación por Hg observada en los organismos estudiados es especialmente preocupante ya que se encuentra por encima del límite para el consumo establecido por la UE y podría constituir un cierto riesgo para la salud humana.

- Los resultados sugieren que los organismos que habitan el cañón de Blanes presentan un riesgo mayor de sufrir efectos adversos asociados a la exposición a COPs comparado con aquellos que se encuentran en el margen continental adyacente, debido a una acumulación mayor de contaminantes, sobre todo en la zona de la cabecera del cañón.

- Los resultados obtenidos a partir del análisis químico y de biomarcadores aplicados en los estudios comparativos entre las zonas seleccionadas fueron totalmente coherentes en sí. Ello indica que la combinación de estudios químicos y bioquímicos es adecuada para evaluar el impacto de la presencia de contaminantes en organismos de gran profundidad. En este contexto, el uso de la expresión génica como biomarcador representa una herramienta prometedora para su aplicación en estudios futuros de monitoreo del mar profundo.

- Se ha observado que, a pesar de la estabilidad estacional de parámetros ambientales como la temperatura y salinidad en el mar profundo, las actividades enzimáticas de los organismos presentan una variabilidad significativa como

consecuencia de la variación de factores naturales como el sexo, tamaño, actividad reproductiva y disponibilidad de alimentos.

- Tanto el análisis químico como de biomarcadores indican diferencias entre especies de peces y entre peces y crustáceos. La gamba roja *A. antennatus* constituye el caso más significativo ya que el estudio de biomarcadores indicó diferencias importantes en su capacidad metabólica comparada con los peces, diferencias que quedaban reflejadas en los perfiles de bioacumulación de COPs. Estos resultados indican que la elección de una especie centinela depende en gran medida del objetivo del estudio, pero en cualquier caso, parece necesario la incorporación en los estudios de vigilancia de especies de crustáceos que complementen los resultados obtenidos con peces.

7. References

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8. Publications

Paper 1

Legacy and emergent persistent organic pollutants (POPs) in NW Mediterranean deep-sea organisms

Samuel Koenig [a;b]*, David Huertas [b] and Pilar Fernández [b]

[a] Institut de Ciències del Mar (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

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Resumen

El trabajo realizado se centró en la determinación de los niveles de bioacumulación de contaminantes orgánicos persistentes (COPs) en tres especies de peces y un crustáceo de zonas de aguas profundas del Mediterráneo noroccidental. Los contaminantes analizados incluyeron compuestos organoclorados usados en el pasado, como los bifenilos policlorados (PCBs), el diclodifeniltricloroetano (DDT) y sus productos de degradación (DDE y DDD), los hexaclorociclohexanos (HCHs), y el penta- y hexaclorobenzeno (PeCB, HCB), así como los polibromodifenil éteres (PBDEs), utilizados en las últimas décadas como retardantes de llama y considerados como contaminantes emergentes. En general, los niveles más altos para todos los contaminantes analizados se detectaron en peces, mientras que la gamba roja, *Aristeus Antennatus*, presentaba los niveles más bajos. Los PCBs y DDTs fueron los compuestos encontrados a concentraciones más altas, que oscilaban entre los 6-8 ng/g ww en *Alepocephalus rostratus* y 1-3 ng/g ww en *Aristeus antennatus*. Los PBDEs se detectaron a niveles un orden de magnitud más bajos que los PCBs y DDTs, entre 0,047 y 0,92 ng/g ww en *A. antennatus* y *A. rostratus*, respectivamente. Los compuestos dominantes en todas las familias de contaminantes estudiados fueron los de peso molecular más elevado, de acuerdo con su mayor hidrofobicidad que implica una tendencia mayor a la bioacumulación en los organismos y un transporte desde las aguas superficiales a las zonas profundas más eficaz, favorecido por su asociación con el material particulado en suspensión presente en la columna de agua. Por otra parte, se han observado perfiles de bioacumulación diferentes entre los peces y la gamba, pero también entre las distintas especies de peces estudiados, probablemente debido a diferencias en la capacidad metabólica y/o en las estrategias de alimentación entre los distintos organismos analizados.

Abstract

The levels and profiles of organochlorine (OC) contaminants, including polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs), hexachlorocyclohexanes (HCHs) and penta- (PeCB) and hexachlorobenzene (HCB), as well as polybrominated diphenyl ethers (PBDEs) were determined in muscle samples of the deep-sea fish *Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion* and the red-shrimp *Aristeus antennatus* from the NW Mediterranean sea. Mean PCB and DDT levels ranged from the highest concentrations in the fish *A. rostratus* (Σ_7PCBs 6.93±0.71 ng/g w.w. and ΣDDTs 8.43±1.10 ng/g w.w.) to the lowest concentrations in the crustacean *A. antennatus* (Σ_7PCBs 1.17±0.24 ng/g w.w. and ΣDDTs 2.53±0.26 ng/g w.w.). The concentrations of ΣHCHs and HCB levels were more than one order of magnitude lower, ranging from 0.07-0.36 ng/g w.w. and 0.03-0.15 ng/g w.w., respectively, while PeCB was only detected in a few samples above the detection limit. PBDE levels were approximately ten times lower than PCB and DDT concentrations, ranging from 0.47 ± 0.20 ng/g w.w. in *A. antennatus* to 0.92 ± 0.13 ng/g w.w. in *A. rostratus*. The high-molecular-weight (HMW) PCBs 153, 138 and 180 represented 69-79 % of Σ_7PCBs in fish and 60 % in the red shrimp. Moreover, in fish, the main DDT compound detected was the metabolite *p,p'*-DDE (70-80 % of ΣDDTs), indicative of old DDT residues. In contrast, o,*p'*-DDE was the main DDT metabolite (49 % of ΣDDTs) in shrimp, while the parent compound *p,p'*-DDT and its metabolite *p,p'*-DDE exhibited similar proportions of 16 % and 21 %, respectively. For PBDEs, the most abundant congeners were BDE 28, 47, 99, 100 and 154 in fish (>70 % Σ_{14}PBDEs), while BDE 153 and 209 were also important in *A. antennatus*, suggesting a different uptake and/or biotransformation rate of PBDEs between fish and crustacea. In this sense, the ratios BDE99/100, BDE153/154, and BDE 47/99 were determined as proxies for BDE metabolization capacities and contrasted among species. Higher BDE 99/100 and BDE 153/154 ratios were observed in shrimp compared to fish and in *C. mediterraneus* compared to the two other fish species. Furthermore, a significant relationship between these ratios was observed. These results indicated differences in metabolic capacities between the crustacean and fish as well as between fish species or differences in feeding strategies among organisms. BDE 47/99 ratio close to one indicates that no biotransformation of BDE 99 to BDE 47 has occurred in these organisms as observed in other studies.

Keywords: persistent organic pollutants (POPs); organochlorine contaminants (OC); polybrominated diphenyl ether (PBDE); deep-sea; fish; crustacea; Mediterranean sea

1. Introduction

The deep-sea has long been considered a pristine environment due to its remoteness from anthropogenic pollution sources. However, there has been growing concern over the impact of anthropogenic contaminants on deep-sea ecosystems (Ramirez-Llodra et al., 2011). In particular, several studies have shown that the deep-sea might act as a sink for highly persistent compounds that enter the marine environment (Kramer et al., 1984; Froescheis et al., 2000; Looser et al., 2000; Scheringer et al., 2004). In this context, persistent organic pollutants (POPs) are of particular concern due to their high hydrophobicity, toxicity and persistence (Scheringer et al., 2009). Because of their hydrophobic nature, POPs present in the aquatic systems have a high affinity to bind to suspended particles and previous findings have suggested a long-term vertical transport of organic contaminants from surface waters to the deep-sea floor (Dachs et al., 2002; Wania and Daly, 2002; Scheringer et al., 2004; Bouloubassi et al., 2006). Indeed, polychlorinated biphenyls (PCBs) and organochlorine (OC) contaminants, including dichlorodiphenyltrichloroethanes (DDTs) and hexachlorocyclohexanes (HCHs), have been found in deep-sea organisms all over the world (Berg et al., 1998; Looser et al., 2000; de Brito et al., 2002; Mormede and Davies, 2003; Ramu et al., 2006; Storelli et al., 2007; Unger et al., 2008; Takahashi et al., 2010; Webster et al., 2011).

Recent research efforts have confirmed that emerging contaminants such as polybrominated diphenyl ethers (PBDEs) are also subject to long-range transport being detected in aquatic organisms from remote areas, including deep-sea fish (Ramu et al., 2006; Covaci et al., 2008; Takahashi et al., 2010; Webster et al., 2011). PBDEs, which are structurally similar to PCBs, have been widely used as flame retardants in a wide array of products, including plastics, textiles and electronic devices. There are three technical mixtures of PBDEs, namely penta-, octa- and decaBDE, however, their production has been phased out under the Stockholm Convention due to their high toxicity and persistence. For instance, recent findings have shown that some PBDE congeners can result in neurotoxicity, reproductive and developmental effects and endocrine disruption, in particular, due to their structural similarity to the thyroid hormone thyroxine (Darnerud, 2003; Birnbaum and Staskal, 2004). In the European Union (EU), the use of the penta- and octa-formulations was banned in 2004, while the production of decaBDE was prohibited in 2008 (de Wit et al., 2010).

In comparison to OC contaminants such as PCBs, DDTs and HCHs, which started to be manufactured during the first half of the 20th century, PBDEs have been released into the environment since the 1970s. This 40 year time lag of emissions could explain why PBDEs levels have increased over the last decades while OC pollutant levels appear to have decreased (Gómez-Gutiérrez et al., 2007; Tanabe et al., 2008; Ross et al., 2009).

The northwestern Mediterranean Sea constitutes a highly industrialized area receiving multiple land-based sources of pollution through river inputs, waste water discharges and continental runoff. Recent studies have shown that the distribution of organic contaminants along the NW Mediterranean continental shelf and slope is closely linked to the dispersion dynamics of organic material and fine-grained particles (Salvadó et al., 2012a; Salvadó et al., 2012b). Moreover, in this region, the transfer of particle-bound contaminants to the deep-sea is further enhanced during episodic climatic events such as dense shelf water cascading (DSWC) (Salvadó et al., 2012c). During these events, which occur every 6-10 years in the NW Mediterranean, cold shelf water masses cascade down the continental slope transporting large amounts of sediment and organic matter to the deep-sea environment (Canals et al., 2006; Company et al., 2008). Hence, the impact of anthropogenic contaminants on deep-sea ecosystems might be relevant within the NW Mediterranean basin. This issue is particularly important considering the increasing interest in deep-sea fisheries due to depleted fish stocks of the world's oceans and the fact that the commercially exploited deep-sea shrimp species *Aristeus antennatus* represents one of the most valuable fishing resources within the region.

Despite of the relevance of pollutant concentrations in deep-sea organism for human and wildlife health, only a limited number of studies have thus far investigated the levels of POP contamination of the NW Mediterranean deep-sea fauna (Escartin and Porte, 1999; Porte et al., 2000; Solé et al., 2001; Borghi and Porte, 2002; Castro-Jiménez et al., 2012). However, none of these studies investigated the levels of emerging compounds such as PBDEs.

The present study aimed to investigate the bioaccumulation of OC and PBDE pollutants in deep-sea organisms from the NW Mediterranean (1) to determine baseline levels of both, legacy and emergent POPs in deep-sea biota; (2) to contrast POP levels among different deep-sea organisms; and (3) to investigate the influence of metabolic capacities on the differences in POP distribution observed among species. To this end,

we determined the levels of the ICES (International Council for Exploration of the Sea) 7 PCB congeners, DDT and its metabolites (DDTs), HCH isomers and penta- (PeCB) and hexachlorobenzene (HCB), as well as 14 BDE congeners in muscle tissue of different organisms from this region. The selected species represent the most abundant megafaunal species in the NW Mediterranean deep-sea and include three fish species belonging to different phylogenetic families, namely *Alepocephalus rostratus* (Alepocephalidae), *Coelorinchus mediterraneus* (Macrouridae) and *Lepidion lepidion* (Moridae), and the red-shrimp *Aristeus antennatus*, which is one of the most highly valuable fishery resources of the area.

2. Materials and Methods

2.1. Sample collection

Samples were collected within the area of the Blanes canyon (BC), NW Mediterranean sea (41◦15 N 2◦50 E) (Figure 1). The BC is one of the largest submarine canyons on the NW Mediterranean continental margin and its upper part is located approximately 4 km from the coastline. The hydrodynamic regime in the region is mainly characterized by the Northern Current, which follows the shape of the western Mediterranean continental slope, flowing in a southward direction. Furthermore, BC receives input of continental sediments via the Tordera River. Animals were caught during two cruises conducted in November 2008 and February 2009 onboard the R/V *Garcia del Cid*, using an OTMS otter trawl at depths ranging from 900-1500 m. Onboard, a portion of muscle tissue was dissected and frozen at -20 ºC until further treatment. Sample details are shown in Table 1.

Table 1 Biological characteristics of deep-sea species. Values shown are mean ± standard error of mean

Species	N	Length (mm)	Weight (g)	Lipid (%)	Feeding strategies [a]
Alepocephalus rostratus	30	311.2 ± 7.4	343.3 ± 28.3	1.32 ± 0.26	Non-migratory macroplankton (gelatinous)
Coelorinchus mediterraneus	25	73.4 ± 2.4*	27.8 ± 2.9	0.48 ± 0.07	Infauna
Lepidion lepidion	20	172.6 ± 6.9	37.9 ± 4.2	0.36 ± 0.04	Active predator supra- and epibenthic fauna
Aristeus antennatus	3 pools	40.4 ± 2.6	n.r.	0.79 ± 0.05	Infauna

[a] Cartes et al. (2002)
* pre-anal length (PAL) recorded
n.r. not recorded

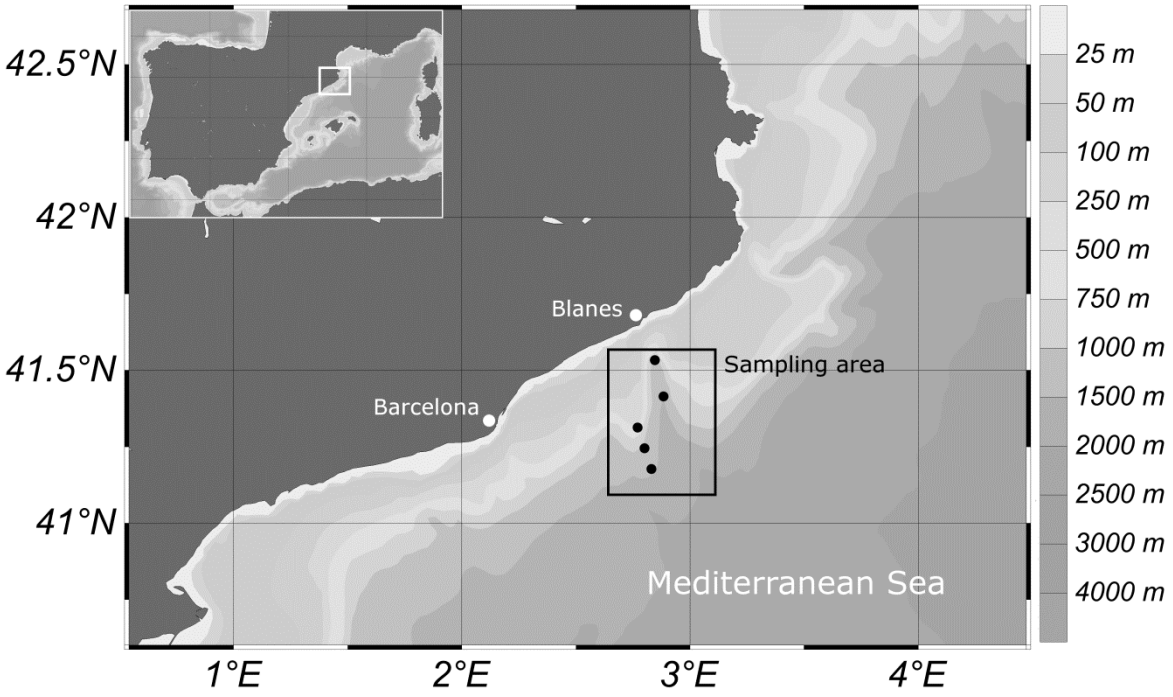

Fig.1 Map of study area within the NW Mediterranean. The map was created using the Ocean Data View (ODV) software package by Schlitzer, R., Ocean Data View, http://odv.awi.de, 2010.

2.2. Sample extraction

Between 20 and 30 individual fish samples of each fish species and 3 pooled samples (5 individuals per pool) of the shrimp *A. antennatus* were analyzed. The extraction of organic pollutants was performed as described in (Koenig et al., 2012a) based on the protocol by Berdié and Grimalt (1998). Briefly, muscle tissue (2-4 g) was ground with anhydrous Na_2SO_4 and soxhlet-extracted with dichloromethane: hexane. Extracts were further purified with sulfuric acid. Tetrabromobenzene (TBB) and PCB 200 were used as recovery standards. The cleaned extracts were concentrated by evaporation and redissolved in 100 µL of PCB 142 in isooctane as internal standard prior to the determination of organochlorine compound levels (*i.e.* CBs, HCHs, PCBs and DDTs). For PBDE analysis, samples were redissolved in 50 µL isooctane containing BDE 118 and [^{13}C]BDE 209 as internal standard.

2.3. Instrumental analysis

To determine levels of PCBs (7 congeners: IUPAC # 18, 52, 101, 118, 138, 153, 180), DDTs (*o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD), Pe-CB, HCB and HCH isomers (α-, β-, γ-, δ-HCH), samples were analyzed using a gas chromatograph (Model HP-6890) equipped with an electron-capture detector (μ-ECD). A 60 m x 0.25 mm I.D. DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5 % diphenylpolydimethylsiloxane (film thickness 0.25 μm) was used for separation. The oven temperature was programmed to increase from 90 °C (holding time 2 min) to 130 °C at a rate of 15 °C min^{-1} and finally to 290 °C at 4 °C min^{-1}, holding the final temperature for 20 min. The injector and detector temperatures were 280 °C and 320 °C, respectively. Injection was performed in splitless mode and helium was used as carrier gas (30.5 psi).

PBDE levels (14 congeners: BDE # 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190, 209) were determined by gas chromatography coupled to negative ion chemical ionization mass spectrometry (GC-MS-NICI) as described in Vizcaino et al. (2009).

2.4. Quality assurance and control

To assess the possible inadvertent contamination of samples during analytical procedures, procedural blanks were performed for every set of six samples. Blanks were used to establish method detection (MDL) and quantification limits (MQL), which were defined as the mean of the blanks plus three times (MDL) or five times (MQL) the standard deviation. They were in the order of 0.03 and 0.05 ng g^{-1} w.w., respectively for organochlorine compounds. For PBDEs MDL and MDQ were in the order of 0.004 and 0.006 ng g^{-1} w.w., respectively, except for congeners 47, 99, 100, and 209 for which they were one order of magnitude higher, namely 0.04 and 0.06 ng/g w.w. respectively. POPs levels were determined by internal standard method. Extraction and analytical performances were evaluated by surrogate standard recoveries, which ranged from 65 % to 90 %. Values reported in this study were corrected based on surrogate recoveries.

3. Results and discussion

3.1. OC levels

The concentrations of OC contaminants are presented in Table 2. Overall, PCB and DDT levels ranged from the highest concentrations in the fish *A. rostratus* (Σ_7PCBs 6.93±0.71 ng/g w.w. and ΣDDTs 8.43±1.10 ng/g w.w.) to the lowest concentrations in the crustacean *A. antennatus* (Σ_7PCBs 1.17±0.24 ng/g w.w. and ΣDDTs 2.53±0.26 ng/g w.w.). The concentrations of ΣHCHs and HCB were more than one order of magnitude lower, ranging from 0.07-0.36 ng/g w.w. and 0.03-0.15 ng/g w.w., respectively, while PeCB was only detected in a few samples above the detection limit. The OC relative abundance follows the sequence PCBs ≈ DDTs >> HCHs ≥ HCB and is generally in accordance with previous studies on deep-sea fish conducted in the Mediterranean (Porte et al., 2000; Storelli et al., 2009), the Atlantic (Berg et al., 1997; Mormede and Davies, 2003) and the Pacific Ocean (Ramu et al., 2006; Takahashi et al., 2010) and also with recent data on deep-sea sediments from the Gulf of Lions (NW Mediterranean) (Salvadó et al., 2012a). These results indicate that the bioaccumulation of HCHs and HCB in deep-sea biota is negligible compared to PCBs and DDTs, which is in agreement with the higher hydrophobicity and bioaccumulation potential of PCBs and DDTs relative to HCHs and HCB. This effect, together with the enhanced vertical transport of these compounds due to their preferential association to suspended particulate matter (Dachs et al., 2002; Scheringer et al., 2009), would explain the dominance of PCBs and DDTs in deep sea species.

Differences in OC levels among species may result from various factors. One important factor is the age of the analyzed specimens, since POP levels have been shown to increase with increasing age in fish (Stow and Carpenter, 1994; Vives et al., 2004). No age determination was done in this study; however, for *A. rostratus* and *C. mediterraneus,* an average age of sampled individuals was estimated based on body length using previously published von Bertalanffy growth curves (Massutí et al., 1995; Morales-Nin et al., 1996). *A. rostratus* is the most long-lived of the analyzed species, with a maximum age of > 20 years (Morales-Nin et al., 1996) and a mean estimated age of 10 years for specimens included in the present study. In contrast, *C. mediterraneus* can reach ages of the order of 10 years (Massutí et al., 1995) and the estimated age for the analyzed fish was 5 years. Although no growth parameters were available for *L.*

lepidion and an average age for the studied specimens could not be calculated, preliminary age estimations have shown that specimens of sizes comparable to those in the present study generally do not exceed ages of 7 years (Morales-Nin, 1990). Similarly, no age could be estimated for the red-shrimp *A. antennatus*, however, this species is thought to live up to no more than 5 years (Company et al., 2008). Based on these estimations, *A. rostratus* would be the oldest organism and *A. antennatus* the youngest, while *C. mediterraneus* and *L. lepidion* exhibit similar and intermediate ages, which is consistent with the differential POP level distributions between species found in the present study.

Another important factor influencing the POP concentrations in biota is the lipid content. When expressing results on a lipid-weight basis, a different trend among species was observed, with highest values of OCs in *L. lepidion* (Σ_7PCBs 2125 ± 332 ng/g l.w. and ΣDDTs 1317 ± 239 ng/g l.w.) due to its low lipid content of muscle tissue (0.36 %, Table 1) and lower OC levels in *A. rostratus* (Σ_7PCBs 721.2 ± 72.8 ng/g l.w. and ΣDDTs 812 ± 102 ng/g l.w.), which also has the highest lipid content (1.3 %, Table 1). In general, organic contaminants are thought to bioaccumulate in relation to tissue lipid content (Hebert and Keenleyside, 1995; Randall et al., 1998), however, if such a relationship does not occur, the normalization of organic contaminant concentrations to lipid content may lead to erroneous conclusions (Hebert and Keenleyside, 1995). In the present study, only *A. rostratus* exhibited a lipid-dependent accumulation of OC compounds (PCB and DDT: Spearman rank $\rho = 0.7$, $p < .0001$). Hence, it is possible that the normalization to lipid-weight only reduced the relative POP levels for *A. rostratus* and thus resulted in a different profile among species as compared to POP concentrations on a wet-weight basis.

POP levels from the present study were compared with previously published results in deep sea organisms as concentrations per wet weight whenever possible or based on lipid weight if applicable. The levels of OC contamination detected in the present study (Σ_7PCBs 1.17-6.93 ng/g w.w., ΣDDTs, 2.53-8.43 ng/g w.w.) are within the range of values previously measured in deep-sea fish from the same study area (*i.e.* NW Mediterranean) by Porte et al. (2000) (Σ_7PCBs 2.5-10.0 ng/g w.w.; ΣDDTs 1.9-10.2 ng/g w.w.) and Solé et al. (2001) (Σ_7PCBs 9.0-16.2 ng/g w.w.; ΣDDTs 7.4-12.6 ng/g w.w.). Although PCB and DDT contamination is thought to have decreased over the last decades, the present findings indicate that OC levels in NW Mediterranean deep-sea

fish have remained relatively similar over the past decade. Based on lipid weight, concentrations of PCBs and ΣDDTs (Σ_7PCBs 145.2-2125 ng/g l.w.; ΣDDTs 321-1317 ng/g l.w.) in NW Mediterranean deep sea organisms appear to be higher than mean values reported in Atlantic deep-sea fish, where Σ_7PCBs ranged from 188 to 792 ng/g l.w. (Webster et al., 2009) and in various deep-sea fish species from the Pacific Ocean, such as the Sulu Sea (Σ_7PCBs 19-110 ng/g l.w.; ΣDDTs 69-270 ng/g l.w.) (Ramu et al., 2006) and off Tohoku, Japan (Σ_7PCBs 34-390 ng/g l.w.; ΣDDTs 36-220 ng/g l.w.) (Takahashi et al., 2010). However, an earlier study conducted in waters off Tohoku found similar OC levels to those described in this study (Σ_7PCBs n.d.-2200 ng/g l.w.; ΣDDTs 14-830 ng/g l.w.) (de Brito et al., 2002). In addition, due to the fact that some of the species analyzed by de Brito et al. (2002) and Takahashi et al. (2010) had high muscle lipid contents, up to 70 % and 25 %, respectively, the conversion of the reported OC concentrations to wet weight resulted in high PCB and DDT levels, reaching 80 ng/g w.w. and 30 ng/g w.w. (see de Brito et al., 2002), one order of magnitude higher than concentrations found in this study.

3.2. PBDE levels

The concentrations of PBDEs detected in the present study are shown in Table 3. They ranged from 0.47 ± 0.20 ng/g w.w. in *A. antennatus* to 0.92 ± 0.13 ng/g w.w. in *A. rostratus* and were approximately one order of magnitude lower than PCB and DDT concentrations, which is in agreement with former studies that simultaneously assessed OC and PBDE contamination in deep-sea fish from the Atlantic (Webster et al., 2009; Webster et al., 2011) and the Pacific Ocean (Ramu et al., 2006; Takahashi et al., 2010). Furthermore, this result is also consistent with sediment contamination data from the NW Mediterranean basin, where PCB and DDT contamination clearly exceeded PBDE levels (Salvadó et al., 2012a; Salvadó et al., 2012b). PCB and DDT levels are generally thought to have decreased in the environment due to the restrictions in their use and production several decades ago, while environmental levels of PBDEs appear to have increased over the last decade due to their relatively recent emissions, even though PCBs and DDTs are still the most dominant contaminants in most marine organisms at present (Gómez-Gutiérrez et al., 2007; Tanabe et al., 2008; Ross et al., 2009).

To our knowledge, only one study has previously measured PBDEs in Mediterranean deep-sea fish (Covaci et al., 2008), however, reported levels were determined in fish

liver and are thus not directly comparable to the results in muscle tissue presented in this study. In comparison to Mediterranean shallow-water species, similar PBDE levels have been detected in the European eel (*Anguilla anguilla*), with a range of Σ_{28}PBDEs 0.08–1.80 ng/g w.w. (including all 14 congeners analyzed in this work) (Labadie et al., 2010). Similarly, Corsolini et al. (2008) determined the sum of 19 PBDEs in swordfish (*Xiphias gladius*), and, although it is noteworthy that the more brominated BDEs (*i.e.* hepta- to decaBDE) were not included, reported values were similar to those found in this study, in the range of 0.04-1.91 ng/g w.w. Finally, significantly higher concentrations of PBDEs (Σ_{23}PBDEs 15.1 ng/g w.w.) have been observed in tuna (*Thunnus thynnus*) from Mediterranean sea (Borghesi et al., 2009).

Σ_{14}PBDE levels expressed on lipid weight basis varied between 61.9 ± 28.9 ng/g l.w. in *A. antennatus* and 188.8 ± 26.6 ng/g l.w. in *L. lepidion.* In comparison, Webster et al. (2009) reported slightly lower levels in deep-sea fish from North Atlantic Scottish waters, ranging from 11.7 to 50.5 ng/g l.w. for Σ_{17}PBDEs, which included all 14 congeners considered in our study except BDE 209. In contrast, the sums of 14 BDE congeners in Pacific deep-sea fish caught in the Sulu Sea (0.9-1.6 ng/g l.w.) (Ramu et al., 2006) and off Tohoku, Japan (1.3-8.5 ng/g l.w.) (Takahashi et al., 2010) were one to two orders of magnitude lower than our results. However, as mentioned earlier, some fish species included in the study by Takahashi et al. (2010) exhibited very high lipid contents in muscle tissue (1.2-25 %). Transforming reported values to wet weight concentrations results in levels in the range of 0.1-0.5 ng/g w.w., which are more similar to the present findings.

Table 2 Organochlorine levels in deep-sea fish and crustacean from NW Mediterranean. Values are mean concentrations (ng/g w.w.) ± standard error of mean (min.-max.). n.d.= not detected. * ng/lipid weight

	A. rostratus **(n = 30)**	***C. mediterraneus*** **(n = 25)**	***L. lepidion*** **(n = 20)**	***A. antennatus*** **(n = 3 pools)**
PeCB	0.02 ± 0.005 (n.d.-0.08)	0.01 ± 0.003 (n.d.-0.04)	n.d.	0.01 ± 0.003 (0.01-0.02)
HCB	0.15 ± 0.02 (n.d.-0.46)	0.05 ± 0.03 (n.d.-0.66)	0.05 ± 0.01 (n.d.-0.09)	0.03 ± 0.01 (0.01-0.04)
α-HCH	0.02 ± 0.01 (n.d.-0.11)	0.04 ± 0.01 (n.d.-0.15)	0.03 ± 0.01 (n.d.-0.09)	0.01 ± 0.01 (n.d.-0.03)
β-HCH	0.07 ± 0.02 (n.d.-0.51)	0.11 ± 0.04 (n.d.-0.68)	n.d.	0.03 ± 0.006 (0.02-0.04)
γ-HCH	0.17 ± 0.02 (n.d.-0.61)	0.019 ± 0.06 (n.d.-0.99)	0.03 ± 0.01 (n.d.-0.09)	0.03 ± 0.01 (n.d.-0.04)
δ-HCH	0.04 ± 0.02 (n.d.-0.51)	0.02 ± 0.01 (n.d.-0.14)	0.004 ± 0.004 (n.d.-0.08)	n.d.
ΣHCHs	**0.30 ± 0.05 (n.d.-1.2)**	**0.36 ± 0.10 (n.d.-1.80)**	**0.07 ± 0.02 (n.d.-0.20)**	**0.07 ± 0.03 (n.d.-0.10)**
ΣHCHs lw*	**49.8 ± 13.7 (n.d.-403.0)**	**130.9 ± 38.8 (n.d.-653.3)**	**20.2 ± 4.4 (n.d.-64.7)**	**8.5 ± 4.3 (n.d.-14.1)**
PCB 28	0.12 ± 0.02 (n.d.-0.35)	0.03 ± 0.01 (n.d.-0.19)	n.d.	0.06 ± 0.04 (0.02-0.13)
PCB 52	0.29 ± 0.05 (n.d.-1.04)	0.56± 0.12 (0.04-2.43)	0.65 ± 0.26 (0.03-4.68)	0.17 ± 0.02 (0.14-0.21)
PCB 101	0.59 ± 0.05 (0.18-1.23)	0.18 ± 0.02 (n.d.-0.35)	0.23 ± 0.04 (0.10-0.88)	0.09 ± 0.02 (0.06-0.12)
PCB 118	0.31 ± 0.05 (n.d.-0.85)	0.34 ± 0.10 (0.10-2.49)	0.37 ± 0.03 (0.18-0.68)	0.10 ± 0.04 (0.06--0.18)
PCB 153	2.36 ± 0.28 (0.42-5.69)	1.44 ± 0.31 (0.35-6.44)	2.53 ± 0.33 (0.83-5-65)	0.21 ± 0.04 (0.12-0.26)
PCB 138	1.83 ± 0.21 (0.35-4.34)	1.17 ± 0.25 (0.31-5.80)	1.41 ± 0.18 (0.52-3.56)	0.44 ± 0.13 (0.18-0.60)
PCB 180	1.44 ± 0.17 (0.26-3.35)	0.77 ± 0.16 (0.17-2.65)	1.03 ± 0.17 (0.12-3.21)	0.12 ± 0.04 (0.05-0.19)
ΣPCBs	**6.93 ± 0.71 (1.70-14.80)**	**4.48 ± 0.79 (1.20-18.10)**	**6.22 ± 0.64 (2.00-11.80)**	**1.17 ± 0.24 (0.70-1.50)**
ΣPCBs lw*	**721.2 ± 72.8 (190-1700)**	**1203 ± 219 (151-4320)**	**2125 ± 332 (463-5100)**	**145.2 ± 23.2 (99-1670)**
p,p'-DDT	1.83 ± 0.21 (0.35-4.34)	0.21 ± 0.03 (0.09-0.56)	0.18 ± 0.01 (0.10-0.31)	0.39 ± 0.24 (0.08-0.87)
p,p'-DDE	6.44 ± 0.91 (0.82.-16.52)	2.18 ± 0.45 (0.65-8.28)	3.38 ± 0.44 (0.37-6.87)	0.50 ± 0.24 (0.24-0.98)
p,p'-DDD	0.50 ± 0.07 (0.05.-1.48)	0.10 ± 0.01 (0.06-0.21)	0.08 ± 0.01 (n.d.-0.12)	n.d.
o,p'-DDT	0.32 ± 0.03 (0.06-0.70)	0.07 ± 0.01 (n.d.-0.15)	0.01 ± 0.01 (n.d.-0.07)	0.22 ± 0.06 (0.13-0.34)
o,p'-DDE	0.08 ± 0.01 (n.d.-0.34)	0.01 ± 0.003 (n.d.-0.05)	n.d.	1.33 ± 0.14 (1.06-1.47)
o,p'-DDD	0.94 ± 0.04 (n.d.-0.94)	0.27 ± 0.01 (0.16-0.43)	0.23 ± 0.02 (0.10-0.46)	0.08 ± 0.01 (0.06-0.09)
ΣDDTs	**8.43 ± 1.10 (1.30-21.20)**	**2.83 ± 0.49 (1.10-9.20)**	**3.86 ± 0.47 (0.70-7.70)**	**2.53 ± 0.26 (2.10-3.00)**
ΣDDTs lw*	**812 ± 102 (200-2635)**	**761 ± 131 (81.9-2197)**	**1317 ± 239 (287-4021)**	**322 ± 32.3 (283-386)**

Table 3 PBDE levels in deep-sea fish and crustacean from NW Mediterranean. Values shown are mean concentrations (ng/g w.w.) ± standard error of mean (min.-max.).

