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Ecotoxicological effects of Barcelona harbor sediments in Capitella teleta

Isa Daniela Luís Gomes

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Trabalho de projeto apresentado à Escola Superior de Turismo e Tecnologia do Mar do Instituto Politécnico de Leiria para a obtenção do grau de Mestre em Biotecnologia dos Recursos Marinhos, realizado sob a orientação científica do Doutor Marco Filipe Loureiro Lemos, Professor Adjunto da Escola Superior de Turismo e Tecnologia do Mar do Instituto Politécnico de Leiria e do Doutor Carlos Barata Martí, Investigador Principal no *Instituto de Diagnóstico Ambiental y Estudios del Agua* (IDAEA) do *Consejo Superior de Investigaciones Científicas de Espanha* (CSIC).

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Title: Ecotoxicological effects of Barcelona harbor sediments in *Capitella teleta.*

Título: Efeitos ecotoxicológicos de sedimentos do porto de Barcelona em *Capitella teleta*.

Aos meus pais, que me ajudaram a concretizar este sonho.

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Resumo

O grande declínio da diversidade biológica tem sido relacionado com atividades antropogénicas. Parte do nosso planeta depende dos oceanos e a ameaça à vida marinha pode surgir de várias formas, salientando-se as descargas incontroladas de resíduos e diferentes fontes de poluição. Os portos marítimos estão permanentemente sujeitos a tais contaminantes e os sedimentos são considerados como o seu repositório final. Organismos que escavam por entre os sedimentos e filtram os mesmos como forma de se alimentar, como as comunidades de poliquetas, são os mais expostos a tal contaminação.

Na mesma linha de raciocínio, são necessários esforços para prever contaminações e evitar efeitos irreversíveis (p.e., morte), que por vezes só se tornam notórios numa fase adiantada do tempo e quando já não é possível tomar qualquer medida de remediação. A análise química é essencial para informar acerca do nível de contaminação do meio ambiente, no entanto pode não ser suficiente, e portanto parâmetros ecotoxicológicos são necessários para complementar tal potencial lacuna de informação. A avaliação dos efeitos dos contaminantes a nível individual, como o crescimento e alimentação, são cruciais, mas uma avaliação ao nível subcelular não deve ser ignorada, visto que os dados bioquímicos poderão dar informação mais detalhada e prévia da contaminação. De entre as respostas bioquímicas possíveis inclui-se a eficiência enzimática, utilizada frequentemente neste âmbito. Poliquetas, como *Capitella teleta*, são organismos filtradores que estão em constante contacto com os sedimentos. A sua adaptação e relevância ecológica, juntamente com a sua fácil cultura e manutenção em laboratório, fazem com que estes organismos sejam cada vez mais utilizados em estudos ecotoxicológicos.

Este estudo teve como objetivo avaliar os potenciais efeitos dos sedimentos do porto de Barcelona (Espanha) na poliqueta *Capitella teleta*. Os sedimentos foram recolhidos de três locais diferentes do porto, com um gradiente positivo de contaminação da zona mais externa para a mais interna do mesmo. O peso corporal (crescimento) e a taxa de egestão (alimentação), bem como as enzimas superóxido dismutase, catalase (resposta antioxidante) e acetilcolinesterase (resposta neurotóxica), foram avaliadas. Uma vez que não existiam dados disponíveis sobre a principal colinesterase (ChE) presente em *C. teleta*, procedeu-se à sua caraterização.

Os resultados mostraram a existência de uma única forma de colinesterase, a acetilcolinesterase. O valor da concentração inibitória mediana (IC_{50}) para o inibidor clorpirifos-oxon foi estabelecido em 60,72 nM. Todos os sedimentos do porto de Barcelona afetaram significativamente *C. teleta*, com uma diminuição significativa do peso corporal e das taxas de egestão e também uma significativa inibição das enzimas estudadas. Além disso, este estudo destaca a relevância de *C. teleta* como biomonitor de interesse para aceder a efeitos de poluição a que estão sujeitos os sedimentos do fundo marinho.

Palavras-chave: Crescimento, Alimentação, Biomarcadores, Acetilcolinesterase, Superóxido dismutase, Catalase, Poliquetas

Abstract

A major decline of the world's biological diversity has been associated to human activities. Life in our planet depends on the oceans and the threat to marine life comes in various forms, noting the uncontrolled waste discharges and the different sources of pollution. Harbor environments are permanently associated to high contamination and bottom sediments are considered as the final repository. Filter-feeding and burrowing organisms, such as polychaete communities, are amongst the most exposed organisms.

Regarding the previous statement, efforts are needed to predict contamination and prevent organisms from irreversible effects (e.g. death or impaired organismal function), which sometimes are only visible at a stage in time when it is too late to take preventive or abatement measures. Chemical analysis provide us with information about the contamination level in the environment, however they may not be enough. Ecotoxicological endpoints are needed to improve this lack of information. Evaluation of individual-level effects, such as growth and feeding, are crucial but an assessment at the sub-cellular level is also quite interesting since biochemical data give a more detailed and earlier evidence of pollution. Such biochemical responses include enzymatic efficiency and are nowadays often used. Polychaetes, such as *Capitella teleta*, are filter-feeding and burrowing organisms. Their high suitability and ecological relevance, together with their easy culture and maintenance in the laboratory, has led them becoming increasingly used in ecotoxicological studies.

This study aimed to assess the potential effects of Barcelona harbor sediments on the polychaete worm *Capitella teleta*. Sediments were collected from three different locations of the Barcelona (Spain) harbor with a positive gradient of pollution from the mouth of the harbor towards the innermost zone. Body weight (growth) and egestion rate (feeding), as well as antioxidant enzymes (catalase and superoxide dismutase) and neurotransmission related enzyme (acetylcholinesterase) were evaluated. Since no data about the main cholinesterase (ChE) present in *C. teleta* was available, a previous ChE characterization assay was performed.

The results showed the existence of only one ChE form, the acetylcholinesterase. The sensitivity of ChE to the model anticholinergic inhibitor chlorpyrifos-oxon was moderate (IC_{50} was 60.72 nM). Sediments from all sites decreased significantly the growth, egestion rates and the studied enzyme activities of *C. teleta*. This study highlights the relevance of ChE characterization before its use as a biomarker in ecotoxicology, and reinforces the use of *C. teleta* as biomonitor for early-warnings of pollution effects on marine soft-bottom macrobentonic communities.

Keywords: Growth, Feeding, Biomarkers, Acetylcholinesterase, Superoxide dismutase, Catalase, Polychaetes

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List of abbreviations

ACh - Acetylcholine
AChE – Acetylcholinesterase
ATCh – Acetylthiocholine iodide
BTCh – *S*-Butyrylthicholine iodide
BW284c51 – 1,5-bis[4-allyldimethylammoniumphenyl]pentan-3-one dibromide
CAT – Catalase
ChE – Cholinesterase(s)
CP – Chlorpyrifos
CPO – Chlorpyrifos-oxon
Dw – dry weight
H_2O_2 - hydrogen peroxide
IC_{50} – Median inhibitory concentration
iso-OMPA – tetra [monoisopropyl] pyrophosphortetramide
$O2^{-}$• - Superoxide free radical
OH• - Hydroxyl free radical
PAH - polycyclic aromatic hydrocarbons
PBC - polychlorinated biphenyls
PTCh – Propionylthiocholine iodide
ROS - Reactive Oxygen Species
SFG – Scope For Growth
SOD – Superoxide dismutase
TBT – Tributyltin
TOC – Total Organic Carbon
UNESCO - United Nations Educational, Scientific and Cultural Organization

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Chapter 1. General Introduction

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1.1. Overview on contamination, pollution and environmental risk assessment

Life in our planet depends on the oceans, based on uncountable ecosystem services, which are the sources of wealth, opportunity, abundance. About 71% of the surface of this planet is covered by salt water (IOC/UNESCO et al., 2011). They provide us food, energy, water and sustain directly the livelihoods of hundreds of millions of people and are the main highway for international trade as well as the main stabilizer of the world's climate (Chiau, 2005). However, as Federico Mayor Zaragoza (Director General, UNESCO) said "Whatever we do, the ocean will survive in one way or another. What is more problematic is whether we shall preserve it in a state that ensures humanity's survival and well-being" (Independent World Commission on the Oceans, 1998).

Human activity is the main responsible for the world's biological diversity decline (Islam and Tanaka, 2004), and marine ecosystems are no exception, being increasingly subject to a multitude of anthropogenic contaminants (Matthiessen and Law, 2002). The threat to marine life comes in various forms, such as overexploitation and harvesting, dumping of waste, pollution, alien species, dredging and global climate change (Derraik, 2002).

Land-based sources (such as agricultural run-off, discharge of nutrients and pesticides and untreated sewage including plastics) account for approximately 80% of global marine pollution (IOC/UNESCO et al., 2011). Besides this, coastal tourism, port and harbor developments, damming of rivers, urban development and construction, mining, fisheries, aquaculture, and manufacturing, among others, are all sources of marine pollution threatening coastal and marine habitats (GESAMP et al., 2009).

Many of the organic and inorganic contaminants released by human activities are concentrated in aquatic sediments (Calow, 1993). The later can sink and be adsorbed by the sediments becoming persistent and reaching levels many times higher than the water column concentrations (Martínez-Lladó et al., 2007). This fact is particularly important in littoral and estuarine ecosystems affected by different contamination sources (Riba et al., 2004). With such high concentrations, the bioavailability of even a minute fraction of the total sediment assumes considerable importance, especially in some filter-feeding and burrowing organisms (Bryan and Langston, 1992). Thus, additionally to water column toxicity it is of major importance to address sediment toxicity in model organisms. This exposure can be performed through spiked and field collected sediments in the laboratory (e.g. Méndez et al., 2013), or *in situ* (e.g. Wang et al., 2012).

There are many pollutants to which aquatic organisms can be exposed, such as PCB (polychlorinated biphenyls), DDTs (dichlorodiphenyltrichloroethanes) and organochlorine and organophosphorus compounds (insecticides), and also to PAH (polycyclic aromatic hydrocarbons), heavy metals, butyltin compounds (as tributyltin, dibutyltin and monobutyltin - TBT, DBT and MBT respectively), among others. Sediments with TBT levels in the range of 326-4,702 µg/kg (dw) found in Barcelona harbor were reported to affect benthic communities, by inducing imposex (Martínez-Lladó et al., 2007). Organochlorine pesticides and DDTs have also been reported in coastal sediments at 3.25 µg/kg and 0.38–1,417 ng/g, respectively (Kilemade et al., 2009; Lofrano et al., 2010), and are also known to act most of the times irreversibly in the neurological system of the organisms, leading to several disorders (Chen, 2012). PAH and different heavy metals (Zn, Cd, Pb, Cu, Fe) have been reported in sediments at concentrations of 0.27-4.25 mg/kg (dw) and 1.5-189,673 mg/kg (dw), respectively, in Huelva estuary (Oliva, Perales, et al., 2012), which are well known compounds that interfered with antioxidant defenses of aquatic organisms by causing oxidative stress (Gravato et al., 2010; Ameur et al., 2012).

Nevertheless, numerous studies have demonstrated that the relationship between contaminant concentration and the damage they cause to biota and ecosystems is not straightforward, due to differences in bioavailability and bioconcentration of these contaminants under different conditions (Casado-Martinez et al., 2009). Hence, there is a renewed and urgent need for comprehensive biological monitoring tools and ecotoxicological research to improve the understanding of the biological impacts of contaminants for estuarine and coastal sediments (Lewis and Watson, 2012), as well as a concern focused towards ecosystems health, and not only towards the impacts on wildlife and the environment.

1.2. Bioassays to assess environmental stress

The ultimate goal of ecotoxicology is to protect ecological systems from adverse effects by xenobiotics – chemical not normally produced or expected to be present in an organism – either synthetic or natural chemicals (e.g. human hormones, toxins produced by algae), or yet by other biotic and abiotic stressors such as temperature, UV light, predation, etc. (Lemos et al., 2010). To accomplish this, ecotoxicologists aim to anticipate where these substances go in the environment (their fate) and what ecological effects they might cause when they reach it (Maltby, 1999). A number of toxicity tests and recommended species have been developed to accommodate regulatory requirements for environmental assessment of pollutants in estuarine and coastal habitats (GESAMP et al., 2009).

1.2.1. Feeding as a classical ecotoxicological endpoint

Efforts to determine sediment toxicity have focused on measurements of acute responses. Survival has been the most common endpoint when assessing dredged material toxicity (Martín-Díaz et al., 2004); but because death is an insensitive endpoint from the ecosystem perspective, and the maximum dose-response gives little information about environmental contamination – except that the lethal dose has been exceeded – physiological endpoints at an individual level have been addressed to measure responses within the normal physiological scope of the organism and correlated with pollutant exposure or effect (Handy and Depledge, 1999; Ramskov et al., 2009).

Avoidance is one of the first physiological response that an organism can show up under stressful conditions to escape to a less stressful location (Pereira et al., 2013). This parameter is an ecologically relevant measurement tool because avoidance reactions affect the energy budget of the organisms (Amorim et al., 2008), and due to its rapidness to screen potentially contaminated environments (Novais et al., 2010).