	A. rostratus **(n = 30)**	***C. mediterraneus*** **(n = 25)**	***L. lepidion*** **(n = 20)**	***A. antennatus*** **(n = 3 pools)**
BDE 17	0.06 ± 0.02 (n.d.-0.29)	0.05 ± 0.02 (n.d.-0.35)	0.001 ± 0.001 (n.d.-0.02)	0.003 ± 0.003 (n.d.-0.01)
BDE 28	0.10 ± 0.02 (n.d.-0.45)	0.12 ± 0.01 (0.03-0.23)	0.13 ± 0.01 (0.07-0.21)	0.06 ± 0.02 (0.03-0.08)
BDE 71	0.01 ± 0.003 (n.d.-0.05)	0.002 ± 0.001 (n.d.-0.01)	0.01 ± 0.005 (n.d.-0.09)	n.d.
BDE 47	0.15 ± 0.03 (n.d.-0.58)	0.14 ± 0.02 (n.d.-0.42)	0.15 ± 0.03 (n.d.-0.49)	0.06 ± 0.01 (0.04-0.08)
BDE 66	0.004 ± 0.004 (n.d.-0.11)	n.d.	n.d.	n.d.
BDE 100	0.14 ± 0.03 (0.03-0.77)	0.08 ± 0.01 (0.03-0.32)	0.10 ± 0.01 (0.03-0.23)	0.02 ± 0.003 (0.01-0.02)
BDE 99	0.16 ± 0.05 (n.d.-1.56)	0.13 ± 0.03 (0.05-0.67)	0.13 ± 0.04 (n.d.-0.66)	0.07 ± 0.03 (n.d.-0.11)
BDE 85	0.04 ± 0.01 (n.d.-0.17)	0.03 ± 0.01 (n.d.-0.15)	0.01 ± 0.002 (n.d.-0.04)	n.d.
BDE 154	0.11 ± 0.03 (0.02-0.79)	0.03 ± 0.01 (n.d.-0.17)	0.02 ± 0.004 (n.d.-0.08)	0.003 ± 0.003 (n.d.-0.01)
BDE 153	0.04 ± 0.01 (0.01-0.24)	0.01 ± 0.004 (n.d.-0.07)	0.001 ± 0.004 (n.d.-0.07)	0.09 ± 0.03 (0.03-0.14)
BDE 138	0.004 ± 0.001 (n.d.-0.03)	n.d.	n.d.	n.d.
BDE 183	0.008 ± 0.002 (n.d.-0.05)	0.002 ± 0.001 (n.d.-0.02)	n.d.	n.d.
BDE 190	0.01 ± 0.002 (n.d.-0.03)	n.d.	n.d.	n.d.
BDE 209	0.11 ± 0.05 (n.d.-1.66)	0.02 ± 0.01 (n.d.-0.09)	0.02 ± 0.01 (n.d.-0.22)	0.17 ± 0.17 (n.d. ± 0.52)
ΣBDEs	**0.92 ± 0.13 (0.29-3.02)**	**0.61 ± 0.07 (0.23-1.97)**	**0.58 ± 0.08 (0.20-1.63)**	**0.47 ± 0.20 (0.18-0.84)**
ΣBDEs lw*	**107.9 ± 15.8 (16.6-349)**	**172.1 ± 23.4 (14.5-501)**	**188.8 ± 26.6 (21.4-448)**	**61.9 ± 28.9 (23.1-1195)**

n.d.= not detected
*ng/lipid weight

3.3. Compound distributions

Organochlorine compounds

PCB profiles in deep sea species were dominated by the high-molecular-weight (HMW) PCBs 153, 138 and 180, which represented 69-79 % of Σ_7PCBs in fish and 60 % in the red shrimp *A. antennatus* (Figure 2). While PCB 153 exhibited the highest abundance in fish, in the shrimp the most abundant congener was PCB 138. The differential PCB accumulation between the fish and the crustacean species has been described in a previous study and is likely related to differences in hepatic cytochrome P450-mediated metabolism of PCBs between fish and crustacea (Koenig et al., 2012b). Overall, the detected PCB profiles are in accordance with the general bioaccumulation patterns of PCBs in deep-sea fish reported in former studies (Porte et al., 2000; Solé et al., 2001; Mormede and Davies, 2003). The predominance of these compounds in biota can be explained by the higher bioaccumulative potential of the more hydrophobic higher chlorinated PCBs (*i.e.* hexa- to octachloro congeners) (McFarland and Clarke, 1989). In addition, as mentioned previously, highly chlorinated congeners have higher sediment affinities than low chlorinated compounds and are thus more prone to particle-bound transport from surface waters to the deep-sea (Dachs et al., 2002; Scheringer et al., 2004).

In fish, the main DDT compound detected was the metabolite *p,p'*-DDE, which comprised on average 70-80 % of ΣDDTs, while the parent compound *p,p'*-DDT contributed only 6-10 % to ΣDDTs (Figure 3). This result is a commonly observed distribution in marine organisms (Voorspoels et al., 2004), including deep-sea fish (Mormede and Davies, 2003; Takahashi et al., 2010) and is indicative of old DDT residues, which progressively degrade in aquatic environments into their even more persistent metabolites, primarily DDE (Wolfe et al., 1977). In shrimp however, o,*p'*-DDE was the main DDT metabolite and represented 49 % of ΣDDTs, while the parent compound *p,p'*-DDT and its metabolite *p,p'*-DDE exhibited similar proportions of 16 % and 21 %, respectively (Figure 3). Hence, the DDT/DDE ratio profile detected in shrimp would indicate a recent input of the parent compound *p,p'*-DDT within the study area, which is in contrast to the results observed in fish. Thus, it seems that, despite the wide use of the DDT/DDE ratio as a means to discriminate between recent and past use of

DDT (Corsolini et al., 2008), these results indicate that it should be applied with caution as it can vary among different organisms.

The technical HCH mixtures, containing all four isomers with a α-HCH/γHCH ratio of 4-7, were gradually replaced by lindane, which is the only component exhibiting significant insecticide activity and still being released, although to a limited extent, into the environment. Accordingly, the most abundant HCH isomer detected in the present study was γ-HCH (lindane), contributing approximately 50 % to ΣHCH in all species. Moreover, α-HCH/γ-HCH ratios ranged between 0.2 in *A. rostratus* and 1.0 in *L. lepidion*, showing a predominance of lindane over the technical mixture.

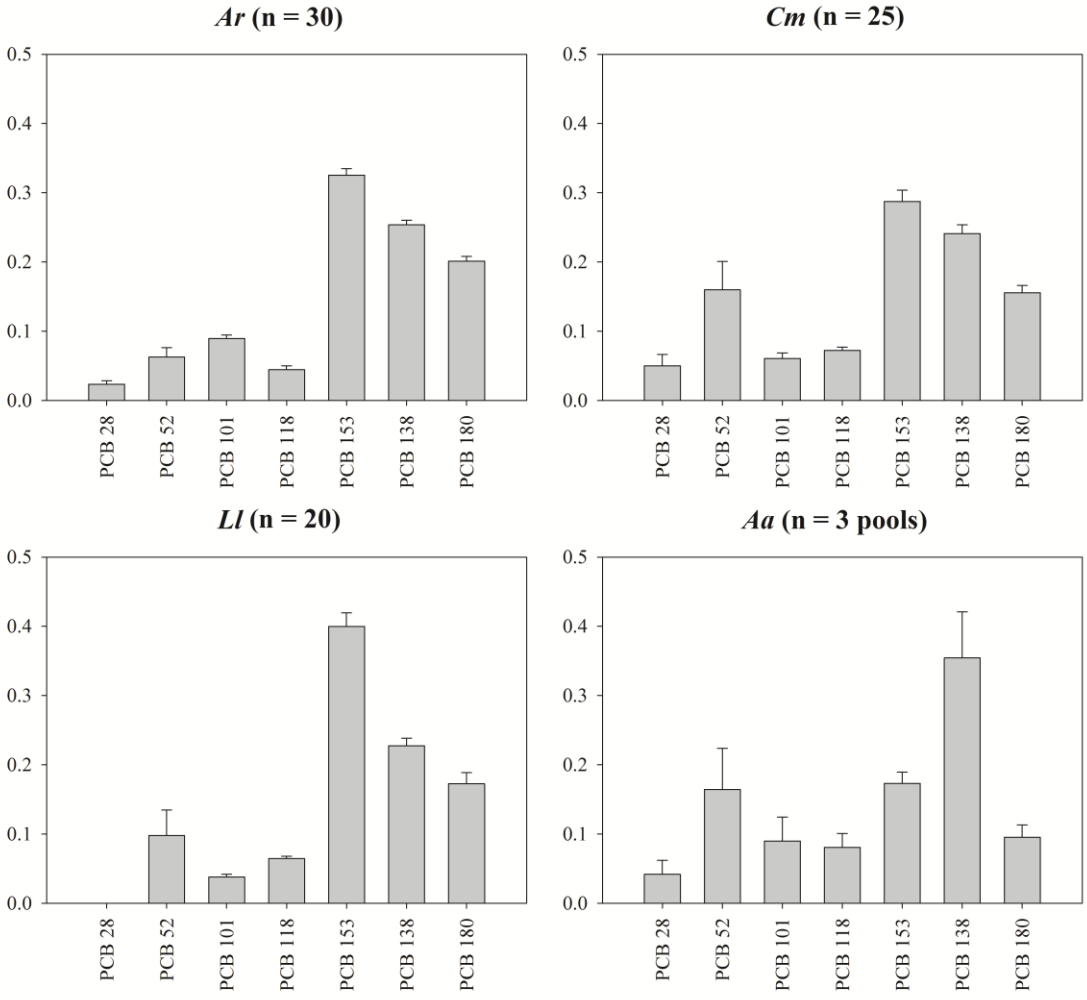

Fig.2 Bioaccumulation profiles of ICES 7 PCB congeners in the three fish species, *Alepocephalus rostratus* (*Ar*), *Coelorinchus mediterraneus* (*Cm*), *Lepidion lepidion* (*Ll*) and the shrimp *Aristeus antennatus* (*Aa*). Values shown are mean proportions (PCB_x/Σ_7PCBs) ± standard error of mean.

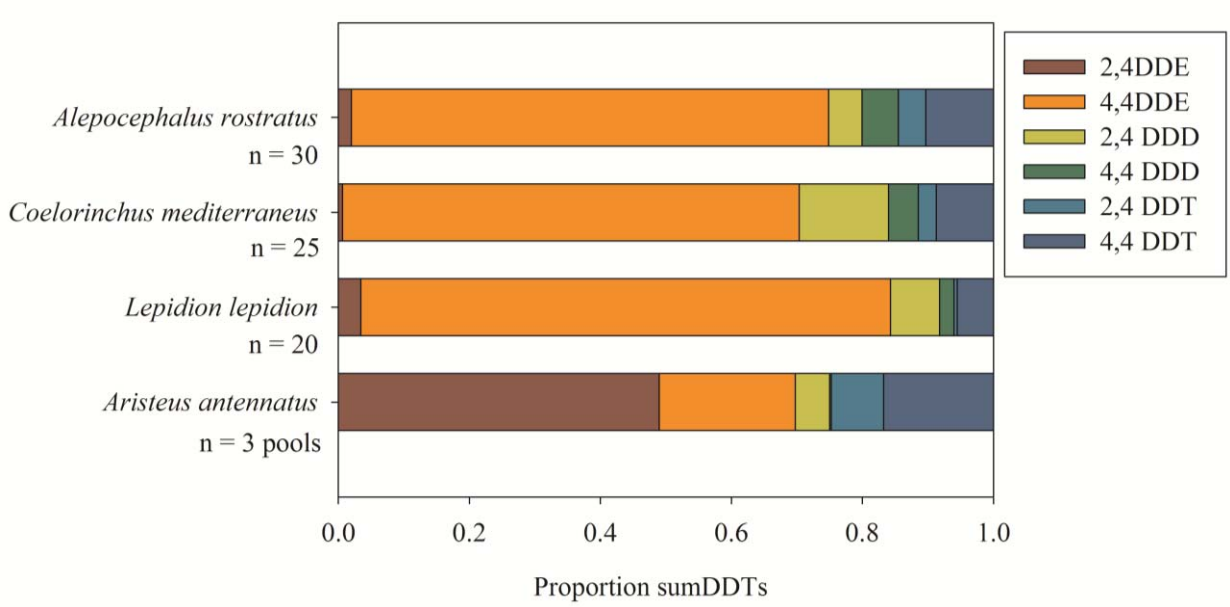

Fig.3 Bioaccumulation profiles of DDT and its metabolites in the deep sea species studied.

PBDE

In fish, the most important PBDE congeners detected were BDE 28, 47, 99 and 100, constituting from 68 % in *A. rostratus* to 89 % in *L. lepidion* of ΣPBDEs (Figure 4), similar to previous results observed in muscle tissue of deep-sea fish (Webster et al., 2009; Takahashi et al., 2010). These congeners are the main components in the commercial penta-BDE formulations (La Guardia et al., 2006). BDE 154 and 209 levels were also significant in all fish species. BDE 154 has been suggested to be a debromination product of BDE 183, the main congener in the technical octa-BDE mixtures (Stapleton et al., 2004; Roberts et al., 2011), while BDE 209 constitutes between 92 to 97 % of the total BDE content in the deca-BDE formulations (La Guardia et al., 2006). Therefore, PBDE composition observed in deep sea organisms are consistent with the composition of the technical mixtures used in Europe. This PBDE profile differs from that reported in deep-sea sediments from a nearby area (Gulf of Lions, NW Mediterranean) (Salvadó et al., 2012b), where BDE 209 was the predominant congener (78 %). These differences can be attributed to differences in bioavailability and biotransformation potential between compounds. BDE 209 is thought to have low bioavalability, in agreement with its high molecular size (Eljarrat et

al., 2004), and it can be metabolized to less brominated congeners in some fish species (Kierkegaard et al., 1999; Stapleton et al., 2006), thus potentially explaining the relatively low proportion of BDE 209 found in the deep sea fish.

In contrast to fish, BDE 153 and 209 were the most abundant congeners in shrimp, although large variability was observed potentially because of the low sample sizes (n = 3 pools) (Figure 4). Previous studies have also observed high concentrations of BDE 209 (Ashizuka et al., 2008; van Leeuwen et al., 2009), as well as BDE 153 (Voorspoels et al., 2003) in shrimp, suggesting a higher uptake or lower biotransformation capacity of these congeners in crustacea.

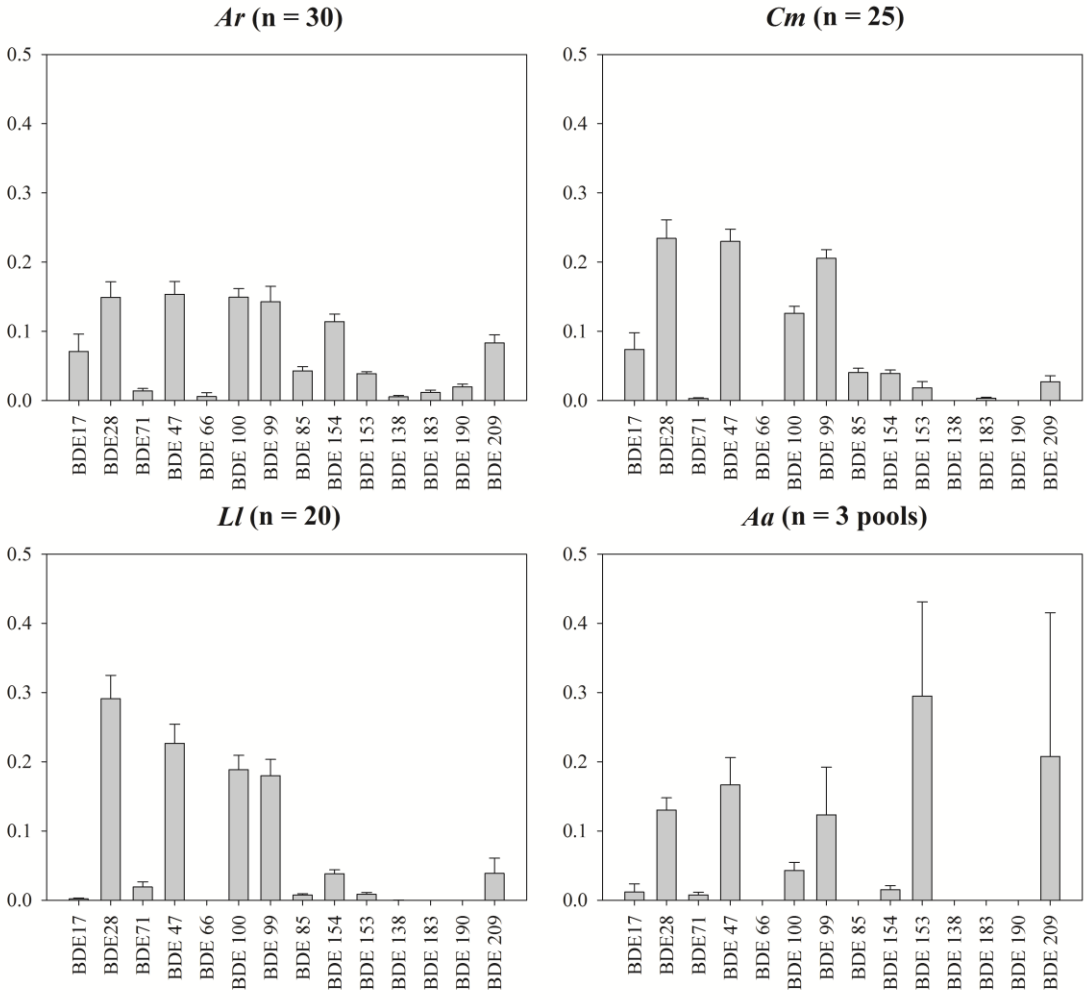

Fig.4 Bioaccumulation profiles of 14 PBDE congeners in the three fish species, *Alepocephalus rostratus* (*Ar*), *Coelorinchus mediterraneus* (*Cm*), *Lepidion lepidion* (*Ll*) and the shrimp *Aristeus antennatus* (*Aa*). Values shown are mean ($PBDE_x/\Sigma_{14}PBDEs$) ± standard error of mean.

Despite the correspondence between PBDE congeners found in deep sea organisms and main components in the technical mixtures, the relative abundance of these compounds differs from that found in the commercial formulations. These results can be explained by differences in metabolic transformation rates between congeners (Roberts et al., 2011). In this sense, BDE 99/100, 153/154 and 47/99 ratios have been used to assess differences in metabolic capacities as well as trophic position among various aquatic organisms (Voorspoels et al., 2003; Xiang et al., 2007; Dickhut et al., 2012). PBDE ratios determined in this study are summarized in Table 4. Usually, high BDE 99/100 ratios, similar to those found in the original commercial pentaBDE mixtures such as Bromkal 70 5-DE (approx. 5.3) or DE-71 (approx. 3.71), are found in sediments and lower organisms such as invertebrates, but decrease through the food chain due to higher biotransformation rate of BDE 99 in higher organisms (Christensen and Platz, 2001; Voorspoels et al., 2003; Xiang et al., 2007; Hu et al., 2010). This ratio varied between 0.99 to 1.91 in deep sea fish species (Table 4), indicating a significant degradation of BDE 99. The higher value found in shrimp (3.3) is in accordance with a number of studies reporting higher ratios in crustacea compared to fish (Voorspoels et al., 2003; Xiang et al., 2007; Hu et al., 2010). The ratio between BDE 153 and BDE 154 has been similarly related to the metabolic capacities of different organisms (Xiang et al., 2007), with higher contributions of BDE 154 reflecting the higher biotransformation of more brominated congeners such as BDE 183 (Roberts et al., 2011). Values were <1 in fish, but shrimp exhibited a very high BDE153/154 ratio, pointing to a lower metabolic capacity of the crustacean in relation to fish, although it is noteworthy that this result is largely based on very high BDE 153 levels and a lack of BDE 154 in shrimp. However, a significant relationship between the ratios BDE99/100 and BDE153/154 in all three fish species ($\rho>0.4$, $p<0.05$) indicates the coherent covariation of these two parameters, reinforcing their use as proxies for the BDE metabolization abilities of different species. Furthermore, BDE 99/100 and BDE 153/154 ratios were highest in the shrimp, but also higher in *C. mediterraneus* compared to the two other fish species. These two species are infaunal feeders, closely associated to the sediment, while *A. rostratus* and *L. lepidion* feed on epibenthic and/or pelagic prey (see Table 1). Hence, it is possible that, in addition to differences in metabolic capacities, these BDE congener ratios also reflect differences in feeding strategies among organisms.

Table 4 Mean PBDE ratios in deep-sea fish and crustacean from NW Mediterranean.

Ratio	*A. rostratus* (n = 30)	*C. mediterraneus* (n = 25)	*L. lepidion* (n = 20)	*A. antennatus* (n = 3 pools)
BDE 99/100	0.99	1.91	1.32	3.33
BDE 153/154	0.37	0.63	0.28	14.0*
BDE 47/99	1.35	1.17	1.36	0.75

* BDE 154 only detected in one pooled sample and thus ratio based on one individual

BDE 47/99 ratios in deep sea fish varied between 1.17 and 1.36, with BDE 47 only representing 15-22 % of ΣPBDEs. This is in contrast to results found in liver of two Mediterranean deep-sea fish species, where BDE 47 contributed approximately 50 % to ΣPBDEs and BDE 99 was clearly depleted (Covaci et al., 2008). BDE 99 has been shown to be metabolized to BDE 47 in carp liver (Stapleton et al., 2004) and the congener ratio BDE 47/99 has been used to assess the level of metabolization of BDE 99 to BDE 47 (Wang et al., 2009). However, a different debromination pathway of BDE 99 has been detected in salmon and trout, suggesting significant differences in efficiency and metabolite formation of BDE 99 debromination among teleost species (Browne et al., 2009; Roberts et al., 2011) and the BDE 47/99 ratio does therefore not necessarily reflect the metabolization rate of BDE 99 in all species. In fact, the similar proportions of BDE 47 and 99 reported in the present study (Table 4) are consistent with BDE 47/99 ratios in the commercial penta-BDE mixtures, which primarily contain these two congeners at equal concentrations, suggesting a lack of debromination of BDE 99 to BDE 47. However, slightly lower BDE 47/99 ratios were observed in the shrimp (0.75) and *C. mediterraneus* (1.17), compared to the two other fish species (1.36), suggesting again the potential existence of different metabolic capacities and/or differences in BDE uptake related to feeding strategies between the two infaunal feeders and the more pelagic species.

These results indicate that metabolism plays an important role in the PBDE congener distributions in aquatic organisms, resulting in a selective accumulation of the lower brominated congeners. This is relevant to human as well as wildlife health, since lower brominated congeners have higher biomagnification potential and toxicological effects.

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Paper 2

New insights into mercury bioaccumulation in deep-sea organisms from the NW Mediterranean and their human health implications

Samuel Koenig [a,b]*, Montserrat Solé [a], Cristal Fernández-Gómez [b] and Sergi Díez [b]

[a] Institut de Ciències del Mar (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

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Resumen

En este estudio se determinaron los niveles de mercurio total (THg) en doce especies de peces y el crustáceo *A. Antennatus*, como indicador de la bioacumulación de metilmercurio (MeHg), ya que estudios previos mostraron que el MeHg representa más del 80 % del THg en musculatura de los organismos. Además de los niveles también se investigó la influencia de diferentes factores como nivel trófico, masa corporal y profundidad de máxima abundancia de cada especie en las concentraciones detectadas. Los resultados indican una buena correlación entre los niveles de THg y el nivel trófico y el peso corporal de cada especie. Al margen de estas relaciones descritas ampliamente en la literatura, también se observó una correlación significativa entre la concentración de THg y la profundidad a la que vive cada especie. En particular, las especies con un rango batimétrico más cercano a la superficie presentaron una acumulación de THg más baja de lo esperado según su nivel trófico y peso. Por el contrario, los peces que se encuentran a más profundidad tenían una tendencia a acumular más THg de lo que les correspondería por nivel trófico y la masa corporal.

Cabe destacar que las concentraciones de THg detectadas sobrepasaron el límite para el consumo humano de 0.5 μg/g peso fresco establecido por la Unión Europea (UE) en prácticamente todas las especies analizadas. Este resultado es especialmente relevante en el caso de la mora común, *Mora moro*, y la gamba roja, *Aristeus antennatus*, que representan un recurso de gran valor comercial en el Mediterráneo, por lo que las concentraciones de THg detectadas podrían constituir un cierto riesgo para la salud humana.

Abstract

A number of studies have found high levels of mercury (Hg) in deep-sea organisms throughout the world's oceans, but the underlying causes are not clear as there is no consensus on the origin and cycling of Hg in the ocean. Recent findings suggested that Hg accumulation may increase with increasing forage depth and pointed to the deep-water column as the origin of most Hg in marine biota, especially its organic methylmercury (MeHg) form. In the present study, we determined total mercury (THg) levels in 12 deep-sea fish species and a decapod crustacean and investigated their relationship with the species' nitrogen stable isotope ratio ($\delta^{15}N$) as an indicator of their trophic level, average weight and habitat depth. THg levels ranged from 0.27 to 4.42 μg/g w.w. and exceeded in all, except one species, the recommended 0.5 μg/g w.w. guideline value. While THg levels exhibited a strong relationship with $\delta^{15}N$ values and to a lesser extent with weight, the habitat depth, characterized as the species' depth of maximum abundance (DMA), had also a significant effect on Hg accumulation. The fish species with a shallower depth range exhibited lower THg values than predicted by their trophic level ($\delta^{15}N$) and body mass, while measured THg values were higher than predicted in deeper-dwelling fish. Overall, the present results point out a particular risk for human health from the consumption of deep-sea fish. In particular, for both, the red shrimp *A. antennatus*, which is one of the most valuable fishing resources of the Mediterranean, as well as the commercially exploited fish *M. moro*, THg levels considerably exceeded the recommended 0.5 μg/g w.w. limit and should be consumed with caution.

Keywords: mercury bioaccumulation, trophic level, habitat depth, deep-sea fish, red shrimp *Aristeus antennatus,* Mediterranean

1. Introduction

Mercury (Hg) is a trace element of natural and anthropogenic origin that can be found throughout the atmosphere, biosphere and geosphere. In aquatic environments, mercury is readily transformed by chemical and biological (*i.e.* bacterially mediated) pathways into organomercury compounds such as methylmercury (MeHg), greatly affecting its solubility, volatility, bioavailability and toxicity (Díez, 2009). Methylmercury, the most toxic mercury species, is known to have numerous adverse effects, including neurotoxicity, genotoxicity and endocrine disruption on a wide range of vertebrates, including fish (Scheuhammer et al., 2007), as well as invertebrate species (Carrasco et al., 2008; Faria et al., 2009; Faria et al., 2010).

The origin and cycling of MeHg in the marine environment is still not fully understood. Some studies have suggested that MeHg could originate from deep-sea sediments (Kraepiel et al., 2003; Ogrinc et al., 2007), while a recent study conducted in open Mediterranean waters concluded that most of the MeHg found in the water column had been generated in situ by planktonic organisms (Cossa et al., 2009). After entering the aquatic food web, MeHg tends to bind to sulfhydryl groups of proteins and biomagnifies in higher trophic level organisms (Mason et al., 2006). The presence of mercury in commercially important seafood, especially in large predatory pelagic fish is of major concern with regard to human consumption. Increasing anthropogenic emissions and growing public awareness of the potential health impacts of mercury have lead to the establishment of advisories and consumption limits for the general population and particularly for sensitive subgroups (*e.g.* pregnant women and young children). The concentration limit for total Hg (THg) in fish for human consumption was set at 1 mg/kg w.w. for predatory fish and 0.5 mg/kg w.w. for non-predatory species (FAO/WHO, 1991; EC, 2001). In general, more attention has been devoted to fish such as tuna, shark, king mackerel, swordfish and tilefish due their high position in the trophic chain; however, less attention has been paid to deep-sea fish and especially deep-sea crustaceans (*e.g.* shrimp).

Deep-sea species are thought to accumulate higher levels of heavy metals than more shallow-water species, possibly as a result of their higher longevity and trophic levels (Mormede and Davies, 2001). In this context, relatively high levels of mercury have been found in a number of deep-sea fish (Monteiro et al., 1996; Cronin et al., 1998;

Mormede and Davies, 2001; Storelli et al., 2002; Chiu and Mok, 2011). However, a previous study conducted in the North Pacific Ocean has also highlighted that foraging depth may also influence mercury accumulation in fish and that mercury levels are higher in deeper-feeding pelagic predators (Choy et al., 2009).

The objectives of the present study were: (1) to determine mercury levels in twelve deep-sea fish species and a deep-sea crustacean and investigate their relationship with the trophic level, size and depth of occurrence of the species (2) to give recommendations regarding the safe consumption of the selected fish species and the red shrimp *Aristeus antennatus*, a very popular seafood and one of the most valuable fishing resources of the Mediterranean.

2. Materials and methods

2.1. Sampling

Sampling cruises were conducted off the coast of Blanes, Catalan Sea (CS) onboard the R/V *Garcia del Cid* (CSIC) in February 2009 and in the southern Balearic Sea in the western basin (WM) and the western Ionean Sea in the central basin (CM) onboard the R/V *Sarmiento de Gamboa* (CSIC) in May 2009 (Fig. 1). Animals were caught using a OTMS otter trawl (Sardà et al., 1998) at depths ranging from 900 m to 2000 m. Onboard, muscle tissue was dissected and stored at -20 °C until further analysis.

2.2. Mercury analysis

THg was determined in pooled samples, consisting of 10 individuals per pool for all fish species and 5 individuals per pool for the crustacean *A. antennatus*, as male and female shrimp were analyzed separately. An equal portion of 0.2 g or 0.4 g were taken from the dorsal muscle tissue of each individual and subsequently homogenized. The measurements were performed using an advanced mercury analyser AMA-254, manufactured by Altec (Prague, Czech Republic) and distributed by Leco (St. Joseph, MI, USA). This instrument is based on catalytic combustion of the sample, preconcentration by gold amalgamation, thermal desorption and atomic absorption spectrometry (AAS). Samples were taken directly from the freezer, cut into 50-150 mg

pieces, precisely weighed in a nickel boat and automatically introduced into the AMA. Replicate analysis were conducted for the samples of each fish species and accepted if the relative standard deviation was lower than 10%, otherwise analysis were run again. The entire analytical procedure was validated by analysing blanks and CRM DORM-2 and DORM-3 samples at the beginning and end of each set of tissue samples (usually 10), ensuring that the instrument remained calibrated during the course of the study. Blanks consisted of an empty boat. Detection and quantification limits were calculated from blank measurements with THg values of 0.2 and 0.7 ng/g w.w., respectively (Diez et al., 2007).

2.3. Statistical analysis

For comparisons of THg levels among species, a linear regression analysis on Log THg (ng/g w.w.) data was performed using the species' habitat depth, trophic level and body mass as continuous variables. The habitat depth was chosen as the depth of maximum abundance (DMA) of each species, which was extracted from the *DeepMed Research Group Database* (ICM-CSIC). This parameter represents the depth at which the population of a given species exhibits its highest abundance within the species' depth range, thus representing the optimal habitat depth of the species across its depth range (Company and Sardà, 1998). It is noteworthy that DMA values do not necessarily coincide with sampling depths as the database extends beyond the depth range sampled in the present study (900-2000 m). The trophic level was characterized as the nitrogen stable isotope ($\delta^{15}N$) values determined by Tecchio et al. (2012), except for *Coelorinchus mediterraneus* and *Nezumia aequalis* data were taken from Polunin et al. (2001). The average weight (g) of the species was used instead of body size because for some fish species (*i.e.* macrouridae) the standard length was recorded as the anal length instead of total body length and the comparison of size among species was therefore not possible. All interaction levels between factors were not significant and were therefore omitted in the final analysis.

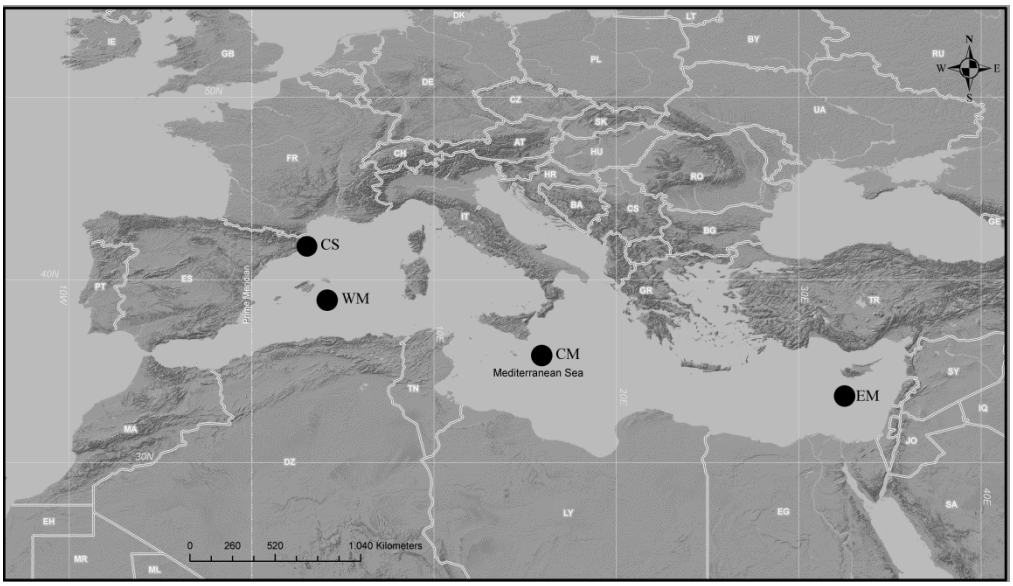

Fig. 1 Map of sampling sites across Mediterranean Sea

3. Results and discussion

3.1. Interspecies comparison

In the present study, THg levels were used as proxy for organic mercury content because MeHg is the major chemical form of mercury stored in fish muscle tissues (80-90 % of the total mercury) (Harris et al., 2003). A similar relationship between THg and MeHg has been demonstrated for the crustacean *A. antennatus*, with an average 85 % MeHg content in muscle tissue (Minganti et al., 1996).

Values for total mercury (THg) concentrations in muscle tissue ranged from 0.27 to 4.42 μg/g w.w. and only one fish species, namely *Lampanyctus crocodilus*, exhibited total mercury (THg) levels below the European limit for safe consumption of 0.5 μg/g w.w. (Table 1). For the six species, for which samples from different sites were collected, no differences were detected among sites across the Mediterranean, but all levels exceeded the recommended consumption limit (Fig. 2). The lack of any clear trend for the comparison among sites across the Mediterranean basin suggests a relatively uniform contamination pattern of the Mediterranean deep-sea. Moreover, this result indicates that the high THg levels recorded in the present study are not due to the

presence of a contamination hot spot in the study area, but represent a general elevated mercury contamination of Mediterranean deep-sea.

Table 1 Biological characteristics and mean total mercury (THg) levels (min.-max-) of 12 deep-sea fish species and the crustacean *A. antennatus*.

Species	N	DMA	$\delta^{15}N$	Weight (g) ± S.E.	Diet	THg (µg/g ww)
Alepocephalus rostratus (*Ar*)	8	1300 m	9.86	370 ± 49	Macroplankton (non-migratory)[a]	0.64 (0.30-1.32)
Bathtypterois mediterraneus (*Bm*)	1	1750 m	11.39	17 ± 1	Benthopelagic plankton (non-migratory)[b]	1.23
Cataetyx laticeps (*Cl*)	1	2800 m	12.75	430 ± 60	Epibenthic prey[c]	4.42
Coelorinchus mediterraneus (*Cm*)	3	1500 m	12.60*	23 ± 2	Infauna[a]	1.46 (1.40-1.56)
Coryphaenoides guentheri (*Cg*)	1	1750 m	11.00	16 ± 1	Benthic feeder[d]	0.92
Coryphaenoides mediterraneus (*Cm**)	1	2700 m	10.97	88 ± 17	Benthopelagic and benthic feeder[d]	1.96
Lampanyctus crocodiles (*Lc*)	1	500 m	8.09	43 ± 4	Macroplankton (migratory)[a]	0.27
Lepidion lepidion (*Ll*)	10	1200 m	11.20	64 ± 12	Benthopelagic and benthic feeder[e]	0.94 (0.21-1.71)
Mora moro (*Mm*)	2	1100 m	11.78	584 ± 48	Nekto-suprabenthos (Active predator)[e]	2.40 (2.38-2.43)
Nezumia aequalis (*Na*)	1	600 m	13.78*	55 ± 4	Nekto-suprabenthos[a,f]	1.79
Nezumia sclerorhynchus (*Ns*)	5	1200 m	12.22	25 ± 3	Nekto-suprabenthos[f]	1.44 (0.97-1.86)
Trachyrhynchus scabrus (*Ts*)	1	900 m	10.25	190 ± 12	Infauna[a]	0.65
Aristeus antennatus (*Aa*)	13	700 m	9.61	17 ± 5	Infauna[a]	1.04 (0.48-2.24)

N: number of pools used to calculate THg
DMA: depth of maximum abundance
$\delta^{15}N$: nitrogen stable isotope values from Tecchio et al. (In prep.) , except values indicated by * from Polunin et al. (2001)
[a] (Cartes et al., 2002); [b] (Carrassón and Matallanas, 2001); [c](Mauchline and Gordon, 1984); [d] (Carrassón and Matallanas, 2002); [e] (Carrassón et al., 1997); [f] (Marques and Almeida, 1998)

The high level of mercury contamination of the Mediterranean Sea has been the subject of extensive studies for the last decades and has resulted in international campaigns such as the UNEP MED POL program, to assess the impact of mercury contamination

on the Mediterranean marine environment (Aston and Fowler, 1985; UNEP/FAO/WHO, 1987; Cossa and Coquery, 2005). Moreover, our results are concordant with previous results of mercury concentrations in Mediterranean deep-sea organisms from the central (Storelli et al., 2002; Drava et al., 2004) and the eastern Mediterranean basin (Hornung et al., 1993). In contrast, mercury levels in deep-sea fish from the Atlantic and Pacific Ocean, including species also analyzed in the present study such as *Nezumia aequalis* (Mormede and Davies, 2001) and *Coryphaenoides guentheri* (Cronin et al., 1998), generally exhibited Hg levels of one order of magnitude lower than those recorded within this work. Several potential explanations for these high mercury concentrations in Mediterranean biota have been proposed, including volcanic activity and higher anthropogenic emission rates (*e.g.* mining), although more recent findings suggest a biological rather than geochemical origin (Cossa and Coquery, 2005). In particular, the oligotrophic nature of the Mediterranean Sea could enhance the methylation of mercury within the water column thus resulting in higher bioaccumulation of MeHg at the base of the food chain, which is subsequently transferred to larger predators (Cossa et al., 2009; Heimbürger et al., 2010; MerMex Group, 2011).