Additionally, the energy available for growth and reproduction is also a field of investigation when assessing pollutants' effects on individuals. A widely used approach related with energy budged is the Scope For Growth (SFG) matrix (Widdows and Donkin, 1991; Mubiana and Blust, 2007; Tankoua et al., 2012). This index is determined by the energy absorbed from ingested food items minus the energy lost through respiration and excretion (Widdows and Johnson, 1988; Tsangaris et al., 2007). The rationale behind that is that when ingesting food, the energy absorbed should be high enough to allow the organisms to grow and to have sufficient energy to reproduce, fulfilling its metabolic needs, ensuring the population growth (Verslycke et al., 2004). However, when facing stressful conditions (Widdows and Johnson, 1988; Halldórsson et al., 2005; Tsangaris et al., 2007, 2010) such as pollutants, the organisms tend to divert their energies to metabolize/eliminate such pollutants if avoidance is not possible, and also, if they do not feed, the energy absorbed from the ingested food is fewer and, consequently, the SFG will be lower. For example, mussels collected from the field sites along a pollution gradient in Langesundfjord (Norway) showed marked decline in the scope for growth, with increasing heavy metals, PAH and PCB concentrations (Widdows and Johnson, 1988). According to Tsangaris and co-workers (2007, 2010), low SFG rates (-5 to 1 J/h/g) of mussels from Larymna and Salaora sites (Greece) showed comparable values with reported in the literature (Widdows et al., 2002) for high levels of pollution stress (< 5 J/h/g), while high SFG values at the reference site Agios Kosmas (21 J/h/g) coincided with those found in pristine environments with minimum contamination (20–25 J/h/g).

Besides avoidance behavior and energy budget, reproduction, feeding and growth rates are also viable tools to assess toxicity at an individual level (Moreira et al., 2006), since reductions in such parameters have been correlated with the presence of toxicants (McLoughlin et al., 2000).

The feeding rate of test organisms is a sensitive indicator of toxic stress in both freshwater and marine species (McLoughlin et al., 2000). In aquatic invertebrates, feeding inhibition is in most cases one of the first reported responses to environmental pollution (Coulaud et al, 2011). The impairment of feeding at the individual level has been demonstrated to have direct effects on growth, reproduction, and eventually survival, which can lead to alteration in life-history traits, clearly demonstrating the ecological relevance of both feeding and growth as toxicity endpoints (Maltby, 1999; Moreira et al., 2006).

The feeding activity can be assessed through two main methods: ingestion rate and egestion rate, by measuring the food consumption and fecal pellets production, respectively (Whitacre, 2010). Both methods are accurate, however when concerning measurement of ingestion rates on organisms which filter sediment as their natural dietary item (e.g. shrimps, polychaetes), there are experimental difficulties in quantifying its consumption since usually the amount of sediment consumed is extremely small compared with the amount of sediment supplied, making gravimetric analysis of ingested sediment difficult (Roast et al., 2000). Therefore, the more readily quantified rate of egestion is used as a surrogate measure of feeding rate, where highly positively correlations between egestion and ingestion rates were achieved and thus validating their use as a measure of feeding rate. Such egestion rates have been used previously to calculate feeding rates of crustaceans (Roast et al., 2000; Alonso and Camargo, 2004) and annelids (Forbes and Lopez, 1990; Hu et al., 2003; Méndez et al., 2013). The choice of the egestion rate as ecological endpoint derived from the fact that are relatively easy to measure and, for deposit-feeders, they constitute a very accurate surrogate measure of the amount of particulate material ingested per unit time (Forbes and Lopez, 1990). Changes at egestion rates could be associated to a response of individual animals to the change of the environment quality (Roast et al., 2000).

1.2.2. Biochemical responses in current ecotoxicological research

When exposed to xenobiotics, the body's major defense mechanism is to minimize exposure by speedily lowering its body burden; to achieve this goal, the body produces a number of enzyme systems involved in detoxification of foreign compounds (Liska, 1998). In many cases, these enzyme responses can be related quantitatively to the extent of exposure to the xenobiotic, and also to a given chemical or class of chemicals, and can be used as a bioassay to detect their presence and their toxic effects (Walker, 1992) – such responses are called "Biomarkers".

Biomarkers were originally defined as any biochemical, histological or physiological alterations or manifestations of environmental stress, as stated by Depledge in 1994 (Hyne and Maher, 2003; Martín-Díaz et al., 2004). However, Gestel and Brummelen in 1996 defined biomarkers as "*any biological response to an environmental chemical below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism*". This approach has been followed by several authors and the term biomarker is now more commonly used in a more restrictive sense, as biological responses at the sub-individual level resulting from individual exposure to xenobiotics. Yet, nowadays there are still very controversial opinions around the "biomarker" definition. Controversy aside, and following Gestel and Brummelen (1996) approach, giving the fact that these biomarkers are measured at the molecular or cellular level, they have been proposed as sensitive "early warning" tools for biological effect measurement in environmental quality assessment (Moore, 1993; Cajaraville et al., 2000; Gil and Pla, 2001; Martín-Díaz et al., 2004; Monserrat et al., 2007; Lemos et al., 2010), so they have the potential to anticipate changes at higher levels of the biological organization (refer to section 1.2.3.).

Different studies for the application of biomarkers to assess the impact of xenobiotics in the environment have been developed. Several studies focus on determining the potential neurotoxicity, mainly through cholinesterases' inhibition (e.g. Kristoff et al., 2006), the activity of biotransformation enzymes (through detoxification metabolism) (e.g. Kilemade et al., 2009), the antioxidant responses (e.g. Faria et al., 2010) and biochemical indices of oxidative and genetic damage (e.g. Jebali et al., 2007).

Ordinarily, xenobiotic compounds existing in the bodies are lipophilic in nature and need to be converted into more-water soluble compounds before being eliminated and excreted from the body; this elimination involves detoxification mechanisms (figure 1) which are important defenses for organisms' survival (Chen, 2012). Two main phases in the detoxification process are considered: phase I and phase II; their enzymes are referred to as Phase I enzymes and Phase II enzymes, respectively.

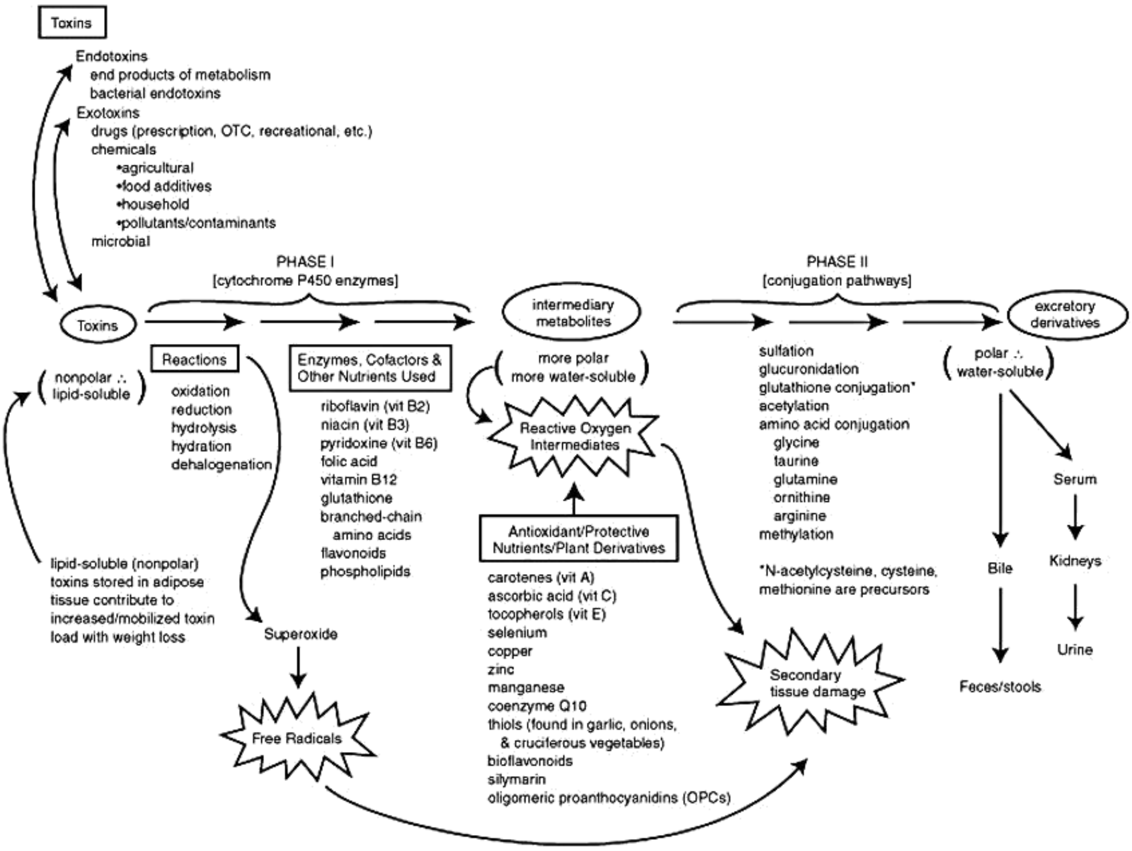

Figure 1. Main detoxification pathways and supportive nutrients (adapted after Liska, 1998)

The Phase I detoxification system is generally the first enzymatic defense against foreign compounds, where a functional group is introduced to the chemical structure of the lipophilic foreign compound, through a reaction such as oxidation, hydrolysis or reduction. This step is defined as “Functionalization” and basically increases the polarity of the xenobiotic, making it ready for the next metabolic step (Chen, 2012). An example of a typical Phase I reaction is mediated by the cytochrome P450 enzyme that uses oxygen and, as a cofactor, NADH, to add a reactive group, such as a hydroxyl radical (Liska, 1998). As a consequence of this step, reactive molecules such as reactive oxygen species (ROS) are produced, which may be more toxic than the parent molecule, causing damage to proteins, lipids, RNA, and DNA within the cell (Martín-Díaz et al., 2004).

In a conjugation reaction (phase II), the functional group of the xenobiotic is combined with a chemical group of a small molecule, frequently the cofactor of an enzyme (Liska, 1998). Glucuronidation, sulfation, and glutathione and aminoacid conjugation are some types of conjugation reactions. The conjugation reaction greatly increases the solubility and excretory potential of a foreign compound, thus facilitating its removal from the body. As these reactions require cofactors, they must be replenished through dietary sources (Liska, 1998). Either phase I or phase II metabolism consists of a variety of enzymes that carry out different catalytic reactions, and their reactions represent critical elements of detoxification mechanisms (Chen, 2012).

In normal metabolism, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and the free superoxide ($O2^{-}$•) and hydroxyl (OH^{-}•) radicals, are produced during regular cellular functions (e.g., mitochondrial electron transport) (Wright and Welbourn, 2002). Without the influence of stress conditions, a balance exists between the generation of ROS and other pro-oxidants, and their detoxification and removal by enzymatic and non-enzymatic antioxidant defense mechanisms (Moreira et al., 2006).

Nonetheless, under stressed conditions the organism focuses on metabolizing the xenobiotic compounds. As a consequence cellular production of pro-oxidant free radicals like ROS can be greatly enhanced by exposing organisms to heavy metals and numerous organic contaminants including PAH and their oxidation products, halogenated hydrocarbons (e.g., PCB and lindane), DDTs and also butyltin compounds (De Luca-Abbott et al., 2005; Viarengo et al., 2007; Ameur et al., 2012) and redox cycling compounds (e.g., quinones, nitroaromatics, nitroamines and bipyridyl herbicides). Superoxide radicals can cause peroxidation of membrane lipids, resulting in loss of membrane integrity and the inactivation of membrane-bound enzymes (Wright and Welbourn, 2002), or can reduce the cellular antioxidant capacity under its overaccumulation (Freitas et al., 2012), resulting in a decrease of antioxidant enzyme activities (Martín-Díaz et al., 2004; Faria et al., 2009). Although not highly reactive, H_2O_2 can also inhibit some antioxidant enzymes, and its fast capacity to penetrate cell membranes creates toxic effects at several different subcellular locations; H_2O_2 can also be the responsible to produce OH^{-}• radicals, which can indiscriminately attack and damage every type of macromolecule in living cells including lipids, proteins, and DNA (Wright and Welbourn, 2002), leading to various disease conditions such as cancer, cardiovascular disease and neurological disorders (Chen, 2012). Therefore, a balance between detoxification metabolism and enzymatic antioxidant defenses is needed for the accurate removal of both xenobiotics and ROS.

With regard to antioxidant enzymes, the main enzymes include: the superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.8.1.7), among others (Oliva, Vicente, et al., 2012). The primary defense against oxygen toxicity involves the enzyme SOD (Ameur et al., 2012). The superoxide free radical (O_2^{-}•) is formed by the transfer of a single electron to oxygen; SOD enzyme is the responsible to catalyze the conversion of this free radical to H_2O_2, which needs, then, to be degraded to H_2O and O_2, by CAT or by GPx (figure 2) (Wright and Welbourn, 2002; Leslie et al., 2013). The SOD-CAT system acts as the key part to fight against oxygen damage and free radicals generated in phase I of xenobiotic detoxification (Yang et al., 2012), and such antioxidant enzymes can be considered as the first line of antioxidant enzymatic defense against ROS (Faria et al., 2009). Yet, if the stress/contamination is extreme, this antioxidant system may not be sufficient to detoxify ROS and to prevent cells from damage, by placing the organism in jeopardy. The cleavage of H_2O_2 into the super reactive hydroxyl radical (OH^{-}•) is catalyzed by a number of trace metals in their reduced form (e.g., Cu^{+}, Fe^{2+}, Ni^{2+}, and Mn^{2+}), causing damage to the cell (Wright and Welbourn, 2002).