In addition, linear regression analyses of data from all species exhibited a significant effect of the species' depth of maximum abundance (DMA), nitrogen stable isotope ratio ($\delta^{15}N$) and weight on THg content in muscle tissue ($R^2 = 0.85$, $P < 0.0001$, Fig. 3a). However, the crustacean *Aristeus antennatus* was an outlier (Fig. 3a) and the fit of the model improved when only the 12 fish species were included in the analysis, with DMA, $\delta^{15}N$ and weight accounting for 96 % of the variability in THg among fish species (Table 2a). Of the three factors included in the analysis, variations in $\delta^{15}N$ explained most of the variability of THg among fish species, while the effect of the DMA was second most important followed by the average weight of the species (Table 2a). The strong relationship between THg and $\delta^{15}N$ observed in the present study (Fig. 3b) is consistent with the general understanding that mercury, in particular MeHg, tends to bioaccumulate in higher tropic level organisms (Senn et al., 2010). Interspecific mercury variation in fish has been attributed to size/mass in numerous studies (Choy et al., 2009), a trend which is also confirmed by the significant effect of weight on THg levels observed in the present study.

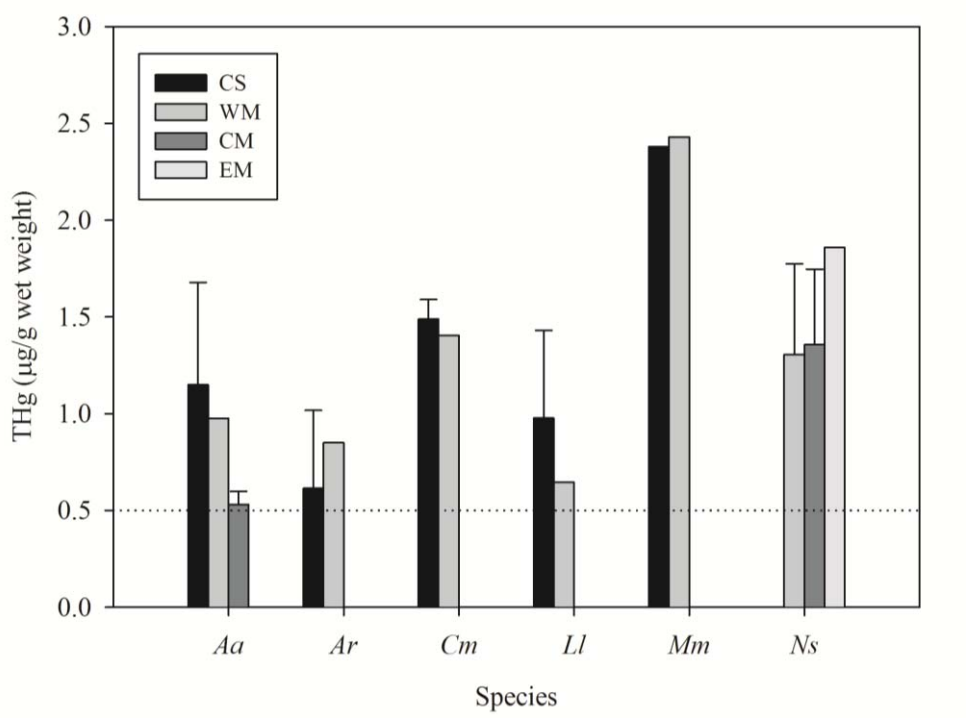

Fig. 2 Mean total mercury (THg) levels ± S.D. (µg/g wet weight) in six deep-sea species from various sampling sites across the Mediterranean Sea (Blanes and theWestern, Central and Eastern Mediterranean areas). For species abbreviation names see Table 1. Dashed line indicates the 0.5 µg/g w.w. recommended value for human consumption.

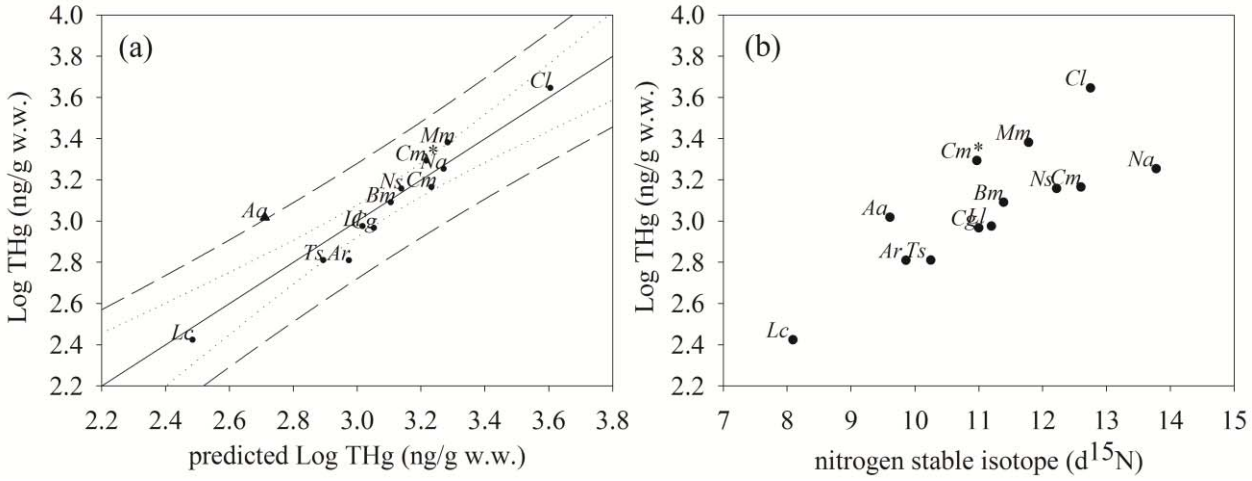

Fig. 3 (a) Measured versus predicted Log THg levels based on the equation for the linear regression for for 12 deep-sea fish species and the red shrimp *Aristeus antennatus*. (b) Relationship between Log THg levels (ng/g wet weight) nitrogen stable isotope values ($\delta^{15}N$). For species abbreviation names see Table 1.

Table 2 Linear regression results and Type III sum of squares of Log THg (ng/g w.w.) values in deep-sea fish (n = 12) as a function of depth of maximum abundance (DMA), nitrogen stable isotope ratio ($\delta^{15}N$) and average weight of the species.

Factors	Source	df	Sum of squares	Mean squares	F	Pr > F
a) DMA, $\delta^{15}N$, Weight	R^2 = 0.96, Log THg = 1.75E-04*DMA + 0.15*$\delta^{15}N$ + 4.75E-04*Weight + 1.04					
	Model	3	1.058	0.353	65.33	<.0001
	Error	8	0.043	0.005		
	Corrected Total	11	1.101			
	DMA	1	0.162	0.162	30.05	0.001
	$\delta^{15}N$	1	0.533	0.533	98.75	<.0001
	Weight	1	0.091	0.091	16.89	0.003
b) $\delta^{15}N$, Weight	R^2 = 0.81, Log THg = 0.17*$\delta^{15}N$ + 5.72E-04*Weight + 1.04					
	Model	2	0.896	0.448	19.62	0.001
	Error	9	0.205	0.023		
	Corrected Total	11	1.101			
	$\delta^{15}N$	1	0.731	0.731	32.02	0.000
	Weight	1	0.136	0.136	5.95	0.037

While the variation of mercury burden among species according to the trophic level and body mass is well understood, the influence of habitat depth on mercury accumulation is less clear. To illustrate the effect of DMA on THg, a linear regression model with $\delta^{15}N$ and weight was fitted (Table 2b) and predicted values were contrasted against measured THg levels in biota across depth ranges (Fig. 4). THg levels encountered in fish were lower than predicted based on $\delta^{15}N$ and weight at 500-1300 m depth and higher than predicted at 1500-2800 m depth (Fig. 4). The present results thus clearly indicate that mercury levels increase with increasing habitat depth in fish, with a particularly pronounced difference between the shallower depth range (500 - 900 m) and the highest depth of 2800 m. This increasing THg trend with depth is in accordance with former studies that reported elevated mercury levels in deep-sea fish from the Mediterranean Sea (Hornung et al., 1993; Storelli et al., 2002), as well the Atlantic (Monteiro et al., 1996; Mormede and Davies, 2001; Bebianno et al., 2007) and Pacific Ocean (Chiu and Mok, 2011). Furthermore, this increasing trend in Hg contamination with depth has been previously described in a study on large pelagic predators such as tuna in the North Pacific Ocean, where species that forage at greater depths accumulated higher mercury loads than species that feed on epipelagic prey (Choy et al., 2009).

In contrast to the deep-sea fish, the crustacean *A. antennatus* did not follow the same trend and the predicted THg value (based on $\delta^{15}N$ and weight, see equation in Table 2b)

was lower than the actual observed level, despite a relatively shallow DMA of 700 m (Fig. 4). This could be due to distinct reasons. For instance, the crustacean might present higher bioaccumulation kinetics for mercury than fish as a result of higher uptake through diet and/or lower metabolism. However, to our knowledge no information exists regarding differential Hg accumulation mechanisms between fish and crustaceans and further research is needed to corroborate this hypothesis. Another potential explanation arises from the difference in the depth profile of juvenile recruitment between the deep-sea fish species and the crustacean *A. antennatus*. Deep-sea fish recruitment takes place at shallower depths and juveniles progressively migrate to greater depths during their lifetime ("bigger-deeper") (*e.g.* Massutí et al., 1995; Morales-Nin et al., 1996; Rotllant et al., 2002). In contrast, the recruitment of *A. antennatus* occurs at greater depths and juveniles become more abundant with increasing depth ("smaller-deeper") (Sardà et al., 2004). Hence, it is possible that although adult *A. antennatus* are most abundant at 700 m, juveniles feed at greater depths, which could result in higher bioaccumulated mercury levels in adult shrimp than predicted by their trophic level.

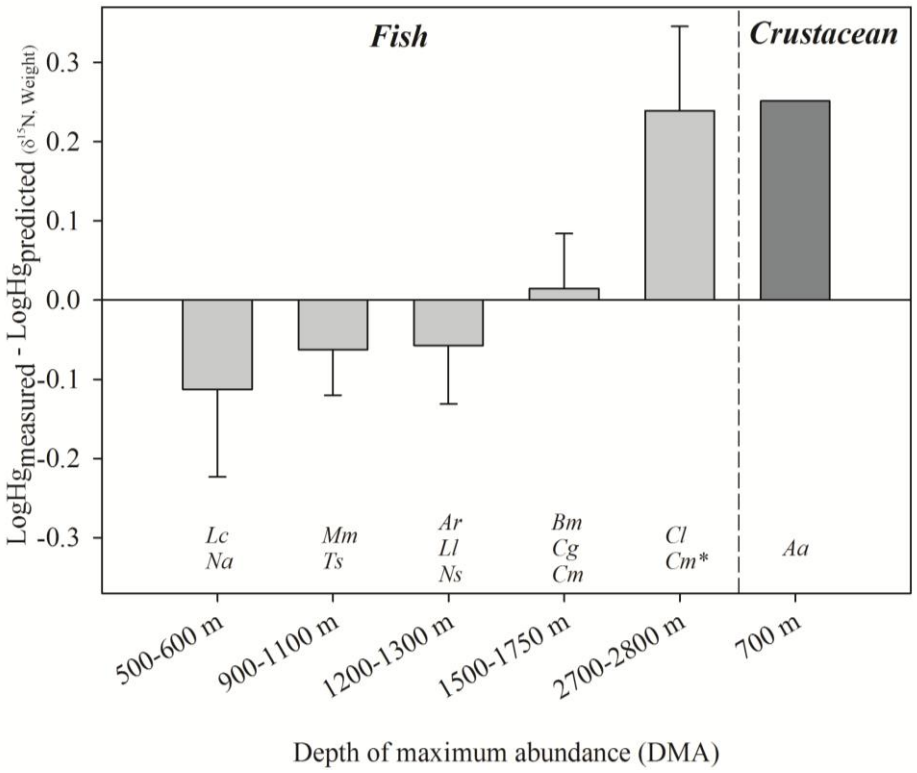

Fig. 4 Difference between measured and predicted Log THg levels (based on $\delta^{15}N$ and weight, see Table 3b) in fish from different depth ranges and the crustacean *A. antennatus.* Full species names and their respective depth of maximum abundance (DMA) are listed in Table 1. Values shown are mean ± S.E.M.

3.2. Public health issues

For the decapod crustacean *Aristeus antennatus* (red shrimp), a species of high commercial value, all samples (n = 13) exceeded the recommended value of 0.5 μg/g w.w. Although previous studies already detected relatively high levels of mercury in this species from the Ligurian Sea (Minganti et al., 1996; Drava et al., 2004), the present findings further stress the need to investigate the mercury content in this species and potentially issue advisories on the human consumption of red shrimp. This issue is particularly important considering the fact that shrimp are reported to generally contain lower mercury levels than most fish species and thus to be safe for human consumption, as recommended for instance by the US Food and Drug Administration (USFDA, 2002) or the Catalan Food Safety Agency (ACSA, 2008). However, these recommendations are based on values detected in shallow-water shrimp species (*e.g.* 0.12 μg/g w.w. in *Penaeus setiferus*; Domingo, 2008), which appear to have lower Hg content than *A. antennatus*.

The US Environmental Protection Agency (USEPA) adopted a revised reference dose (RfD) for MeHg of 0.1 μg mercury per kg body weight per day (USEPA, 1997). In 2006, a provisional tolerable weekly intake (PTWI) of 1.6 μg MeHg/kg body weight/week was established in the 67st meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006). We assumed that the concentration of total Hg is equal to that of MeHg (Bloom, 1992) and a body weight of 70 kg for adult males was also assumed. Therefore, based on these assumptions, the guideline value calculated from RfD of US EPA and PTWI of JECFA was 7.0 μg Hg/day and 16.1 μg Hg/day, respectively. Based on the mean Hg concentration in red shrimp in Table 1 (1.04 μg/g w.w.) and the shrimp consumption rate of 3.5 g/day in Catalonia, Spain (Domingo, 2008), dietary Hg exposure from shrimp consumption was estimated at 3.64 μg Hg/day, which was significantly lower than the US EPA and JECFA guidelines. However, the estimated exposure value represents 52 % of the RfD and 23 % of the PTWI for adult males, in contrast to the reported 2 % of PTWI deduced from Hg levels detected in shallow-water shrimp species (Domingo, 2008).

Moreover, the deep-sea fish species *Mora moro* (commom mora) is also commercially exploited and mercury levels in samples from the NW Mediterranean and the Central Mediterranean exceeded by far the 0.5 μg/g w.w. limit (Fig. 2). Although the other fish

species included in the present study are currently not relevant for human consumption, the fact that all except one were found to have higher mercury levels than the recommended value indicates that mercury contamination might be of particular concern for deep-sea fisheries. This issue is particularly relevant considering the fact that deep-water fisheries, which are of relatively modern origin, are continuously expanding and deep-sea fish are becoming increasingly important as a human food resource (Morales-Nin and Panfili, 2005; Ramirez-Llodra et al., 2011).

4. Conclusions

The present study has shown that apart from the species' trophic position and body mass, the habitat depth also significantly influences the THg accumulation in deep-sea fish. Fish species with a shallower depth distribution exhibited lower than expected THg concentrations while the contrary was observed for deeper-dwelling species. Thus, when taking into the account the trophic level and weight of each species, THg contamination appears to increase with habitat depth. This trend is concordant with observations from other studies on Hg accumulation in deep-sea fish throughout the world's oceans, and appears to be a general phenomenon rather than an exception. These findings are particularly relevant considering the fact that the exploitation of deep-sea waters is becoming increasingly important as a fisheries resource and the consumption of deep-sea organisms might be of particular concern with regard to human health. As shown in the present study, THg exceeded in all except one species the recommended 0.5 μg/g w.w. guideline value, including the highly valuable red shrimp *A. antennatus* and the commercially exploited fish *M. moro*.

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Paper 3

Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms

Samuel Koenig [a,b]* and Montserrat Solé [a]

[a] Institut de Ciències del Mar (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

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Resumen

La actividad enzimática se ha utilizado comúnmente como un biomarcador de efectos adversos en organismos acuáticos asociados a la presencia de contaminantes de origen antropogénico. Sin embargo, estas respuestas enzimáticas están sujetas a fluctuaciones estacionales importantes provocadas por la variabilidad natural de factores tanto bióticos (*e.g.* reproducción, tamaño, sexo) como abióticos (*e.g.* temperatura, salinidad). En este sentido, las aguas profundas del Mediterráneo noroccidental se consideran un medio muy estable con escasas fluctuaciones de temperatura y salinidad, sobre todo a profundidades superiores a los 200 m, por lo que las variaciones en las actividades enzimáticas en peces de gran profundidad relacionadas con factores abióticos son mínimas. En base a ello, el objetivo principal de este estudio fue determinar la variabilidad estacional de seis biomarcadores hepáticos: etoxiresorufina-O-deetilasa (EROD) o pentoxiresorufina-O-deetilasa (PROD), glutatión-S-transferasa (GST), carboxilesterasa (CbE), glutatión peroxidasa (GPX), glutatión reductasa (GR) y catalasa (CAT), en dos especies de peces, *Alepocephalus rostratus* y *Lepidion lepidion*, y la gamba roja, *Aristeus antennatus,* con el fin de estudiar la influencia de factores bióticos como el sexo, tamaño y actividad reproductiva de las especies, en las respuestas enzimáticas. Los resultados obtenidos mostraron fluctuaciones significativas de los biomarcadores estudiados en las tres especies, relacionadas sobre todo con la variación estacional del desarrollo gonadal y la disponibilidad de alimento. También se realizó un estudio de la variación de las respuestas enzimáticas con la profundidad, pero no se detectó ninguna tendencia clara, por lo que se concluyó que las fluctuaciones en profundidad no estaban relacionadas con ninguno de los factores estudiados.

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Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms

Samuel Koenig [a,b,*], Montserrat Solé [a]

[a] Instituto de Ciencias del Mar (ICM-CSIC), Paseo Marítimo de la Barceloneta 37-49, 08003 Barcelona, Spain
[b] Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain

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ABSTRACT

Biomarker assays are widely used as proxies for contaminant-induced effects in aquatic organisms. However, in many cases, their intrinsic natural variability due to exogenous and endogenous factors makes the interpretation of biomarker data difficult. In the present study, we investigated the natural fluctuations of six hepatic biomarkers, namely ethoxyresorufin-*O*-deethylase (EROD) in fish and pentoxyresorufin-*O*-deethylase (PROD) in crustacea, catalase (CAT), carboxylesterase (CbE), glutathione-*S*-transferase (GST), total glutathione peroxidase (GPX) and glutathione reductase (GR) in two deep-sea fish species, namely *Alepocephalus rostratus* and *Lepidion lepidion* and the decapod crustacean *Aristeus antennatus*. The NW Mediterranean deep-sea environment is characterized by very stable temperature and salinity conditions, allowing the exclusion of these two factors as potential sources of interference with biomarker activities. Biomarker results exhibited a clear influence of reproductive processes on enzyme activities, in particular in *A. rostratus*, which presented a pronounced seasonal pattern linked to variations in the gonadosomatic index (GSI). In addition, other factors such as food availability may also have influenced the observed variability, in particular in specimens of *L. lepidion*, which did not exhibit variations in reproductive activity throughout the sampling period. Depth-related variability did not exhibit a clear trend and fluctuations across sampling depths were not attributable to any specific factor. Body size had also a significant influence on some biomarkers, although allometric scaling of certain enzyme activities appears to be species-specific. The present work has thus shown that despite the lack of fluctuations of abiotic parameters such as temperature and salinity, biomarker activities in deep-sea organisms still exhibit significant variability, mainly as a result of reproductive processes and food availability.

1. Introduction

Biomarkers have been defined as measures of changes in biological parameters resulting from contaminant exposure and their use has been advocated as a means to provide early detection of exposure and adverse effects of pollutants on aquatic organisms (Peakall, 1992). In this context, a number of parameters have been investigated to assess chemical-induced disturbances of biological functions (van der Oost et al., 2003). However, it is very unlikely that a single biomarker response can unequivocally provide a measure of environmental degradation and the use of a suite of biomarkers has thus been advocated (Handy et al., 2003; Galloway et al., 2004). In particular, biomarkers are susceptible to natural variability due to abiotic (*e.g.* temperature, salinity, dissolved oxygen) and biotic factors (*e.g.* gender, age, size, reproductive stage) (Whyte et al., 2000; van der Oost et al., 2003; Martínez-Álvarez et al., 2005). These confounding factors can sometimes mask the effect of contaminant-induced stress signals and impede the interpretation of biomarker results (Sheehan and Power, 1999). For the practical application of biomarkers there are several options to minimize their variability such as the careful experimental design of field studies, data normalization and the characterization of confounding environmental and biological factors (Flammarion and Garric, 1999; Handy et al., 2003; Sanchez et al., 2008). To be able to implement biomarkers in environmental monitoring studies it is thus crucial to previously establish baseline levels and characterize the natural variability of these assays.

The suite of hepatic biomarkers used in the present study included xenobiotic metabolism enzymes such as ethoxyresorufin-*O*-deethylase (EROD) in fish and pentoxyresorufin-*O*-deethylase (PROD) in crustacea, glutathione-S-transferases (GST) and carboxylesterases (CbE) as well as enzymatic antioxidant defenses, such as

* Corresponding author. Instituto de Ciencias del Mar (ICM-CSIC), Paseo Marítimo de la Barceloneta 37-49, 08003 Barcelona, Spain. Tel.: +34 93 230 95 00; fax: +34 93 230 95 55.
E-mail address: koenig@icm.csic.es (S. Koenig).

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catalase (CAT), glutathione-peroxidase (GPX) and glutathione reductase (GR). The EROD and PROD assays are commonly used as proxies for CYP1A- and CYP2B-mediated phase I metabolism, respectively (Goksøyr and Förlin, 1992; Koenig et al., 2012b), which is responsible for the biotransformation (mainly oxidation) of numerous endogenous and exogenous compounds in fish and crustacea, respectively. CbEs are also categorized as phase I drug metabolizing enzymes involved in the hydrolysis of ester-containing chemicals (Satoh and Hosokawa, 2006; Wheelock et al., 2008). GST enzymes form part of the phase II metabolism, which involves the conjugation of the xenobiotic compound or its metabolite with an endogenous molecule (*e.g.* glutathione) to facilitate excretion (Nimmo, 1987). Moreover, these enzymes can also function as antioxidant enzymes catalyzing the reduction of organic hydroperoxides (Wang and Ballatori, 1998). Other antioxidant enzymes that inhibit the formation of reactive oxygen species (ROS) are CAT, which is responsible for the reduction of H_2O_2, GPX, which catalyzes the reduction of peroxides to their corresponding alcohols and GR, which maintains the homeostasis between GSH/GSSG under oxidative stress conditions (Winston and Di Giulio, 1991).

The Mediterranean deep-sea (>400 m) is characterized by fairly stable temperatures, and salinity (Danovaro et al., 2010), although episodic events can cause pronounced fluctuations in hydrological parameters and particle fluxes (Heussner et al., 2006; López-Fernández et al., 2012). In particular, episodic dense-shelf water cascading (DSWC) events have been shown to take place in the NW Mediterranean every 6–10 years (Canals et al., 2006; Company et al., 2008). During these events, cold shelf water masses cascade down the continental slope transporting large amounts of sediment and organic matter, resulting in an increased particle-associated contaminant input to the deep-sea environment (Salvadó et al., 2012). In addition, previous work has shown that deep-sea organisms dwelling within submarine canyons in the NW Mediterranean are particularly at risk of experiencing adverse contaminant-induced effects (Koenig et al., 2012a). These findings further stress the need for the implementation of regular environmental monitoring studies in these areas.

The species selected for the present study include the deep-sea fish *Alepocephalus rostratus* (Alepocephaliform), *Lepidion lepidion* (Gadiform) and the crustacean *Aristeus antennatus* (Decapoda). *A. rostratus* can be found in the eastern Atlantic and north-western Mediterranean from 500 m up to 2300 m depth, with maximum aggregations at midslope depths between 1000 m and 1450 m (Morales-Nin et al., 1996). *L. lepidion* is mainly found in the NW Mediterranean and has a wide bathymetric distribution (500–2300 m), although it is most abundant at the lower depths of the continental slope (Rotllant et al., 2002). *A. Antennatus* is a eurybathic species with a known depth range from 80 m to 3300 m, which can be found throughout the Mediterranean Sea and along the NW African coast. This shrimp species is also one of the most valuable fishery resources in the Mediterranean (Company et al., 2008). All three species have been previously used in environmental monitoring studies conducted in Mediterranean deep-sea habitats (Escartin and Porte, 1999; Porte et al., 2000; Antó et al., 2009; Solé et al., 2009, 2010).

The main objective of the present study was to characterize baseline levels and the natural variability of selected hepatic biomarkers in two deep-sea fish and a crustacean species. We determined EROD or PROD, respectively, GST, CbE, CAT, GPX and GR activities in the fish *A. rostratus* and *L. Lepidion* and the crustacean *A. antennatus* from four seasonal sampling periods and different sampling depths. Furthermore, the relationship between biomarker activities and biological parameters (*e.g.* size, gender, sexual maturity) of the sampled organisms was investigated.

2. Materials and methods

2.1. Collection of animals and sampling site

Seasonal sampling cruises were carried out off the coast of Blanes, north-western Mediterranean (41°15′N 2°50′E) onboard the R/V *Garcia del Cid* in winter (February), spring (May), summer (September) and autumn (November) in 2009. Fish were caught using a OTMS otter trawl (Sardà et al., 1998) at various water depths ranging from 900 m to 2000 m (Fig. 1). The OTMS is a benthic trawling net with a cod-end mesh size of 40 mm fitted with two divergent doors and a single warp cable. Total trawl times, including net deployment and retrieval, ranged between 1.5 and 3 h depending on sampling depth (winch speed 70 m/s), with bottom haul times of 40–60 min. Only animals dissected within 2 h of net retrieval were considered for biochemical analyses. Body size, weight and sex were recorded and the liver/hepatopancreas was dissected and frozen in liquid nitrogen and stored at −80 °C until further analysis. GSI values were only available for *A. rostratus* as the gonad weight could not be recorded for *L. lepidion* and *A. antennatus* due to technical limitations onboard the vessel. Number of individuals sampled for each season and depth are shown in Tables 1 and 3, respectively.

2.2. Sample preparation

A portion of liver/hepatopancreas (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing for fish liver 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMFS), 1 mM ethylenediaminetetraacetic acid (EDTA) and for crustacean hepatopancreas 100 mM KCl, 1 mM EDTA, 0.1 mM phenanthroline and 0.1 mg/L trypsin inhibitor. The homogenate was centrifuged at 10,000 g for 30 min and the obtained supernatant (S9) was stored at −80 °C until further biochemical analyses.

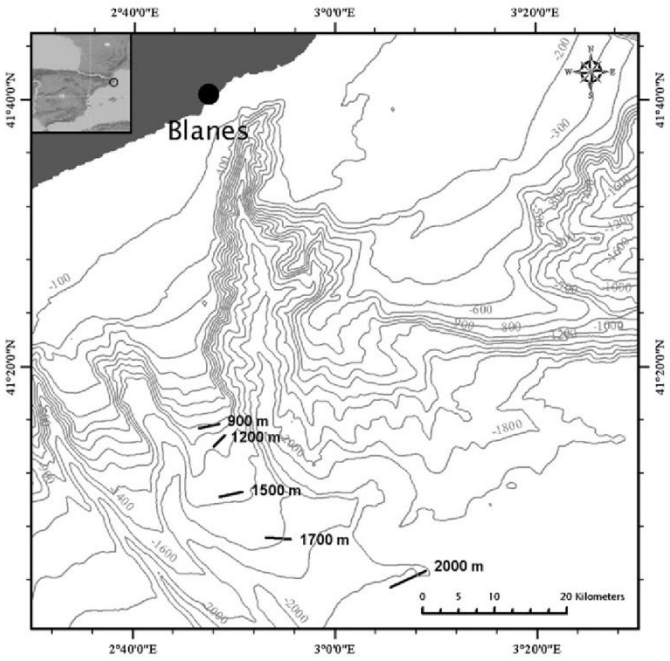

Fig. 1. Location of sampling sites off the coast of Blanes, NW Mediterranean. Map created by J.A. García, using ESRI® ArcMap™ 9.3 and bathymetric data from Canals et al. (2004).

Table 1
Seasonal variation of biological parameters of selected deep-sea species. Values are shown as mean ± S.E.M. Different letters denote significant differences between seasons based on Tukey's HSD multiple comparisons ($P < 0.05$).

Species	Parameter	Sex	Winter	Spring	Summer	Autumn
A. rostratus	Sample size	M	8	8	7	9
		F	7	9	9	9
	Size (mm)	M	298.4 ± 16.2^{ab}	326.4 ± 13.0^{a}	301.3 ± 9.1^{ab}	265.8 ± 13.6^{b}
		F	338.5 ± 15.7^{b}	387.1 ± 4.1^{a}	338.8 ± 5.1^{b}	315.9 ± 14.7^{b}
	GSI (%)	M	1.09 ± 0.18^{c}	1.45 ± 0.33^{c}	7.56 ± 0.85^{a}	4.91 ± 0.70^{b}
		F	2.08 ± 0.75^{b}	1.02 ± 0.15^{b}	7.57 ± 1.81^{a}	2.85 ± 1.01^{b}
L. lepidion	Sample size		8	10	10	10
	Size (mm)		161.1 ± 6.2^{b}	207.0 ± 4.2^{a}	201.8 ± 3.5^{a}	197.3 ± 5.3^{a}
A. antennatus	Sample size		30	30	30	30
	Size (mm)		45.1 ± 2.3^{a}	47.9 ± 2.0^{a}	41.3 ± 2.2^{a}	32.2 ± 1.9^{b}

2.3. Biochemical analysis

All assays were carried out in triplicate at 25 °C in 96-well format using a TECAN™ Infinite M200 microplate reader. For each assay, blank samples were analyzed in triplicate, which were used to correct for background activity. Prior to analysis, assay conditions were optimized for each species by determining the appropriate dilution of the S9 supernatant (protein content 5–10 mg/mL) for each assay to ensure constant linearity of the measured activity (dilutions for each species shown below in parenthesis for each assay). All reaction mixtures, except for catalase, contained 100 mM phosphate buffer pH 7.4.

Catalase (CAT) activity was measured in a UV-transparent microplate (Greiner UV-Star®) as absorbance decrease at 240 nm for 1 min using 50 mM H_2O_2 as substrate ($\varepsilon = 40$ mol^{-1} cm^{-1}) and a 100 mM phosphate buffer pH 6.5 (Aebi, 1974). Sample volume used was 10 μL (*Ar* 1:400, *Ll* 1:200, *Aa* 1:2) in a total volume of 210 μL.

Glutathione reductase (GR) activity was measured as decrease in absorbance at 340 nm for 3 min using 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) ($\varepsilon = 6.22$ mmol^{-1} cm^{-1}) and 0.9 mM oxidized glutathione (GSSG) as substrate (Carlberg and Mannervik, 1985). Sample volume used was 20 μL (not diluted) in a total volume of 200 μL.

Total glutathione-peroxidase (GPX) activity was determined as decrease in absorbance at 340 nm during 3 min using 2.5 mM reduced glutathione (GSH), 1 mM glutathione reductase (GR), 0.625 mM cumene hydroperoxide (CHP) and 0.3 mM NADPH ($\varepsilon = 6.22$ mmol^{-1} cm^{-1}) (Günzler and Flohe, 1985). Sample volume used was 10 μL (not diluted) in a total volume of 240 μL.

Glutathione-S-transferase (GST) activity was measured as increase in absorbance at 340 nm for 3 min using 1 mM 1-chloro-2,4- dinitrobenzene (CDNB) ($\varepsilon = 9.6$ mmol^{-1} cm^{-1}) and 1 mM GSH as substrate (Habig et al., 1974). Sample volume used was 25 μL (*Ar* 1:20, *Ll* 1:20, *Aa* 1:20) in a total volume of 225 μL.

Carboxylesterase (CbE) activity was determined as increase in absorbance at 405 nm during 5 min using 0.18 mM 5,5-dithio-bis-2-nitrobenzoate (DTNB) ($\varepsilon = 13.6$ mmol^{-1} cm^{-1}) and 0.67 mM S-phenylthioacetate as substrate (Ellman et al., 1961). Sample volume used was 25 μL (*Ar* 1:5, *Ll* 1:5, *Aa* 1:20) in a total volume of 225 μL.

7-Ethoxyresorufin-O-deethylase (EROD) activity for fish and *7-Pentoxyresorufin-O-deethylase* (PROD) activity in crustacea were measured kinetically as increase in fluorescence at 537 nm excitation and 583 nm emission over 10 min based on the procedure by Burke and Mayer (1974). Substrates used include 7-ethoxyresorufin (3 μM) and 7-pentoxyresorufin (5 μM), respectively and 0.2 mM NADPH with a seven-point curve of resorufin sodium salt standard. Sample volume used was 50 μL (not diluted) in a total volume of 250 μL.

Protein content was determined according the method by (Bradford, 1976), using bovine serum albumin as standard (BSA 0.1–1 mg/ml). Sample volume used was 10 μL (*Ar* 1:20, *Ll* 1:40, *Aa* 1:20) in a total volume of 260 μL.

2.4. Statistical analysis

Data were checked for normality (Shapiro–Wilk's test) and homogeneous variance (Levene's test) and were $\log_{10}(x)$-transformed for parametric *t*-test/ANOVA/ANCOVA analyses followed by Tukey's HSD test for multiple comparisons. Correlations were determined using Pearson's correlation coefficient. Differences at the 5% significance level were considered significant. In the case of significant correlations between enzyme activities and body size, ANCOVA tests were performed introducing size as covariable in the model. The interaction factors (*e.g.* Sex*Size) were only included in the model if significant (unequal slopes) (Engqvist, 2005).

3. Results

Temperature and salinity exhibited very little seasonal fluctuations with a maximum variation of 0.2 °C temperature and 0.04 PSU salinity across seasons. The depth profile exhibited a slight increase in salinity from 900 m to 1500 m depth (approx. 0.3 PSU), while temperature was 0.2 °C higher at 900 m compared to all lower depths (Tecchio et al., 2012).

3.1. Biomarkers in A. rostratus

Male and female *A. rostratus* differed significantly in size, with females being larger than males (*t*-test, $t = 4.79$, $P < 0.0001$). Moreover, males and females exhibited significant seasonal differences in body size and GSI (Table 1). Because some biomarker activities varied between sexes (*i.e.* EROD and GST), seasonal comparisons were conducted for males and females separately. Due to the segregated sex distribution of *A. rostratus* at different depths, comparisons among depths could not be performed for this species. All statistical results are given in Table 2.

Overall, a negative correlation was observed between EROD, CbE and GPX activity and body size. EROD, GST, CbE and GPX differed significantly between sexes, although in the case of CbE and GPX this difference was mainly due to size. Moreover, EROD and GST activities were significantly correlated negatively with the GSI in females, while CbE and CAT exhibited a negative correlation with the GSI in both sexes. All biomarkers, except CAT, exhibited seasonal variations in females, whereas CbE, GR and CAT fluctuated significantly in males (Fig. 2 and Table 2).

3.2. Biomarkers in L. lepidion

There was no significant difference in size between male and female *L. lepidion*, but size differed among seasons (Table 1) and

Table 2
Details for statistical analyses of biomarker data. Table shows Pearson's correlation for size and GSI, as well *t*-test/ANCOVA for contrasts between sex, and ANOVA/ANCOVA for seasonal and depth comparisons. In the case of significant correlations between enzyme activities and size, ANCOVA tests were performed introducing size as covariable in the model. Effect size is reported as partial eta-squared values (η^2) based on Type III sum of squares.

Species	Factor		EROD		GST		CbE		GPX		GR		CAT	
Alepocephalus rostratus														
	Size	($n = 120$)	$R = -0.37^{***}$		n.s.		$R = -0.26^{**}$		$R = -0.38^{***}$		n.s.		n.s.	
	Sex	($n = 120$)	ANCOVA***	$F = 20.19$	*t*-test*	$t = 2.11$	ANCOVA**	$F = 5.23$	ANCOVA***	$F = 12.14$	*t*-test	n.s.	*t*-test	n.s.
			Sex**	$\eta^2 = 0.11$	**Sex** *	$\eta^2 = 0.03$	Sex	n.s.	Sex*	$\eta^2 = 0.03$	Sex	n.s.	Sex	n.s.
			Size*	$\eta^2 = 0.04$	Size	n.i.	**Size***	$\eta^2 = 0.04$	**Size****	$\eta^2 = 0.08$	Size	n.i.	Size	n.i.
	GSI	Males ($n = 60$)	n.s.		n.s.		$R = -0.31^{*}$		n.s.		n.s.		$R = -0.62^{***}$	
		Females ($n = 60$)	$R = -0.37^{*}$		$R = -0.48^{**}$		$R = -0.30^{*}$		n.s.		n.s.		$R = -0.55^{***}$	
	Season	Males	ANCOVA	n.s.	ANOVA	n.s.	ANCOVA***	$F = 9.43$	ANCOVA	n.s.	ANOVA*	$F = 3.49$	ANOVA***	$F = 8.84$
		1200 m	Season	n.s.	Season	n.s.	**Season****	$\eta^2 = 0.54$	Season	n.s.	**Season***	$\eta^2 = 0.27$	**Season****	$\eta^2 = 0.49$
		($n = 32$)	Size	n.s.			Size	n.s.	Size	n.s.				
		Females	ANCOVA*	$F = 3.48$	ANOVA***	8.90	ANCOVA*	$F = 5.75$	ANCOVA***	$F = 7.18$	ANOVA**	$F = 5.10$	ANOVA	n.s.
		1500 m	**Season***	$\eta^2 = 0.48$	**Season****	$\eta^2 = 0.48$	**Season***	$\eta^2 = 0.28$	**Season****	$\eta^2 = 0.34$	**Season****	$\eta^2 = 0.35$	Season	n.s.
		($n = 34$)	Size	n.s.			Size	n.s.	Size	n.s.				
Lepidion lepidion														
	Size	($n = 78$)	n.s.		$R = 0.39^{***}$		n.s.		n.s.		n.s.		n.s.	
	Sex	($n = 78$)	*t*-test	n.s	ANCOVA	n.s	*t*-test	n.s	*t*-test	n.s	*t*-test	n.s	*t*-test	n.s
	Season	1200 m	ANOVA***	$F = 14.18$	ANCOVA***	$F = 9.57$	ANOVA***	$F = 47.58$	ANOVA***	$F = 10.29$	ANOVA***	$F = 14.03$	ANOVA***	$F = 10.08$
		($n = 38$)	**Season****	$\eta^2 = 0.56$	**Season****	$\eta^2 = 0.49$	**Season****	$\eta^2 = 0.81$	**Season****	$\eta^2 = 0.49$	**Season****	$\eta^2 = 0.55$	**Season****	$\eta^2 = 0.47$
					Size	n.s.								
	Depth	autumn	ANOVA	n.s.	ANCOVA*	$F = 3.01$	ANOVA***	$F = 24.87$	ANOVA	n.s.	ANOVA**	$F = 4.11$	ANOVA	n.s.
		($n = 50$)	Depth	n.s.	Depth	n.s.	**Depth****	$\eta^2 = 0.69$	Depth	n.s.	**Depth****	$\eta^2 = 0.27$	Depth	n.s.
					Size**	$\eta^2 = 0.17$								
			PROD		GST		CbE		GPX		GR		CAT	
Aristeus antennatus														
	Size	($n = 138$)	n.s.		n.s.		$R = -0.40^{***}$		n.s.		$R = -0.23^{**}$		$R = 0.29^{**}$	
	Sex	($n = 138$)	*t*-test	n.s.	*t*-test	n.s.	ANCOVA***	$F = 13.26$	*t*-test	n.s.	ANCOVA*	$F = 3.80$	ANCOVA**	$F = 5.57$
							Sex	n.s.			Sex	n.s.	Sex	n.s.
							Size**	$\eta^2 = 0.13$			**Size***	$\eta^2 = 0.04$	**Size***	$\eta^2 = 0.05$
	Season	($n = 120$)	ANOVA	n.s.	ANOVA***	$F = 10.36$	ANCOVA***	$F = 21.82$	ANOVA***	$F = 6.34$	ANCOVA	n.s.	ANCOVA***	$F = 17.79$
			Season	n.s.	**Season****	$\eta^2 = 0.21$	Season***	$\eta^2 = 0.22$	**Season****	$\eta^2 = 0.14$	Season	n.s.	**Season****	$\eta^2 = 0.59$
							Size**	$\eta^2 = 0.35$			Size	n.s.	Size	n.s.
	Depth	autumn	ANOVA*	$F = 3.24$	ANOVA***	$F = 6.30$	ANCOVA***	$F = 18.12$	ANOVA***	$F = 12.26$	ANCOVA	n.s.	ANCOVA	n.s.
		($n = 48$)	**Depth***	$\eta^2 = 0.24$	**Depth****	$\eta^2 = 0.37$	**Depth****	$\eta^2 = 0.32$	**Depth****	$\eta^2 = 0.54$	Depth	n.s.	Depth	n.s.
							Size***	$\eta^2 = 0.09$			Size	n.s.	Size	n.s.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
n.s.; not significant ($P > 0.05$).

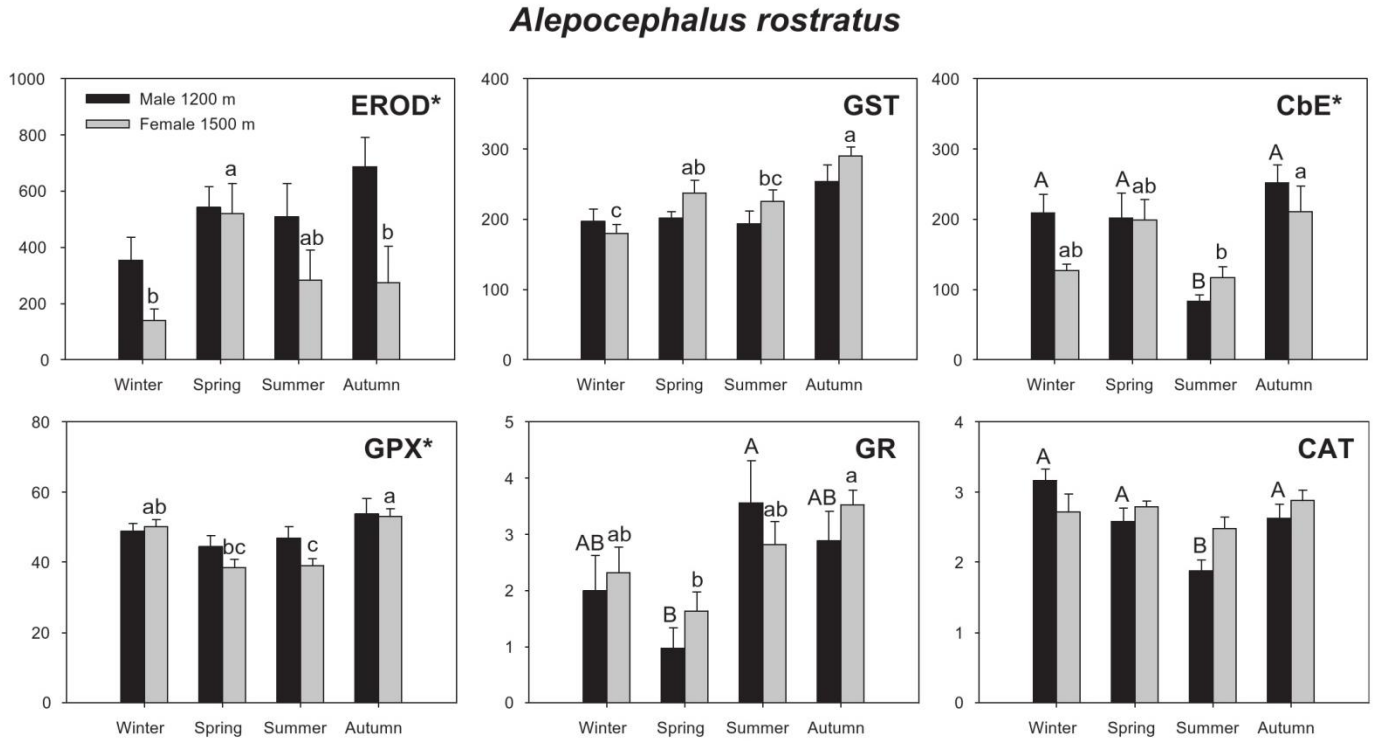

Fig. 2. Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in male (black) and female (gray) *A. rostratus* in winter (M: $n = 8$; F: $n = 7$), spring (M: $n = 8$; F: $n = 9$), summer (M: $n = 7$; F: $n = 9$) and autumn (M: $n = 9$; F: $n = 9$). All activities are expressed as nmol min^{-1} mg $protein^{-1}$ except for EROD (fmol min^{-1} mg $protein^{-1}$). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

depths (Table 3). Seasonal variation was investigated in samples from 1200 m depth and the depth-related variability was assessed in fish collected during autumn from 900 m to 2000 m depth (Table 3). Details for statistical analyses are also given in Table 2.

Out of the six biomarkers analyzed, only GST activity exhibited a significant relationship with body size. Moreover, no differences in enzyme activities were detected between sexes and results are thus presented together regardless of sex. However, all enzyme activities, except GPX, varied seasonally (Fig. 3). EROD and CbE activity exhibited a peak in spring, while GST, CAT and GR activities were lower during summer (Fig. 3). Moreover, CbE and GR activities differed significantly among sampling depths, while the depth-related variations in GST activity were due to differences in body size (Table 3).

3.3. Biomarkers in A. antennatus

Carapace size differed significantly between sexes and female *A. antennatus* were significantly larger than males (*t*-test, $t = 8.47$, $P < 0.0001$). Moreover, carapace size also differed among seasons (Table 1) and depths (Table 3). Seasonal variations were assessed combining data from several depths (*i.e.* 900 m, 1200 m and 1500 m), while the effect of depth was determined in samples collected in autumn (Table 3). Details for statistical analyses are also given in Table 2.

CbE and GR activities exhibited a negative correlation with carapace size, whereas for CAT it was positive. These three biomarkers presented differences in activities between sexes, but in all three cases the variability was attributed to differences in size.

Table 3
Biomarker data (mean ± S.E.M.) for the fish *Lepidion lepidion* and the crustacean *Aristeus antennatus* sampled at different depths during autumn 2009. All activities are expressed as nmol min^{-1} mg $protein^{-1}$ except for EROD (pmol min^{-1} mg $protein^{-1}$), PROD (fmol min^{-1} mg $protein^{-1}$) and CAT (mmol min^{-1} mg $protein^{-1}$). Different letters denote significant differences between depths based on Tukey's HSD multiple comparisons ($P < 0.05$).

Species	Depth (m)	Size (mm)	EROD	GST	CbE	GPX	GR	CAT
L. lepidion								
$n = 10$	900	185.9 ± 5.8 b	8.2 ± 2.7	335.1 ± 22.5	50.1 ± 2.2 a	51.9 ± 2.9	3.6 ± 0.7 b	0.9 ± 0.1
$n = 10$	1200	197.3 ± 5.3 b	4.0 ± 0.7	301.6 ± 18.4	24.2 ± 1.4 b	45.5 ± 2.2	6.3 ± 0.5 a	1.2 ± 0.2
$n = 10$	1500	217.6 ± 14.4 ab	4.1 ± 2.2	345.2 ± 14.9	31.8 ± 3.2 b	34.6 ± 1.7	3.8 ± 0.4 b	1.0 ± 0.1
$n = 10$	1700	244.8 ± 16.9 a	4.1 ± 0.9	469.2 ± 40.2	35.4 ± 3.6 b	43.1 ± 3.4	4.0 ± 0.3 ab	0.9 ± 0.1
$n = 10$	2000	224.0 ± 10.3 ab	3.9 ± 1.1	387.1 ± 43.2	75.3 ± 7.9 a	48.5 ± 2.6	4.3 ± 0.5 ab	1.1 ± 0.1
	Depth (m)	Size (mm)	PROD	GST	CbE	GPX	GR	CAT
A. antennatus								
$n = 10$	900	43.0 ± 3.1 a	155.7 ± 16.9 ab	67.5 ± 11.4 b	360.8 ± 23.6 a	138.6 ± 8.0 c	0.9 ± 0.3	7.7 ± 1.2
$n = 10$	1200	29.0 ± 1.4 b	204.0 ± 20.6 a	89.2 ± 21.5 b	700.1 ± 69.9 a	220.2 ± 20.2 a	1.3 ± 0.2	n.a.
$n = 10$	1500	24.5 ± 1.6 b	197.5 ± 62.5 a	241.4 ± 70. a	795.4 ± 81.0 a	310.8 ± 48.5 a	1.8 ± 0.4	3.1 ± 1.4
$n = 8$	1700	26.6 ± 2.4 b	183.5 ± 79.7 ab	268.6 ± 88.8 a	464.1 ± 34.8 ab	208.8 ± 19.2 ab	1.6 ± 0.4	6.9 ± 0.7
$n = 10$	2000	33.5 ± 3.1 ab	101.7 ± 15.4 b	118.4 ± 20.3 ab	306.9 ± 39.9 b	149.2 ± 13.3 bc	1.5 ± 0.5	6.4 ± 1.4

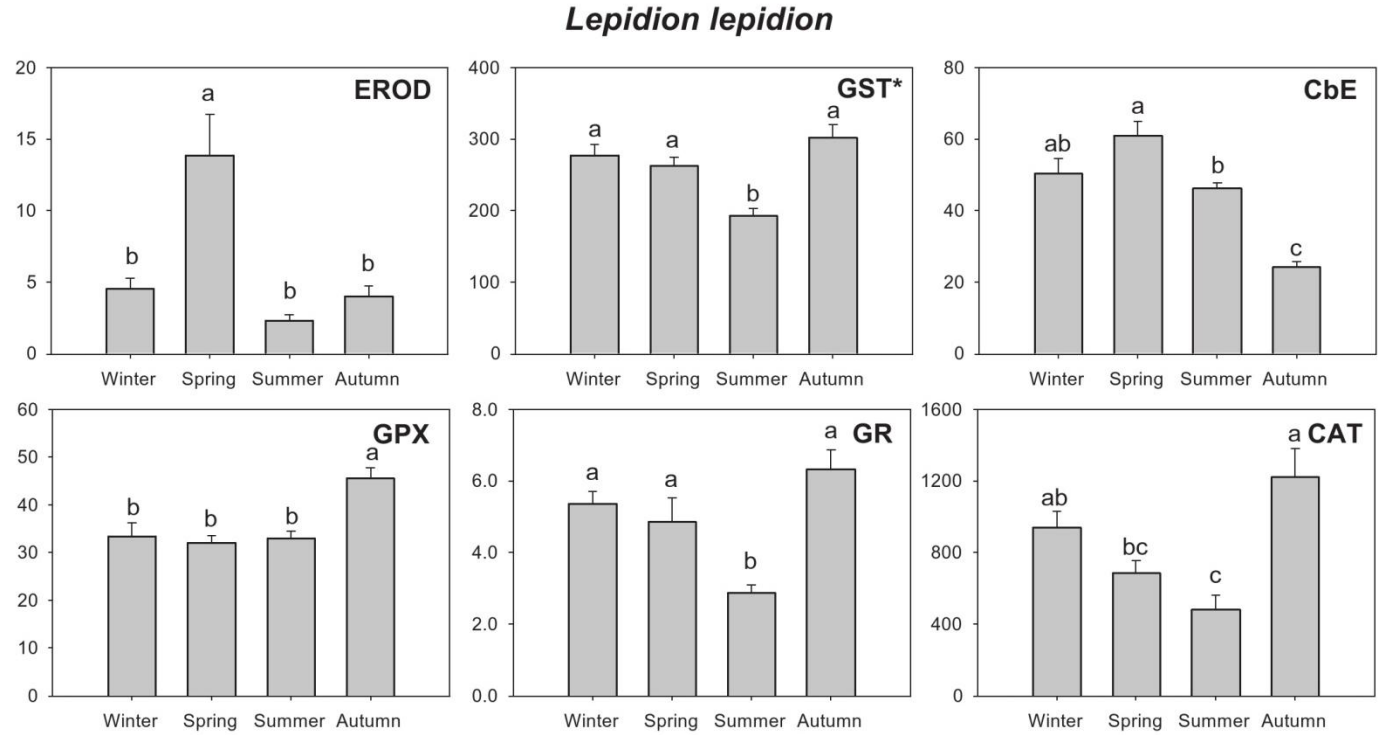

Fig. 3. Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in *Lepidion lepidion rostratus* in winter ($n = 8$), spring ($n = 10$), summer ($n = 10$) and autumn ($n = 10$). All activities are expressed as nmol min^{-1} mg $protein^{-1}$ except for EROD (pmol min^{-1} mg $protein^{-1}$) and CAT (μmol min^{-1} mg $protein^{-1}$). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

PROD, CbE, GPX and CAT presented a peak in activity in spring, while a significant decline in GST activity was observed in autumn (Fig. 4). ANCOVA results indicated that seasonal variations in CbE activity were mainly due to differences in body size. PROD, GST, CbE and GPX varied significantly among sampling depths, although no clear pattern was observed (Table 3).

4. Discussion

Seasonal and depth-related fluctuations of water temperature and salinity in this deep-sea environment were minimal and up to two orders of magnitude lower than the variations of these parameters reported for studies that observed seasonal variations of biomarkers in fish from coastal areas of the Baltic Sea (Kopecka and Pempkowiak, 2008), the Adriatic Sea (Pavlović et al., 2010) or the Arctic Ocean (Nahrgang et al., 2010). Hence, the influence of these two abiotic parameters on the variability of biomarker activities among seasons and sampling depths is likely to be negligible. Furthermore, as the biomarkers included in the present study are used as proxies for contaminant exposure, variations in contamination levels could also have influenced the presented results. However, as shown by Gómez-Gutiérrez et al. (2007), organic contaminant levels in Mediterranean offshore sediments (>1000 m) are considered background contamination levels for the region due to the remoteness from pollution sources and exhibit low temporal variability. Moreover, although the transfer of pollutants from NW Mediterranean surface waters to the deep-sea has been shown to increase during episodic dense-shelf water cascading (DSWC) events (Salvadó et al., 2012), no such event occurred in 2009 when the present study was conducted, with the last one registered during the winter 2005/06. In addition, chemical analyses of biota from the study area did not reveal any seasonal changes in contamination levels (author's unpublished data) and it is thus assumed that biomarker results from the present work are likely not affected by variations in pollution levels.

4.1. A. rostratus

Numerous studies have shown that differences in sex and body size can influence enzymatic activities and complicate the interpretation of biomarker results (van der Oost et al., 2003). As female *A. rostratus* were significantly larger than males, it is important to determine whether the observed gender-related differences were actually due to differential enzyme activities or resulted from the above-mentioned sexual dimorphism. In the present study, EROD, GST, CbE and GPX activities varied significantly between sexes and all, except GST, were higher in males than females. Moreover, significant overall correlations with body size were observed for all the above-mentioned biomarkers, except GST, suggesting that for the latter enzyme, differences between sexes cannot be attributed to size. The gender-dependent EROD activity, with significantly higher activities in liver of male fish, is a well documented phenomenon (Whyte et al., 2000). CYP1A-related EROD activity has been shown to be suppressed in mature females by 17β-estradiol and a decline of CYP1A activity is usually observed from the onset of ovulation until spawning (Whyte et al., 2000). Moreover, EROD activity showed a negative relationship with GSI in females, which is consistent with other studies (Flammarion et al., 1998; Kopecka and Pempkowiak, 2008). Although mature individuals of *A. rostratus* were found all year round, a peak in maturation usually occurs from summer to autumn, when spawning activity is highest (Morales-Nin et al., 1996; Follesa et al., 2007). Accordingly, the individuals analyzed in the present study presented the highest GSI during summer and autumn, while at the end of the spawning period in spring the GSI was at its lowest (Table 1). In fact, the end

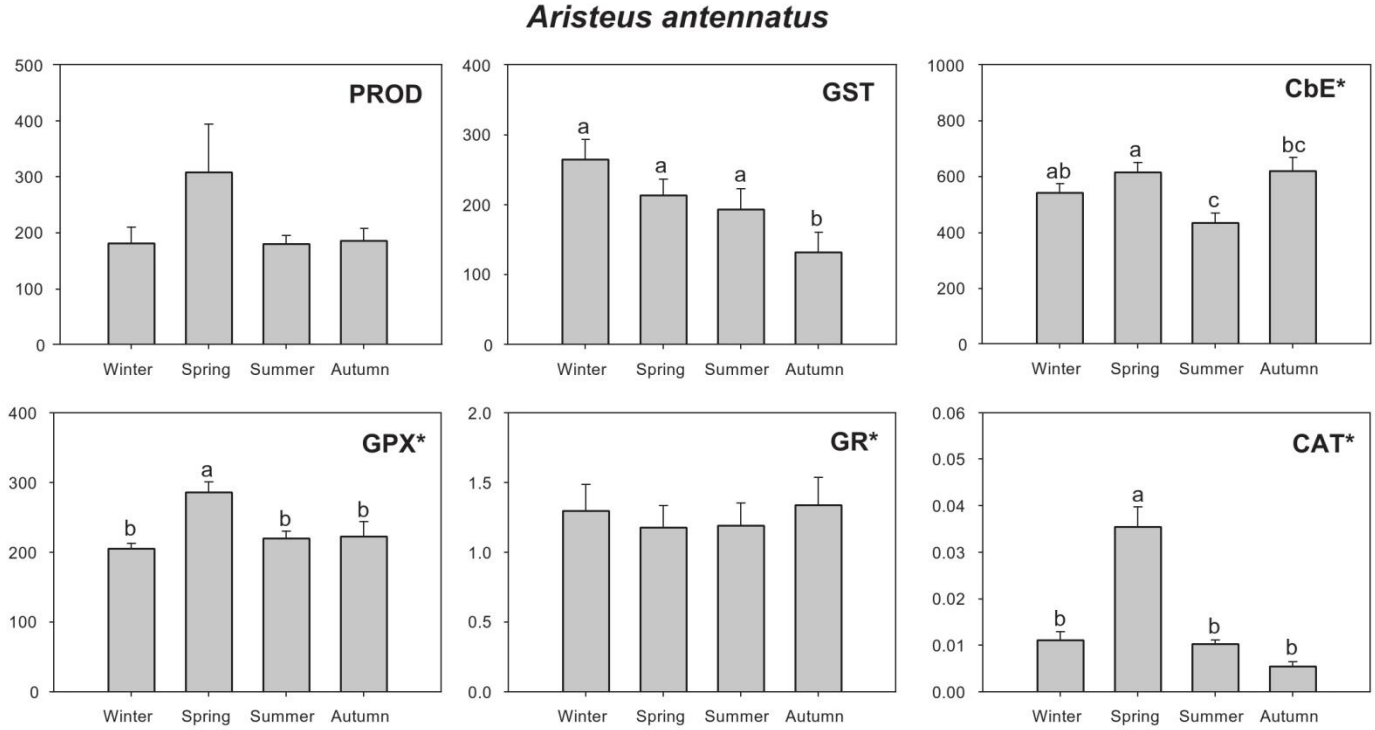

Fig. 4. Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in *A. antennatus* in winter ($n = 30$), spring ($n = 30$), summer ($n = 30$) and autumn ($n = 30$). All activities are expressed as nmol min^{-1} mg $protein^{-1}$ except for PROD (fmol min^{-1} mg $protein^{-1}$) and CAT (mmol min^{-1} mg $protein^{-1}$). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

of the spawning period coincided with in an increase in EROD activity in females, reaching activity levels similar to males. Moreover, the increase in GSI in summer coincided with a decline in EROD activity in females, while seasonal variations of EROD were absent in males.

The conjugating enzyme GST also exhibited seasonal fluctuations only in females, a trend that has also been reported in other studies (Ronisz et al., 1999). Furthermore, females exhibited a negative correlation between GST activity and the GSI, suggesting that female sex hormones might also influence this enzyme activity. In contrast to GST, ANCOVA results indicated that gender-related differences in CbE activity were likely due to the sexual dimorphism as, once adjusted for body size, CbE activity did not differ between sexes. This size-dependence of CbE activity is in accordance with results presented for other fish species such as the Senegalese sole, further supporting the assumption that CbEs behave like other esterase enzymes (*e.g.* cholinesterases) and that the activity decreases with increasing body size (Solé et al., 2012). Contrarily, CbE activity in rainbow trout has been shown to be independent of body size (Barron et al., 1999), suggesting that the allometric scaling of CbE activity is species-dependent. Alpuche-Gual and Gold-Bouchot (2008) also reported a significant correlation with size as well as gender-dependent differences in CbE activity in the reef fish *Haemulon plumieri*, but the influence of size on the gender-related differences in CbE activity was not addressed. CbE enzymes are involved in reproductive processes such as lipid metabolism and bioinactivation of specific hormones (Leinweber, 1987), which potentially explains the negative correlation between CbE and the GSI in both sexes and the significant decline of CbE activity during summer, when the GSI was highest.

The difference in GPX activity between sexes was mainly due to the difference in body size. However, the fact that seasonal variation of GPX activity was only observed in females suggests that some sex-related factor potentially affected GPX activity, which is consistent with previous studies (Ronisz et al., 1999; Sanchez et al., 2008). The significant relationship of CAT activity with the GSI in both genders and the concordant activity decline in summer are also in accordance with the observations made by Ronisz et al. (1999) and suggest the influence of reproductive processes on CAT activity. In contrast, GR activity was lowest in spring when reproductive activity is low and highest in summer and autumn during the spawning period. Furthermore, it should be noted that all glutathione-dependent enzymes, namely the antioxidant enzymes GPX and GR, as well as GST, presented highest activities during autumn.

In addition to reproductive processes, food availability has also been shown to influence biotransformation enzyme activities such as EROD and CbE (Leinweber, 1987; Bucheli and Fent, 1995; Whyte et al., 2000) and antioxidant enzymes including GST, GPX, GR and CAT (Martínez-Álvarez et al., 2005). In this context, the simultaneous study by López-Fernández et al. (2012) on particle fluxes in the study area revealed a peak in particulate matter input from autumn to spring, while during summer particle fluxes were lower. However, although the influence of food availability on antioxidant enzymes is well described, the direction of change of these responses (increase or decrease) can be variable (Martínez-Álvarez et al., 2005). For instance, brown trout (*Salmo trutta*) antioxidant defenses such as CAT, GPX and GR increased as a result of food deprivation, while GST decreased (Bayir et al., 2011). Moreover, Pascual et al. (2003) showed that the direction of variation of some antioxidant activities such as GPX and GST may vary according to the level and duration of the food deprivation period. The same study showed that an increased level of lipid peroxidation due to prolonged starvation could have opposite effects on CAT and GR activities. This trend is also apparent in the present study in which GR was significantly lower in spring than in summer and CAT the other way round.

4.2. L. lepidion

In contrast to *A. rostratus, L. lepidion* did not exhibit any sex-related differences in biomarkers, which is consistent with the lack of sexual dimorphism in this species (*i.e.* equal body size) and the fact that no fully mature individuals were caught throughout the sampling period (all individuals were classified as maturity stage II). However, all biomarker activities varied significantly among seasons, with most enzymes exhibiting a decline in activity during summer, a peak in metabolizing enzymes EROD and CbE in spring and high antioxidant activities in autumn. Hence, the seasonal variability of enzymatic activities observed for *L. lepidion* is probably not related to fluctuations in reproductive activity, but results from other factors such as the above-mentioned variations in food availability. Indeed, higher particle fluxes in spring may be responsible for the increase in biotransformation enzyme activities (*i.e.* EROD and CbE) (Leinweber, 1987; Bucheli and Fent, 1995; Whyte et al., 2000), while the lower antioxidant activities in summer could be related to lower particle fluxes during that time. Coinciding with *A. rostratus*, antioxidant activities were elevated in autumn, indicating that similar factors might affect these parameters in both species. Biomarkers in specimens collected at different sampling depths only differed significantly in CbE and GR activities and no clear trend was apparent.

4.3. A. antennatus

Biomarker data for *A. antennatus* exhibited gender-dependent activity for GPX, CbE and CAT, although the inclusion of size in the model canceled out the effect of sex for CbE and CAT. Hence, it seems that differences in CbE and CAT activities between male and female shrimp result from the pronounced sexual dimorphism in this species. These results are in accordance with previous findings that reported sex-related differences in GPX, but not CAT activity in freshwater gammarids (Sroda and Cossu-Leguille, 2011). In the present study, GPX and CAT exhibited opposite correlation patterns with size, which is similar to observations in brain tissue of *A. antennatus*, (Mourente and Díaz-Salvago, 1999). Furthermore, GPX and GR activities exhibited a significant overall negative relationship with size, which is consistent with the general idea of metabolic scaling of antioxidant activities as a result of decreasing oxygen consumption (and associated ROS production) with increasing size (Amérand et al., 2010).

A clear peak in GPX and CAT activities was observed during spring, coinciding with the reproductive period of *A. antennatus*. Sexual development of adult *A. antennatus* reaches its maximum from May to September, accompanied by increased molting activity during this period (Demestre, 1995). Thus, the peak in antioxidant defenses during spring (late May) might result from increased reproductive and associated molting activity, which is in accordance with previous studies on other crustacean species (Sroda and Cossu-Leguille, 2011). Moreover, a peak in CAT activity during June 2007 was recorded in a previous study conducted on the same species (Antó et al., 2009), supporting the idea of enhanced antioxidant activities during the reproductive period. The increase in CAT activity was also more pronounced than for GPX activity. This difference in response amplitude can potentially be explained by the fact that both enzymes have complementary roles in hydrogen peroxide detoxification as well as different cellular localizations (Janssens et al., 2000; Barata et al., 2005). A peak in activity during spring was also evident for CbE, suggesting the involvement of CbE in the sexual development of *A. antennatus*. In fact, CbEs are thought to be involved in the regulation of physiological processes in crustaceans such as molting and reproduction (*i.e.* catabolism of juvenile hormone) (Ezhilarasi and Subramoniam, 1984; Reddy et al., 2004; Lee et al., 2011). Similarly, CYP450 enzymes are thought to be involved in crustacean molting and reproductive processes (James and Boyle, 1998; Rewitz et al., 2006) and PROD activity was also highest in spring, although not statistically significant due to high inter-individual variability. Moreover, the lack of GST activity increase during spring is consistent with previous results reporting that molting did not alter GST activity in crabs (Hotard and Zou, 2008).

Contrasts among sampling depths exhibited significantly lower PROD, CbE and GPX activities at 2000 m compared to 1200 m and 1500 m depth. As mentioned previously, these three enzymes are likely influenced by the reproductive and molting cycle. Moreover, reproductively active adult *A. antennatus* have been shown to aggregate at shallower depths (Sardà et al., 2004). Hence, it is possible that individuals caught at shallower depths exhibit higher reproductive and associated molting activities than deeper-dwelling specimens. In contrast, GST activity was higher at greater depths, confirming the lack of influence of molting on GST.

5. Conclusions

The present work has shown that despite the lack of seasonal and depth-related fluctuations in temperature and salinity, which are characteristic for most deep-sea habitats, biomarker activities in deep-sea organisms still exhibit significant variability. All three species experienced seasonal variations of enzyme activities as a result of fluctuations of endogenous factors such as reproductive processes and/or exogenous factors such as food availability. However, the fact that *A. rostratus* exhibited higher gender-related seasonal variability than *L. lepidion* indicates that *L. lepidion* might be a more adequate sentinel species for future monitoring studies. Furthermore, allometric scaling of enzymatic activities was not consistent among species, indicating that these relationships need to be investigated on a species-specific level. In this context, the use of appropriate statistical analyses such as the ANCOVA test, which allow the assessment of the covariation of biomarkers with body size, is highly recommended.

Acknowledgments

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Paper 4

Differences in cytochrome P450 enzyme activities between fish and crustacea: relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs)

Samuel Koenig [a,b], Pilar Fernández [b], Montserrat Solé [a,*]

[a] Institut de Ciències del Mar (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

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Resumen

Las enzimas que forman parte del citocromo P450 (CYP) están relacionadas con el metabolismo de un gran número de contaminantes antropogénicos, entre los que se incluyen la familia de los bifenilos policlorados (PCBs). Este grupo de contaminantes orgánicos está formado por 209 congéneres con diferente grado de cloración, por lo que cabe esperar que diferencias en la actividad de esas enzimas entre especies dieran lugar a perfiles de bioacumulación de estos compuestos distintos. Sin embargo, aunque estudios previos sugirieron que las diferencias en la composición de PCBs observadas entre peces y crustáceos podrían ser debidas a diferencias en los enzimas existentes en los CYPs de ambas especies, la relación entre la acumulación de PCBs y las actividades enzimáticas de los CYPs no ha sido evaluada en estos organismos.

En este estudio se investigaron las actividades catalíticas en microsomas hepáticos usando seis sustratos fluorescentes de CYPs, a saber 7-etoxiresorufina (ER), 7-pentoxiresorufina (PR), 7-benziloxiresorufina (BR), 3-cyano-7-etoxicumarina (CEC), dibenzilfluoresceina (DBF) y 7-benziloxi-4-trifluorometilcoumarina (BFC), en tres especies de peces, *Alepocephalus rostratus*, *Coelorinchus mediterraneus* y *Lepidion lepidion*, y la gamba roja *Aristeus antennatus*. Estas actividades metabólicas se relacionaron con los perfiles de acumulación de 41 congéneres de PCBs determinados en tejido muscular de estos organismos. Los resultados obtenidos indicaron diferencias muy marcadas en los perfiles de CYPs entre peces y crustáceo. Los microsomas hepáticos de las tres especies de peces mostraron capacidad para metabolizar los seis sustratos CYP estudiados. Los resultados indicaron que los enzimas responsables de la actividad metabólica observada pertenecerían principalmente a las familias de CYP1A y CYP3A. Por el contrario, los microsomas de hepatopancreas de *A. antennatus* solo presentaron actividades medibles para los sustratos PR y DBF, ambos relacionados con enzimas de tipo CYP2B en mamíferos. Los resultados de los análisis químicos mostraron perfiles de PCBs totalmente coherentes con los estudios bioquímicos, con niveles más altos de los congéneres típicamente metabolizados por enzimas del tipo CYP1A (*i.e.* PCB 28, 52, 118, 138, 158, 169) en gamba y concentraciones más bajas de aquellos metabolizados por enzimas CYP2B (*i.e.* PCB 87, 149, 153, 170, 180, 183, 194, 206).

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Differences in cytochrome P450 enzyme activities between fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs)

Samuel Koenig[a,b], Pilar Fernández[b], Montserrat Solé[a,*]

[a] Instituto de Ciencias del Mar (ICM-CSIC), Paseo Marítimo de la Barceloneta 37-49, 08003 Barcelona, Spain
[b] Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain

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ABSTRACT

Variations in cytochrome P450 enzyme (CYPs) distribution and function between animal groups could result in differential metabolism and elimination kinetics for certain contaminants. Although a number of studies have suggested that differences in polychlorobiphenyl (PCB) accumulation profiles between crustacea and fish might result from differential CYP patterns, the relationship between PCB bioaccumulation and CYP capacities has not been demonstrated in these organisms. In the present study we investigated the hepatic microsomal catalytic activities in three deep-sea fish species, *Alepocephalus rostratus* (Alepocephalidae), *Coelorinchus mediterraneus* (Macrouridae), and *Lepidion lepidion* (Moridae), and the decapod crustacean *Aristeus antennatus* (Decapoda), using six fluorescent CYP-mediated substrates, namely ER (7-ethoxyresorufin), PR (7-pentoxyresorufin), BR (7-benzyloxyresorufin), CEC (3-cyano-7-ethoxycoumarin), DBF (dibenzylfluorescein) and BFC (7-benzyloxy-4-trifluoromethylcoumarin). Furthermore, we related the metabolic activities to the accumulation patterns of 41 PCB congeners in the muscle of these organisms. The results indicated a marked difference in the presence and activities of CYP isoforms between fish and the crustacean *A. antennatus*. Liver microsomes of the three selected fish species were capable of metabolizing all six CYP-mediated substrates and enzymes were identified as primarily belonging to CYP1A and CYP3A subfamilies. In contrast, hepatopancreas microsomes from *A. antennatus* only showed activity for PR and DBF substrates, generally related to mammalian CYP2-like enzymes. Furthermore, a direct relationship between metabolic activities and PCB accumulation profiles could be established. Results revealed that *A. antennatus* accumulated significantly higher proportions of PCBs 28, 52, 118, 138, 158 and 169 than fish, which is in accordance with the previously observed lack of CYP1A-like biotransformation capacities. Moreover, *A. antennatus* exhibited lower levels of PCBs 87, 149, 153, 170, 180, 183, 194 and 206 indicating that this crustacean is able to metabolize congeners considered mammalian CYP2B inducers. Hence, the present findings highlight the role of CYP-mediated metabolism in the congener-specific accumulation of PCBs in aquatic organisms and stress the need to further investigate quantitative and qualitative differences in xenobiotic metabolism among animal groups.