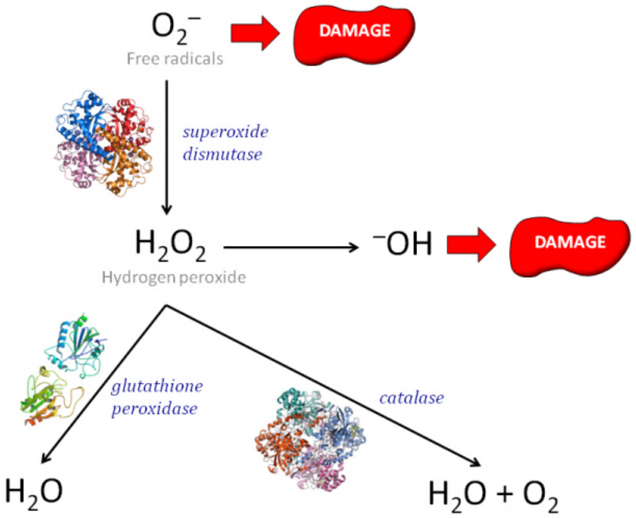

Figure 2. Superoxide dismutase and catalase mechanism of action (adapted after Leslie et al., 2013)

Although theoretically it is expected that antioxidant enzymes increase their activities under oxidative stress, in practice this not always happens. Previous studies about antioxidant enzymes indicated contrasting variations caused by pollutants with increases (Rodríguez-Ariza et al., 1993; Geracitano et al., 2004; Alla et al., 2006; Moreira et al., 2006; Douhri and Sayah, 2009; Bouraoui et al., 2010; Faria et al., 2010; Gorokhova et al., 2010; Benedetti et al., 2012), decreases (Gravato et al., 2010; Ameur et al., 2012; Oliva, Vicente, et al., 2012) and biphasic responses (Regoli et al., 2004; Sun and Zhou, 2008; Suriya et al., 2012; Won et al., 2012). This is not contradictory, it depends on several aspects according to the susceptibility of the exposed living species, chemicals (single or mixed contaminants, bioavailability) and intensity of exposure (Regoli et al., 2002, 2003; Bocchetti et al., 2008; Faria et al., 2009). Furthermore, it is well known that antioxidant enzyme levels can vary among seasonal and physiological factors, sexual conditions, gametogenesis period, and behaviors (Sun and Zhou, 2008), so the consistency of the factors mentioned above should be taken into account in laboratory and field studies.

Besides these enzymes, there are others also very important as biomarkers, but that are not directly related to detoxification systems which indicate other forms of effects. Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme involved in the regulation of the transmission of nerve impulses (Bouraoui et al., 2009), being found on the post-synaptic membrane of cholinergic synapses. Acetylcholine (ACh) binds to a postsynaptic cell, where the nerve impulse is conducted to the next axon. Then, ACh is hydrolyzed by AChE to acetate and choline, inactivating it and interrupting the nerve impulse (figure 3) (Payne et al., 1996). If this enzyme is inhibited, ACh is not hydrolyzed and will accumulate in the synaptic cleft. Consequently, the nerve impulse is not interrupted, leading to an overstimulation of cholinergic receptors, neuromuscular paralysis and uncoordinated movements which can result in the organism's death (Chen, 2012; Pereira et al., 2013).

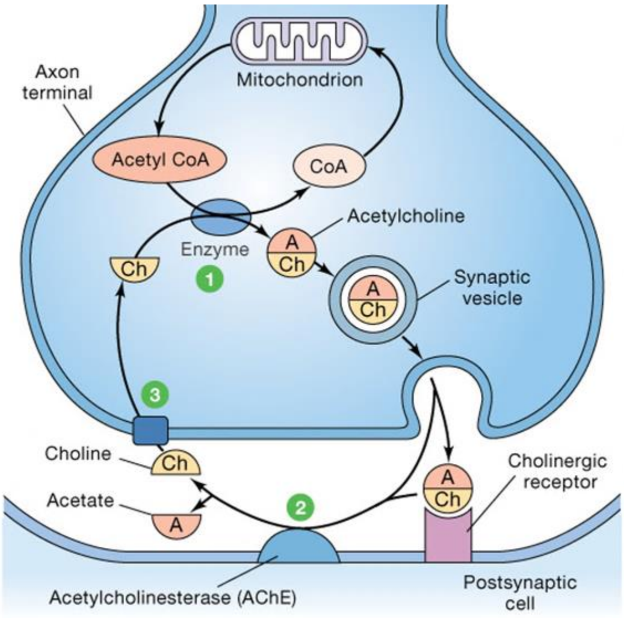

Figure 3. The mechanism of action of acetylcholinesterase (adapted after Soreq and Seidman, 2001)

AChE inhibition is linked directly with the mechanism of toxic action of organophosphorus and carbamate insecticides (Day and Scott, 1990; Cajaraville et al., 2000), thus this knowledge of the mode of action has been used to detect and measure the biological effects of organophosphates and carbamates in the marine environment (Cajaraville et al., 2000; Botté et al., 2012).

Because enzyme systems are age, gender, and species dependent and are affected by genetic polymorphisms and environmental factors (Chen, 2012), it is necessary to understand the pathways behind each specific circumstance. Although the utility of a biomarker approach may be reduced because of the lack of specificity regarding contaminants, biomarkers still hold value when attempting to improve risk assessments (McLoughlin et al., 2000). Preferably, the biomarker-based toxicity assessments should be associated with alternative bioassay techniques, like the use of sublethal physiological endpoints (as growth, reproduction and feeding rate) to gauge toxicity and to establish the link to ecologically relevant endpoints.

1.2.3. Linking different levels of biological organization: from the molecule to the community - giving ecological relevance to bioassays

A variety of organisms are exposed to the contaminants in sediments, either directly or indirectly. They can be organisms that ingest sediments or particulate detritus as food, or while seeking food, directly exposed, or individuals from higher trophic levels that feed on such organisms from benthic food webs (Calow, 1993).

One of the approaches to assess water or sediment toxicity is direct determination of the ecological and biological changes caused in nature by the contaminants. Some studies have successfully correlated chemical contamination in marine sediments with community or population changes, especially where contaminant gradients were identified by chemical analysis (Martínez-Lladó et al., 2007).

However, sometimes irreversible damage at the population or community level has already occurred and lower-level biological organization responses would be preferable to prevent such episodes. As referred before, responses at the biochemical level are lower order responses to contaminants that are relatively easy to detect and might be contaminant specific, and are good indicators of exposure to contaminants (Won et al., 2012). Besides, they may have the potential to anticipate changes at higher levels of the biological organization (i.e., population, community or ecosystem) (McLoughlin et al., 2000). Thus these “early warning” biomarkers can be used in a predictive way, allowing the initiation of remediation or abatement strategies before irreversible environmental damage of ecological consequences occurs (Martín-Díaz et al., 2004).

The ‘‘biomarker approach’’ assumes that effects seen at higher levels of biological organization (populations and communities) are the consequence of the sum of effects on individuals, which resulted from impacts at the cellular and molecular levels (figure 4). This makes these lower biological level and early responding endpoints potential tools for rapid diagnoses of toxicity (Lemos et al., 2010).

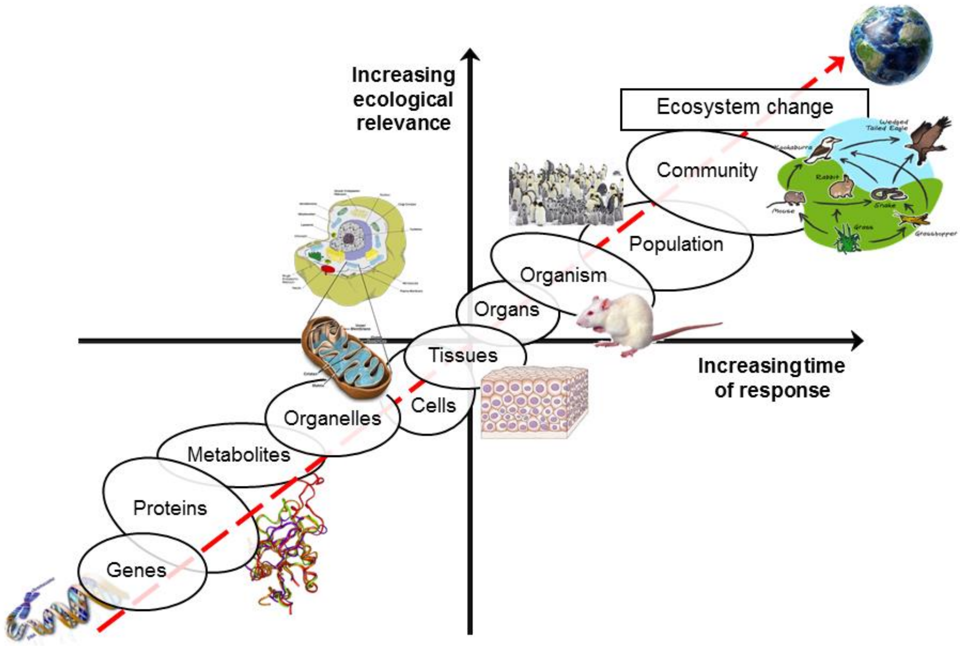

Figure 4. Relationship between ecological relevance, time of response and the different levels of biological organization after stress exposure (redrafted after Lemos et al., 2010)

Thus, the ecotoxicologist goal is to develop and validate tools that are rapidly responsive, but despite having low ecological relevance per se, can be linked to higher-level of biological organization responses and thus confer these prospective tools a potential do address at an early stage, effects that are ought to happen later in time and at the population and community level.

1.3. Using *Capitella* in ecotoxicology: its role in environmental assessment

Benthic organisms have been especially useful in applied research and are good indicators of environmental stress, and sediment toxicology is the most used strategy because they are in constant contact with such organisms. As stated by Montagna (2005) “…sediments are the «memory» of the ecosystem. Because they live and feed in sediments, benthic organisms are usually the first organisms affected by pollution or stress”.

Polychaetes are segmented worms common throughout the aquatic environment, but are especially common in soft marine sediments where they constitute 35-70% of the macroinvertebrate population (Lewis and Watson, 2012). Intertidal polychaetes are exposed to highly dynamic environments, and are under increasing pressure from urbanization due to 50% of the world's population living near the coast (Shain, 2009). These animals usually live in the sediments or attached to hard surfaces and, while their larvae may be capable of long distance transport, the adults are relatively inert - this relative immobility ensures chronic exposure to any toxic materials in the environment rather than the episodic exposures of a more vagile organism. Moreover, any long-term change in the wellbeing of the benthos should be reflected in the polychaete community (Dean, 2008).

The endobenthic deposit feeder *Capitella* is a cosmopolitan polychaete (Phylum Annelida) that is often associated with marine polluted environments, and has been widely used as a bioindicator of organic pollution (e.g. Reish, 1970; Rossi et al., 1976; Warren, 1976; Selck et al., 1998; Solis-Weiss et al., 2004; Méndez et al., 2008). The genus *Capitella* comprises multiple species that are morphologically similar as adults, but are differentiated reproductively (e.g. in ovum size, sperm characteristics, spawning habits and development), and their life cycle includes both a short-lived larval stage and an adult stage (Shain, 2009).

Females of *Capitella* genus constructs brood tubes with fecal materials, substrate and potential food, which are open at both ends. According to Reish (1970), after copulation, the female lays the fertilized eggs around the inner surface of the brood tube where incubation occurs, remaining there until the metatrochophore stage. According to Seaver and colleagues (2005), at 19ºC, animals of the specie *Capitella* sp. I (later defined by Blake et. al (2009) as *Capitella teleta*) emerge from the brood tube approximately 8 days following fertilization (figure 5, St. 9), and within 30-40 days they reach the adult stage, with a 6-12 months longevity in culture (Shain, 2009). Briefly, the trochophore larvae is characterized by the appearance of two eye spots, segmentation and two cilliary rings (prototroch and telotroch), which allow free movement inside the tube (figure 5, St. 4-5). The metatrochophore stage shows 13 segments and a visible ventral stomodeal concavity (figure 5, St. 9), and then larvae emerge from the brood tube (Seaver et al., 2005). The juvenile stage of *Capitella* has a vermiform shape with a complete segmentation and a clear distinction between thorax and abdomen. The mature males are characterized by the presence of genital spines between the 8th and 9th thoracic setigers, while oocytes are visible in the ventral coelomic cavity of mature females. Oocytes mature once they enter the coeloma, and became spherical and creamy-white to pale yellow (Méndez, 2002; Blake et al., 2009; Shain, 2009).

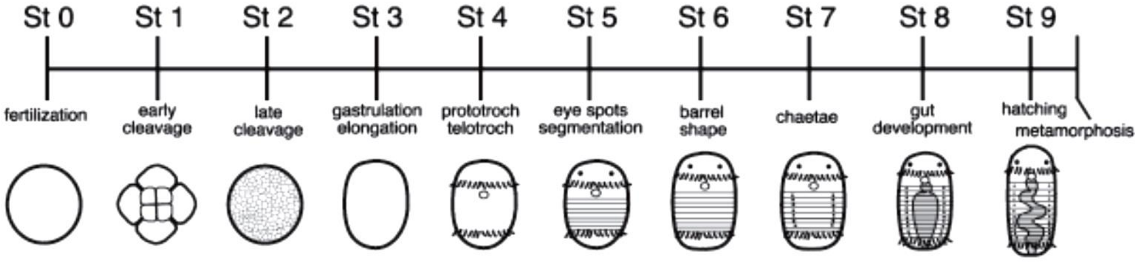

Figure 5. Developmental stages in Capitella sp. I. St. 0 – fertilization, day zero; St. 1 - early cleavage; St. 2 - late cleavage; St. 3 – gastrulation and elongation; St. 4 - appearance of trochal bands, segmentation begins; St. 5 - trochophore stage with eyespots, segmentation apparent; St. 6 - segmentation is clearly visible, larva more elongate than in previous stage; St. 7 - midbody completely segmented, chaetae present; St. 8 - midgut clearly visible and greenish; St. 9 - segmented metatrochophore ready to emerge from brood tube (adapted after Seaver et al., 2005).

Furthermore, *Capitella teleta* has been considered opportunistic and numerically dominant specie due to its rapid growth rate in organically enriched areas. Thus it has been the subject of several investigations around the world (Grémare et al., 1988; Pechenik et al., 2001; Méndez and Baird, 2002; Borja et al., 2003; Hu et al., 2003; Seaver et al., 2005; Thiyagarajan et al., 2005; Blake et al., 2009; Horng et al., 2009; Lewis and Watson, 2012). This specie was reported to process high densities of contaminated sediments, particularly with polycyclic aromatic hydrocarbons (e.g. Méndez et al., 2001) and heavy metals (e.g. Selck et al., 1998), the most abundant compounds at the contaminated sites like harbors and sewage discharges, but also organophosphorus compounds from agriculture fields (e.g. Méndez et al., 2008), alkylphenols (e.g. Hansen et al., 1999), among a few others.