1. Introduction

Over the last decades, numerous synthetic chemicals have been produced and released into the aquatic environment. Among these man-made xenobiotics, persistent organic pollutants (POPs) are of particular concern due to their high resilience to degradation and toxicity (Scheringer et al., 2009). These compounds have been shown to accumulate along the food chain due to their high affinity for lipids and their octanol–water partition coefficient (K_{ow}) is often used as proxy for their bioconcentration potential. However, the K_{ow} only reflects the uptake of contaminants from water, sediment and/or diet, without taking into account that many POPs can interact with animal physiology and be subject to metabolism by biotransformation enzymes, which is a key process governing their concentrations and pattern in biota (Brown, 1992; Elskus et al., 1994; Livingstone, 1998).

The biotransformation of organic contaminants is a complex process that involves xenobiotic-metabolizing phase I and phase II enzymes. The enzymes of the phase I metabolism generally introduce a functional group (*e.g.* –OH, –COOH, $–NO_2$) into the compounds, to which phase II enzymes attach a large moiety (*e.g.*

Abbreviations: ER, 7-ethoxyresorufin; PR, 7-pentoxyresorufin; BR, 7-benzyloxyresorufin; CEC, 3-cyano-7-ethoxycoumarin; DBF, dibenzylfluorescein; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; EROD, 7-ethoxyresorufin-*O*-deethylase; PROD, 7-pentoxyresorufin-*O*-deethylase; PCB, polychlorobiphenyl; CYP, cytochrome P450; α-NF, α-naphthoflavone; Keto, ketoconazole; Chl, chloramphenicol.

* Corresponding author. Tel.: +34 93 230 95 00; fax: +34 93 230 95 55.
E-mail address: msole@icm.csic.es (M. Solé).

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glutathione, glucuronide) to increase their water solubility and thus facilitate their excretion. Cytochrome P450s (CYPs) constitute a superfamily of monooxygenase enzymes that are involved in the phase I of the biotransformation of a variety of endogenous and exogenous compounds and have been found in eukaryotic as well as prokaryotic organisms (Nelson, 2003; Nelson and Strobel, 1987; Rewitz et al., 2006; Snyder, 2000). In mammals, isoenzymes belonging to the first four families (CYP1-CYP4) are generally associated with xenobiotic metabolism, but there is a lack of information on non-mammalian drug metabolism pathways (Goksøyr and Förlin, 1992; Smith and Wilson, 2010), especially with regard to invertebrates (James and Boyle, 1998; Rewitz et al., 2006; Solé and Livingstone, 2005).

The expression of CYP genes can be induced by xenobiotics that bind to receptor proteins, which in turn results in an increase in protein synthesis and related enzyme activity (Honkakoski and Negishi, 2000). Variations in CYP profiles are commonly used as indicators of exposure to environmental contaminants (Andersson and Förlin, 1992; Stegeman and Lech, 1991). However, the affinity of a certain compound to bind to a specific receptor and consequently induce or repress the respective CYP enzyme is determined by its chemical structure/properties. In fish, the response of CYP1A isoforms to exogenous inducers such as polycyclic aromatic hydrocarbons (PAHs) and other aryl hydrocarbon receptor agonists (*e.g.* dioxins, some polychlorobiphenyl (PCB) congeners) has been extensively studied (as reviewed in Goksøyr and Förlin, 1992; Whyte et al., 2000). With regard to PCBs, congeners that lack *ortho*-Cl substitutions retain a planar configuration similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and thus have a high affinity to bind to the aryl hydrocarbon receptor. Hence their categorization as dioxin-like PCBs, which denotes their high potential to induce CYP1A (Whyte et al., 2000). However, most PCBs are considered phenobarbital-like inducers, which are mainly metabolized by mammalian CYP2B-type enzymes (Connor et al., 1995).

In this context, a number of studies have reported different contaminant accumulation patterns between fish and crustacean species. In these studies, crustacea exhibited a higher capacity to metabolize PCBs than fish, especially congeners typically metabolized by mammalian CYP2B-type enzymes (Bodin et al., 2007, 2008; Brown, 1992; Goerke and Weber, 2001; Kannan et al., 1995; Porte and Albaiges, 1993). Conversely, other studies showed that crustacea accumulated higher levels of dioxins (Abalos et al., 2010), a contaminant type mainly metabolized by CYP1A in vertebrates (Whyte et al., 2000). There is however no consensus on the presence and inducibility of CYP2B-like enzymes in fish and CYP1A enzymes in invertebrates (Goksøyr and Förlin, 1992; Goldstone et al., 2010; James and Boyle, 1998; Rewitz et al., 2006).

The characterization of the quantitative and qualitative differences in biotransformation capacities between animal groups is important from an ecological and toxicological perspective. In particular, knowledge on biotransformation processes is necessary for the design of toxicity tests, the development and application of biomarkers, the design of field sampling strategies and the understanding of the fate of chemicals in ecosystems and their effects on community structures (Livingstone, 1998). Previous studies have highlighted the importance of studying the metabolism of, for instance, benzo[a]pyrene (BaP) in sentinel species to ensure their suitability as sensitive indicators (McElroy et al., 2000; Rust et al., 2004). Furthermore, biotransformation processes not only affect residual contaminant body burden but can also alter the toxicity of certain chemicals (*e.g.* certain PAH compounds such as BaP), for which metabolites can be more toxic than the parent compound (Buhler and Williams, 1988; Lech and Bend, 1980).

The main objective of the present study was to compare the catalytic activities of fish (liver) and crustacean (hepatopancreas) microsomal CYPs and to subsequently relate these metabolic activities to the PCB accumulation patterns observed in these organisms. For that purpose, we determined CYP activities in three deep-sea fish species, *Alepocephalus rostratus* (Alepocephalidae), *Coelorinchus mediterraneus* (Macrouridae), and *Lepidion lepidion* (Moridae), and the decapod crustacean *Aristeus antennatus* (Decapoda), using six fluorescent CYP-mediated substrates, namely ER (7-ethoxyresorufin), PR (7-pentoxyresorufin), BR (7-benzyloxyresorufin), CEC (3-cyano-7-ethoxycoumarin), DBF (dibenzylfluorescein) and BFC (7-benzyloxy-4-trifluoromethylcoumarin). In addition, we evaluated in vitro the substrate-specificity of these isoenzymes using three specific mammalian CYP inhibitors (α-naphthoflavone for CYP1A, chloramphenicol for CYP2B and ketoconazole for CYP3A). The study was completed by the analysis of 41 PCB congeners in the muscle tissue of these organisms in order to investigate the potential relationship between biotransformation capacities and PCB bioaccumulation profiles, which has thus far only been reported in higher vertebrate species including birds (Borgå et al., 2005; van den Brink et al., 2000) and marine mammals (Li et al., 2003; Routti et al., 2008; Wolkers et al., 1999).

2. Materials and methods

2.1. Sample collection

Animals were collected from the NW Mediterranean sea (41°15′N 2°50′E) in February 2009 using an OTMS otter trawl at 1500 m water depth. Onboard, a portion of muscle tissue and the liver/hepatopancreas were dissected, immediately frozen in liquid nitrogen and stored at −20 °C and −80 °C, respectively, until further treatment. Species were chosen based on availability in trawls. Further details on sampled individuals are provided in Table 1. It should be noted that only for *A. rostratus* liver sizes permitted individual analyses of microsomal catalytic activities. For the two other fish and the crustacean species, 2–4 individuals of the same sex were pooled to obtain enough sample volumes (1 g).

2.2. Catalytic assays

Microsomes were prepared in a 100 mM phosphate buffer pH 7.4 containing 150 mM KCl, 1 mM Dithiothreitol (DTT), 0.1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 mM Ethylenediaminetetraacetic acid (EDTA) and 20% glycerol, based on the method published by Förlin and Andersson (1985).

Catalytic activities were determined using six fluorescent substrates and assay conditions were optimized in our lab based on the method described in Smith and Wilson (2010) as listed in Table 2. All reactions were run using 100 μg of microsomal protein per well at 30 °C in transparent 96-well format in a TECAN Infinite 200 microplate reader using kinetic assays (Magellan software v.6.2).

Due to limited sample material, tissue from the same species was pooled ($n = 4$) and incubated in duplicate for 30 min with three specific mammalian CYP inhibitors, ketoconazole, chloramphenicol and α-naphthoflavone at a concentration of 10 μM dissolved in methanol, ethanol and DMSO respectively. Final solvent concentration in reaction mixture was 0.04% (v/v). As chloramphenicol is a mechanism-based inhibitor, additional tests were performed where samples were preincubated with NADPH and chloramphenicol for 20 min to facilitate the CYP-mediated conversion of chloramphenicol to an active inhibitor that irreversibly binds the CYP enzyme (Li et al., 2003).

Protein content was determined according to the method by Bradford (1976), using bovine serum albumin as standard (BSA 0.1–1 mg/ml).

Table 1
Sample details.

	Species	Length (mm)	Sex ratio	Maturity stage[c]
Fish	*Alepocephalus rostratus*	346 ± 26	2:2	3
	Lepidion lepidion	165 ± 32	2:2	1–2
	Coelorinchus mediterraneus	71 ± 8[a]	2:2	2
Crustacea	*Aristeus antennatus*	38 ± 11[b]	2:2	2

[a] Pre-anal length (PAL).
[b] Carapace length (CL).
[c] Based on five-point scale by Brown-Peterson et al. (2011).

Table 2
Assay conditions for fluorometric substrates to determine microsomal CYP activity.

Substrate		Metabolite	Ex/Em (nm)	Substrate concentration (μM)[a]	Mammalian CYPs[b]
ER	7-ethoxyresorufin			3	1A1 > 1A2, 1B1
PR	7-pentoxyresorufin	Resorufin sodium salt	537/583	5	2B
BR	7-benzyloxyresorufin			3	1A1, 2B, 3A
CEC	3-cyano-7-ethoxycoumarin	3-cyano-7-hydroxy-4-methylcoumarin	408/455	10	1A2, 2C9, 2C19
BFC	7-benzyloxy-4-trifluoromethylcoumarin	7-OH-4-trifluoromethylcoumarin	410/538	500	3A4, 2C19
DBF	Dibenzylfluorescein	Fluorescein	485/538	1	2C8, 2C9, 2C19, 3A4

[a] NADPH [200 μM] in plate for all substrates except for BFC [20 μM].
[b] Smith and Wilson (2010).

2.3. Chemical analysis

Individual fish ($n = 6$) and pooled *A.* antennatus ($n = 4$ pools of 5 individuals) muscle tissue samples (equal number of males and females) were prepared following the method published by Berdié and Grimalt (1998) and subsequently analyzed for PCB levels by gas chromatography equipped with an electron-capture detector (GC μ-ECD, Agilent Technologies, model 6890N) as described by Cabrerizo et al. (2009). 41 PCB congeners, namely IUPAC# 18, 28, 31, 33, 44, 49, 52, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 158, 169, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 201, 205, 206, 208, and 209 were determined by the internal standard method, response factors referred to PCB 142. In the instrumental conditions used, the following PCB congeners coeluted: 99–101, 132–105, 171–156, and 201–199. Reported levels for these compounds refer to all coeluting congeners. PCB concentrations were only considered if they exceeded the method quantification limit (MQL), calculated as the mean blank values plus five times the standard deviation.

2.4. Statistical analysis

Data were analyzed using the software package JMP 7.0 (SAS Institute, Cary, NC, USA) for univariate and PRIMER^{TM} software package v6.0 with the PERMANOVA+ add-on (Clarke, KR, Gorley, RN, 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth) for multivariate statistical analyses. Differences at the 5% significance level were considered significant. For univariate ANOVA, data were log-transformed. For multivariate analyses, data were standardized (by total ΣPCBs). The multivariate PERMANOVA and ANOSIM routines were conducted on a Euclidian distance resemblance matrix.

3. Results and discussion

The aim of the present study was to characterize the CYP-mediated activities of fish and crustacea hepatic microsomes using fluorescent substrates and subsequently relate these activities to PCB bioaccumulation profiles. Although the uptake of organic contaminants from the water column, sediment and/or diet is an important factor that has to be taken into account when comparing bioaccumulation profiles, the present study was conducted on deep-sea organisms, for which differences in trophic level are minimal due to a single primary food source supporting these communities. In fact, stable isotope analyses confirmed that the trophic levels of the species selected in this study are very similar, indicating that the influence of differential food sources on contaminant uptake among them is minimal (Polunin et al., 2001).

3.1. Cytochrome P450 activities

The results obtained by measuring the catalytic activities of hepatic microsomes using six CYP-mediated fluorescent substrates indicated a marked difference in the presence and activities of CYP isoforms between fish and the crustacean *A. antennatus*.

Liver microsomes of the three selected fish species were able to metabolize all six CYP-mediated substrates (Fig. 1). The specificity of these substrates was further evaluated by incubating microsomes with three mammalian CYP inhibitors (Table 3). Although it should be noted that the use of pooled samples reduces the statistical power of the inhibition results, we observed strong inhibitory effects on ER- and CEC-related activities for incubations with α-naphthoflavone (mammalian CYP1A inhibitor), as well

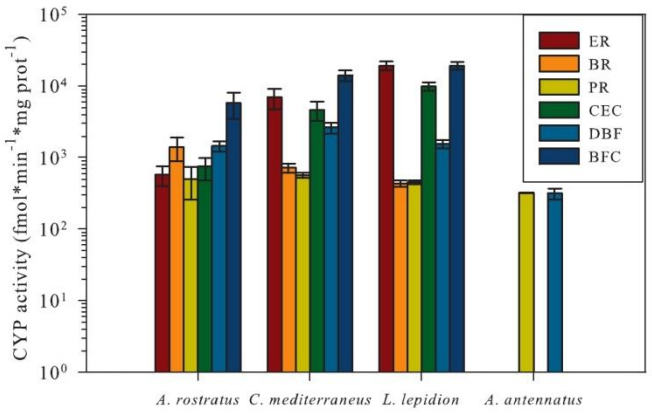

Fig. 1. CYP activity (fmol × min^{-1} × mg prot^{-1}) of fish (*Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*) and crustacean (*Aristeus antennatus*) hepatic microsomes measured using six fluorescent substrates.

Table 4
Structure and induction capacity of the PCB congeners exhibiting significant differences (ANOVA $p < 0.05$) in proportional levels between fish and the crustacean species.

Congener	Structure	*m*, *p*-H	2,4,5-Cl	*ortho*-Cl	Inducer type[a]	PB induction[b]	Present study (metabolized by)
28	2,4,4′	–	–	1	n.d.	+	Fish
52	2,2′,5,5′	2	–	2	Weak 2B	+	Fish
118	2,3′,4,4′,5	–	1	1	1A + 2B	+	Fish
138	2,2′,3,4,4′,5′	–	1	2	1A + 2B	n.d.	Fish
158	2,3,3′,4,4′,6	–	–	2	n.d.	n.d.	Fish
169	3,3′,4,4′,5,5′	–	–	–	1A	n.d.	Fish
87	2,2′3,4,5′	1	–	2	2B	n.d.	Crustacea
149	2,2′3,4′,5′,6	1	1	3	n.d.	n.d.	Crustacea
153	2,2′,4,4′,5,5′	–	2	2	2B	+++	Crustacea
170	2,2′,3,3′,4,4′,5	–	1	2	1A + 2B	n.d.	Crustacea
180	2,2′,3,4,4′,5,5′	–	2	2	2B	+++	Crustacea
183	2,2′,3,4,4′,5′,6	–	1	3	2B	++	Crustacea
194	2,2′,3,3′,4,4′,5,5′	–	2	2	2B	+++	Crustacea
206	2,2′,3,3′,4,4′,5,5′,6	–	2	3	2B	n.d.	Crustacea

n.d., not determined.
CYP activity (fmol × min^{-1} × mg $prot^{-1}$) of fish (*Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*) and crustacean (*Aristeus antennatus*) hepatic microsomes measured using six fluorescent substrates.
PCB congener profile (mean % Σ_{41}PCBs ± standard error) in muscle samples of three fish species ($n = 6$), *Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*, and the crustacean *Aristeus antennatus* ($n = 4$).
Ordination plot of principal component analysis based on proportional levels (% ΣPCBs) of 41 PCB congeners in samples from three fish species, *Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*, and the crustacean *Aristeus antennatus*.

[a] McFarland and Clarke (1989).
[b] Connor et al. (1995); Phenobarbital (PB) induction of PROD activity: (+), <20%; (++), 20–60%; (+++), >60%.

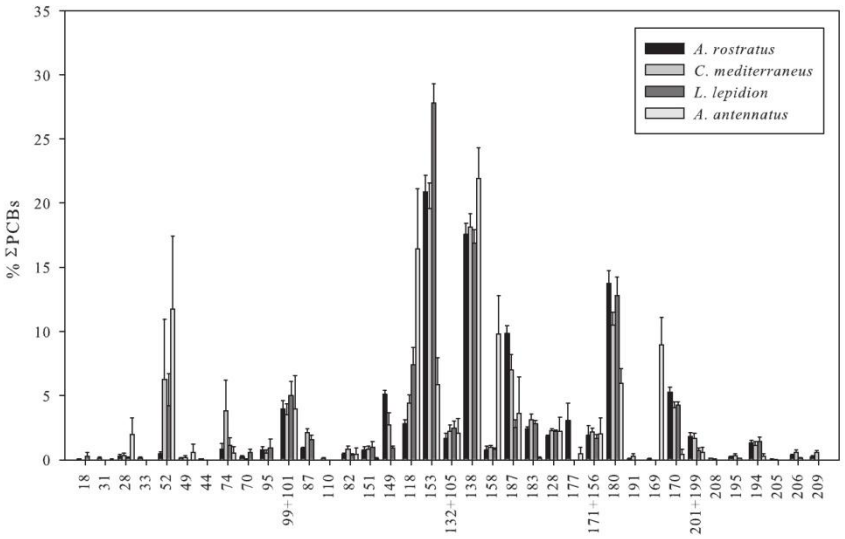

Fig. 2. PCB congener profile (mean % Σ_{41}PCBs ± standard error) in muscle samples of three fish species ($n = 6$), *Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*, and the crustacean *Aristeus antennatus* ($n = 4$).

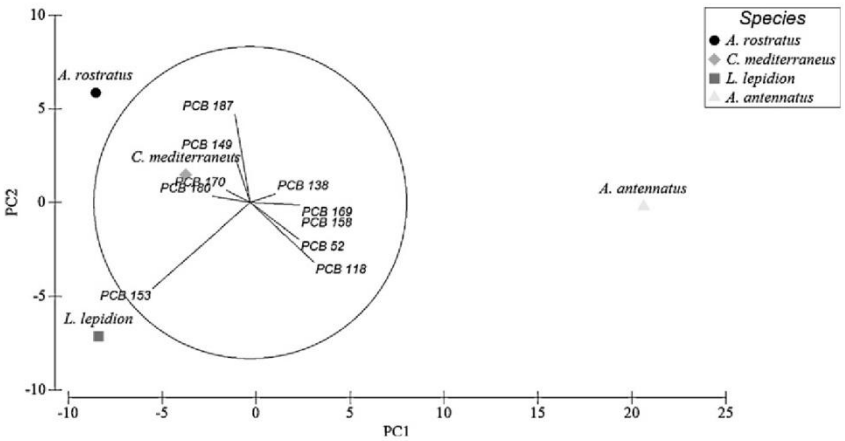

Fig. 3. Ordination plot of principal component analysis based on proportional levels (% ΣPCBs) of 41 PCB congeners in samples from three fish species, *Alepocephalus rostratus*, *Coelorinchus mediterraneus* and *Lepidion lepidion*, and the crustacean *Aristeus antennatus*.

metabolism of PCB 169 by *A. antennatus* further supports the idea that the ability to bind dioxin-like compounds was acquired during vertebrate evolution (Hahn, 2001) and that the CYP1 family is vertebrate specific (Rewitz et al., 2006). Moreover, this result is in accordance with the fact that in a previously mentioned study, *A. antennatus* accumulated higher levels of dioxins than fish, including congeners that are usually not found in biota (Abalos et al., 2010). The mono-*ortho* PCB 118 is also considered a dioxin-like congener and has been shown to bind with intermediate affinity to the Ah receptor (Safe, 1994). However, PCB 118 has also been described as a mixed inducer (Hansen, 1998; Kannan et al., 1995) and a recent study showed that it is mainly metabolized by human CYP2C and CYP3A isoenzymes (McGraw and Waller, 2009). The fact that various CYP families are responsible for the metabolism of PCB 118 in humans potentially explains why the difference in relative abundance of this congener is less marked between fish and crustacea as compared to PCB 169. For instance, the previously suggested presence of CYP2C-like enzymes in *A. antennatus* (see Section 3.1) might account for PCB 118 metabolism in crustacea, while in fish PCB 118 is likely metabolized by CYP1A- and CYP3A-related proteins. PCB 28 is also thought to be metabolized by CYP1A due to the lack of *meta*, *para*-vicinal H-atoms and the presence of only one *ortho*-substituted Cl-atom (Boon et al., 1994; Wolkers et al., 2006).

Although some studies have considered PCB 52 as a CYP2B inducing agent due to the presence of *meta–para* vicinal H-atoms in both rings (Boon et al., 1994; McFarland and Clarke, 1989), its potency to induce CYP2B in rats was found to be very low (Connor et al., 1995). Moreover, in a study conducted on seals, PCB 52 metabolism was significantly inhibited by ketoconazole, suggesting the involvement of CYP3A enzymes (Li et al., 2003). The lower rate of biotransformation of PCB 52 in *A. antennatus* therefore corroborates the previously observed absence of CYP3A activity in this species (Section 3.1). For PCB 138 and 158 the classification as CYP inducer is less clear and they are generally regarded as very persistent. However, in a study by Buckman et al. (2006) PCB 158 was the only highly chlorinated congener that rainbow trout juveniles were able to metabolize, indicating that this PCB is biotransformed in fish although the underlying mechanism remains unclear. Thus, the PCB profiles clearly show that congeners that are more recalcitrant to biotransformation in *A. antennatus* are those primarily metabolized by CYP1A-like and to a lower extent by CYP3A-like proteins, which is clearly consistent with the lack of CYP1A- and CYP3A-related activity observed in this species.

The congeners that were significantly (ANOVA, $p < 0.05$) more abundant in fish compared to *A. antennatus* included PCBs 87, 149, 153, 170, 180, 183, 194 and 206 (Table 4). All of these compounds are generally considered mammalian CYP2B inducers as they all contain *m,p* vicinal H-atoms and/or 2,4,5-trichloro substitutions in at least one of the two phenyl rings (Connor et al., 1995; Hansen, 1998; McFarland and Clarke, 1989). This includes highly chlorinated congeners such as PCBs 153 and 180, which are generally considered as very recalcitrant to metabolism and are often used to normalize PCB levels in biota (Boon et al., 1994; Kannan et al., 1995). Results from the present study indicate that, in comparison to fish, the crustacean *A. antennatus* possesses a strong capacity to metabolize these compounds.

4. Conclusion

The present findings suggest that the differences in congener-specific accumulation of PCBs between fish and crustacea are mainly due to variations in CYP-mediated metabolism. Although fish microsomes metabolized all six fluorescent substrates, CYP1A and CYP3A-like proteins accounted for the main part of the measured activities and results indicated a lack of CYP2B-like enzymes. In contrast, the crustacean *A. antennatus* appeared to lack CYP1A- and CYP3A-like biotransformation capacities, while exhibiting activities for enzymes related to the mammalian CYP2 family. These differences in metabolism between fish and crustacea were consistent with PCB bioaccumulation profiles. *A. antennatus* clearly accumulated CYP1A inducing congeners, but metabolized congeners that are known mammalian CYP2B inducers. However, additional studies are necessary to further identify the isoenzymes present in these animal groups and to corroborate that these results can be extrapolated to other crustacean species. The fact that most of the existing 209 PCB congeners are mainly metabolized by CYP2B-like enzymes supports the idea that crustacea generally accumulate lower levels of PCBs than fish. Furthermore, their greater capacity to metabolize PCBs also indicates a greater potential responsiveness of their CYPs to PCBs. Hence, future studies should address the question if crustacean species might be better candidates than fish for biomarker studies on PCB exposure. In contrast, fish seem to accumulate PCBs at higher rates than crustacea, emphasizing their use in chemical monitoring studies. Finally, the fact that CYPs are involved in the metabolism of many xenobiotics stresses the need to further investigate qualitative as well as quantitative differences in CYP-mediated metabolism among animal groups.

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Paper 5

Are deep-sea organisms dwelling within a submarine canyon more at risk from anthropogenic contamination than those from the adjacent open slope? A case study of Blanes canyon (NW Mediterranean)

Samuel Koenig [a,b]*, Pilar Fernández [b], Joan B. Company [a], David Huertas [b], Montserrat Solé [a]

[a] Institut de Ciències del Mar (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

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Resumen

Debido a su proximidad a la costa y sus características geomorfológicas, los cañones submarinos del margen continental del Mediterráneo noroccidental podrían actuar como conductos naturales de transporte de contaminantes asociados a partículas desde las aguas superficiales al mar profundo. En consecuencia, los organismos que habitan esas zonas podrían presentar un mayor riesgo asociado a la presencia de contaminación antropogénica. En base a esta hipótesis, el estudio realizado pretendía contrastar la bioacumulación de contaminantes orgánicos persistentes (COPs) y la actividad de varios biomarcadores hepáticos, indicadores de efectos negativos debido a la exposición a compuestos tóxicos, entre organismos que habitan en el cañón de Blanes (BC) y aquellos que se encuentran en el margen continental adyacente (OS). Con este fin, se tomaron muestras de dos especies de peces, *Alepocephalus rostratus* y *Lepidion lepidion,* y un crustáceo, *Aristeus antennatus*, a profundidades entre 900 m y 1500 m en la zona del cañón de Blanes y plataforma continental adyacente. En dichas muestras se determinaron los niveles de varias familias de COPs, incluyendo bifenilos policlorados (PCBs), diclodifeniltricloroetano y sus derivados (DDTs) y hexaclorociclohexanos (HCHs), junto con las actividades de los biomarcadores etoxiresorufina-O-deetilasa (EROD) o pentoxiresorufina-O-deetilasa (PROD), glutatión-S-transferasa (GST), carboxilesterasa (CbE), glutatión peroxidasa (GPX), glutatión reductasa (GR) y catalasa (CAT). Los resultados obtenidos indicaron concentraciones más elevadas de COPs tanto en *L. lepidion* como en *A. antennatus* dentro del cañón a 900 m, que coincidían con una mayor respuesta de las enzimas de metabolismo de xenobióticos EROD (en peces) o PROD (crustáceo), y los antioxidantes CAT o GPX, en peces o gamba, respectivamente. Sin embargo, estas diferencias en contaminación y biomarcadores entre BC y OS no se detectaron en los organismos recogidos a 1500 m, lo que indica que el gradiente de contaminación entre el interior y el exterior del cañón es menos marcado a mayor profundidad. Los resultados sugieren que los organismos que habitan la zona de la cabecera del cañón de Blanes presentan un riesgo mayor de sufrir efectos adversos asociados a la exposición a COPs.

Abstract

Due to their geomophormological structure and proximity to the coastline, submarine canyons may act as natural conduit routes for anthropogenic contaminants that are transported from surface waters to the deep-sea. Organisms dwelling in these canyon environments might thus be at risk of experiencing adverse health effects due to higher pollution exposure. To address this question, chemical and biochemical analyses were conducted on two of the most abundant deep-sea fish species in the study area, namely *Alepocephalus rostratus* and *Lepidion lepidion*, and the most abundant deep-sea commercial decapod crustacean *Aristeus antennatus* sampled inside Blanes canyon (BC) and on the adjacent open slope (OS). Persistent organic pollutants (POPs) levels, including polychlorinated biphenyl (PCB), dichlorodiphenyltrichloroethane (DDT) and derivatives, hexachlorocyclohexanes (HCHs) and hexaclorobenzene (HCB) were determined in muscle tissue of selected samples from 900 m and 1500 m depth. Potential effects resulting from contaminant exposure were determined using hepatic biomarkers such as ethoxyresorufin-*O*-deethylase (EROD), pentoxyresorufin-*O*-deethylase (PROD), catalase (CAT), carboxylesterase (CbE), glutathione-*S*-transferase (GST), total glutathione peroxidase (GPX), glutathione reductase (GR) and superoxide-dismutase (SOD) enzyme activities and lipid peroxidation levels (LP). *L. lepidion* and *A. antennatus* tissues exhibited higher POP levels inside BC compared to the OS at 900 m depth. These findings were consistent with biomarker data (*i.e.* enzymatic response to presence of contaminant agents). Elevated xenobiotic-metabolizing (EROD and PROD) and antioxidant enzymes (CAT and GPX) indicated higher contaminant exposure in both species caught within BC. No difference in POP accumulation between sites was observed in *L. lepidion* at 1500 m depth, nor in biomarker data, suggesting that the pollution gradient was less pronounced at greater depths. This trend was further corroborated by the results obtained for *A. rostratus* at 1500 m depth. Hence, the present findings suggest the, at least temporary, existence of a pollution gradient between Blanes canyon and the open slope at shallower depths and this resulted in alterations of the physiology of deep-sea organisms dwelling within this area.

Keywords: persistent organic pollutants (POPs), biomarkers, bioaccumulation, deep-sea organisms, submarine canyon, Blanes canyon, NW Mediterranean

1. Introduction

Deep-sea habitats have been considered the last regions on Earth that have remained untouched from anthropogenic disturbance. However, in recent years there has been increasing concern that the deep-sea might act as a global sink for contaminants that enter the marine environment (Ballschmiter et al., 1997; Froescheis et al., 2000; Dachs et al., 2002; Wania and Daly, 2002; Scheringer et al., 2004a). Because of their geomorphological structure and proximity to the coastline, the role of submarine canyons in the transport of contaminants from surface waters to the deep-sea might be of particular importance. Previous studies investigating the dispersal of anthropogenic contaminants in canyons at the Portuguese margin have shown that pollution input occurs mostly at the canyon heads with a progressive dilution in offshore direction (Richter et al., 2009). Furthermore, in comparison to the adjacent shelf and slope, canyons appear to function as preferential conduits for pollutants from coastal zones to the deep-sea (Paull et al., 2002; Hartwell, 2008; Richter et al., 2009; Jesus et al., 2010; Castro-Jiménez et al., 2012). In the NW Mediterranean Sea, a number of hydrodynamic processes such as storms, water advection and dense shelf water cascading (DSWC) may result in an increased transport of sediments to the deep-sea, channeled through the NW Mediterranean submarine canyons (Canals et al., 2006; Palanques et al., 2006; Company et al., 2008; López-Fernández et al., 2012; Sanchez-Vidal et al., 2012). Moreover, recent studies have shown that the distribution of organic contaminants in sediments along the NW Mediterranean continental shelf and slope is closely linked to the dispersion dynamics of organic material and fine-grained sediments (Salvadó et al., 2012a) and have underlined the importance of major hydrodynamic events such as DSWC as transport mechanism of POPs to the deep-sea (Salvadó et al., 2012b). Considering the fact that sediments and organic material are preferentially channeled through canyons, canyon environments might be subject to higher input of particle-bound contaminants than the adjacent open slope areas. Deep-sea ecosystems within these submarine canyons might consequently be particularly at risk from experiencing adverse effects due to anthropogenic pollution.

In this context, persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane and derivatives (DDTs) could be of major concern because of their high hydrophobicity (sediment affinity), persistence and

toxicity (Scheringer et al., 2009). Several studies have highlighted the important role of the deep-sea as a sink of POPs, which affects their long-term behavior and impact (Dachs et al., 2002; Wania and Daly, 2002; Scheringer et al., 2004b). Adverse effects resulting from POP exposure include neurotoxicity, endocrine disruption, impaired behavior, immunotoxicity and genotoxicity (Vasseur and Cossu-Leguille, 2006). It has been reported that POP exposure can even induce perturbations in population structure and dynamics (Vasseur and Cossu-Leguille, 2006). Although the impacts of marine pollution at the population, community and ecosystem level are of major concern, they are generally too complex and far removed from the causative events to be of much use as ecotoxicological tools (Clements, 2000; Moore et al., 2004). In contrast, distress signals measured at lower levels of biological organization (*e.g.* molecular, biochemical) are thought to precede disturbances at the population, community or ecosystem levels and could potentially serve as 'early warning' prognostic indicators of pollution effects (Schlenk, 1999; Clements, 2000; Moore et al., 2004; Vasseur and Cossu-Leguille, 2006). In this context, the use of biomarkers, defined as functional measures of exposure to stressors expressed at the sub-organismal, physiological or behavioral level, has been advocated as a means to determine adverse effects resulting from contaminant exposure in marine organisms (Handy et al., 2003; Galloway et al., 2004).

Enzymes involved in the detoxification of xenobiotics and their metabolites, including biotransformation and antioxidant enzymes, are the most extensively studied biomarkers (van der Oost et al., 2003). The biotransformation of xenobiotics mainly takes place in the liver/hepatopancreas and usually consists of an alteration (oxidation, reduction or hydrolysis) of the original foreign molecule (Phase I), followed by a conjugation reaction (Phase II) where a large endogenous molecule (*e.g.* glucoronid, glutathione, sulphate) is added to increase its water-solubility and facilitate excretion. Enzymes involved in the Phase I metabolism of xenobiotics include cytochrome P450 (CYP) enzymes (oxidation) and carboxylesterases (hydrolysis), while glutathione-*S*-transferase (GST) plays an important role in the Phase II metabolism. Moreover, the exposure of aquatic organisms to anthropogenic contaminants can result in the production of reactive oxygen species (ROS), potentially causing adverse effects related to oxidative stress (Winston and Di Giulio, 1991). An increase in oxidative stress can result in the induction of antioxidant enzymes such as catalase (CAT), glutathione

peroxidase (GPX) and superoxide-dismutase (SOD) as a protection mechanism against the generation of oxidative radicals (Winston and Di Giulio, 1991; Valavanidis et al., 2006). However, when this protective mechanism is overwhelmed, lipid peroxidation (LP) (*i.e.* the oxidation of polyunsaturated fatty acids) can occur (Winston and Di Giulio, 1991; Valavanidis et al., 2006).

Even though a broad range of anthropogenic contaminants have been shown to accumulate in deep-sea biota from different regions of the world (Kramer et al., 1984; e.g. Berg et al., 1998; Solé et al., 2001; de Brito et al., 2002; Storelli et al., 2004; Ramu et al., 2006; Storelli et al., 2007; Unger et al., 2008; Webster et al., 2009), few studies have investigated the potential effects of these pollutants on the physiology of deep-sea organisms (Stegeman et al., 1986; Porte et al., 2000; Solé et al., 2008). In a previous study, biomarker response patterns suggested that fish caught at 600 m inside Blanes submarine canyon were exposed to higher contaminant levels compared to individuals from the adjacent open slope, although this trend was not reflected in the chemical body burden of these same organisms (Solé et al., 2009).

The present study aimed to investigate the accumulation and potential effects of POPs in deep-sea organisms from inside the Blanes canyon (BC) and the adjacent open slope (OS) in the NW Mediterranean, combining chemical (*i.e.* contaminant concentration) and biochemical (*i.e.* physiological response to contaminant exposure) analyses. We determined levels of POPs in muscle tissue of selected species including the most abundant deep-sea fish *Alepocephalus rostratus* and *Lepidion lepidion* and the commercially exploited decapod crustacean *Aristeus antennatus* from inside and outside the Blanes canyon at 900 m and 1500 m depth. At the same time, potential adverse effects resulting from contaminant exposure were assessed using six hepatic biomarkers.

2. Materials and Methods

2.1. Collection of animals and sampling site

Sampling cruises were conducted onboard the R/V *Garcia del Cid* in 2009 during winter (February), summer (September) and autumn (November) off the coast of

Blanes, northwestern Mediterranean sea (Figure 1). Individuals of the deep-sea fish *Alepocephalus rostratus* and *Lepidion lepidion* and the decapod crustacean *Aristeus antennatus* were caught using a OTMS otter trawl (Sardà et al., 1998) at depths ranging from 900 m to 1500 m inside Blanes canyon (BC) and on the open slope (OS) (Figure 1). Previous to fish dissection on board, size, weight and sex of each animal were recorded. To avoid chemical contamination of samples while dissecting, muscle tissue samples were wrapped in acetone precleaned aluminum foil and dissection tools were cleaned with acetone and rinsed with Milli-Q water after each fish. Muscle tissue and the liver/hepatopancreas of fish and crustacean, respectively, were immediately frozen in liquid nitrogen and stored at -20 °C and -80 °C, respectively, until further analysis.

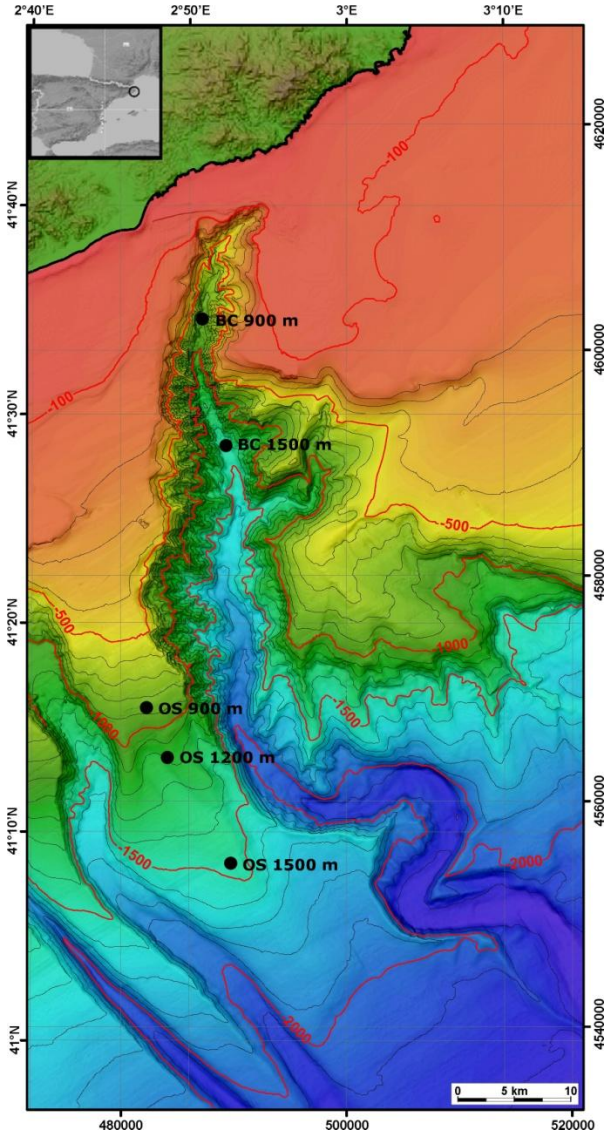

Fig. 1 Location of sampling sites within Blanes canyon (BC) and on the adjacent open slope (OS) in the NW Mediterranean. Bathymetric data from Canals et al. (2004) and Lastras et al. (2011).

2.2. Selected species

The selected species are the most abundant fish and crustacean at these depths in the study area (Company et al., 2004; D'Onghia et al., 2004). The fish species *A. rostratus* is a non-migratory macroplancton feeder with a wide depth-distribution (500 - 2200 m) found in the northwestern Mediterranean and eastern Atlantic (Morales-Nin et al., 1996). Similarly, *L. lepidion* is mainly found in the northwestern Mediterranean at depths ranging from 500m to 2800 m. This species has been shown to mainly feed on benthopelagic and benthic prey (Carrassón et al., 1997). The crustacean *A. antennatus* is considered a eurybathic species (80 - 3300 m) found throughout the Mediterranean Sea and along the northwestern African coast, feeding on infaunal species (Sardà et al., 2004).

2.3. Chemical analysis

2.3.1. Sample extraction

For *A. rostratus* and *L. lepidion*, individual samples were analyzed for BC (n = 10) and OS (n = 10) at 1500 m depth, while samples were pooled in the case of individuals from 900 m depth as well as for individuals caught at 1200 m depth. For *A. antennatus*, only pooled samples were analyzed. Extraction of organic pollutants was performed as described in Berdié and Grimalt (1998). Muscle tissue (2-4 g) of individual fish was ground with activated anhydrous sodium sulphate (Na_2SO_4) until a fine powder was obtained. The mixture was spiked with tetrabromobenzene (TBB) and PCB 200 as recovery standards and Soxhlet-extracted with 100 mL *n*-hexane-dichloromethane (4:1) for 18 hours. The extract was concentrated by vacuum rotary evaporation (20 °C, 20 Torr) to 2 mL and 2 mL of sulphuric acid were added. The mixture was vigorously stirred using a Vortex-mixer (2 min) and centrifuged (4000 rpm, 5 min) to remove any foam in the interphase and the sulphuric acid phase was discarded. This clean-up step was repeated until a colorless transparent acid layer was obtained (3-5 times).

The final sulphuric acid mixture was re-extracted with *n*-hexane (2 x 2 mL) and all *n*-hexane solutions were combined and passed through an anhydrous sodium sulphate column. Subsequently, the *n*-hexane solutions were concentrated by vacuum rotary evaporation (20 °C, 20 Torr) to small volumes (ca. 300 µL) and transferred to vials

before further evaporated almost to dryness under a gentle stream of nitrogen (10-20 °C). The cleaned extract was redissolved in 50 μL of PCB 142 in isooctane as internal standard for instrumental analysis. Lipid content was determined gravimetrically after drying to constant weight an aliquot of the organic extracts.

2.3.2. Instrumental analysis for chemical contaminants in animal tissue

To determine levels of PCBs (7 congeners: IUPAC # 18, 52, 101, 118, 138, 153, 180) (Bachour et al., 1998), DDTs (2,4'-DDT, 4,4'-DDT, 2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 4,4'-DDD), hexachlorobenzene (HCB), and hexachlorocyclohexane isomers (α-, β-, γ-, δ-HCH), samples were analyzed using a gas chromatograph (Model HP-6890) equipped with an electron-capture detector (μ-ECD). The separation was achieved with a 60 m x 0.25 mm I.D. DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5 % diphenylpolydimethylsiloxane (film thickness 0.25 mm). The oven temperature was programmed from 90 °C (holding time 2 min) to 130 °C at 15 °C min^{-1} and finally to 290 °C at 4 °C min^{-1}, keeping the final temperature for 20 min. The injector and detector temperatures were 280 °C and 320 °C, respectively. Injection was performed in splitless mode. Helium was the carrier gas (30.5 psi). To determine levels of 41 individual PCB congeners (IUPAC # 18, 28, 31, 33, 44, 49, 52, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 158, 169, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 201, 205, 206, 208, and 209) instrument conditions were set as described in Cabrerizo et al. (2009).

2.3.3. Quality assurance and control

Procedural blanks were analyzed for every set of six samples to assess the possible inadvertent contamination of samples during analytical procedures. Blanks were used to establish method detection (MDL) and quantification limits (MQL), which were defined as the mean of the blanks plus three times (MDL) or five times (MQL) the standard deviation. They were in the order of 0.03 and 0.05 ng g^{-1} w.w., respectively. Extraction and analytical performance were evaluated by surrogate standard recoveries, which ranged from 65 % to 90 %. Values reported in this study were surrogate recovery corrected.

2.4. Biomarker analysis

2.4.1 Sample preparation

A portion of liver/hepatopancreas (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing for fish liver 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMFS), 1 mM ethylenediaminetetraacetic acid (EDTA) and for crustacean hepatopancreas 100 mM KCl, 1 mM EDTA, 0.1 mM phenantroline and 0.1 mg L^{-1} trypsin inhibitor. The homogenate was centrifuged at 10,000 g for 30 min and the obtained supernatant (S10) was stored at -80 °C until further biochemical analyses.

2.4.2. Biomarker assays

Assays were carried out in triplicate at 25 °C in 96-well format using a TECAN™ Infinite M200 microplate reader as described in (Antó et al., 2009) if not further specified.

Catalase (CAT) activity was measured as absorbance decrease at 240 nm for 1 min using 50 mM H_2O_2 as substrate ($\boldsymbol{\varepsilon}$ = 40 M^{-1} cm^{-1}).

Glutathione reductase (GR) activity was measured as decrease in absorbance at 340 nm for 3 min using 0.9 mM oxidized glutathione as substrate (GSSG) and 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) ($\boldsymbol{\varepsilon}$ = 6.22 mM^{-1} cm^{-1}) .

Total glutathione-peroxidase (GPX) activity was determined as decrease in absorbance at 340 nm during 3 min using 2.5 mM reduced glutathione (GSH), 1 unit of glutathione reductase (GR), 0.625mM cumene hydroperoxide (CHP) and 0.3mM NADPH ($\boldsymbol{\varepsilon}$ = 6.22 mM^{-1} cm^{-1}).

Glutathione-S-transferase (GST) activity was measured as increase in absorbance at 340 nm for 3 min using 1 mM GSH as substrate and 1 mM 1-chloro-2,4- dinitrobenzene (CDNB) ($\boldsymbol{\varepsilon}$ = 9.6 mM^{-1} cm^{-1}) .

Carboxylesterase (CbE) activity was determined as increase in absorbance at 405 nm during 5 min using 0.67 mM S-phenylthioacetate as substrate and 0.18 mM 5,5-dithio-bis-2-nitrobenzoate (DTNB) (ε = 13.6 M^{-1} cm^{-1}).

7-Ethoxyresorufin-O-deethylase (EROD) activity in fish and *7-Pentoxyresorufin-O-deethylase* (PROD) activity in the crustacea were measured kinetically as increase in fluorescence at 537 nm excitation and 583 nm emission over 10 min as described in (Koenig et al., 2012). Substrates used include 7-ethoxyresorufin (3 μM) and 7-pentoxyresorufin (5 μM), respectively and 0.2 mM NADPH as cofactor. A standard curve of seven resorufin sodium salt concentrations (0-160 nM) was used to quantify activities.

Superoxide dismutase was measured following the method for microplate format as described in Kopecka-Pilarczyk and Correia (2009). SOD activity was determined as inhibition of the reduction of cytochrome c at 550 nm. The reaction contained 50 μM hypoxanthine, 1.8 $mUml^{-1}$ xanthine oxidase and 10 μM cytochrome c. SOD activity was expressed as units mg $protein^{-1}$, where one SOD unit represents the amount of sample causing a 50 % inhibition of the cytochrome c reduction. A seven point standard curve of commercial SOD (1-40 U mL^{-1}) was used for quantification.

Lipid peroxidation (LP) levels were determined using 200 μL of liver/hepatopancreas sample mixed with 650 μL of 1-methyl-2-phenylindole in acetonitrile:methanol (3:1) and 150 μL of HCl. This mixture was incubated for 40 minutes at 45 ºC and subsequently centrifuged at 13,000 rpm x 10 minutes to precipitate proteins. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated in the same way. LP content was expressed as nmol malondialdehide g^{-1} wet weight.

Protein content was determined according the method by , using bovine serum albumin as standard (BSA 0.1–1 mg ml^{-1}).

2.5. Statistical analysis

All data were tested for normality using the Shapiro-Wilk's test and for homogeneity of variance according to Levene's test. Data did not satisfy the assumptions of normality and homogeneity of variance and a non-parametric Mann-Whitney U analysis (MWU) by mean ranks was performed ($P < 0.05$). Spearman's rank correlation coefficient was used to analyze the correlation between variables. In case significant differences in body length existed, a General Linear Model (GLM) was performed on logarithmic transformed biomarker data using the factor body length as covariate to confirm that site differences were not due to differences in size.

3. Results

3.1. Chemical analysis

Contaminant analysis in muscle tissue revealed significantly higher DDT (MWU, $U = 19$, $n_1 = 9$, $n_2 = 10$, $P = 0.037$) and PCB (MWU, $U = 20$, $n_1 = 9$, $n_2 = 10$, $P = 0.045$) concentrations for *A. rostratus* inside Blanes canyon (BC) than on the open slope (OS) at 1500 m depth during winter (Table 1 and Figure 2). However, a significant positive correlation between PCB and DDT concentrations and lipid content in fish muscle was also observed (Spearman's rank correlation: $\rho = 0.69$, $P = 0.001$ and $\rho = 0.67$, $P = 0.002$, respectively). The muscle tissue of fish from the BC showed the highest % of lipid; therefore PCB and DDT levels did not differ between BC and OS when concentrations were expressed on lipid weight basis (ng g^{-1} lipid weight) ($P > 0.05$, data not shown). In contrast, organisms from the OS exhibited higher concentrations of HCHs than samples from BC (MWU, $U = 4$, $n_1 = 9$, $n_2 = 10$, $P < 0.001$) (Table 1). No differences in HCB concentrations were detected between sites. In addition, POP concentrations in OS appeared to increase with depth, although this trend could not be statistically confirmed because of the use of pooled samples at 900 m and 1200 m depth, respectively. Also, *A. rostratus* caught at 900 m depth were significantly smaller than fish individuals from 1500 m depth (MWU, $U = 20$, $n_1 = 9$, $n_2 = 10$, $P = 0.025$) (Table 1).

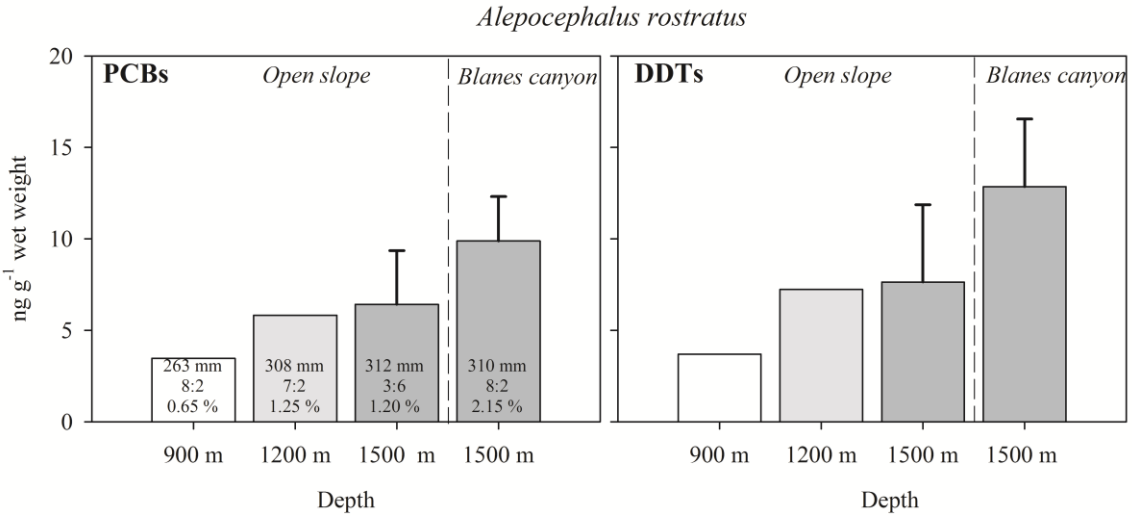

Fig.2 Mean PCB and DDT levels (ng·g^{-1} wet weight) in *Alepocephalus rostratus* from the open slope (900 m, 1200 m and 1500 m depth) and inside Blanes canyon at 1500 m depth during winter (February). Samples from 900 m and 1200 m depth were analysed as pooled samples. Numbers inside bars indicate average length (mm), sex ratio (M:F) and lipid content (%).

Contaminant levels measured in *L. lepidion* did not exhibit any significant differences between BC and OS at 1500 m depth during winter, except HCHs, which were detected at higher concentrations in OS (MWU, U = 22, $n_1 = n_2 = 10$, P = 0.032) (Table 1), similar to the results found for *A. rostratus*. In contrast, higher PCB, DDT and HCB concentrations were found in *L. lepidion* within BC compared to OS at 900 m depth during autumn, although no statistical analysis could be performed because of the use of pooled samples (Table 1 and Figure 3). It should also be noted that individuals caught within BC at 900 m depth were significantly smaller in size than those from the OS (MWU, U = 2, $n_1 = n_2 = 10$, P < 0.001) (Table 1). In addition, POP levels in BC at 900 m in autumn were similar to those measured in winter at 1500 m at both sites. Similarly, pooled samples of *A. antennatus* appeared to have higher PCB and DDT levels within BC in comparison to OS during autumn at 900 m depth, while HCHs showed again the opposite trend (Table 1 and Figure 3). There was no significant difference in size of *A. antennatus* at 900 m depth during autumn (P > 0.05).

Table 1 Biological characteristics and mean organic contaminant concentrations in deep-sea organisms from inside Blanes canyon (BC) and the adjacent open slope (OS). Numbers marked in bold indicate significant differences between sites samples (Mann-Whitney U, $P < 0.05$).

Species	Season	Site	Depth (m)	Length[a] (mm)	Sex ratio[b] M:F:Im	Lipid (%)	POP (ng g^{-1} w.w.)				
							ΣPCB_7	ΣPCB_{41}	ΣDDT	ΣHCHs	HCB
A. rostratus	Winter (February)	OS	900*	263 ± 14	8:2:0	0.65	3.5	n.a.	6.9	0.09	0.06
			1200*	308 ± 14	8:2:0	1.25	5.8	n.a.	5.8	0.34	0.08
			1500	312 ± 12	3:6:0	1.20 ± 0.4	**6.4 ± 1.3**	n.a.	**7.6 ± 1.8**	**0.36 ± 0.08**	0.16 ± 0.04
		BC	1500	310 ± 9	8:2:0	2.15 ± 0.7	**9.9 ± 1.1**	n.a.	**12.9 ± 1.6**	**0.09 ± 0.02**	0.17 ± 0.04
L. lepidion	Winter (February)	OS	1500	**156 ± 11**	5:4:1	0.36 ± 0.02	5.6 ± 1.0	n.a.	3.7 ± 0.6	**0.09 ± 0.01**	0.05 ± 0.01
		BC	1500	**188 ± 5**	0:6:4	0.36 ± 0.07	6.8 ± 0.8	n.a.	4.1 ± 0.7	**0.04 ± 0.02**	0.04 ± 0.01
	Autumn (November)	OS	900*	**186 ± 5**	3:6:1	0.77	2.0	2.8	2.7	0.10	0.09
		BC	900*	**107 ± 9**	1:1:8	0.60	3.8	7.4	5.8	0.09	0.18
A. antennatus	Autumn (November)	OS	900*	51.9 ± 2	0:5:0	0.71	0.7	1.0	2.1	0.11	0.03
		BC	900*	49.5 ± 3	0:5:0	0.78	1.3	2.9	3.0	0.02	0.01

n.a., not analyzed

* pooled samples

[a] Fish: total length and *A. antennatus*: carapace length

[b] M = male, F = female and Im = immature

ΣPCB_7, sum of seven individual congeners; ΣPCB_{41}, sum of 41 individual congeners; ΣDDT, sum of DDTs, DDEs and DDDs; ΣHCHs, sum of α-, β-, γ-, δ-HCHs

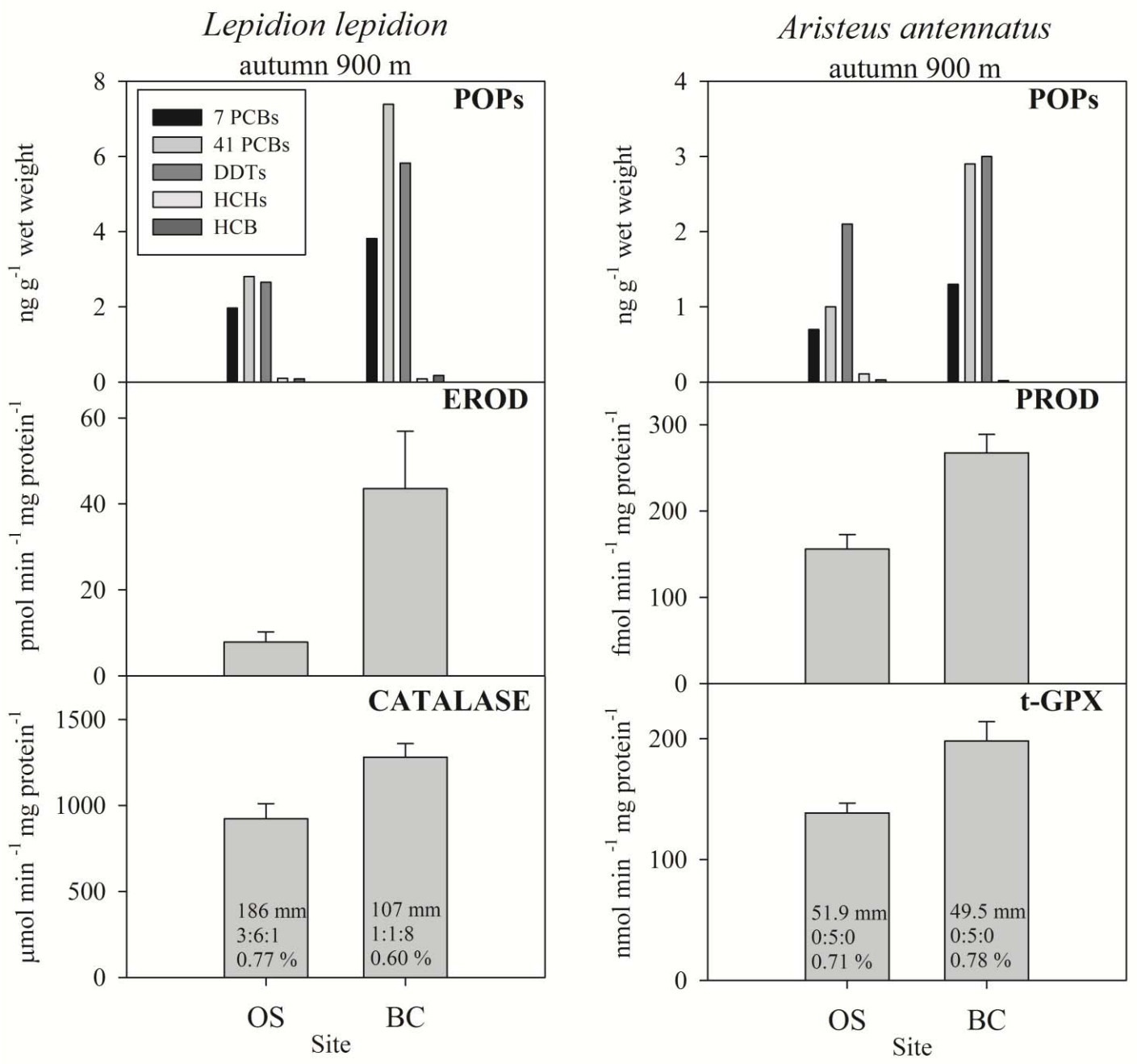

Fig. 3 Contrast between samples from open slope (OS) and Blanes canyon (BC) for *Lepidion lepidion* and *Aristeus antennatus* at 900 m depth during autumn (November). Reported results include organic contaminant levels (ΣPCBs 7 and 41 congeners and ΣDDTs) cytochrome P450 (EROD/PROD), and antioxidant (CAT/GPX) enzymes activities. Numbers inside bars indicate average length (mm), sex ratio (M:F) and lipid content (%).

Figure 4 illustrates the difference in the relative proportion of each PCB group (based on their number of chlorine atoms) between OS and BC for the three species selected in this study. The plotted value represents the difference between the percentages a given PCB group contributed to the sum of PCBs in samples from OS and from BC, calculated as followed

$\%\Sigma PCBs_{(OS)} - \%\Sigma PCBs_{(BC)}$, where $\%\Sigma PCBs = \frac{[PCBgroup]}{[\Sigma PCBs]} \times 100$

For *A. rostratus*, differences in the relative proportion of each PCB group between OS and BC were low, 5 % at the most. These differences involved higher proportion of tetrachloro- and hexachlorobiphenyls in OS and BC, respectively. Similar results were observed for *L. lepidion* in winter (maximum 3.6 % differences between OS and BC). In contrast, *L. lepidion* and *A. antennatus* exhibited significant differences in PCB groups between OS and BC in autumn at 900 m depth. For both species, low-molecular-weight compounds (3-5 chlorine atoms) were more abundant in the samples from OS than in BC, while higher chlorinated PCBs, particularly hexachlorobiphenyls (*i.e.* PCBs 153 and 138), contributed 30 % more to the total sum of PCBs in samples from BC.

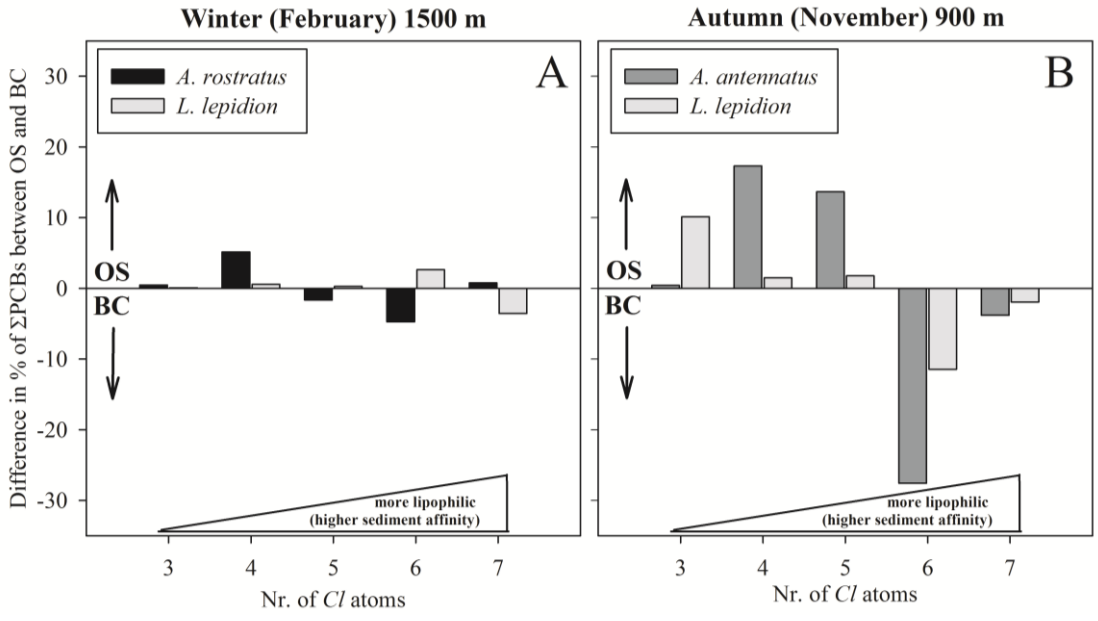

Fig. 4 Difference in relative proportion of PCB groups (according to their number of chlorine atoms) between open slope (OS) and Blanes cayon (BC). Data are shown for (A) *A. rostratus* and *L. lepidion* at 1500 m depth in winter (February) and (B) *Aristeus antennatus* and *L. lepidion* at 900 m depth in autumn (November). The plotted value is the difference of the percentage each PCB group contributed to the total sum of PCBs between samples from OS and BC.

3.2. Biomarker analysis

For *A. rostratus*, hepatic biomarker responses were contrasted between samples from BC and OS caught in winter and autumn at 1500 m depth. Biomarker data did not exhibit any significant differences between sites, except for SOD activity, with higher values in samples from OS during autumn (Table 2 and 3).

For *L. lepidion*, hepatic biomarker responses were contrasted between sites in autumn at 900 m and 1500 m depth (Table 2 and 3). At 900 m depth, results indicated significantly higher EROD and CAT activities in BC compared to OS (Figure 3), while CbE activity was significantly lower within BC. Although fish length was also different between sites at 900 m depth, the former characterization of these enzyme activities has shown that the above-mentioned enzyme activities do not exhibit a significant relationship with body length in *L. lepidion* (n = 80, unpublished data). Thus, the difference in length between fish from BC and OS does not have any effect on EROD, CbE and CAT activities in this species.

Hepatopancreas biomarker activities were contrasted between sites for *A. antennatus* during three seasons: in winter at 1500 m, in summer at 900 m and in autumn at both 900 m and 1500 m depths (Table 2 and 3). No significant differences between sites (*i.e.* BC and OS) were observed in winter at 1500 m depth. In summer, CbE activity was significantly higher in OS, but carapace size was also significantly smaller for individuals from BC compared to OS. In autumn, PROD and GPX activities were significantly higher for samples from BC than from OS at 900 m depth (Figure 3). At the same time, CbE and GST activities were significantly lower within BC at 1500 m depth, although it is noteworthy that carapace size was also bigger for these individuals. As CbE and GST have been shown to be influenced by carapace size in *A. antennatus* (n = 140, unpublished data), a GLM with carapace size as covariate was used to confirm the site differences in CbE activity in summer at 900 m depth and CbE and GST activities in autumn at 1500 m depth. The GLM revealed that the factor length, and not site, has a significant effect on the differences in CbE and GST activities, indicating that the differences in enzyme activities between sites were due to the differences in size.

Table 2 Biomarker responses (mean ± S.E.) for *Alepocephalus rostratus, Lepidion lepidion* and *Aristeus antennatus* in samples from open slope (OS) and Blanes canyon (BC). Values marked in bold denote significant differences between OS and BC (Mann-Whitney U, $P < 0.05$). Values are shown for ethoxyresorufin-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD), carboxylesterase, glutathione S-transferase (GST), total glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), superoxide-dismutase (SOD) activities and lipid peroxidation levels (LP).

Species	Season	Site	Depth (m)	Length (mm)	Sex ratio M:F:Im	EROD/PROD[a]	CbE[b]	GST[b]	GPX[b]	GR[b]	CAT[c]	SOD[d]	LP[e]
A. rostratus	Winter (February)	OS	1500	318.6 ± 12.7	3:7:0	256.6 ± 84.6	142.3 ± 11.4	179.9 ± 10.3	50.9 ± 1.7	2.9 ± 0.4	2708.1 ± 178.3	12.9 ± 1.2	n.a.
		BC	1500	310.0 ± 9.1	8:2:0	390.8 ± 94.7	167.2 ± 15.2	208.0 ± 10.4	50.0 ± 1.8	1.7 ± 0.3	2829.5 ± 151.6	12.0 ± 0.7	n.a.
	Autumn (November)	OS	1500	315.1 ± 13.2	1:9:0	280.9 ± 116.1	230.4 ± 37.2	288.4 ± 11.8	52.9 ± 2.1	3.6 ± 0.2	2815.7 ± 151.1	**21.9 ± 1.5**	63.5 ± 8.7
		BC	1500	292.3 ± 10.2	7:3:0	452.8 ± 112.2	225.2 ± 32.8	289.7 ± 33.7	50.7 ± 4.1	3.1 ± 0.5	2572.8 ± 111.2	**14.7 ± 1.0**	96.1 ± 24.6
		Mean		309.0 ± 5.7	19:21:0	345.3 ± 51.0	191.3 ± 14.1	241.5 ± 12.1	51.1 ± 1.3	2.8 ± 0.2	2731.5 ± 73.9	15.4 ± 0.8	79.8 ± 13.3
L. lepidion	Autumn (November)	OS	900	**186.3 ± 5.2**	3:6:1	**7836.1 ± 2403.4**	**52.8 ± 3.3**	324.5 ± 22.8	51.9 ± 2.9	3.6 ± 0.6	**922.9 ± 88.6**	n.a.	21.8 ± 3.9
		BC	900	**107.1 ± 9.7**	1:1:8	**43533.1 ± 13353.5**	**30.5 ± 6.6**	276.7 ± 23.8	43.2 ± 1.8	2.1 ± 0.8	**1281.9 ± 78.2**	n.a.	20.0 ± 2.4
		OS	1500	217.6 ± 14.4	4:6:0	4133.7 ± 2214.9	31.8 ± 3.2	345.2 ± 14.9	34.6 ± 1.7	3.8 ± 0.4	**1031.3 ± 72.3**	10.7 ± 1.9	22.7 ± 2.8
		BC	1500	215.7 ± 7.7	1:7:2	2118.7 ± 426.2	28.6 ± 2.4	380.9 ± 22.3	36.2 ± 1.7	3.1 ± 0.4	**632.4 ± 42.3**	19.5 ± 5.9	18.7 ± 2.5
		Mean		181.7 ± 8.6	9:20:11	13656.9 ± 4059.7	35.9 ± 2.6	331.8 ± 11.9	40.9 ± 1.7	3.3 ± 0.3	967.2 ± 51.1	15.1 ± 3.2	20.6 ± 1.4
A. antennatus	Winter (February)	OS	1500	30.2 ± 2.6	4:6:0	173.9 ± 50.3	648.1 ± 74.0	203.1 ± 46.1	200.5 ± 15.5	1.5 ± 0.4	6.5 ± 1.2	49.7 ± 22.8	n.a.
		BC	1500	34.4 ± 2.2	12:3:0	265.9 ± 35.9	713.1 ± 74.9	198.4 ± 24.3	197.5 ± 13.1	1.3 ± 0.2	6.9 ± 1.2	66.8 ± 23.7	n.a.
	Summer (September)	OS	900	**44.1 ± 3.7**	1:9:0	191.3 ± 16.1	**341.4 ± 44.8**	212.0 ± 62.2	183.6 ± 16.4	0.9 ± 0.3	9.4 ± 1.4	n.a.	35.4 ± 4.9
		BC	900	**31.9 ± 1.5**	0:10:0	157.5 ± 11.3	**687.4 ± 49.6**	187.0 ± 37.3	221.1 ± 14.2	1.0 ± 0.3	7.1 ± 1.1	n.a.	27.2 ± 1.4
	Autumn (November)	OS	900	43.0 ± 3.1	5:5:0	**155.7 ± 16.9**	360.8 ± 23.6	67.5 ± 11.4	**138.6 ± 8.0**	0.9 ± 0.3	7.7 ± 1.2	89.1 ± 22.7	n.a.
		BC	900	37.7 ± 4.3	5:5:0	**267.3 ± 21.4**	324.7 ± 31.6	71.3 ± 8.9	**198.2 ± 15.9**	1.3 ± 0.4	4.4 ± 0.9	54.1 ± 19.0	n.a.
		OS	1500	**24.5 ± 1.6**	5:5:0	197.5 ± 62.5	**795.4 ± 81.0**	**241.4 ± 70.6**	310.8 ± 48.5	1.8 ± 0.4	3.1 ± 1.4	46.8 ± 12.8	n.a.
		BC	1500	**34.0 ± 4.0**	8:2:0	241.6 ± 25.9	**592.9 ± 50.3**	**83.1 ± 9.9**	222.6 ± 18.8	1.0 ± 0.2	2.4 ± 0.6	81.5 ± 25.8	n.a.
		Mean		35.0 ± 1.2	40:45:0	209.8 ± 12.8	567.1 ± 28.0	160.4 ± 15.1	208.4 ± 8.7	1.2 ± 0.1	6.0 ± 0.5	63.8 ± 9.4	31.3 ± 2.9

n.a., not analyzed
[a] fmol min^{-1} mg $protein^{-1}$
[b] nmol min^{-1} mg $protein^{-1}$
[c] µmol min^{-1} mg $protein^{-1}$
[d] a.U mg $protein^{-1}$
[e] nmol MDA g^{-1} wet weight

Table 3 Details for pair-wise comparisons (Mann-Whitney U, $P < 0.05$) of biomarker responses between samples from Blanes canyon (BC) and open slope (OS). In the case of A. antennatus, a General Linear Model (GLM) was performed using length as covariate if length was significantly different between sites.