The high suitability and ecological relevance of polychaetes, together with their easy culture and maintenance in the laboratory (relatively fast reproduction and growth), has them led to become increasingly used in ecotoxicological studies. Besides that, sediment processing by the *Capitella* species may have important ecological implications in the recovery of polluted sediments via sediment turnover (Méndez et al., 2008). However, a few species have dominated the literature to date and there remains a paucity of data on their ecological and biochemical responses to toxic insult. Thus such knowledge gaps limit ability to assess the true impact of environmental contaminants on real world populations (Lewis and Watson, 2012).

Deposit feeders as *Capitella* obtain their nutrients from the sediment and are the important secondary producers in wetland ecosystems. They play a central role in transferring both energy and material from primary producers and detritus to organisms, such as fishes and birds, in the higher trophic levels. The sediment nutrients can influence the feeding behavior of deposit feeders. This, in turn, affects the growth and reproduction, thus the fitness of the animal (Hu et al., 2003). Recent studies on the use of biomarkers in polychaetes, as for example to oxidative stress of heavy metal pollutants, has shown to be promising (Dean, 2008). Including ecological and biochemical parameters seems to be very important, in order to combine them to obtain more specific responses. Nevertheless, further work is needed to overcome the lack of specificity of these methods.

1.4. Environmental impact of harbors: the role of ecotoxicological studies

Anthropogenic contaminants discharged into water-bodies can cause detrimental effects to marine ecosystems, which above certain threshold levels can lead to species loss, fishing restrictions and dietary restrictions on seafood (Lepland et al., 2010). Harbors are economic engines of vital importance not only for the harbor area but for a wide zone of influence (“the hinterland”). Nowadays, a trend exists in harbor cities to recover waterfront zones for leisure use by citizens and for the installation of nautical clubs and tourist activities (Grifoll et al., 2011). Being the major interface between coastal cities and the sea, harbor areas are heavily stressed by human activity, since they are the fate of a wide range of contaminants coming from industrial, domestic and shipping activities (Díez et al., 2006; Kerambrun et al., 2012). Regardless whether discharges originate from air, rivers, urban runoff or effluent pipes, the question is that many contaminants show high persistence, hydrophobic nature, and low solubility in water, and consequently they are adsorbed to the particulate matters, and accumulate in muddy sediments (Galanopoulou et al., 2005; Lepland et al., 2010). Consequently, low indices of water quality can negatively reverberate on the development of human activities. In this context, European harbor authorities are responsible for monitoring environmental pollution. Therefore, management of the water quality in harbor areas is of vital importance from an economic, social and environmental point of view (Grifoll et al., 2011). Although water quality in harbors can be degraded by several factors, Islam and Tanaka (2004) suggest that anthropogenic discharges, even accidental or not, are likely to be the most important factors involved in water quality degradation in a harbor. As a result, some marine species living in these areas could evidence acute or chronic effects even at low concentrations (Díez et al., 2006).

Sediments in harbors can be contaminated by hundreds of individual compounds. In the Mediterranean, coastal sediments are particularly contaminated with heavy metals (Cd, Zn, Pb) and organic compounds such as butyltins (TBT, DBT, MBT) from anti-fouling paints, PAH from fossil fuels, and PCB mainly present in sewage effluents and plastics (Mamindy-Pajany et al., 2011). The occurrence of PAH, TBT, heavy metals and PCB in sediments of Barcelona harbor and their impact on benthic communities were evaluated in previous studies by Díez and co-workers (2006), Martínez-Lladó and co-workers (2007) and Gibert and co-workers (2009), showing a moderate to highly contamination of the sediments, which could represent a serious environmental threat for the benthic community in the Barcelona harbor. However, other persistent and potentially harmful contaminants such as organochlorine pesticides, organophosphates and carbamates have been reported to exert adverse effects on the benthic fauna (Hai et al., 1997; Canty et al., 2007; Patetsini et al., 2013) and should not be overlooked since their presence in coastal waters is common, from agriculture practice.

The Port of Barcelona (Spain) has a 2000-year history and great contemporary commercial importance. It is Catalonia's region largest port and Europe's ninth largest container port, with a trade volume of 1.94 million TEU's (Twenty-Foot Equivalent Units) in 2010, according to AAPA (American Association of Port Authorities). It is also one of the most important ports in the Mediterranean and it is managed by the Port Authority of Barcelona. Its 1,066.75 hectares are divided into three zones: Port Vell (the Old Port) destined to personal and touristic boats, the commercial/industrial port and the logistics port (Barcelona Free Port). The port is still undergoing an enlargement that will double its size, according to Port of Barcelona data (Port of Barcelona, 2013).

Therefore, a multitude of xenobiotics contaminate the harbor environment and although chemical analyses are able to measure many of these compounds qualitatively and quantitatively, complex mixtures of these chemical pollutants cannot be fully assessed. Aside to measure the potential impacts of these contaminants in the organisms inhabiting this area, there is a major challenge in current ecotoxicological research to identify suitable biomarkers and monitoring methods for these purposes (Kerambrun et al., 2012).

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Chapter 2. Aim of the study

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The main purpose of this study was to assess the potential ecological impact of sediments from Barcelona harbor (Spain) in *Capitella teleta* polychaetes, under laboratory conditions. To fulfill this objective:

(a) The effects on *Capitella teleta* at an individual biological level were assessed, through physiological response experiments such as growth (body weight) and feeding activity (egestion rate).

(b) Potential biomarkers, linked to neurotoxicity and antioxidant enzymes, were evaluated as a tool for ecotoxicological assessment. For this purpose, these biochemical markers protocols were optimized, and cholinesterases' were characterized, since no data was available.

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Chapter 3. In vitro **characterization of cholinesterases as potential biomarker in the polychaete *Capitella teleta***

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In vitro characterization of cholinesterases as potential biomarker in the polychaetes *Capitella teleta*

Abstract

The application of biomarkers to assess the impact of xenobiotics is increasingly nowadays. Marine ecosystems are subject to a multitude of anthropogenic contaminants and an important advantage of biomarkers in evaluating the impact of such contaminants is the inherent capability of detecting early occurrence of various stress conditions within the organism and monitoring temporal progression (or regression) of the disturbance, at various levels of biological organization.

Nevertheless, few studies were performed with the polychaete *Capitella teleta*, a filter-feeding and burrowing organism. The high suitability and ecological relevance of polychaetes, together with their easy culture and maintenance in the laboratory, has led them to becoming increasingly used in ecotoxicological studies, demanding for the need to characterize their cholinesterases to address neurotoxicity biomarkers in further studies.

Thus, the present study was aimed to evaluate the cholinesterases in *Capitella teleta*, using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and *S*-butyrylthiocholine iodide) and four known esterase inhibitors (eserinehemisulfate, BW284C51, iso-OMPA and chlorpyrifos-oxon). The results showed the existence of only one cholinesterase form, the acethylcolinesterase. The IC_{50} for chlorpyrifos-oxon was 60.72 nM. The study highlights the relevance of ChE characterization before its use as a biomarker in ecotoxicology and biomonitoring studies.

Keywords: Acetylcholinesterase, BW284c51, Chlorpyrifos-oxon, Eserine hemisulfate, *iso*-OMPA, Median inhibitory concentration

3.1. Introduction

With respect to neural and muscular functions, cholinesterase activities (ChE) have been widely used as markers of neural and muscular functions (Howcroft et al., 2011). They can be distinguished functionally, according to their preference for specific substrate and to their susceptibility to selective inhibitors (Monteiro et al., 2005). Within the substrates specificity, acetylcholinesterase (AChE, EC 3.1.1.7) have a strong preference for acetylcholine; butyrylcholinesterase (BChE, EC 3.1.1.8), also known as pseudocholinesterase, prefer butyrylcholine; propionylcholinesterase (PChE, EC 3.1.1.8) is better at degrading propionylcholine (Nunes, 2011). Predominant types of ChE varied across species. Vertebrate species usually possess two distinct molecular forms of the enzymes, acetylcholinesterase and butyrylcholinesterase (Massoulié and Bon, 1982; Massoulié and Toutant, 1988), whereas the ChE preference for different substrates is a common feature in invertebrates (Cacciatore et al., 2012). For example AChE and PChE have been detected as major ChE in the clam *Corbicula fluminea* (Mora et al., 1999), AChE and BChE in the oyster *Ostrea edulis* (Valbonesi et al., 2003), and PChE in the bivalves *Arca noae* and *Venus verrucosa* (Perić et al., 2013). Additionally, ChE may also have a broad distribution across several types of tissue, including serum, liver, heart, brain, vascular endothelia and in the nervous system (Leticia and Gerardo, 2008; Valbonesi et al., 2011). BChE was found in plasma, liver, muscle and other tissues in vertebrates and invertebrates (Monteiro et al., 2005; Rault et al., 2007; Nunes, 2011). Therefore, the high variability and complexity of ChE requires their characterization before their use as biomarkers.

AChE is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firing, which is vital for normal functioning of sensory and neuromuscular systems (Payne et al., 1996). Indeed, the inhibition of AChE activity is one of the main targets of organophosphorus and carbamate pesticides that are widely used to control insect pests (Key and Fulton, 2002; Caselli et al., 2006; Valbonesi et al., 2011). In non-target organisms, the inhibition of AChE activity has been considered as a tool in diagnosing organophosphate and carbamate poisoning in various *taxa* (Stenersen, 1979; Day and Scott, 1990; Sanchez-Hernandez and Walker, 2000; Jokanović, 2001; Varó et al., 2002; MacGregor et al., 2005; Rendón-von Osten et al., 2005; Čolović et al., 2011; Botté et al., 2012; Cacciatore et al., 2012). Depression of the activity of this enzyme may indicate sublethal toxicity and thus provide an early warning for detrimental effects on populations and communities (Day and Scott, 1990).

The endobenthic deposit feeder *Capitella capitata* (Fabricius, 1780) is a cosmopolitan polychaete that consists of a complex of non-interbreeding but morphologically similar sibling species which are distinguishable by protein variation, developmental and reproductive features, ecophysiological characters and respiration rate(Méndez, 2006). Among the described *Capitella* sibling species, *Capitella* sp. I, recently designed as *Capitella teleta* (hereafter termed this way) by Blake and co-workers (2009), has been considered as an opportunistic specie due to its rapid growth rate and numerical dominance in organically enriched areas (Blake et al., 2009), being especially useful as a bioindicator of environmental stress (Forbes et al., 1996; Dean, 2008; Méndez et al., 2013). Common in mainly soft marine sediments, with few species present in freshwater environments (Shain, 2009), polychaetes are relatively inert and this relative immobility ensures chronic exposure to any toxic materials in the environment rather than the episodic exposures of a more vagile organism, thus any long-term changes in the wellbeing of the benthos should be reflected in the polychaete community (Dean, 2008). Besides this, deposit feeders as *Capitella* obtain their nutrients from the sediment and are the important secondary producers in wetland ecosystems, playing a central role in transferring both energy and material from primary producers and detritus to organisms, such as fishes and birds, in the higher trophic levels (Hu et al., 2003). So far, however, to our knowledge, there are no reported studies on *C. teleta* ChE.

The aims of this study were to (i) characterize the cholinesterases present in *C. teleta* tissues using different substrates and selective cholinesterase inhibitors and (ii) to determine the *in vitro* sensitivity of ChE to be inhibited by the model organophosphorous oxon chlorpyrifos-oxon.

3.2. Methods

3.2.1. Chemicals

Homogenization, along with protein assays were performed with potassium phosphate dibasic (K2HPO4); potassium phosphate monobasic (KH2PO4); potassium chloride (KCl); ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA); bovine serum albumin (BSA), all purchased from Sigma-Aldrich (St. Louis, MO, USA). The ChE characterization was evaluated with the substrates acetylthiocholine iodide (ATCh), *S*-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh), and with the inhibitors eserine hemisulfate, 1,5-bis[4-allyldimethylammoniumphenyl]pentan-3-one dibromide (BW284c51), tetra [monoisopropyl] pyrophosphortetramide (*iso*-OMPA) and chlorpyrifos-oxon (CPO; O,O-diethylO-3,5,6-trichloro-2-pyridyl phosphate, CAS no.5598-15-2). 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB) was used for the ChE quantification (Ellman’s reagent), and the referred chemicals were also purchased from Sigma-Aldrich (St. Louis, MO, USA). The rest of chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

3.2.2. Experimental animals and culture conditions

Individuals of *C.teleta* were originally collected in Setauket Harbor (New York, USA) in 1984, and later identified by Judith P. Grassle (Méndez et al., 2013), and have been cultured in the Department of Life Sciences and Chemistry of the Roskilde

University (Denmark) for several generations (Ramskov et al., 2009). Worms were held in culture at the Institute of Environmental Assessment and Water Research (CSIC, Barcelona) one year prior to the experiments.

Plastic aquarium tanks (2000 ml) with 400 g (dw) of clean sediment and 1500 ml of aerated seawater adjusted to a salinity of 30±2‰, both from Sitges (NE Spain, 41º 14' 22.7'' N, 1º 51' 44.6'' E), were used for the cultures. The sediment was previously oven-dried (60°C) and sieved to under 250 µm, and the seawater filtered (<30 µm). The sediment organic matter content was 8.23±0.16% (n=3; loss on ignition method by Dean, 1974). Stock cultures were maintained at 20±2°C in the dark, following Horng and co-workers (2009) for the cultures developed in the Roskilde laboratory. Organisms were fed weekly with 0.15 g of artificial food (sieved to under 250 µm) consisting in an equal part-mixture of commercial fish food (Wardley-Hartz Company, New Jersey USA), baby cereal (Nestlé, Switzerland) and dried (60ºC) spinach (Forbes et al., 1996).