Species	Season	Depth	Length	EROD/PROD	CbE	GST	GPX	GR	CAT	SOD	LP
A. rostratus	Winter	1500	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.a.
	Autumn	1500	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	U = 9 $n_1 = n_2 = 10$ P = 0.002	n.s.
L. lepidion	Autumn	900	U = 2 $n_1 = n_2 = 10$ $P < 0.001$	U = 20 $n_1 = 9, n_2 = 10$ P = 0.045	U = 18 $n_1 = n_2 = 10$ P = 0.017	n.s.	n.s.	n.s.	U = 18 $n_1 = n_2 = 10$ P = 0.017	n.a.	n.s.
		1500	n.s.	n.s.	n.s.	n.s.		n.s.	U = 7 $n_1 = n_2 = 10$ P = 0.001	n.s.	n.s.
A. antennatus	Winter	1500	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.a.
	Summer	900	U = 12 $n_1 = n_2 = 10$ P = 0.005 GLM	n.s.	U = 5 $n_1 = n_2 = 10$ $P < 0.001$ site P = 0.47 length P = 0.002 site*length n.i.	n.s.	n.s.	n.s.	n.s.	n.a.	n.s.
	Autumn	900	n.s.	U = 4 $n_1 = n_2 = 10$ $P < 0.001$	n.s.	n.s.	U = 7 $n_1 = n_2 = 10$ P = 0.001	n.s.	n.s.	n.s.	n.a.
		1500	U = 16 $n_1 = n_2 = 10$ P = 0.01 GLM	n.s.	U = 21 $n_1 = n_2 = 10$ P = 0.031 site P = 0.56 length P = 0.004 site*length P = 0.003	U = 11 $n_1 = n_2 = 10$ P = 0.004 site P = 0.54 length P = 0.086 site*length P = 0.069	n.s.	n.s.	n.s.	n.s.	n.a.

n.a., not analyzed
n.s., not significant
n.i., not included

4. Discussion

Chemical and biomarker results indicated that organisms dwelling within Blanes canyon (BC) are exposed to higher pollution levels in comparison to the adjacent open slope (OS), although this trend was not evident at all depths, suggesting that the existence of a pollution gradient between sites could vary spatially. At 900 m depth, the fish *L. lepidion* and the crustacean *A. antennatus* exhibited higher organic contaminant accumulation as well as increased biomarker responses within the canyon (Figure 3). Differences appeared to be more pronounced in *L. lepidion* than in *A. antennatus*, suggesting differential bioaccumulation dynamics (uptake and excretion) between the two species. This is in accordance with previous results showing that *L. lepidion* has lower CYP-mediated PCB metabolizing capacities than *A. antennatus* and thus accumulates PCBs more readily (Koenig et al., 2012). In accordance with chemical results, both species, namely *L. lepidion* and *A. antennatus*, exhibited significantly higher activities of cytochrome P450-related EROD and PROD enzymes, respectively, as well as antioxidant enzymes CAT and GPX, respectively (Figure 3). A multitude of chemicals have been shown to be able to induce CYP enzymes and numerous studies have linked increases in EROD/PROD activity to exposure to environmental stressors (Goksøyr and Förlin, 1992; Whyte et al., 2000; van der Oost et al., 2003). *L. lepidion* exhibited a 600 % increase in CYP1A-related EROD activity, while *A. antennatus* CYP2B-related PROD activity increased by approximately 100 %. As reviewed in van der Oost et al. (2003), an increase in EROD activity is considered as strong if the activity is 500 % of the control value. In this study, the strong increase in EROD activity in significantly smaller juvenile fish within BC is particularly remarkable considering the fact that former studies revealed that the inducibility of EROD, as a result of contaminant exposure, is lower in smaller fish (Whyte et al., 2000).

Concomitant with the above-mentioned increase in CYP activity, both species experienced an increase in antioxidant enzyme activities (Figure 3). This finding is consistent with the fact that the CYP-mediated biotransformation of organic contaminants may enhance the production of reactive oxygen species (ROS), potentially causing an increase in oxidative stress and subsequent antioxidant responses (*i.e.* CAT and GPX in this case) (Winston and Di Giulio, 1991). The lack of increase in LP levels for *L. lepidion* samples from BC suggests that oxidative stress did not result in the

peroxidation of lipids. The fact that different antioxidant enzymes responded in the fish and the crustacean species might reflect a potentially different role of CAT and GPX between these organisms. As seen in other deep-sea fish (Janssens et al., 2000), *A. rostratus* and *L. lepidion*, exhibited low GPX activities, whereas CAT activities were almost one order of magnitude higher than those of fish dwelling at shallower depths (Solé et al., 2010). In this context, it has been postulated that GPX is mainly used as defense mechanism against metabolically produced ROS, while CAT could be essentially responsible for eliminating exogenously-generated H_2O_2 in deep-sea fish (Janssens et al., 2000). Thus, in deep-sea fish, CAT might exhibit a more marked response than GPX as a result of xenobiotic exposure, which is also in accordance with other studies (Lemaire et al., 2010). In contrast to fish, *A. antennatus* exhibited low CAT and high GPX activity, indicating that crustaceans potentially rely on different antioxidant mechanisms than fish to respond to oxidative stress. This, in turn, would explain why in *A. antennatus* GPX, and not CAT, enzymes responded to the putative increase in pollutant-induced ROS production.

In addition, CbE activity was significantly lower in *L. lepidion* from BC. In general, CbE activity is believed to increase in the presence of xenobiotics due to its role in the metabolism of ester-containing compounds (Wheelock et al., 2005). However, certain chemicals such as tributyltins **(**TBTs) and organophosphate pesticides are known to inhibit CbE activity (Al-Ghais et al., 2000; Wheelock et al., 2005). In particular, elevated levels of TBTs have been found in deep-sea fish within the same region, indicating the presence of this contaminant at great depths (Borghi and Porte, 2002). The significant inhibition of CbE within BC could thus reflect higher exposure to the above-mentioned pollutant classes. This trend is however not reflected in *A. antennatus,* for which CbE activities did not differ between sites at 900 m depth. The fact that other biomarker responses applied in the present work (*i.e.* GST, GR, SOD and LP) did not reveal any significant differences between sites in autumn at 900 m depth could be due to their lower sensitivity as pollution biomarkers compared to, for instance, the EROD assay (van der Oost et al., 2003).

The pollution gradient between OS and BC detected at 900 m was not evidenced in POP levels and biomarker responses in *L. lepidion* at 1500 m depth. Moreover, the fact that at 900 m depth in autumn relatively small juvenile *L. lepidion* exhibited POP levels

within the same range as the values observed for larger individuals at 1500 m depth in winter corroborates that a significant pollution occurred within the canyon at 900 m depth. Accordingly, at 1500 m depth, biomarker data also did not indicate any clear differences between sites (Table 2). In the case of the fish *A. rostratus*, significant differences in POP levels between individuals from OS and BC at 1500 m depth were observed (Figure 2). However, they cannot be unequivocally attributed to the existence of a pollution gradient because of confounding biological parameters between individuals from the different sampling sites, mainly sex distribution and lipid content (Table 1). For instance, when normalized for lipid content, PCB and DDT levels did not differ between sites at 1500 m depth, because fish from BC had a higher lipid content than those from OS. Previous studies have shown that lipid content can differ between sexes, as females are known to invest stored lipid reserves into ovarian egg production, which are then released when spawning (Tocher, 2003). Thus, the difference in POP levels between specimens from OS and BC at 1500 m could simply be due to the influence of sex on the accumulation of POPs, as sex ratio differed between sites. This hypothesis is however not confirmed when taking into account the results for *A. rostratus* sampled on the OS at 1200 m depth, as lipid content was similar in male fish from 1200 m and females from 1500 m depth, indicating that sex did not affect the muscle lipid content in *A. rostratus* (Table 1 and Figure 2). Moreover, when contrasting POP levels between fish from the OS at 1200 m depth and BC 1500 m depth, both composed of males of comparable sizes, it confirms that the lipid content, regardless of sex, is the main confounding factor responsible for the observed differences.

The detected relationship between POP levels and lipid content in *A. rostratus* is in accordance with the general agreement that the bioaccumulation of organic contaminants is related to tissue lipid content (Hebert and Keenleyside, 1995; Randall et al., 1998). The lipid content of muscle tissue is usually related to the feeding condition in teleost fish and increased levels could indicate higher food availability within the canyon (Tocher, 2003; Lloret et al., 2005). In the case of the BC, higher organic matter deposition has been shown to occur from the vertical transport of organic material from surface waters as well as the lateral transport of organic matter as a result of re-suspension of sediments by water currents generated during meteorological events (López-Fernández et al., 2012). Hence, the higher lipid content in *A. rostratus* from BC at 1500 m may have resulted from higher organic matter deposition inside the canyon,

which is accordance with hydrodynamic data showing that organic matter flux was higher within BC at 1500 m depth than on the open slope at any depth (López-Fernández et al., 2012). In addition, for *A. rostratus* pollutant concentrations appeared to increase with depth (Figure 2), but this trend is most likely due to changes in fish size distribution with depth. As shown by Morales-Nin et al. (1996), this species presents a size/age stratification with depth in the NW Mediterranean, with smaller, younger fish mainly concentrating at the shallower depths of the species' distribution range. Hence, lower POP levels in smaller individuals from 900 m depth compared to larger fish from 1500 m depth is consistent with the age-dependent accumulation of organic contaminants observed in other fish species (Stow and Carpenter, 1994; Vives et al., 2005).

Hence, while POP levels and biomarker results provided strong evidence that the accumulation rate of organic contaminants was higher in organisms within the upper canyon (900 m depth) compared to the open slope, the difference was less clear at 1500 m depth. This is in accordance with previous studies conducted on submarine canyons off the Portuguese coast, which concluded that anthropogenic contaminants are preferentially channeled through the canyons as compared to the adjacent open slope and tend to accumulate at the head of the canyon (Richter et al., 2009; Jesus et al., 2010). These findings are also consistent with hydrodynamic data from the same area showing that particle flux within the canyon axis was highest at shallower depths (*i.e.* < 1200 m) during the study period (López-Fernández et al., 2012), which further supports the above-mentioned idea that the pollution gradient was more pronounced in the upper canyon region as compared to the lower part of the canyon. Moreover, this hypothesis is further corroborated by the fact that PCB accumulation profiles for *L. lepidion* and *A. antenntaus* caught inside the canyon at 900 m, but not for *L. lepidion* and *A. rostratus* sampled at 1500 m depth, exhibited higher proportions of the more lipophilic high-molecular weight congeners (Figure 4), suggesting that higher contaminant input to the upper canyon may be associated to sediment transport. This trend was not as prominent for *A. rostratus* at 1500 m depth, which is in accordance with the highest particle deposition rate within the canyon at 900 m depth (López-Fernández et al., 2012). This is also consistent with the higher concentrations of HCHs found in the OS for all organisms. In contrast to the preferentially particle-bound POPs such as PCBs and DDTs, HCHs are mainly present in the water column in the dissolved phase due to their

higher water solubility (Xiao et al., 2004), therefore their transport and fate in aquatic systems are not related to the suspended particle dynamics.

Due to the high hydrophobicity of POPs, the spatial and temporal patterns of the accumulation of particle-bound compounds within the canyon are likely dependent on the amplitude of sediment transportation and re-suspension processes, exhibiting a dynamic rather than constant character. Seasonal variations in particle flux may thus cause a depth-related and temporal variability of contaminant input in the NW Mediterranean deep-sea, closely linked to the deposition of organic matter and the fine sediment fraction (Salvadó et al., 2012a). It is thus possible that higher pollution loads are transferred to the canyon during episodic events of higher particle deposition associated with local meteorological forcing events, which are known to principally occur from late autumn until early spring (Heussner et al., 2006; Zúñiga et al., 2009; López-Fernández et al., 2012). The head of the canyon may thus act, at least temporarily, as a trap for organic contaminants, which consequently bioaccumulate in deep-sea biota that dwell within the upper region of BC. Considering that studies on the life history of the deep-sea species analysed in the present work have shown that they perform short-term (Aguzzi and Company, 2010) and/or long-term migrations across bathymetric depths (Sardà and Cartes, 1993; Morales-Nin et al., 1996; Rotllant et al., 2002), most individuals probably experience varying degrees of contaminant exposure during their life time. Thus, the assessment of spatial differences in POP accumulation and the effects resulting from chronic exposure to anthropogenic contaminants may be hard to detect, which further underlines the relevance of the present findings. The combined analysis of long-term parameters such as the bioaccumulation of POPs in muscle tissue and short-term biomarkers such as those applied in the present study and a higher temporal resolution in sampling, especially with regard to particular meteorological events, may be a good approach for monitoring the impact of environmental pollutants in the Blanes canyon area.

5. Conclusions

The findings of the present study give further support to the hypothesis that submarine canyons act as natural conduits for anthropogenic contaminants, potentially posing a threat to deep-sea organisms living within these environments. Chemical analyses showed that the fish *Lepidion lepidion* and the crustacean *Aristeus antennatus* caught within Blanes canyon accumulated higher amounts of POPs at 900 m depth. This result was further confirmed by biomarker results for both species. The consistency of chemical and biomarker results in these two species belonging to different phylogenetic groups (*i.e.* fish and crustacean), provides strong evidence that higher pollution levels were present inside BC compared to the adjacent open slope at 900 m depth. Additional chemical and biomarker data did not exhibit clear differences between sites, i.e. BC and OS, at 1500 m depth , suggesting the existence of a depth-related pattern in contaminant input. On some occasions, confounding factors such as differential sex-ratio and lipid content between sites impeded the interpretation of data. The fate of hydrophobic pollutants in aquatic environments, like those included in this study, is closely linked to particle deposition and sediment transport. Thus, seasonal storm-like events might result in pulses of contaminant loads temporarily accumulating at shallower depths within the head of the Blanes canyon, resulting in higher bioaccumulation of POPs and potentially causing adverse effects in organisms dwelling within that area.

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Paper 6

Biliary PAH and alkylphenol metabolites, biomarker enzyme activities and gene expression levels in the deep-sea fish *Alepocephalus rostratus*

Samuel Koenig [a,b]*, Cinta Porte [b], Montserrat Solé [a] and Joachim Sturve [c]

[a] Institute of Marine Science (ICM-CSIC). Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain.

[b] Institute of Environmental Assessment and Water Research (IDAEA-CSIC). C/ Jordi Girona 18, 08034 Barcelona, Catalonia, Spain.

[c] Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE 40530 Göteborg, Sweden

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Resumen

En el presente estudio se investigaron los niveles de contaminación y los posibles efectos negativos asociados a ellos en una especie de pez de aguas profundas, *Alepocephalus rostratus*, capturado en dos zonas diferentes del Mediterráneo noroccidental: el margen continental de la costa catalana (CS) y en mar abierto cerca de las Islas Baleares (WM). En las muestras estudiadas se evaluaron los niveles de metabolitos de hidrocarburos aromáticos policíclicos (PAHs) y alquilfenoles (AP) en bilis, junto con la expresión génica y actividades enzimáticas de varios biomarcadores en hígado. Este trabajo constituye el primer estudio en el que se aplica la expresión génica como herramienta ecotoxicológica en una especie de pez abisal. Los genes que se pudieron secuenciar y, posteriormente, cuantificar usando la técnica de la reacción en cadena de la polimerasa (PCR**)** son el citocromo P450 1A (CYP1A), la catalasa (CAT), la glutatión reductasa, la superóxido dismutasa (SOD) y la vitelogenina (Vtg). Además, también se midieron en hígado las actividades enzimáticas correspondientes a estos genes (menos Vtg) (*i.e.* EROD, CAT, GR, SOD). Los metabolitos de los APs, nonilfenol (NP) y octilfenol (OP), pudieron ser detectados en muestras de WM recogidas hasta los 2000 m de profundidad, indicando la importancia del transporte vertical de estos compuestos hacia zonas marinas profundas y su capacidad de bioacumulación en los organismos que habitan dichas zonas. Por otro lado, se detectaron niveles más elevados de APs en bilis de los organismos de WM comparado con los de CS, indicando una exposición reciente mayor a estos compuestos en la región de las Islas Baleares. La mayor exposición a APs observada en las muestras de WM se confirmó con los resultados de los estudios biológicos que indicaron una expresión génica más elevada en los organismos de esta zona. En particular, el gen de la vitelogenina, indicador de efectos de disrupción endocrina en peces machos, presentó una inducción 35 veces más alta en machos capturados en WM que en CS. Sin embargo, no se observaron diferencias a nivel de respuestas enzimáticas entre los dos puntos de muestreo, indicando que la expresión de genes biomarcadores podría representar una herramienta mejor para detectar efectos adversos en hábitats como el mar profundo.

Abstract

Biliary polycyclic aromatic hydrocarbon (PAH) and alkylphenol (AP) metabolites, hepatic gene expression profiles and corresponding enzyme activities were determined in the deep-sea fish *Alepocephalus rostratus* from two sites within the Mediterranean. The biliary metabolites were quantified using gas chromatography-mass spectrometry electron impact mode GC-MS-EI and included the hydroxylated PAH metabolites (OH-PAHs) 1-naphthol, 2-naphthol, 9-fluorenol, 9-phenantrol and 1-pyrenol and the APs 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP). Five biomarker genes, namely cytochrome P450 1A (CYP1A), vitellogenin (Vtg), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) were successfully cloned and quantified using qRT-PCR. In addition, corresponding enzyme activities (EROD, CAT, SOD and GR) were also determined and potential relationships between gene expression and catalytic activities were investigated. The ΣOH-PAHs detected ranged from 21.1-300.3 ng/g bile and were mainly comprised (>90 %) of 1-naphthol. Both, NP and OP metabolites were also detected in all samples with concentrations of 17.4-107.2 ng/g bile and 4.9-17.3 ng/g bile, respectively. Moreover, significantly higher AP levels were detected in samples from the western Mediterranean (WM) compared to those from the Catalan slope (CS). In accordance with chemical results, gene expression was significantly induced in fish from the WM, however, these results were not reflected in enzyme activity levels. In particular, male fish caught at 2000 m in the WM exhibited a 35-fold Vtg gene induction compared to those from the CS, suggesting that endocrine-disrupting effects may potentially be occurring in such remote environments as the deep-sea.

Keywords: gene expression; biomarkers; vitellogenin (Vtg); alkylphenols (AP); polycyclic aromatic hydrocarbons (PAH); bile metabolites, deep-sea fish; Mediterranean

1. Introduction

For a long time the deep-sea was considered a relatively pristine environment due to its remoteness from anthropogenic pollution sources. However, over the last decades research has shown that the deep-sea may actually act as a sink for persistent contaminants that enter the marine environment (Ballschmiter et al., 1997; Froescheis et al., 2000; Dachs et al., 2002; Wania and Daly, 2002; Scheringer et al., 2004). Among these contaminants, polycyclic aromatic hydrocarbons (PAHs) have been shown to be transported to deeper waters by sinking particles (Bouloubassi et al., 2006), where they are taken up by deep-sea organisms (Escartin and Porte, 1999; Solé et al., 2001). PAHs are a unique class of ubiquitous persistent pollutants, originating from natural and anthropogenic sources such as incomplete combustion processes (pyrogenic) and the release of crude oil and petroleum products (petrogenic).

Alkylphenols (APs) represent a class of nonionic surfactants that are extensively used in detergents, pesticide formulations, fuel, lubricants and many other industrial products, with octylphenol and nonylphenol ethoxylates being the most common surfactants applied. APs are released via municipal wastewater or directly discharged into the environment, where they degrade to more persistent and toxic shorter-chain compounds such as nonylphenol (NP) and octylphenol (OP) (Ying et al., 2002). Many studies have found AP metabolites in a wide range of environmental samples (Ying et al., 2002; David et al., 2009) and there has been growing concern on the toxicity of these compounds due to their ability to mimic natural hormones by interfering with the estrogen-receptor (Nimrod and Benson, 1996). However, little is known about the environmental fate of APs in the marine environment and only few studies have addressed the issue of AP exposure in marine organisms (David et al., 2009). Moreover, the presence of APs has thus far not been investigated in deep-sea biota, although a study by Kannan et al. (1998) detected a peak of NP in Japanese waters at 1000 m depth.

Once taken up by an organism, xenobiotic compounds including PAHs and APs undergo a series of biotransformation processes, before being excreted through the bile as hydroxylated and/or conjugated metabolites. Thus, biliary metabolites of these contaminants can be used as exposure markers in fish (Beyer et al., 2010). Furthermore, the activation of enzymatic biotransformation systems and subsequent effects on, for

instance, antioxidant responses can serve as ecotoxicological tools to assess the exposure to these pollutants (van der Oost et al., 2003).

The cytochrome P450 (CYP) system plays a key role in the Phase I metabolism of many xenobiotics. In fish, CYP1A induction has been used as a robust biomarker to assess the exposure to organic contaminants such as PAHs and polychlorinated biphenyls (PCBs) (Goksøyr and Förlin, 1992; Whyte et al., 2000). Moreover, the exposure to organic pollutants can also cause oxidative stress due to the generation of reactive oxygen species (ROS) (Winston and Di Giulio, 1991). Increased oxidative stress can lead to the induction of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) as a protective mechanism against these oxidative radicals (Winston and Di Giulio, 1991; Valavanidis et al., 2006). The induction of vitellogenin (Vtg) has been used as a marker to assess the exposure of aquatic organisms to xenoestrogens (Matozzo et al., 2008). Vitellogenins are the precursors of the egg-yolk proteins vitellins and their production is under the control of the estrogen receptor in vertebrates. Vtg levels generally increase during sexual maturation of females, but are usually undetectable in males (Copeland et al., 1986). However, exposure to estrogenic compounds including APs, can lead to the expression of Vtg genes in males, as has been observed in laboratory as well as field studies on freshwater and marine fish (Sumpter and Jobling, 1995; Arukwe et al., 2001; Min et al., 2003; Vetillard and Bailhache, 2006; Arukwe and Røe, 2008).

The objectives of the present study were to compare PAH and AP bile metabolite levels, biomarker enzyme activities and gene expression profiles in the deep-sea fish *Alepocephalus rostratus* captured at two sites within the western Mediterranean basin. In particular, we determined hepatic enzyme activity levels of EROD, CAT, GR and SOD using spectro- and fluorimetric assays as well the gene expression of CYP1A, CAT, GR, SOD and Vtg using qRT-PCR. The present work is the first study to assess biliary AP metabolites and to apply quantitative gene expression biomarker techniques to deep-sea fish.

2. Material and Methods

2.1. Sample collection

Specimens of *A. rostratus* were collected from the Catalan slope (CS) during February 2009 and the western Mediterranean (WM) during May 2009 (Figure 1), using an OTMS otter trawl (Sardà et al., 1998) at 1500 m depth on the CS and 1200 m and 2000 m in the WM. Sample details are provided in Table 1. *A. rostratus* is a demersal fish species with a relatively wide depth distribution (300-2300 m), with an abundance peak between 1000 m and 1500 m depth. It is mainly found in the western Mediterranean, where it represents one of the most abundant megafauna species and mainly feeds on gelatinous macroplankton (Cartes et al., 2002). Previous studies have found mature individuals of *A. rostratus* all year round, although a clear peak in spawning activity was observed during summer and autumn (Morales-Nin et al., 1996). Onboard, the bile and liver were dissected and immediately frozen at -20 ºC and -80 ºC, respectively, until further treatment.

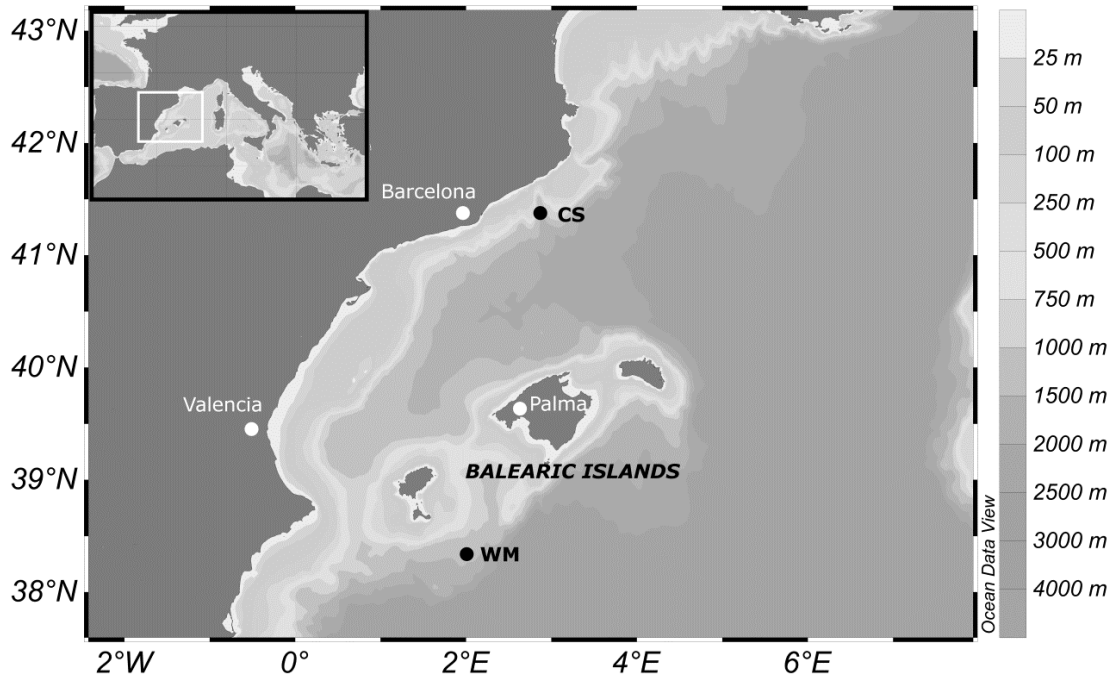

Fig. 1 Map of sampling sites within the Mediterranean Sea. The map was created using the Ocean Data View (ODV) software package by Schlitzer, R., Ocean Data View, http://odv.awi.de, 2010.

Table 1 Biological characteristics and sample sizes (N) of *Alepocephalus rostratus* sampled at two sites within the Mediterranean sea. Maturity scale are given as range based on five-point scale by Brown-Peterson et al., (2011)

Site	Sex	Depth (m)	Maturity	N	Size (mm)
Catalan slope (CS)	F	1500	2-4	9	326 ± 15
	M	1500	2-4	10	302 ± 7
Western Mediterranean (WM)	F	1200	2-4	5	320 ± 24
	M	2000	1-3	6	223 ± 17

2.2. Analysis of hydroxylated PAHs and alkylphenol metabolites in bile

Bile samples were analyzed as described in (Fernandes et al., 2008). Bile liquid of several individuals were pooled if obtained samples volumes were not sufficient (<100 mg) and sample sizes were OS (n = 12) and BC (n = 9). Briefly, 100 mg of bile was incubated for 1 h at 40 ºC in 1 mL 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 U of β-glucuronidase and 50 U sulfatase. Before analysis, hydrolyzed samples were derivatized by the addition of 100 µL bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), left to stand overnight at room temperature. Analyses were carried out by gas-chromatography-mass spectrometry (GC-MS-EI). The derivatized ions used for the quantification of hydroxylated-PAHs were: m/z 216 and 201 for 1-naphthol; m/z 254 and 165 for 9-fluorenol; m/z 266 and 251 for 9-phenanthrol; and m/z 290 for 1-pyrenol. The ions used for monitoring and quantification of APs were: m/z 207 and 193 for 4-nonylphenol (NP) and m/z 207 for 4-tert-octylphenol (OP). Concentrations are expressed as ng/g bile. Detection limits were calculated as signal to noise ratio of 3:1 and were in the order of 1-5 pg for OH-PAHs and 10 pg for NP and 7 pg OP.

2.3. Enzymatic activities

2.3.1. Sample preparation

A portion of liver (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing 150 mM KCl, 1 mM dithiotreitol (DTT), 0.1 mM

phenylmethylsulfonyl fluoride (PMFS) and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 g for 30 min and the supernatant (S9) was used for biochemical analyses.

2.3.2. Biomarker assays

All assays were carried out in triplicate in a 96-well format using a TECAN™ Infinite M200 microplate reader as described in (Koenig et al., 2012). Briefly, CAT activity was measured as absorbance decrease at 240 nm for 1 min using 50 mM H_2O_2 as substrate (ε = 40 $mol^{-1} * cm^{-1}$) and a 100 mM phosphate buffer pH 6.5 (Aebi, 1974). GR activity was measured as decrease in absorbance at 340 nm for 3 min using 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) (**å** = 6.22 * $mmol^{-1} * cm^{-1}$) and 0.9 mM oxidized glutathione (GSSG) as substrate (Carlberg and Mannervik, 1985). SOD activity was determined as inhibition of the reduction of cytochrome c at 550 nm, following the method for microplate format as described in Kopecka-Pilarczyk and Correia (2009). The reaction contained 50 μM hypoxanthine, 1.8 $mUml^{-1}$ xanthine oxidase and 10 μM cytochrome c. EROD activity was measured kinetically based on the procedure by Burke and Mayer (1974), using 7-ethoxyresorufin (3 μM) as substrate and 0.2 mM NADPH. Protein content was determined according the method by Bradford (1976), using bovine serum albumin as standard (BSA 0.1–1 mg/ml).

2.4. Gene expression analysis

2.4.1 RNA extraction

Hepatic RNA was extracted using the RNeasy® Plus kit (Qiagen). RNA concentration, quality and purity were analyzed using a Nanodrop® ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was further analyzed using the Experion™ RNA StdSens Analysis Kit (BIORAD Laboratories, Inc.). All samples passed quality criteria (RQI > 7).

2.4.2. cDNA synthesis

Total RNA (1μg) was used for cDNA synthesis using the iScript First Strand Synthesis kit (BIORAD Laboratories, Inc.) in a total volume of 20μL and an additional control was run using RNAse-free MilliQ H_2O. In addition, a pooled RNA sample was used to

control for genomic DNA contamination by running the cDNA synthesis protocol without reverse transcriptase (NoRT control).

2.4.3. Sequencing and primer design

No published mRNA sequences were available for *A. rostratus.* Conserved sequence regions were derived from the alignment of at least four known fish sequences (*i.e. Salmo salar, Oncorhynchus mykiss, Danio rerio*) using the EMBL-EBI ClustalW2 alignment tool (Larkin et al., 2007; Goujon et al., 2010). Based on the alignment score, either existing primer pairs that had been successfully used in other species (i.e. *Oncorhynchus mykiss*, *Zoa viviparous*) were used or degenerate consensus primers were designed using the Primaclade web-based application (Gadberry et al., 2005) (Table 2). PCR products were run on gels and sequenced by Eurofins MWG Operon. Obtained sequences were tested for homology to existing gene sequences from other fish species using the NCBI-BLAST package (Altschul et al., 1990). Gene-specific primer pairs for qPCR were designed using Beacon Designer software 7.02. (PREMIER Biosoft International) using the SYBR® Green Design settings. Sequences and amplicon size for qPCR primers are shown in Table 3.

Table 2 Primer pairs used for gene identification based on multispecies sequence alignment scores

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sequenced amplicon size (bp)
β-actin	TGGCATCACACCTTCTAC (*Om*)	CCATACCAAGGAAGGAAGG (*OreoM*)	521
EF1a	TGCTGGACAAGCTGAAGG (*Om*)	AGGGTGGTTCAGGATGATGAC (*Om*)	795
12S rRNA	ATCCTCACGGCTGTTATAC (*Zv*)	GCTGATGCTCGTAGTTCC (*Zv*)	144
CYP1A	CTGGARGARCAYRTCWGCAA	SGAGTCWGTRATRTCWCGGAT	334
VTG	TCAARGAGAARTTYMWGGCTG	GGACTTSATGTGRGARTADG	575
CAT	AGGGCAACTGGGAYCTKAC	CCCTGCAGCATCTTGTCNGG	594
SOD	CATGGHTTCCAYGTCCATGC	GYGATGCCDATVACWCCACA	298
GR	CSCGBTTYGATTTTCTRGTG	GCCATYTCCACWGCAATATA	497

Om: Oncorhynchus mykiss

OreoM: Oreochromis mossambicus

Zv: Zoa viviparus

Table 3 Primer sequences used for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
β-actin	TGGCATCACACCTTCTAC	GATACCAGTGGTACGACC	199
EF1a	CGGTATCTCCAAGAACGG	TTGACTCCAACGATGAGC	85
12S rRNA	ATCCTCACGGCTGTTATAC	GCTGATGCTCGTAGTTCC	137
CYP1A	CGTGTCGGTTGCCAATGTCATC	CCCACCACCTGCCCAAACTC	114
VTG	GCAAAGGTTGACTTGGCTGAAATC	TTGGCTCCTTCTTAGCAATGTTCC	184
CAT	TCATTCACTCTCAGAAGCGTAACC	TGGGAGCCGTAGCCGTTC	163
SOD	GGCGGACCCACTGATGAAG	TCACCATAGTTCTCCCAATGATG	142
GR	ATCACGCAGACTACGGCTTTG	TACTTCTTCCCATTGACCTCCAC	199

2.4.4. Quantitative real-time PCR (qPCR)

A standard curve containing six dilution series of pooled cDNA samples (40 ng-1.25 ng) was used to ensure an optimized assay for each primer pair (efficiency 100 % ± 5%). The qPCR was performed in an iQ5 Thermal cycler (Bio-Rad) on a 96-well qPCR plate (4titude). The amplification reaction was performed in duplicates using the fluorescent dye iQ™ SYBR®Green Supermix (Bio-Rad) with 20 ng sample cDNA resulting in a final reaction volume of 20 µl/well. Respective primer had a 'non template control' (NTC) in the amplification reaction to control for primer and mastermix contamination. PCR was achieved by a 1 min activation step at 95 °C, followed by 40 cycles of a 20 s denaturing step at 95 °C, a 20 s primer annealing step at 60 °C and a 30 s elongation step at 72 °C. A melting curve was performed at each run in order to confirm specific amplification of each sample. An accepted Ct-value of NTC and NoRT samples was set to be either non-detectable or >32 cycles. Housekeeping gene expression was used to normalize quantitative PCR cycle threshold (Ct) data (Livak and Schmittgen, 2001). However, previous studies have shown that the expression of commonly used reference genes can also be altered by toxicological processes (Arukwe, 2006; Filby and Tyler, 2007). Therefore, in the present work the geometric mean of three reference genes, namely β-actin, elongation factor 1a (EF1a) and ribosomal RNA 12S, was used to minimize the potential variability of housekeeping gene expression. The calculated geometric mean of reference gene values did not differ between sites ($p = 0.9$).

2.5. Statistical analysis

Data were tested for normality (Shapiro-Wilk's test) and homogeneous variance (Levene's test). For bile metabolites, data were found to satisfy both assumptions and site comparisons were performed using the parametric Student's t-test. For biomarker and gene expression data, the assumptions of normality and homogeneous variance were not satisfied and differences between sites were analyzed using the Mann-Whitney U test. Correlations were determined using the Spearman's rank correlation (ρ) to account for non-linear relationships. Differences at the 5% significance level were considered significant.

3. Results and discussion

3.1. Bile metabolites

The levels of biliary ΣOH-PAHs detected in *A. rostratus* varied between 21.1 and 300.3 ng/g bile (Table 4). Of the five PAH metabolites analyzed, 1-naphthol represented on average 90 % of ΣOH-PAHs, with levels ranging from 15.5 to 287.3 ng/g bile, while the concentrations for 2-naphthol, 9-fluorenol, 9-phenantrol and 1-pyrenol were relatively low or below detection limits (Table 4). Although pyrenol has been regarded as the key PAH metabolite in fish (Ruddock et al., 2002), this compound was almost not detectable in the present study. The high abundance of naphthol indicates a petrogenic origin of PAH contamination, similar to results found in two fish species sampled along the northern Iberian shelf off Galicia (Fernandes et al., 2008), but in contrast to previous results on PAH metabolites in *A. rostratus* from the NW Mediterranean, where pyrenol was the dominant biliary metabolite (Escartin and Porte, 1999). ΣOH-PAHs levels were slightly higher than those measured for fish from the northern Iberian shelf, Atlantic Sea (Fernandes et al., 2008), but similar to previous results reported for *A. rostratus* from the NW Mediterranean (Escartin and Porte, 1999).

To our knowledge, the present study is the first to determine AP metabolites in deep-sea fish. NP and OP were present in all bile samples, with concentrations of OP approximately 5 times lower than NP (Table 4). Only a limited number of field studies have measured AP metabolites in fish bile and the levels of NP and OP detected in the

present study are within the same range as concentrations found in the previously-mentioned study from the northern Iberian shelf (Fernandes et al., 2008), but lower than the concentrations observed in bile of the red mullet (*Mullus barbatus*) from the NW Mediterranean (Martin-Skilton et al., 2006). The relatively short half-life of APs in fish bile suggests that biliary AP residues represent signals of ongoing and not historic exposure to these compounds (Beyer et al., 2011), indicating recent AP exposure in the analyzed deep-sea fish. This result is particularly important considering that APs were detected in fish sampled at up to 2000 m depth, showing that these contaminants are transported to the deep-sea and potentially accumulate in deep-sea organisms.

Table 4 Biliary metabolites detected in *Alepocephalus rostratus* at two sampling sites within the Western Mediterranean basin: Catalan slope (CS) and western Mediterranean (WM). Values represent mean (ng/g bile) ± S.E.M. (min.-max.). Asterisk denotes significant difference between sites based on Student's t-test ($p < 0.05$).