3.2.3. Experimental procedure

3.2.3.1. Samples homogenization

Adult polychaetes (length ranging from 6 to 10 mm) were collected from culture boxes and observed under a stereo dissecting microscope. Abnormal organisms and pregnant females were discarded. Pools of 50 individuals were homogenized in 10 ml of ice-cold homogenization buffer - 100mM phosphate buffer pH 7.4, containing 100mM KCl and 1mM EDTA – with the assistance of a sonicator (Branson Sonifier® W-150, G. Heinemann - SchwäbischGmünd, Germany) and then centrifuged for 10min at 10,000g, 4ºC. Carefully the S9 fraction (supernatant) was collected and used in the biochemical assays. All the steps were performed on ice to prevent enzyme degradation.

3.2.3.2. Enzymatic assays

Biochemical measurements were carried out on a Synergy 2 Multi-Mode Microplate Reader (BioTek® Instuments – Vermont, USA) spectrophotometer. The ChE was determined using the Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), with some modifications and run at least in triplicate. The ChE activites were measured in the presence of 40µl of sample (S9 fraction), 60µl of homogenization buffer and 200µl of reaction mixture that included different concentrations of substrates – from 0.0033 to 3.3 mM – and DTNB at 0.17 mM (final concentrations). The increase of absorbance measured at 405 nm within the first

10 minutes, due to the hydrolysis of the studied substrates and subsequent complexation with DTNB, was used to determine enzyme activity. Appropriate blanks with homogenization buffer were subtracted to each sample before result interpretation. The enzymatic activity was expressed as nmoles of hydrolyzed substrate per minute per mg of protein (nmol/min/mg protein), using the extinction coefficient 13.6 mM^{-1} cm^{-1}. The protein quantification was determined according to the Bradford method (Bradford, 1976), using bovine serum albumine as protein standard.

3.2.3.3. Cholinesterases characterization

Cholinesterases characterization was performed according to Monteiro and co-workers (2005), with some modifications. For each step of the characterization a minimum of 3 biological replicates were used. Firstly, ATCh, BTCh and PTCh were used as substrates at the follow final concentrations: 0.0033, 0.033, 0.066, 0.165, 0.33, 0.66, 1.32, 1.98 and 3.3 mM. Once an optimal substrate and its concentration selected (ATCh at 0.33mM), samples were exposed to different concentrations of the following selective inhibitors. In independent experiments, eserine hemisulfate, BW284c51 and *iso*-OMPA were used as selective inhibitors for all the ChE, AChE and BChE, respectively. Stock solutions of eserine hemisulfate and BW284c51 were prepared in ultrapure water while stock solution of *iso*-OMPA was prepared in ethanol and diluted in water prior to its use. For each inhibitor, 5 µl of a stock solution was incubated, for 30 minutes at 25ºC, with 495 µl of S9 fraction. Final concentrations for each inhibitor were 10 and 100 µM of eserine and BW284c51, and 1 and 10 µM of *iso*-OMPA. Ultrapure water was added to controls and an additional control with ethanol (0.1%) was used in the experiments with *iso*-OMPA. After the incubation period, AChE activity, was determined as describe above (section: 3.2.3.2.) using 0.33mM of ATCh.

3.2.3.4. *In vitro* IC_{50} of chlorpyrifos-oxon

The organophosphate metabolite chlorpyrifos-oxon (CPO) was used as a known potent inhibitor of AChE activity (Amitai et al., 1998; Koenig et al., 2013). A stock solution of CPO was prepared in ethanol, and samples were exposed to final concentrations ranging from 6 to 600 nM, for 30 min at 25ºC. For controls, only ethanol was added. Carrier concentration was normalized across samples to 0.1%. After the incubation period, AChE activity was determined as describe above (section: 3.2.3.2.), using 0.33mM of ATCh.

3.2.4. Statistical analysis

The enzyme kinetic parameters maximal velocity (Vmax), Michaelis–Menten constant (Kd) and their ratio (Vmax/Km) – which indicate the catalytic efficiency of the enzyme – were estimated by fitting experimental data to Michaelis-Menten equation (1), where X is the substrate concentration and F is the enzyme activity.

$$F = \frac{V_{\max} x\ X}{K_d + X} \qquad (1)$$

One way analysis of variance (ANOVA), followed by Pairwise Multiple Comparison Procedures (Fisher LSD Method) was performed to assess differences between treatments. The *in vitro* IC_{50} of CPO for *C. teleta* ChE was determined using equation 2, where X is the toxic contaminant concentration and h the Hill coefficient.

$$Inhibition = \frac{1}{1+\left(\frac{X}{IC_{50}}\right)^h} \qquad (2)$$

All the mentioned analyses were performed by SigmaPlot software for Windows Version 11.0 (Systat Software, 2008).

3.3. Results

3.3.1. Substrate affinity

Kinetic curves of the different tested substrates are described in Figure 6, and the respective kinetic parameters are described in the Table 1. All regression fittings had $r^2>0.92$ ($P<0.001$). Vmax for the ATCh was 10 to 31 fold greater than that of PTCh and BTCh, respectively. Furthermore the ATCh had the highest Vmax/Kd ratio followed by BTCh and PTCh. This means that ATCh was the preferred substrate having the greatest enzyme catalytic efficiency. No activity inhibition by excess of substrate was observed, no matter the concentration used. Based on these findings, the substrate with better affinity for ChE present in *C. teleta* was the acetylthiocholine iodide, thus all the next enzymatic assays were performed with ATCh at 0.33mM.

Table 1. Values of the maximal velocity (Vmax), Michaelis–Menten constant (Kd) and the catalytic efficiency of *Capitella teleta* cholinesterases (ratio Vmax/Kd) for the three tested substrates. Values are expressed as mean ± standard error.

	N	ATCh	BTCh	PTCh
Vmax (nmol/min/mg protein)	18	366.93±5.53	16.70±0.36	37.8±0.70
Kd (mM)	18	0.19±0.02	0.01±0.002	0.05±0.005
Ratio Vmax/Kd	18	1931	1670	756

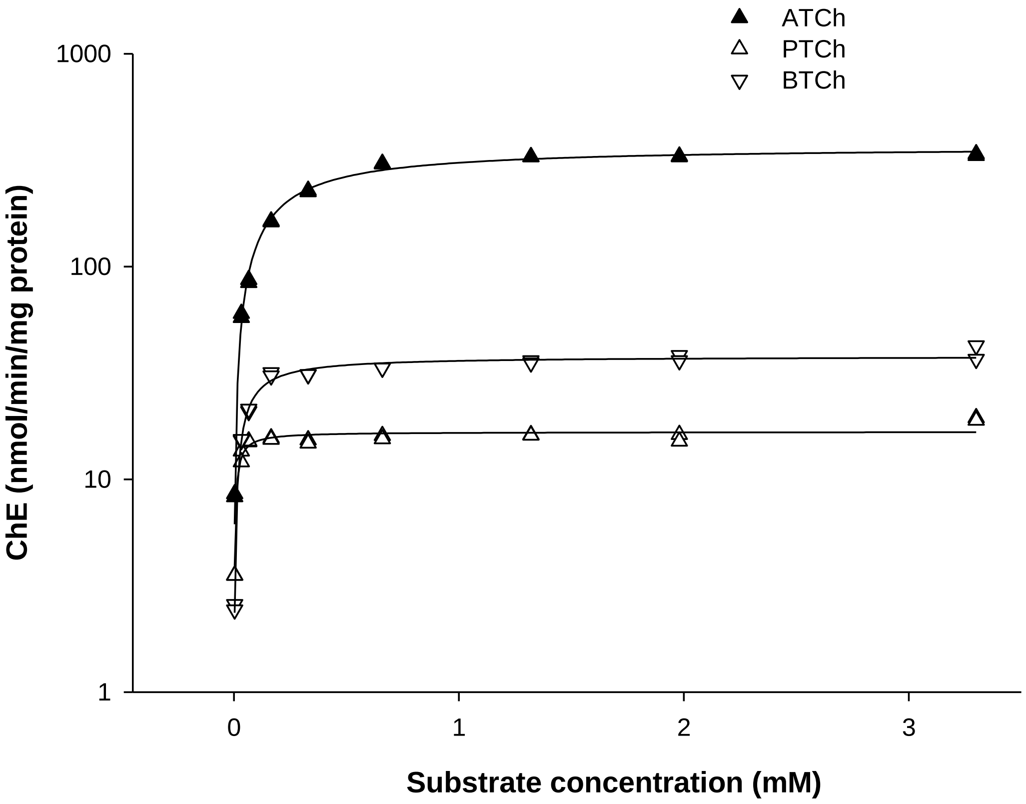

Figure 6. Cholinesterase (ChE) activity in *Capitella teleta* as a function of acetylthiocholine iodide (ATCh), *S*-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh). Each point is a single replicate determination. For clarity ChE axis is in log scale.

3.3.2. Inhibitory effect

All tested model inhibitors except iso-OMPA reduced significantly ChE ($F_{7,17}$=19393,7; P<0.001) (Figure 7). Eserine hemisulfate and BW284c51 inhibited 88.25 and 99.44 % ChE of control treatments. This means that most of the hydrolysis of ATCh was conducted by AChE with a negligible contribution of BChE or nonspecific esterases.

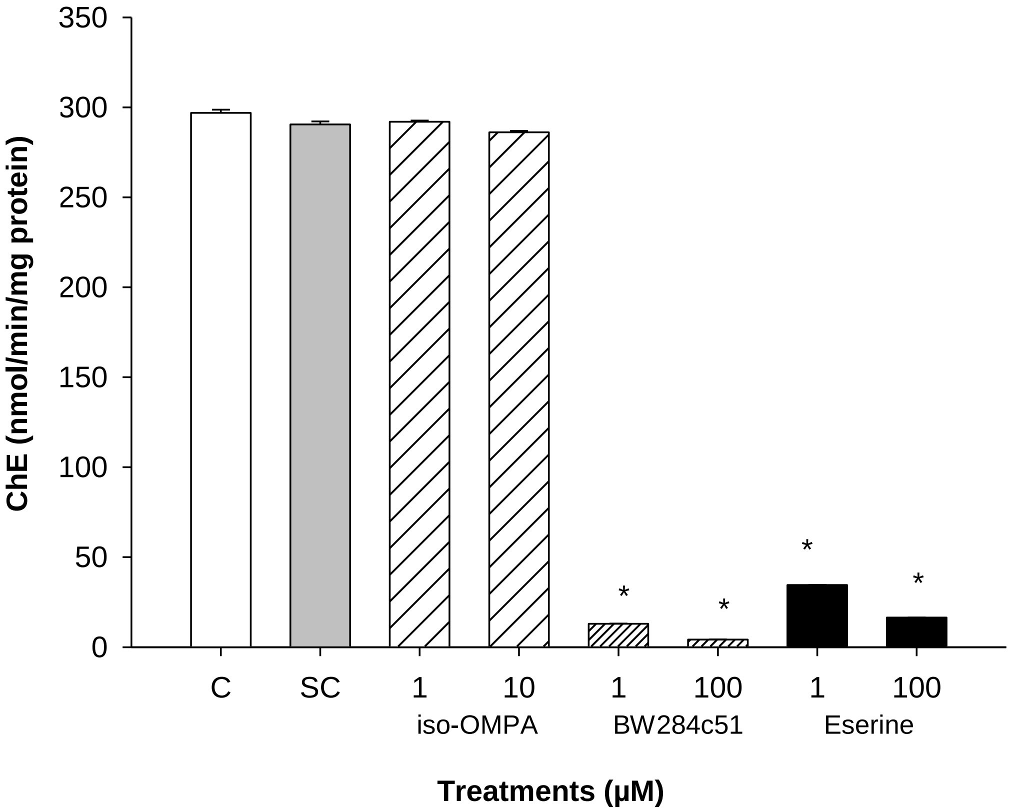

Figure 7. Effect of eserine hemisulfate, BW284c51 and iso-OMPA on cholinesterase (ChE) activity (nmol/min/mg protein) of *Capitella teleta*, using acetylthiocholine iodide (0.33mM) as substrate. Results are expressed as the mean ± SEM (n=4). * means significantly (P<0.05, based on ANOVA and Dunnets tests) different from the control (C) for BW284c51 and eserine and from solvent control (ethanol, SC) for iso-OMPA.

3.3.3. *In vitro* IC_{50} of chlorpyrifos-oxon

Proportional inhibition responses across CPO concentrations depicted in Figure 8 were accurately adjusted to the Hill equation ($r2= 0.93$, $P<0.001$; $n=20$) and a median inhibitory concentration (IC_{50} ± SE) of CPO *in vitro* was estimated to be 60.7 ± 7.4 nM (20.31 µg/L).

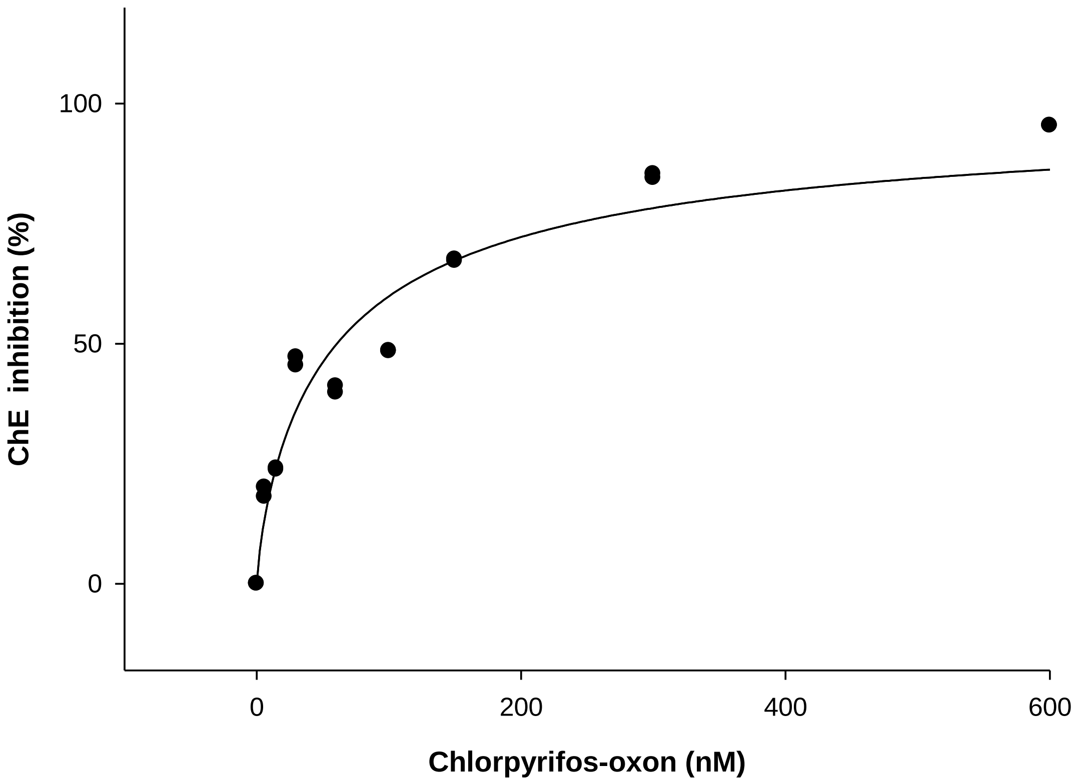

Figure 8. In vitro cholinesterase (ChE) inhibition proportional responses to chlorpyrifos-oxon (CPO) of *Capitella teleta* homogenates, using acetylthiocholine iodide (0.33mM) as substrate.

3.4. Discussion

Characterization of ChE is vital when using it as potential biomarker, because tissues may contain several non-specific esterases that can contribute to the measured activity, leading to failures in the assessment and interpretation of results in ecotoxicological studies (Ferreira et al., 2010; Howcroft et al., 2011). A first characterization of *C. teleta* cholinesterases was conducted using kinetic assays that showed that ChE had the greatest efficiency hydrolyzing acetylthiocholine. A further characterization involved the use of specific inhibitors. The distinction between ChE from non-specific esterases was estimated using eserine hemisulfate, a organophosphorus compound well-known as general inhibitor of ChE at low concentrations (Jokanović, 2001; Nunes, 2011). Our results indicated that most of the measured activity was related to ChE since enserine inhibited almost completely enzyme activity. The distinction between BChE and AChE was conducted using AChE and BChE specific inhibitor BW284c51 and iso-OMPA, respectively. Results obtained in Figure 7 showed that whereas iso-OMPA hardly impaired ChE, BW284c51 inhibited almost completely enzymatic activities. This means that measured ChE was mainly AChE.

ChE forms in aquatic invertebrate species have not been widely studied (Rault et al., 2007). So far no data is available about another species of the genus *Capitella*. When considering other polychaetes species, Scaps and co-workers (1996) showed that AChE was the predominant ChE in *Nereis diversicolor*, with 3.61±0.27, 1.70±0.12 and 0.32±0.09 enzymatic activities (nmol/min/mg protein) for ATCh, PTCh and BTCh, respectively. However, in the same study, the authors found a different behavior for the oligochaete *Eisenia fetida*, where the substrates preferentially hydrolyzed were PTCh and ATCh and then BTCh (enzymatic activities of 24.30±4.52, 13.61±2.60 and 7.63±1.95 nmol/min/mg protein, respectively), with a significant inhibition when incubating *E. fetida* tissues with iso-OMPA (Scaps et al., 1996). The ChE activity in body tissues of the polychaete *Arenicola marina* was also dominated by PChE, with 59.37 µmol/min/mg protein, being that esterase activity the highest recorded in a polychaete worm (Hannam et al. 2008). Concerning oligochaetes, several ChE characterization studies have been performed. In the oligochaetes *Lumbriculus variegates* Kristoff and co-workers (2006) showed that both AChE and BChE forms of ChE were present, with enzymatic activities of 320 and 266 nmol/min/mg protein, respectively. Yet, the authors did not study the presence of PChE. Indeed, *Lumbricus terrestris* and *Lumbricus castaneus* hydrolyzed ATCh and PTCh better than BTCh – recording activities of 209 and 260 nmol/min/mg protein with ATCh for *L. terrestris* and *L. castaneus*, respectively, and similar activities when using PTCh as substrate (Rault et al., 2007). Unfortunately, for the above referred works authors did not provide kinetic data on Vmax and Kd values. Vmax values of 46.52 and 40.83 nmol/min/mg protein and Kd values of 0.18 and 0.14 mM for PTCh and ATCh, respectively, were recorded in *Eisenia andrei.* Similar substrate preference (Kd) and maximum velocity (Vmax) were observed for the oligochaete *Enchytraeus albidus* for ATCh, BTCh and PTCh, with Vmax values of 15.32, 19.70 and 23.36 nmol/min/mg protein and Kd values of 0.16, 0.24 and 0.17 mM, respectively (Howcroft et al., 2011).

Regarding other invertebrates, there are some studies about their ChE. The gastropod *Potamopyrgus antipodarum* showed two main ChE, PChE and AChE, while *Valvata piscinalis* gastropod showed AChE as main ChE, with ChE activities of 31.6 and 17.3 nmol/min/mg protein, respectively. Vmax values for *P. antipodarum* and *V. piscinalis* were between 13.4 and 29.6 nmol/min/mg protein, Kd values were between 0.13 and 3.19 mM, and their Vmax/Km ratios were between 4.2 and 227.7 (Gagnaire et al., 2008). Low ChE activities, Vmax, Km and Vmax/Km ratio values were also observed for the oysters *Ostrea edulis* and *Mytilus galloprovincialis*, and the clam

Tapes philippinarum (Mora et al., 1999; Valbonesi et al., 2003), as well as for the clam *Corbicula fluminea* (Mora et al., 1999) and for two different species of *Artemia* (*A. salina* and *A. parthenogenetica*) (Varó et al., 2002).

This substantial variety of esterase characteristics and activities in invertebrate species, make it difficult to compare with those found in *C. teleta*. In addition, *C. teleta* AChE activities are by far greater than those of other polychaetes species, for example, *Nereis diversicolor* which also preferred both ATCh over the other substrates showed AChE activities 100 fold lower than *C. teleta*. On the other hand, curiously similar ChE Michaelis-Menten behavior to that of *C. teleta* was found in the brain of the reef fish *Haemulon plumieri*, with Vmax and Km values of 237.8 nmol/min/mg protein and 0.31 mM, respectively, with consequent Vmax/Km ratio of 767.1 (Leticia and Gerardo, 2008) for AChE activity. Besides, other ChE characterization studies performed with fish species revealed enzymatic kinetic parameters more identical to those obtained in this study than the studies with invertebrates (Monserrat and Bianchini, 2001; de la Torre et al., 2002; Monserrat et al., 2002; Rodríguez-Fuentes and Gold-Bouchot, 2004). For example the AChE of the *Cnesterodon decemmaculatus* and *Cyprinus carpio* fishes showed Vmax values of 464.6 and 482 nmol/min/mg protein and Km values of 0.17 and 0.23 mM, for each one respectively (de la Torre et al., 2002). Overall the previous results indicated that our measured ChE enzymatic activities were quite high compared with those reported in other annelid worms but below those of *A. marina*, and *Lumbricus* species were the only invertebrates with the closest ChE activities to those obtained with *C. teleta*. It is important to emphasize that both enzymatic activities and kinetic parameters of the polychaete studied where were identical to reported studies in vertebrates, more precisely in some fish species.

Chlorpyrifos (CP) is an organophosphorus insecticide (EPA toxicity class II, CAS no. 2921-88-2) that has been used extensively in pest control (Sparling and Fellers, 2007). This pesticide needs to be oxidized to its toxic metabolite chlorpyrifos-oxon (CPO) to act in the organism, a reaction that is accomplished by Phase I P450 monooxogenase enzymes (Barata et al., 2004; Cacciatore et al., 2012). Oxon compounds are capable of disrupting the nervous system development (neuronal cell proliferation, differentiation and apoptosis as well as the proliferation and differentiation of glia) (Stenersen, 1979), through the phosphorylation of the serine hydroxyl group located in the active site of AChE, resulting in a stable inactive enzyme (Kousba et al., 2004). Moreover, the oxon actions occur at concentrations that are neurodevelopmentally relevant and very potent, for example CPO and diazoxon could be 10–1000 times stronger than CP and diazinon, respectively (Flaskos, 2012). Therefore, studies have to be performed with the oxon metabolites.

The results of our study show that CPO inhibited *C. teleta* ChE having an IC_{50} of 60.7 nM, after 30 min of incubation. To our knowledge, there is scarce information about CPO IC_{50} in invertebrates. The freshwater fish *Ictalurus punctatus* exhibited ChE IC_{50} values between 28 and 33 nM, depending on the studied tissue – brain, gill, liver, muscle or plasma – when exposed to CPO for 15 minutes, (Straus and Chambers, 1995). Other freshwater fish, *Gambusia affinis*, showed different IC_{50} values of approximately 50 nM (Boone and Chambers, 1997) and 64 nM (Carr et al., 1997) when exposed *in vitro* to CPO also for 15 minutes. Carr and co-workers (1997) also found ChE IC_{50} values of 25, 28 and 52 nM for the freshwater fishes *Micropterus salmoides*, *Lepomis macrochirus* and *Notemigonus crysoleucas*, respectively. The marine fish *Solea solea* showed an *in vitro* ChE IC_{50} of 75 nM, while for *Solea senegalensis* it was 38 nM (Koenig et al., 2013), with a 30 minutes-incubation. *Xeropicta derbentia*, a terrestrial snail, exhibited a ChE IC_{50} of 38 nM (Laguerre et al., 2009), while the freshwater snail *Planorbarius corneus* exhibited a higher IC_{50}, about 100 nM (Cacciatore et al., 2012), both after 30 minutes of incubation with CPO. Therefore the sensitivity observed for CPO of ChE from *C. teleta* was similar to those reported elsewhere. Unfortunately, no data about ChE IC_{50} for CPO exposure is available for other polychaetes species. In fact the IC_{50} value of ChE inhibition by CPO of *C. teleta* was more approximate to those of vertebrate species (mainly fish) than invertebrates. The same trend was seen with AChE kinetic parameters. All these former observations reveal that cholinesterases could be very sensitive markers of OP pesticide exposure and give support to their use since their responses were highly dependent on the pesticide and also on the polychaete specie assayed.

This study is the first work in providing original data concerning an enzymological characterization of ChE activity in *C. teleta*. Current results demonstrate that ChE activity is primarily due to AChE. The study supports the notion that ChE activity in *C. teleta* may be a suitable biomarker, since a great enzymatic catalytic efficiency and an *in vitro* sensitivity to a known insecticide were observed. Despite this, further studies both under laboratory and field conditions, with other inhibitors and under different conditions, are necessary in order to corroborate and strengthen these results.

Chapter 4. Effects of Barcelona harbor sediments in biological responses of *Capitella teleta*

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Effects of Barcelona harbor sediments in biological responses of *Capitella teleta*

Abstract

Marine ecosystems are increasingly exposed to a multitude of anthropogenic contaminants. Harbor environments are permanently subjected to such contaminants and bottom sediments are considered as the final repository. Filter-feeding and burrowing organisms, such as polychaete communities, are amongst the most exposed organisms. Since chemical analysis may not be enough to predict effects on organisms, individual- and subcellular-level responses are crucial to assess those effects.

This study aimed to assess the toxicity of Barcelona harbor sediments to the soft sediment dwelling polychaete worm *Capitella teleta*. Sediments were collected from three different locations of the Barcelona (Spain) harbor with a positive gradient of pollution from the mouth of the harbor towards the innermost zone. Body weight (growth) and egestion rate (feeding) were the individual-level responses selected, while subcellular-level responses measured consisted in antioxidant (catalase and superoxide dismutase) and neurotransmission related (acetylcholinesterase) enzymes.

Sediments from all sites affected significantly individual- and subcellular-level response of *C. teleta* worms when compare with those of controls. Barcelona's sediment impaired growth, egestion rates and also inhibited the activities of neurotoxic and antioxidant enzymes, indicating pollution impact along the Barcelona harbor. These results indicate that biological responses of *C. teleta* worm can be used as early-warning tools to assess pollution effects on marine soft-bottom macrobentonic communities.

Keywords: Acetylcholinesterase, Biomarkers, Catalase, Feeding, Growth, Superoxide dismutase, Sediments

4.1. Introduction

Life in our planet depends on the oceans which are based on uncountable ecosystem services, providing us with sources of wealth, opportunity and abundance. Anthropogenic activities are related to the major decline of the world's biological diversity (Islam and Tanaka, 2004), and marine ecosystems are no exception, being increasingly subject to a multitude of anthropogenic contaminants (Matthiessen and

Law, 2002). Hence, there is a renewed and urgent need for comprehensive biological monitoring tools and ecotoxicological research to improve the understanding of the biological impacts of contaminants for estuarine and coastal sediments (Lewis and Watson, 2012).

Survival has been the most common endpoint when assessing dredged material toxicity (Martín-Díaz et al., 2004), but provides little information on sub-lethal responses that are likely to be relevant in marine ecosystem that often are exposed to lower concentrations of pollutants than surface waters (Verslycke et al., 2004). Physiological endpoints, such as growth and feeding, have been used to measure individual-level responses within the normal physiological scope of the organism and correlated with pollutant exposure and detrimental toxic effects (Handy and Depledge, 1999; Ramskov et al., 2009; Méndez et al., 2013). The egestion rate, as an indirect measure of ingestion, can represent the response of individual animals to the change of the environment quality (Roast et al., 2000). In aquatic invertebrates, feeding inhibition is in most cases one of the first reported responses to environmental pollution (Coulaud et al., 2011). The impairment of feeding has been demonstrated to have direct effects on growth, reproduction, and eventually survival, which can lead to alteration in life-history traits, clearly demonstrating the ecological relevance of both feeding and growth as toxicity endpoints (Maltby, 1999; Moreira et al., 2006).