Bile metabolite	CS (n = 16)	WM (n = 9)
1-naphthol	74.4 ± 8.2 (15.5 - 122.3)	107.7 ± 26.6 (33.9 - 287.3)
2-naphthol	1.7 ± 0.5 (n.d. - 3.5)	3.2 ± 0.6 (n.d. - 6.0)
9-fluorenol	4.1 ± 0.7 (n.d. - 7.7)	5.5 ± 0.6 (3.7 - 8.3)
9-phenantrol	n.d.	n.d.
1-pyrenol	0.6 ± 0.3 (n.d. - 4.6)	n.d.
ΣOH-PAHs	80.9 ± 8.2 (21.1 - 122.3)	116.4 ± 27.4 (40.1 - 300.3)
4-nonylphenol*	42.5 ± 5.8 (17.4 - 76.0)	67.1 ± 6.7 (42.3 - 102.7)
4-*tert*-octylphenol*	7.9 ± 0.7 (4.9 - 15.1)	14.2 ± 0.8 (9.5 - 17.3)

Statistical analyses revealed that biliary NP ($t = -2.7$ $p = 0.012$) and OP levels ($t = -5.6$ $p < 0.0001$) were significantly higher in samples from WM compared to CS. Although ΣOH-PAH levels also appeared higher at WM compared to CS, no significant differences in OH-PAH levels were detected between the two regions (Table 4). These results, at least for APs, indicate higher contaminant exposure in fish collected at WM compared to those from the CS, potentially reflecting a higher contaminant input to that area. This apparent pollution difference between the two sites is somewhat surprising considering that the WM area is relatively distant from the coastal mainland and is thought to present relatively low contamination levels as no major industrial pollution sources are known to exist in that area (Baumard et al., 1998). The main pollution sources at the Balearic Islands include shipping-, agricultural- and tourism-related activities (Box et al., 2007), potentially causing the release of APs into the aquatic

environment through the use of surfactant-containing pesticides, cleaning products, detergents as well as household and personal care products (David et al., 2009).

However, it is also noteworthy that samples from CS and WM were collected at different times, namely in February and in May 2009, respectively. To what extent this time lag may have influenced the pollutant exposure levels in *A. rostratus* is difficult to assess and seasonal variations in contaminant input cannot be discarded.

3.2. Gene expression and biomarker responses

Gene expression of EROD, CAT, GR and SOD exhibited significant differences between CS and WM, with a 49-109% induction in samples from WM (Figure 2). The transcriptomic response pattern is in accordance with the biliary PAH and AP metabolite profile, indicating higher pollution exposure in fish from WM compared to those from CS. Previous studies reported an induction of the gene expression of CYP1A (George et al., 2004; Roling et al., 2004; Benedetti et al., 2007; Bilbao et al., 2010), as well as antioxidants, such as CAT and SOD, in fish exposed to PAHs (Nahrgang et al., 2009). Moreover, APs have also been shown to enhance oxidative stress in Atlantic cod (Hasselberg et al., 2004a), suggesting that the induced antioxidant responses at the gene transcription level could reflect the higher PAH and/or AP exposure in fish from the WM.

However, it should be noted that the observed induction levels are relatively low, as compared to, for instance, a 5-fold difference in CYP1A expression between mummichogs (*Fundulus heteroclitus*) from a PAH contaminated and a reference site (Roling et al., 2004). Similarly, George et al. (2004) observed 50-fold difference in CYP1A mRNA levels between European flounders (*Platicthys flesus*) collected from an unpolluted and a heavily-contaminated estuary in the UK. However, both of these field studies were conducted in heavily polluted estuarine environments. A less marked difference in gene transcript levels between sites in this study is in accordance with the small difference in biliary PAH metabolites. In addition, previous laboratory experiments have shown that NP exposure can result in a reduction of CYP1A gene expression, as well as EROD activity and CYP1A protein expression (Hasselberg et al., 2004b; Sturve et al., 2006; Olsvik et al., 2009). A downregulation of CYP1A expression by estrogenic compounds such as APs is thought to occur by a crosstalk between the

estrogen receptor (ER) and the aryl hydrocarbon receptor (AhR) (Arukwe et al., 1997; Navas and Segner, 2001; Sturve et al., 2006; Celander, 2011). In this study however, CYP1A mRNA levels were induced in fish from WM, which also exhibited higher AP levels, thus suggesting a lack of CYP1A downregulation, although a potential interference due to the simultaneous presence of ER and AhR agonists cannot be excluded.

To our knowledge, the present study is the first attempt to simultaneously investigate biliary AP metabolites and hepatic Vtg mRNA levels in deep-sea fish. A 35-fold induction of Vtg was observed in males from WM compared to those from CS, while females exhibited similar levels at both sites (Figure 3). The fact that Vtg expression in male fish is usually related to exposure to estrogenic contaminants (Sumpter and Jobling, 1995; Hutchinson et al., 2006) suggests that endocrine-disrupting effects may have occurred in male *A. rostratus* up to depths of 2000 m as a result of higher AP exposure. The lack of Vtg induction in female fish following AP exposure suggests that the presence of estrogenic sex hormones potentially masked the contaminant exposure signal. In some fish species the female Vtg levels increases up to a million-fold during the seasonal reproductive cycle (Sumpter and Jobling, 1995), which is also reflected in the 3 to 4 orders of magnitude higher levels in female *A. rostratus* compared to males (Figure 3). However, the response amplitude of Vtg seen in male fish is comparable to previous results from field studies conducted in heavily impacted freshwater and coastal environments. For instance, the previously mentioned study by George et al. (2004) found 5-20 times higher Vtg mRNA levels in male flounders from a highly polluted estuary. Similarly, Burki et al. (2006) observed no induction of Vtg mRNA in feral, but a 10-fold increase in caged brown trout (*Salmo trutta)* sampled in a Swiss river downstream of a sewage treatment plant. These findings further highlight the importance of the present results, indicating a significant exposure of male *A. rostratus* from WM to estrogenic compounds.

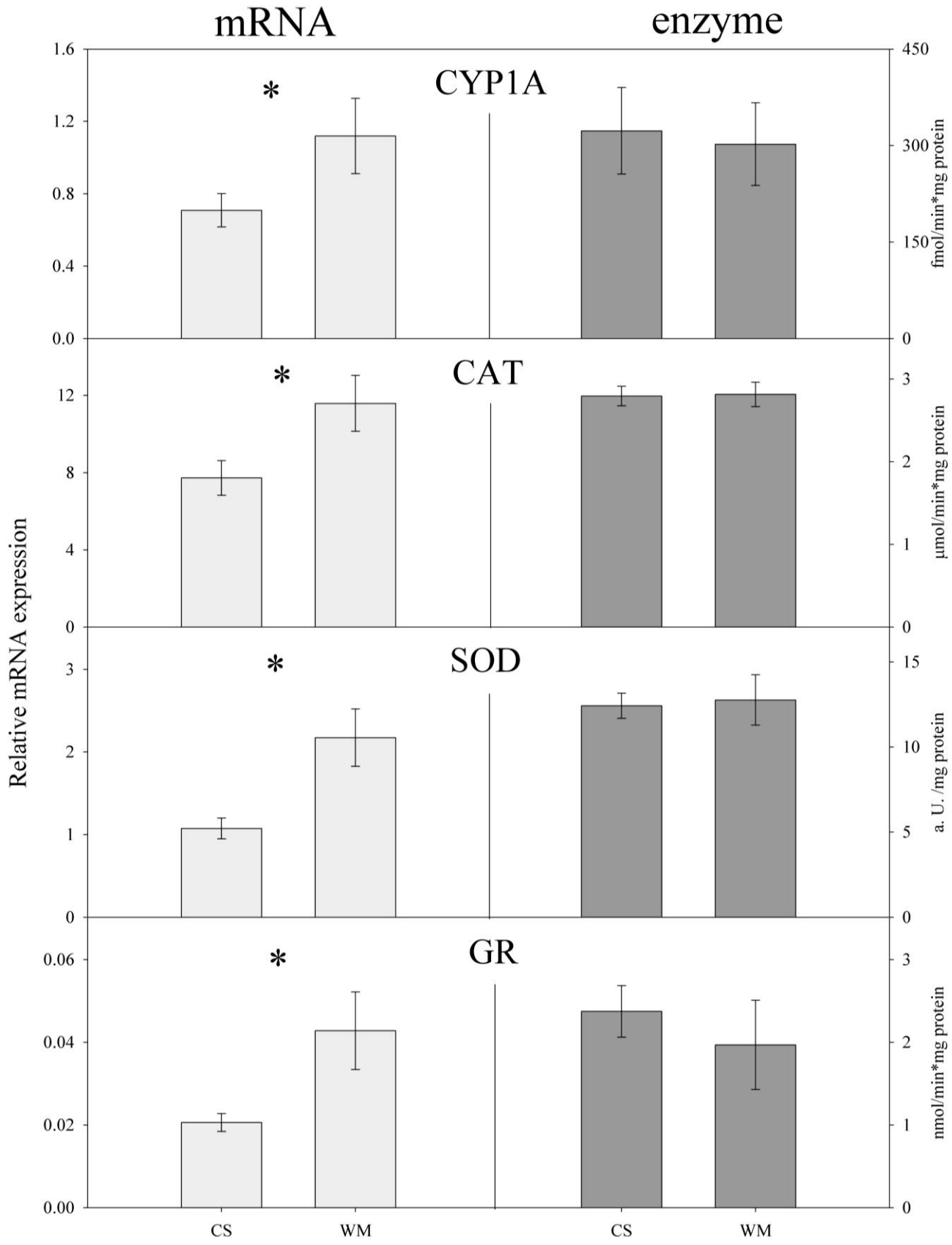

Fig.2 Gene expression and enzyme activities for *A. rostratus* from the Catalan slope (CS) (n=19) and the Western Mediterranean (WM) (n=11) areas. Values shown are mean ± S.E.M. Asterisk denotes significant difference between sites ($p < 0.05$).

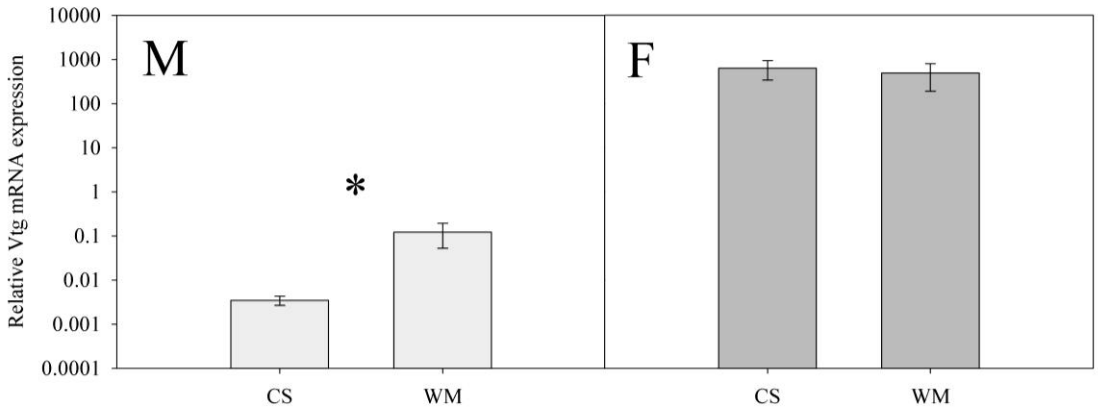

Fig.3 Vtg mRNA expression in male and female *A. rostratus* from the Catalan slope (CS (M: n=10; F: n=9) and the Western Mediterranean (WM) (M: n=6; F: n=5) areas. Values shown are mean ± S.E.M. Asterisk denotes significant difference between sites ($p < 0.05$).

In addition, male fish also showed a more marked transcriptomic stress response than females for the previously discussed biomarker genes, particularly for the antioxidants CAT and SOD (Figure 4). In this context, previous studies reported different effects of estrogenic compounds on male and female fish biomarker responses. In particular, Seo et al. (2006) reported gender differences in the expressions of androgen and estrogen receptor genes in killfish (*Rivulus marmoratus*) after NP exposure. Moreover, alkylphenol treatment resulted in different redox status modulations in male and female Atlantic cod (*Gadus morhua*) (Hasselberg et al., 2004a). Similarly, the exposure of fathead minnow (*Pimephales promelas*) to the environmental estrogen 17β-estradiol resulted in differential response patterns between sexes for a number of genes (Filby et al., 2006). Another interesting finding is the fact that expression levels of all five biomarker genes exhibited significant positive relationships in males, while in females the antioxidant genes CAT, SOD and GR were correlated among each other and a significant relationship between CYP1A and CAT was observed (Table 5). Moreover, Vtg did not correlate with any other biomarker gene in females, suggesting that, in contrast to males, its expression in females was not related to estrogenic contaminant exposure due to the overriding signal of gonad developmental processes. Overall, this result further highlights the importance of determining potential differences in pollution responses between sexes when conducting biomonitoring studies. In our case, male *A. rostratus* appear to be more adequate for monitoring purposes than females due to their higher responsiveness at the gene expression level and the fact that Vtg mRNA in males can be used as marker for estrogenic exposure.

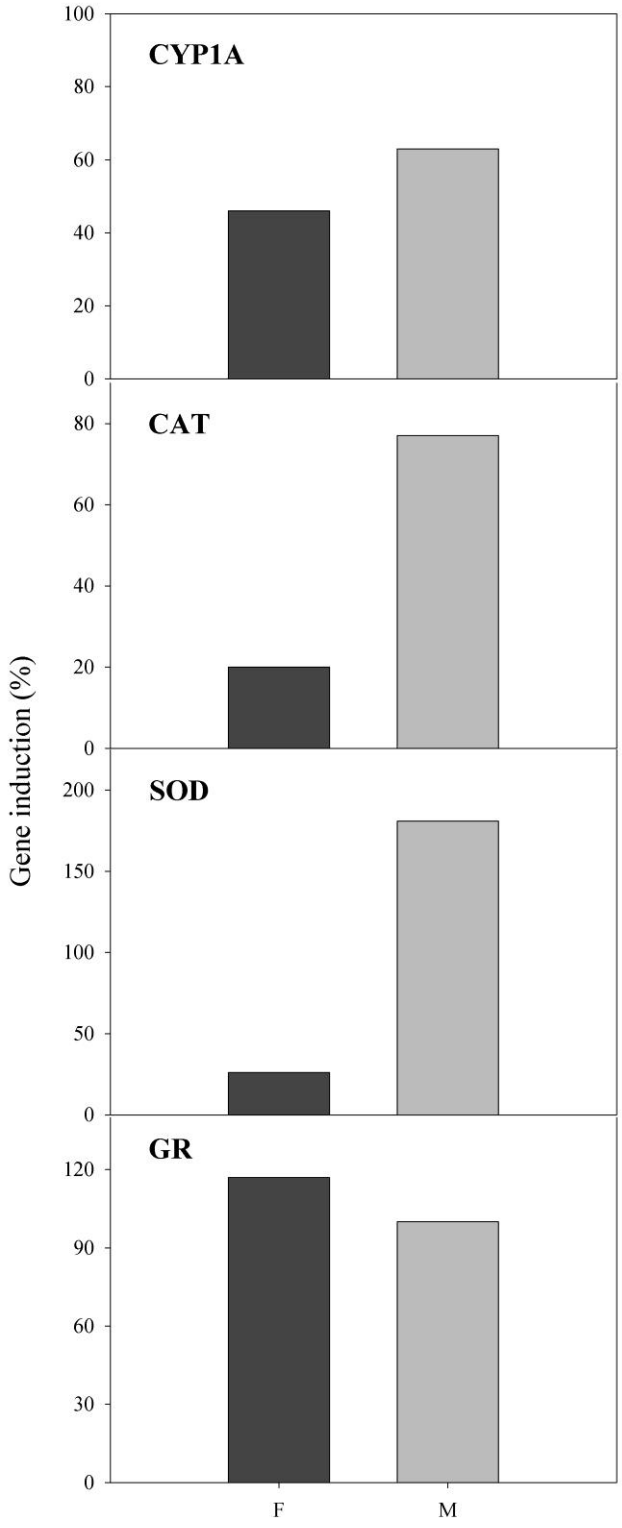

Fig.4 Differences in gene induction between male and female *A. rostratus* at the contaminanted site (WM)

Table 5 Spearman rank correlations between mRNA levels of 5 different biomarker genes in male (light gray) and female (white) *A. rostratus*

Gene	CYP1A	CAT	SOD	GR	Vtg
CYP1A		ρ = 0.78***	ρ = 0.71**	ρ = 0.55*	ρ = 0.63***
CAT	ρ = 0.78**		ρ = 0.76***	ρ = 0.61*	ρ = 0.69**
SOD	n.s.	ρ = 0.86 ***		ρ = 0.81 ***	ρ = 0.78***
GR	n.s.	ρ = 0.65 *	ρ = 0.63 *		ρ = 0.56*
Vtg	n.s.	n.s.	n.s.	n.s.	

* p<0.05; **p<0.01, ***p<0.001
n.s. = not significant

In contrast to gene expression results, biomarker enzyme activities did not differ significantly between sites (Figure 2). Furthermore, no significant relationships were detected between gene expression and catalytic activities of a specific biomarker. In general, both of these biomarker responses vary correspondingly and correlate between each other, with modulations at the gene expression level usually preceding changes in enzymatic activities (Piña et al., 2007). However, some studies have reported a lack of correlation between transcriptomic and catalytic responses, potentially due to differential regulatory mechanisms and the delayed timing of post-transcriptional responses and protein synthesis following pollutant exposure (Regoli et al., 2011). Particularly in field conditions, where organisms are typically exposed to complex contaminant mixtures over long time-periods, the dissociation between transcriptional and catalytic responses may even be more pronounced. For instance, several studies have documented differential response trends between transcript expression of genes and the catalytic activities of their respective products, including CYP1A gene expression and EROD activity (Tom et al., 2003; George et al., 2004; Kammann et al., 2008; Trisciani et al., 2011) as well as antioxidant gene expression and their enzyme activities (Nahrgang et al., 2010; Regoli et al., 2011). Thus, the lack of response of enzyme activities to the putative pollution gradient observed in this work indicates that contaminant exposure in samples from WM could have been too low and/or occurred relatively recently to significantly modify the response patterns at the enzyme activity level. The fact that one would expect contaminant levels to be relatively low in such remote environments as the deep-sea, further advocates the use of molecular biomarker techniques such as the assessment of gene expression profiles as a sensitive tool to detect potential adverse effects resulting from low exposure levels.

Furthermore, the time lag between sampling periods may have also influenced the biomarker response pattern. In fact, in a previous study on the natural variability of biomarkers in different deep-sea organisms, including *A. rostratus*, we found significant seasonal variation in enzyme activities of *A. rostratus*, including EROD and antioxidant responses (Koenig and Solé 2012). However, the seasonal pattern appeared to be mainly related to fluctuations in reproductive activity and was mainly observed in females. In the present study, maturity stages were similar between the individuals sampled at both sites, particularly in females no differences in gonad development between CS and WM were observed (see Table 1). Thus, it is unlikely that the difference in timing of sampling affected the biomarker levels in *A. rostratus*.

The present study has shown that deep-sea fish dwelling at depths up to 2000 m were exposed to PAHs and APs, with higher metabolite levels detected in bile samples from the WM region. To our knowledge, this is the first study to determine biliary AP levels in a deep-sea fish species. Moreover, five biomarker genes and three housekeeping genes were successfully identified and quantified. In accordance with chemical results, gene transcript levels also indicated higher pollutant exposure in fish sampled at the WM compared to fish from the CS area. In particular, Vtg mRNA levels were significantly induced in males from WM, showing that endocrine-disrupting effects, potentially related to AP exposure, may be occurring in such remote environments as the deep-sea. However, biomarker enzyme levels did not exhibit any significant differences between sites, showing that transcriptional and catalytic responses can have different response trends.

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9. Appendix

9

Appendix - Materials and Methods

9.1 Sampling

Deep-sea animals were collected using an benthic otter trawl Maireta system (OTMS) fitted with a cod-end mesh of 40 mm (Figure 9.1). The otter trawl is attached to two divergent doors and a single warp cable. Total trawl times, including net deployment and retrieval, ranged from 1.5 h to 3 h, depending on the sampling depth, with bottom haul times of approximately 40-60 min.

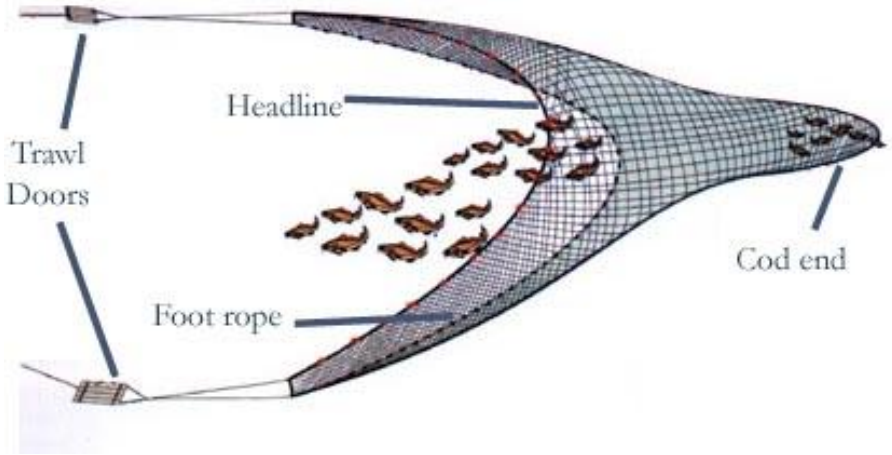

Fig. 9.1 Benthic otter trawl

Upon net retrieval, animals were identified and separated by species and size, weight and sex of each individual were recorded prior to dissection. Onboard, a portion of muscle tissue was dissected, wrapped in an acetone-precleaned aluminium foil for POPs and in clean plastic tubes for Hg analysis and stored at -20 ºC for chemical analyses. Bile bladders were also stored in Eppendorf tubes at -20 ºC. The liver/hepatopancreas was collected and immediately frozen in liquid nitrogen and later transferred to a -80 ºC freezer for biochemical analyses. Dissection material was cleaned with detergent and rinsed with Milli-Q water after each fish to avoid cross contamination.

Fig. 9.2 Identification of species and dissection of fish onboard R/V *García del Cid*

9.2 Chemical analyses

Materials and reagents

Analysis grade isooctane (SupraSolv®), n-hexane, dichloromethane and acetone were purchased from Merck (Darmstadt, Germany). All laboratory materials were first washed with soap and subsequently cleaned by ultrasound sonication using Extran® detergent and finally rinsed with Milli-Q water. Material that could not be cleaned (*e.g.* glass Pasteur pipettes) was heated overnight in a muffle furnace at 400 ºC. Sodium sulphate was cleaned in soxhlet and heated to 400 ºC to convert it to its anhydrous form before its use. Glassware material, cellulose cartridges and glass wool used for the soxhlet extraction were precleaned by extraction during 24 h.

9.2.1 Organohalogenated Compounds

Extraction and clean-up

Muscle tissue (2-4 g) of individual fish was ground with activated anhydrous sodium sulphate (Na_2SO_4) until a fine powder was obtained. The mixture was spiked with TBB (tetrabromobenzene) and PCB 200 as recovery standards and Soxhlet-extracted with 100 mL *n*-hexane-dichloromethane (4:1) for 18 hours. An aliquot of the organic extract was concentrated until dryness, and weighed to determine muscle lipid content. The solvent extract was concentrated by vacuum rotary evaporation (20 °C, 20 Torr) to 2 mL. Subsequently, 2 mL of sulphuric acid were added for clean-up and the mixture was vigorously stirred using a Vortex-mixer (2 min) and centrifuged (4000 rpm, 5 min) to remove any foam in the interphase. The sulphuric acid phase was then discarded and this clean-up step was repeated until a colorless transparent acid layer was obtained (3-5 times).

The final sulphuric acid mixture was re-extracted with *n*-hexane (2 x 2 mL) and all *n*-hexane solutions were passed through an anhydrous sodium sulphate column and combined. The combined *n*-hexane solutions were concentrated by vacuum rotary evaporation (20 °C, 20 Torr) to small volumes (ca. 300 µL), which were then transferred to vials and further evaporated to almost dryness under a gentle stream of nitrogen (10-20 °C). The cleaned extract was redissolved in 100 µL of PCB 142 [25 ppb] in isooctane as internal standard for instrumental analysis prior to the determination of organochlorine POPs (*i.e.* PCBs, DDTs, HCHs, CBs). For PBDE analysis, sample extracts were redissolved in 50 µL isooctane containing BDE 118 and [^{13}C]BDE 209 as internal standards.

Instrumental analyses

To determine levels of PCBs (7 congeners: IUPAC # 18, 52, 101, 118, 138, 153, 180) (Bachour et al., 1998), DDTs (2,4'-DDT, 4,4'-DDT, 2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 4,4'-DDD), pentachlorobenzene (PeCB), hexachlorobenzene (HCB), and hexachlorocyclohexane isomers (α-, β-, γ-, δ-HCH), samples were analyzed using a gas chromatograph (Model HP-6890) equipped with an electron-capture detector (µ-ECD). The separation was achieved with a 60 m x 0.25 mm I.D. DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5 % diphenylpolydimethylsiloxane (film thickness 0.25 µm). The oven temperature was programmed from 90 °C (holding time 2

min) to 130 °C at 15 °C min^{-1} and finally to 290 °C at 4 °C min^{-1}, keeping the final temperature for 20 min. The injector and detector temperatures were 280 °C and 320 °C, respectively. Injection was performed in splitless mode. Helium was the carrier gas (30.5 psi).

To determine levels of 41 individual PCB congeners (paper 4) (IUPAC # 18, 28, 31, 33, 44, 49, 52, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 158, 169, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 201, 205, 206, 208, and 209) the same instrumental conditions as described above were used, except the oven temperature program, which started at 90 °C (held for 2 min) to 190 at 20 °C/min, and then to 310 at 3 °C/min (holding time 18 min), as described in Cabrerizo et al. (2009). PCBs 99 and 101, 105 and 132, 156 and 171, 199 and 201 coeluted and could thus not be quantified individually.

PBDE levels (14 congeners: BDE # 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190, 209) were determined by gas chromatography (Agilent 6890N) coupled to negative ion chemical ionization mass spectrometry (Agilent 5975N) (GC-MS-NICI). The separation was achieved using a 15 m x 0.25 mm low bleed SGE-BPX5 MS fused silica capillary column (film thickness 0.10 μm). The initial oven temperature was held at 90 ºC for 1.5 min, followed by a first ramp up to 200 ºC at 20 ºC/min, a second ramp up to 275 ºC at 5 ºC/min and a third ramp up to 300 ºC at 30 ºC/min, with a final holding time of 10 min. Injection was performed in splitless mode and helium was used as carrier gas. For MS analysis, ammonium was used as ionization gas. Quantification was performed in selected ion monitoring mode (SIM) using the bromine ions *m/z* 79/81 for all congeners, except BDE 209 *m/z* 487/489 and [^{13}C]BDE 209 *m/z* 487/489.

Quality assurance and control

To account for the possible inadvertent contamination of samples during analytical procedures, procedural blanks were analyzed for every set of six samples. Blank samples were used to establish method detection (MDL) and quantification limits (MQL), which were defined as the mean of the blanks plus three times (MDL) or five times (MQL) the standard deviation. They were in the order of 0.03 and 0.05 ng g^{-1} w.w., respectively for organochlorine compounds. For PBDEs MDL and MDQ were in the order of 0.004 and 0.006 ng g^{-1} w.w., respectively, except for congeners 47, 99, 100, and 209 for which they were one order of magnitude higher, namely 0.04 and 0.06 ng/g

w.w. respectively. POPs levels were determined by internal standard method. Extraction and analytical performances were evaluated by surrogate standard recoveries, which ranged from 65 % to 90 %. Values reported in this study were corrected based on surrogate recoveries.

Calibration curves

Organochlorine standards were purchased from Dr. Ehrenstofer (Augsburg, Germany). Eight-point linear calibration curves ($R^2 > 0.99$) were used to quantify OC compounds, ranging from 0.5 to100 ng/g. The standard PBDE solution EO-5103 was purchased from Cambridge Isotope Labs (Andover, MA, USA). Seven-point linear calibration curves ($R^2 > 0.99$) were performed for PBDE quantification, with concentrations ranging from 0.25 to 25 ng/g.

9.2.2 Biliary alkylphenol and PAH metabolites

Bile liquid (100 mg) was incubated for 1 h at 40 ºC in 1 mL 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 U of β-glucuronidase and 50 U sulfatase. The hydrolyzed mixture was extracted with 1mL ethyl acetate (3x) and the extract was reduced to 100 µL under a gentle nitrogen stream. Before analysis, hydrolyzed samples were derivatized by the addition of 100 µL bis-(trimethylsilyl)trifluoroacetamide (BSTFA), left to stand overnight at room temperature. Analyses were carried out by gas-chromatography-mass spectrometry (GC-MS-EI) in selective ion monitoring mode (SIM). The ions used for the quantification of derivatized hydroxylated-PAHs were: m/z 216 and 201 for 1-naphthol; m/z 254 and 165 for 9-fluorenol; m/z 266 and 251 for 9-phenanthrol; and m/z 290 for 1-pyrenol. The ions used for monitoring and quantification of APs were: m/z 207 and 193 for 4-nonylphenol (NP) and m/z 207 for 4-tert-octylphenol (OP). Concentrations are expressed as ng/g bile. Detection limits were calculated as signal to noise ratio of 3:1 and were in the order of 1-5 pg for OH-PAHs, 10 pg for NP and 7 pg OP. Four-point standard calibration curves were used for the quantification of PAHs (10 ng/g – 1 µg/g), NP (10– 100 ng/g) and OP (5-30 ng/mL).

9.3 Biochemical analyses

9.3.1 Enzyme activities

Sample preparation

A portion of liver/hepatopancreas (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing for fish liver 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMFS), 1 mM ethylenediaminetetraacetic acid (EDTA) and for crustacean hepatopancreas 100mM KCl, 1mM EDTA, 0.1 mM phenanthroline and 0.1 mg/L trypsin inhibitor. The homogenate was centrifuged at 10,000 g for 30 min and the obtained supernatant (S9) was stored at -80 °C until further biochemical analyses.

Assays

All assays were carried out in triplicate at 25 °C in 96-well format using a TECAN™ Infinite M200 microplate reader. For each assay, blank samples were analyzed in triplicate, which were used to correct for background activity. Prior to analysis, assay conditions were optimized for each species by determining the appropriate dilution of the S9 supernatant (protein content 5-10 mg/mL) for each assay to ensure constant linearity of the measured activity (dilutions for each species shown below in parenthesis for each assay). All reaction mixtures, except for catalase, contained 100 mM phosphate buffer pH 7.4.

Catalase (CAT) activity was measured in a UV-transparent microplate (Greiner UV-Star®) as absorbance decrease at 240 nm for 1 min using 50 mM H_2O_2 as substrate ($\boldsymbol{\varepsilon}$ = 40 $mol^{-1} * cm^{-1}$) and a 100 mM phosphate buffer pH 6.5. Sample volume used was 10 µL (*Ar* 1:400, *Ll* 1:200, *Aa* 1:2) in a total volume of 210 µL.

Glutathione reductase (GR) activity was measured as decrease in absorbance at 340 nm for 3 min using 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) ($\boldsymbol{\varepsilon}$ = 6.22 * $mmol^{-1} * cm^{-1}$) and 0.9 mM oxidized glutathione (GSSG) as substrate. Sample volume used was 20 µL (not diluted) in a total volume of 200 µL.

Total glutathione-peroxidase (GPX) activity was determined as decrease in absorbance at 340 nm during 3 min using 2.5 mM reduced glutathione (GSH), 1 mM glutathione reductase (GR), 0.625mM cumene hydroperoxide (CHP) and 0.3mM NADPH ($\boldsymbol{\varepsilon}$ = 6.22

* $mmol^{-1}$ * cm^{-1}). Sample volume used was 10 µL (not diluted) in a total volume of 240 µL.

Glutathione-S-transferase (GST) activity was measured as increase in absorbance at 340 nm for 3 min using 1 mM 1-chloro-2,4- dinitrobenzene (CDNB) (**ε** = 9.6 * $mmol^{-1}$ * cm^{-1}) and 1 mM GSH as substrate. Sample volume used was 25 µL (*Ar* 1:20, *Ll* 1:20, *Aa* 1:20) in a total volume of 225 µL.

Carboxylesterase (CbE) activity was determined as increase in absorbance at 405 nm during 5 min using 0.18 mM 5,5-dithio-bis-2-nitrobenzoate (DTNB) (**ε** = 13.6 * $mmol^{-1}$ * cm^{-1}) and 0.67 mM S-phenylthioacetate as substrate. Sample volume used was 25 µL (*Ar* 1:5, *Ll* 1:5, *Aa* 1:20) in a total volume of 225 µL.

7-Ethoxyresorufin-O-deethylase (EROD) activity for fish and *7-Pentoxyresorufin-O-deethylase* (PROD) activity in crustacea were measured kinetically as increase in fluorescence at 537 nm excitation and 583 nm emission over 10 min. Substrates used include 7-ethoxyresorufin (3 µM) and 7-pentoxyresorufin (5 µM), respectively and 0.2 mM NADPH with a seven-point curve of resorufin sodium salt standard. Sample volume used was 50 µL (not diluted) in a total volume of 250 µL.

Superoxide dismutase activity was determined as inhibition of the reduction of cytochrome c at 550 nm. The reaction contained 50 µM hypoxanthine, 1.8 $mUml^{-1}$ xanthine oxidase and 10 µM cytochrome c. SOD activity was expressed as units mg $protein^{-1}$, where one SOD unit represents the amount of sample causing a 50 % inhibition of the cytochrome c reduction. A seven point standard curve of commercial SOD (1-40 U mL^{-1}) was used for quantification.

Lipid peroxidation (LP) levels were determined using 200 µL of liver/hepatopancreas sample mixed with 650 µL of 1-methyl-2-phenylindole in acetonitrile:methanol (3:1) and 150 µL of HCl. This mixture was incubated for 40 minutes at 45 ºC and subsequently centrifuged at 13,000 rpm x 10 minutes to precipitate proteins. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated in the same way. LP content was expressed as nmol malondialdehide g^{-1} wet weight.

Protein content was determined using bovine serum albumin as standard (BSA 0.1–1 mg/ml). Sample volume used was 10 µL (*Ar* 1:20, *Ll* 1:40, *Aa* 1:20) in a total volume of 260 µL.

9.3.2 Gene expression analysis

RNA extraction

Hepatic RNA was extracted using the RNeasy® Plus kit (Qiagen). RNA concentration, quality and purity were analyzed using a Nanodrop® ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was further analyzed using the Experion™ RNA StdSens Analysis Kit (BIORAD Laboratories, Inc.). All samples passed quality criteria (RQI > 7).

cDNA synthesis

Total RNA (1µg) was used for cDNA synthesis using the iScript First Strand Synthesis kit (BIORAD Laboratories, Inc.) in a total volume of 20µL and an additional control was run using RNAse-free MilliQ H_2O. In addition, a pooled RNA sample was used to control for genomic DNA contamination by running the cDNA synthesis protocol without reverse transcriptase (NoRT control).

Sequencing and primer design

No published mRNA sequences were available for *A. rostratus.* Conserved sequence regions were derived from the alignment of at least four known fish sequences (*i.e. Salmo salar, Oncorhynchus mykiss, Danio rerio*) using the EMBL-EBI ClustalW2 alignment tool. Based on the alignment score, either existing primer pairs that had been successfully used in other species (i.e. *Oncorhynchus mykiss*, *Zoa viviparous*) were used or degenerate consensus primers were designed using the Primaclade web-based application. PCR products were run on gels and sequenced by Eurofins MWG Operon. Obtained sequences were tested for homology to existing gene sequences from other fish species using the NCBI-BLAST package. Gene-specific primer pairs for qPCR were designed using Beacon Designer software 7.02. (PREMIER Biosoft International) using the SYBR® Green Design settings.

Quantitative real-time PCR (qPCR)

A standard curve containing six dilution series of pooled cDNA samples (40 ng-1.25 ng) was used to ensure an optimized assay for each primer pair (efficiency 100 % ± 5%). The qPCR was performed in an iQ5 Thermal cycler (Bio-Rad) on a 96-well qPCR plate (4titude). The amplification reaction was performed in duplicates using the fluorescent dye iQ™ SYBR®Green Supermix (Bio-Rad) with 20 ng sample cDNA resulting in a final reaction volume of 20 µl/well. Respective primer had a 'non template control' (NTC) in the amplification reaction to control for primer and mastermix contamination. PCR was achieved by a 1 min activation step at 95 °C, followed by 40 cycles of a 20 s denaturing step at 95 °C, a 20 s primer annealing step at 60 °C and a 30 s elongation step at 72 °C. A melting curve was performed at each run in order to confirm specific amplification of each sample. An accepted Ct-value of NTC and NoRT samples was set to be either non-detectable or >32 cycles. The geometric mean of three reference genes, namely β-actin, elongation factor 1a (EF1a) and ribosomal RNA 12S, was used to normalize quantitative PCR cycle threshold (Ct) data.