Besides individual level traits, there are responses that can be measured at the sub-cellular level. Those responses are called “biomarkers” and have been proposed as sensitive “early warning” tools for biological effect measurement in environmental quality assessment (Moore, 1993; Cajaraville et al., 2000; Gil and Pla, 2001; Martín-Díaz et al., 2004; Monserrat et al., 2007; Lemos et al., 2010), with the potential to anticipate changes at higher levels of the biological organization (i.e., population, community or ecosystem). Being used in a predictive way, biomarkers allow the initiation of amendment strategies before irreversible environmental damage of ecological consequences occurs. Regarding biochemical responses, antioxidant enzymes are used to neutralize, remove, or scavenge the ROS (reactive oxygen species) produced by the normal organisms’ oxidative metabolism as well as during detoxification of many pollutants (Ameur et al., 2012). A set of antioxidant enzymes attempt to decrease the oxidative stress induced by these ROS (Douhri and Sayah, 2009) and include the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), among others (Oliva, Vicente, et al., 2012). The increase of their activity in an organism is usually indicative of oxidative stress, having an important antioxidant role by balancing the levels of ROS in the cells (Faria et al., 2009). When ROS is exceeded antioxidant defenses oxidative damage occurs within cellular macromolecules of DNA, proteins and lipids (Geracitano et al., 2004; Patetsini et al., 2013). Additionally, there are other enzymes also widely used as biomarkers, but that are not directly related to detoxification systems. Acetylcholinesterase (AChE) is an enzyme involved in the regulation of the transmission of nerve impulses, through the degradation of acetylcholine (ACh) in choline and acetate (Bouraoui et al., 2009). Organophosphorous and carbamate pesticides act by inhibiting this enzyme, which can lead to an impairment of the neurological system of the exposed organism leading to alterations in behavior or even death (Chen, 2012). This knowledge of the mode of action has been used to detect and measure the biological effects of organophosphates and carbamates in the marine environment (Cajaraville et al., 2000; Botté et al., 2012).

Polychaetes are segmented worms common throughout the aquatic environment, but are especially abundant in soft marine sediments (Lewis and Watson, 2012). The endobenthic deposit feeder *Capitella* is a cosmopolitan polychaete (Phylum Annelida) often associated with marine sediments enriched with organic material, and has been widely used as a bioindicator of organic pollution (e.g. Reish, 1970; Rossi et al., 1976; Warren, 1976; Selck et al., 1998; Solis-Weiss et al., 2004; Méndez et al., 2008). More specifically, *Capitella teleta* has been considered as an opportunistic and numerically dominant specie due to its rapid growth rate in organically enriched areas. Thus it has been the subject of several ecological and experimental investigations around the world (Grémare et al., 1988; Pechenik et al., 2001; Méndez and Baird, 2002; Borja et al., 2003; Hu et al., 2003; Seaver et al., 2005; Thiyagarajan et al., 2005; Blake et al., 2009; Horng et al., 2009; Lewis and Watson, 2012). This specie was reported to process high densities of contaminated sediments, particularly with polycyclic aromatic hydrocarbons (e.g. Méndez, Linke-Gamenick, Forbes, & Baird, 2001) and heavy metals (e.g. Selck et al., 1998), the most abundant compounds at the contaminated sites like harbors and sewage discharges, but also organophosphorus compounds from agriculture fields (e.g. Méndez et al., 2008), alkylphenols (e.g. Hansen, Forbes, & Forbes, 1999), amongst others. Previous studies have shown the usefulness of using individual-level responses such as egestion rates in *C. teleta* to assess detrimental effects of pollutants in soft sediment fauna (Méndez, 2006; Méndez et al., 2013; Ramskov et al., 2009; Selck et al., 1999; Selck et al., 1998). Biomarker responses such as oxidative stress and neurotoxic related markers have been used in different polychaeta species to monitor pollution effects of pesticides, PAH and metals (Bocchetti et al., 2004; Catalano et al., 2012; Durou et al., 2007; Geracitano et al., 2004; Li et al., 2008; Macrae et al., 2012; Monserrat et al., 2007; Moreira et al., 2006; Pérez et al., 2004; Ramos-Gómez et al., 2011; Rhee et al., 2011; Sandrini et al., 2008;

Sun and Zhou, 2008; Suriya et al., 2012). Nevertheless few of those biomarkers have been developed in *Capitella* (Bach et al., 2005; Suriya et al., 2012). Thus such knowledge gaps limit our ability to assess impacts of environmental contaminants on organic enriched soft bottom sediments (Lewis and Watson, 2012).

Being the major interface between coastal cities and the sea, harbor areas are heavily stressed by human activity, since they are the fate of a wide range of contaminants coming from industrial, domestic and shipping activities (Díez et al., 2006; Kerambrun et al., 2012). Sediments in harbors can be contaminated by hundreds of individual compounds being the most abundant heavy metals (Cd, Zn, Pb) and organic compounds such as butyltins (TBT, DBT, MBT) derivate from anti-fouling paints, PAH from fossil fuels, and PCB mainly present in sewage effluents and plastics (Barakat et al., 2002; Díez et al., 2006; Gibert et al., 2009; Kilemade et al., 2009; Guo et al., 2010; Lofrano et al., 2010; Grifoll et al., 2011; Mamindy-Pajany et al., 2011; Chen et al., 2012). Regardless whether discharges originate from air, rivers, urban runoff, effluent pipes, ship discharges or other harbor activities, the question is that main contaminants show high persistence, hydrophobic nature, and low solubility in water, consequently they accumulate in muddy sediments (Galanopoulou et al., 2005; Lepland et al., 2010). The Port of Barcelona (Spain) has a 2000-year history and great contemporary commercial importance. It is Catalonia's largest port and Europe's ninth largest container port, according to AAPA (American Association of Port Authorities). Its 1,066.75 hectares are divided into three zones: Port Vell (the Old Port) destined to personal and touristic boats, the commercial/industrial port and the logistics port (Barcelona Free Port).

This study aimed to assess the potential effects of Barcelona harbor sediments in the polychaete worm *Capitella teleta*. Sediments were collected from three different locations of the Barcelona (Spain) harbor with an increasing gradient of pollution from the mouth of the harbor towards the innermost zone. Body weight (growth) and egestion rate (feeding) were the individual-level responses selected, and antioxidant enzymes, CAT and SOD, and the neurotransmission related enzyme, AChE, were the subcellular-level responses measured.

4.2. Methods

4.2.1. Chemicals

Homogenization, along with enzymatic activities and protein assays were performed with potassium phosphate dibasic (K_2HPO_4); potassium phosphate monobasic (KH_2PO_4); potassium chloride (KCl); ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA); bovine serum albumin (BSA); 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB); hydrogen peroxide (H_2O_2 30%); superoxide dismutase standard (SOD); cytochrome C (from equine heart); xanthine; xanthine oxidase (from bovine milk) from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

4.2.2. Experimental animals and culture conditions

Specimens of *C. teleta* were originally collected in Setauket Harbor (New York, USA) in 1984, and later identified by Judith P. Grassle (Dai et al., 2012), and have been cultured in the Department of Life Sciences and Chemistry of the Roskilde University (Denmark) for several generations (Ramskov et al., 2009). Worms were held in culture at the Institute of Environmental Assessment and Water Research (CSIC, Barcelona) for one year prior to the experiments.

Worms were cultured in the laboratory in plastic aquarium tanks (2000 ml) containing 400 g (dw) of clean natural sediment and 1500 ml of aerated seawater adjusted to a salinity of 30±2‰. Natural sediment and sea water were collected from a nearby unpolluted sandy beach in Sitges (NE Spain, 41º 14' 22.7" N, 1º 51' 44.6" E). The sediment was previously oven-dried (60°C) and sieved to under 250 µm, and the seawater was filtered (<30 µm). The sediment organic matter content was 2.14±0.13% (n=3; loss on ignition method by Dean (1974)). Stock cultures were maintained at 20±2°C, in the dark following Horng and co-workers (2009) procedures. Organisms were fed weekly with 0.15 g of artificial food (sieved to under 250 µm) consisting in an equal part-mixture of commercial fish food (Wardley-Hartz Company, New Jersey USA), baby cereal (Nestlé, Switzerland) and dried (60ºC) spinach (Forbes et al., 1996).

4.2.3. Experimental sediments

In November 2005, sediment samples were collected from Barcelona harbor (NE Spain, 41º 20' 43.27" N, 2º 10' 6.75" E). For this study, three of those samples collected from three different locations inside the port of Barcelona were used: one from the touristic Port Vell, other from the Northern Mouth, and another from the Southern Mouth, later denominated as H, M and L respectively, due to their high, medium and low contamination levels (figure 9). The reference sediment was collected on March 2013 from a beach of Sitges (NE Spain, 41º 14' 22.7" N, 1º 51' 44.6" E). The sediments were freeze-dried (Lyophilizer ©Telstar S.A.) during three days and sieved to less than 63 µm. Before being used for the tests, the total organic carbon (TOC) was evaluated in all sediment samples through loss on ignition methodology (Dean, 1974).

For that, three replicates of each sediment were placed individually on crucibles and weighed before and after ignition. Ignition was performed in a muffle furnace and heated to 550°C for one hour. After cooling to room temperature, the weight percent loss by difference was determined, since the difference between the first weight and the dry weight is the amount of organic carbon ignited. Contaminant analyses of the Barcelona harbor sediments were obtained from Gibert and co-workers (2009), while the reference sediment was previously characterized by Sánchez-Avila and co-workers (2012).

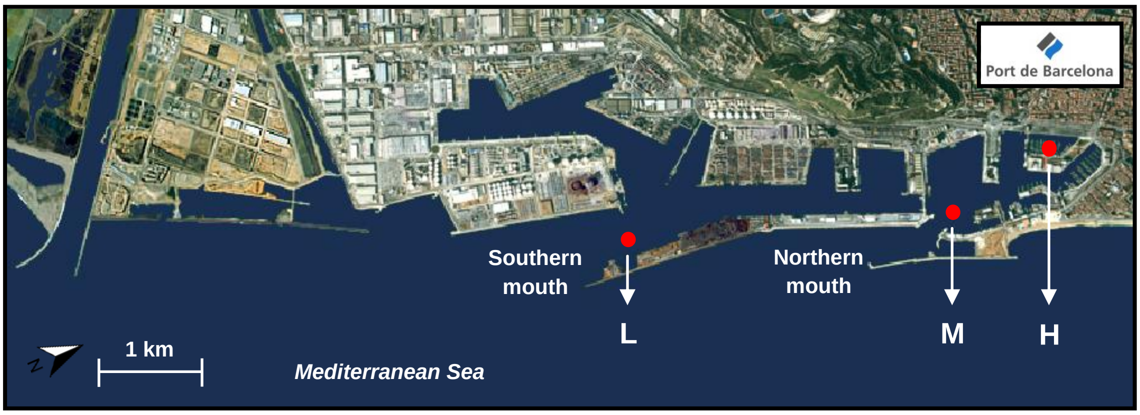

Figure 9. Map of the Barcelona harbor in 2005 with the three sampling stations: Port Vell (H), Northern Mouth (M) and Southern Mouth (L) (in *Port de Barcelona*).

4.2.4. Experimental design

The stock cultures of *C. teleta* were initiated with 2-3 days old juveniles, three months prior to the experiments. It is known that *C. teleta* juveniles can reach the adult stage after about 33 days (Méndez et al., 2000), and the presence of reproductive organs (genital spines or oocytes) confirms it. In this study, only the adult organisms were selected for the experiments, the presence of reproductive organs was detected under a stereo dissecting microscope.

Initial polychaete sizes ranged from (4.94 to 11.50 mm) and were similar to those used in the study performed by Méndez and co-workers (2013) (length averages of 10.3 and 7.73 mm, respectively). It is important to select individuals of similar sizes in order to ensure that all are of same age class.

Before and after exposure tests, gut clearance was performed by placing adults worms individually in dishes containing 7 ml of filtered (<30 µm) seawater (30±2‰ salinity) and lefted for 24-h in the dark, at 20±2ºC (Méndez et al., 2013). After gut clearance, polychaetes were placed individually in 3.5 cm diameter plastic dishes and exposed to 0.5 g of contaminated sediment (<63 µm), 7 ml of filtered (<30 µm) seawater (30±2‰ salinity) and 0.001 g (<63 µm) of artificial food – the same as in cultures. Previous toxicity tests showed that each undiluted sediment were not lethal to *C. teleta* (data not shown). These sediment portions contained food in excess and were not completely processed from one census-day to the next, as previously tested (data not shown). The dishes were maintained at 20±2°C in the dark, and placed in a moisture saturated room to prevent evaporation.

Two independent experiment were performed, one to test individual-level responses (n=16 replicates in total) and one to test subcellular-level responses (n=20 replicates in total).

4.2.5. Individual-level response experiments

The exposure period for the feeding activity test was 21 days and every 7 days (census-day), animals were removed and sediment and water renewed (with the addition of new artificial food). At each census-day, the remaining bulk sediments (pellets, tubes and mucus) were collected and fixed in 75% ethyl alcohol for further analyses of fecal pellets (Selck et al., 1998). On day 21, animals were placed in seawater for 24-h for gut clearance and the pellets accumulated in seawater were added to the corresponding sediment samples. Sediment samples were sieved through a 132 µm mesh, tubes and mucus were removed under the stereo dissecting microscope and retained fecal pellets were collected. Pellets were freeze-dried for 24-h and then weighed (dw) (Selck et al., 1998).

Worms were photographed (three times) using a digital camera (*Canon Powershot SX230HS*), at the beginning and at the end of the exposure time, to later assess body weights. Body length and body area of each polychaete were measured using computer-assisted software *Photoshop CS5*®-extended. Body volumes (*V*) were expressed in mm^3 and were calculated according to Méndez and co-workers (2013) (equation 1).

$$V = \frac{\pi A^2}{4L} \qquad (1)$$

Where *A* is the area and *L* the length of the polychaete. Body weight of each worm, expressed in mg (dw), was estimated by extrapolation, considering volume values, from the linear function $y=0.2128x+0.3392$ obtained by Méndez and co-workers (2013), where *y* is the body weight in mg (dw) and *x* is the volume in mm^3.

Egestion rates were defined as mg of pellets (dw) produced by each individual over each census-day. The percentages of sediment processed over the experiment were calculated considering the total dry weight of sediment processed on days 7, 14 and 21 and the bulk sediment (0.5 g) offered to each individual worm. Since a previous study by Méndez and co-workers (2013) positively correlated significantly egestion rates and body weights through the power function y=72.736$x^{3.1448}$ (where x is the body weight in dw and y the pellets in dw), daily size-specific egestion rates were calculated as the sum of the egestion rates produced during the 21 days, divided by the mean body weight over the 21 days, divided by 21 days.

4.2.6. Subcellular-level response experiments

Biochemical responses were determined at two different times of exposure, 3 and 7 days. Exposures were conducted as explained in section 4.2.4. After exposures and a gut clearance period of 24-h, worms were frozen in liquid nitrogen and stored at -80ºC until further analysis. Later, they were defrosted and pools of 2-3 whole-organisms were homogenized at final volume of 0.25ml. The homogenization of the samples was executed with ice-cold homogenization buffer - 100mM phosphate buffer pH 7.4, containing 100mM KCl and 1mM EDTA – with the assistance of a sonicator (Branson Sonifier® W-150, G. Heinemann - Schwäbisch Gmünd, Germany) and then centrifuged for 10min at 10000g, 4ºC. Carefully the supernatant (S9 fraction) was collected and this fraction was the one used in the biochemical assays. All the steps were performed on ice to prevent enzyme degradation.

Protein quantification was determined according to the Bradford method (Bradford, 1976) adapted to microplate, in quadruplicate and using bovine serum albumine as protein standard. The absorbance was read at 570 nm and the protein content (mg/ml) was determined by quadratic regression of the standard curve.

The ChE was determined using the Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), with some modifications and run at least in triplicate. Preliminary studies confirmed that AChE is the main ChE present in *C. teleta* (Chapter 3), therefore from here after ChE activities will be referred to AChE. AChE activities were measured in the presence of 40µl of sample (S9 fraction), 60µl of homogenization buffer and 200µl of reaction mixture constituted by acetylthiocholine iodide at 0.33 mM and DTNB at 0.17 mM (final concentrations), and the increase of absorbance was measured at 405 nm. Appropriate blanks were performed with homogenization buffer instead of S9 fraction and subtracted to each sample before result interpretation. The enzymatic activity was expressed as nmoles of hydrolyzed substrate per minute per mg of protein (nmol/min/mg protein), using the extinction coefficient 13.6 mM^{-1} cm^{-1}. SOD activity was determined indirectly (with modifications) according to McCord and Fridovic (1969), based on the measurement of the inhibition degree produced by the reduction of cytochrome C through O^{2-}• released by the xanthine oxidase/xanthine reaction. In each well the following quantities were added: 25 µl of S9 fraction, 125 µl phosphate buffer (50mM, pH 7.8, 0.1mM EDTA), 5 µl of cytochrome C (0.06 mM), 5 µl of xanthine (0.14 mM) and 5 µl ml of xanthine oxidase (0.01092 U/ml). The reaction was measured at 550 nm during 10 min and, after blank discount (blanks performed with homogenization buffer instead of S9 fraction), the SOD content (U/ml) was determined by quadratic regression of the standard curve (SOD units were determined using a standard curve of 0 to 1.5 SOD units/ml). Final results were normalized by total protein content and expressed as U/mg of total protein. CAT activity was determined following the procedure of on Aebi (1984), with some modifications (Faria et al., 2009). The assay mixture contained 600 µl of 80 mM phosphate buffer pH 6.5, 350 µl of 52.5 mM H_2O_2 30% and 50 µl of S9 fraction. The decrease of the absorbance of the mixture was recorded at 240nm, through cuvette assay, for 60 seconds. CAT activity was defined as µmoles hydrolyzed substrate per minute per mg of protein (µmol/min/mg protein), using the extinction coefficient 40 M^{-1} cm^{-1}. Enzymatic assays were performed in a 96-well microplate reader (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments – Vermont, USA), except CAT activity, that was measured in a quartz cuvette using a UV spectrometer (Life Science UV/Vis Spectrophotometer DU® 730, Beckman Coulter – Fullerton, CA, USA).

4.2.7. Data analysis

One-way and repeated measurement two-way analysis of variance (ANOVA), followed by pairwise Multiple Comparison Procedures (Fisher LSD Method) and Tukey's post-hoc Multiple Comparison tests were performed to assess differences between treatments. Data was log transformed prior to analyses to meed ANOVA assumptions of normality and variance homoscedasticity. All the mentioned analyses were performed by SigmaPlot software for Windows Version 11.0 (Systat Software, 2008).

4.3. Results

4.3.1. Chemical analysis

Contaminant and TOC levels of the studied sediments are depicted in Table 2. Contaminant results of samples from Barcelona harbor were obtained from Gibert and co-workers (2009) and Sánchez-Avila and co-workers (2012). Barcelona harbor sediments showed a relative increase degree of contamination from the outermost (Southern Mouth, L; Northern Mouth, M) to the innermost part (Port Vell, H) of the harbor. The Southern Mouth (L) presented contamination values smaller than Northern Mouth (M). About the reference sediment, it presents overall chemical values far below the Barcelona harbor sediments. Besides, the total organic carbon (TOC) existing in the studied sediment samples were similar except those from Port Vell (H) sediment that had the highest percentage of TOC (18.46%).

Table 2. Physicochemical characterization of the studied sediments, performed by Gibert and co-workers (2009) in their study, comprising organic matter content (TOC, %), heavy metals and arsenic (As) (µg/g dw) and PCB (ng/g dw).

	Reference	**Southern Mouth (L)**	**Northern Mouth (M)**	**Port Vell (H)**
TOC[a]	9.39	9.83	7.42	18.46
As	6	20	26	36
Cd	0.3	0.9	1.1	2.1
Cr	24	64	73	117
Cu	8	69	175	664
Zn	50	186	290	834
Hg	<0.5	1.2	3.3	7.7
Pb	nd[b]	78	151	431
Ni	11	30	28	42
PCB	21.4	54	134	428

[a] TOC *total organic carbon*, estimated by loss on ignition (Dean, 1974)

[b] nd *not determined*

4.3.2. Individual-level responses

The initial length of worms ranged from 4.94 to 11.50 mm (7.73 ± 1.54 mm, mean ± SEM; n=60). Initial body weight of worms estimated from biovolume measurements (0.495 ± 0.006 mg; mean ± SEM; n=60) were similar across treatments ($P>0.05$; $F_{3,56} = 1.5$) across treatments. Barely any mortality was recorded during the 21 days of exposure, with one death at sediments L and M, and two deaths at sediment H, these mortalities resulted from improper handling and therefore were not considered relevant in this study.

C. teleta body weight, egestion rate and daily size-specific egestion rate per worm across sediments and census-days are shown in Figure 10. Repeated measurements ANOVA denoted significant ($P<0.05$) effects of census-days, sediment and their interaction on body weight and egestion rate (Table 3). Worms increased body weight during the studied period of 21 days, with those cultured in reference and L sediments having the greatest increase within the studied period and those exposed to the H sediment the lowest (Figure 10A). Worms in the reference sediment processed and egested a similar amount of sediment during the studied period, whereas those cultured in contaminated sediments L, M and H diminished their egestion rates across the census-days (Figure 10B). Reference polychaetes processed about (Mean ± SEM, N=14-16) 16.14 ± 0.70% of sediment during the studied 21 days-period, whereas exposed polychaetes processed significantly ($P<0.05$; $F_{3,56} = 133.54$) less sediment (4.31 ± 0.76%, 3.45 ± 0.52% and 0.67 ± 0.11% for sediments L, M, H, respectively).

As a result, daily size-specific egestion rates (Figure 10C) calculated during the entire 21-days period were significantly lower ($P<0.05$, $F_{3,56} = 90.6$; data not shown) in treated worms relative to un-treated worms.

Table 3. Two-way repeated measurement ANOVA of the effects of sediment (fixed factor) and census-days (repeated measure) on body weight and egestion rate. *df*, *F*, *P* are degrees of Freedom, fisher's coefficient and significant value, respectively.

	Body weight			**Egestion rate**		
	df	F	P	df	F	P
Census-days	1,56	236,161	<0.01	1,41	34,870	<0.01
Sediment	3,56	4,328	<0.01	3,41	50,773	<0.01
Interaction	3,56	19,615	<0.01	3,41	11,210	<0.01

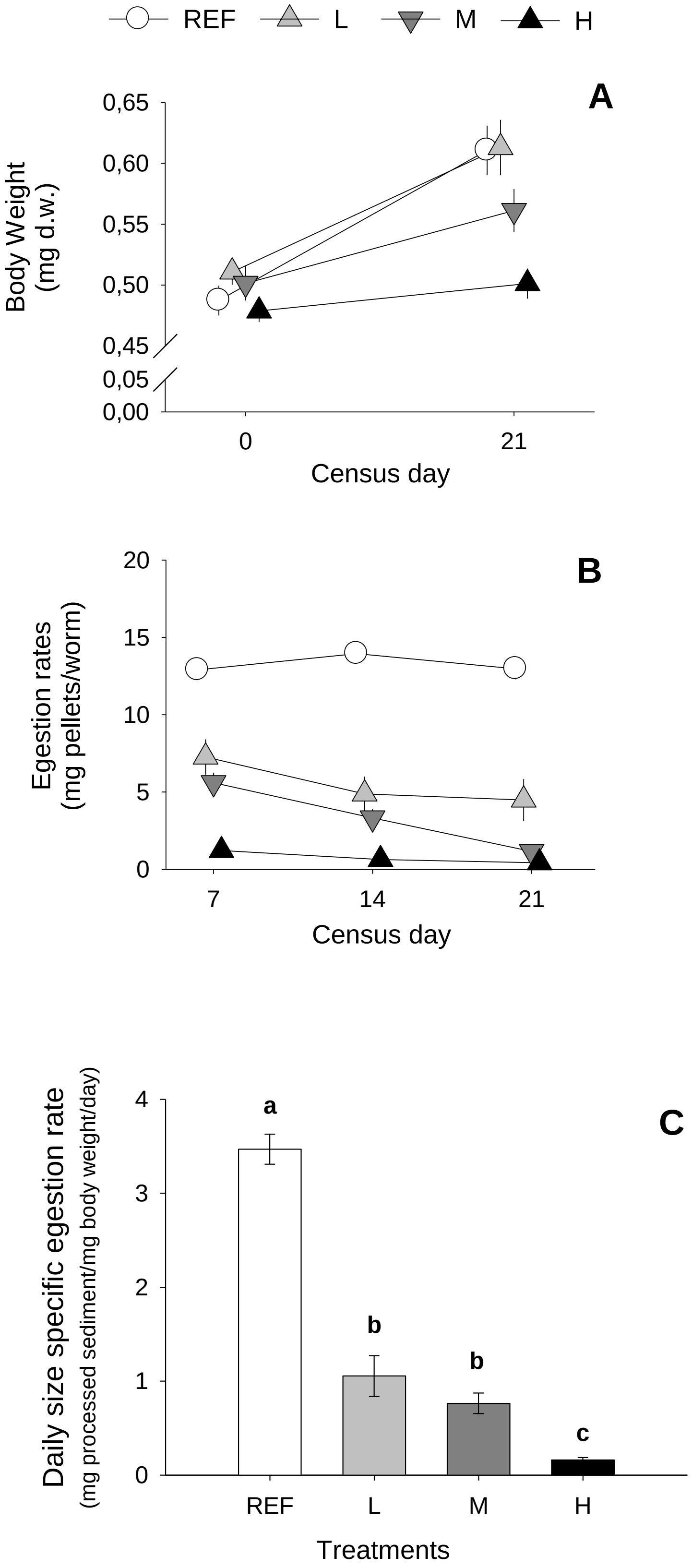

Figure 10. Body weight (A), egestion rate (B) and daily size-specific egestion rates (C) of *Capitella teleta* exposed to reference (REF) and contaminated sediments Southern Mouth (L), Northern Mouth (M) and Port Vell (H), across time. Bars represent Mean ± SEM (n=14-16). Different letters in graph C denote significant differences among treatments (P<0.05 following ANOVA and Tukey's post hoc multiple comparison tests).

4.3.3. Subcellular-level responses

The studied enzymatic activities did not vary significantly ($P<0.05$) in worms exposed to the studied sediments at day 3 but did so at day 7 (Figure 11). This behavior accounted for the substantial census-days, sediment or interaction effects, depicted in Table 4. Exposure time affected the enzymatic responses of AChE and SOD, but not of CAT. Besides this, the sediment and the interaction between sediment and census-days had significant effects in all of the enzymatic activities (Table 4). At day 7 AChE activities of worms exposed to contaminated sediments were lower than those from the reference sediment (Figure 11A). SOD activities decreased significantly ($P<0.05$) in worms exposed to sediments L and M but not in H compared to those cultured in the reference sediment (Figure 11B). Worms cultured in sediments L and H had significantly ($P<0.05$) lower CAT activities than those reared in the reference one (Figure 11C). Despite that CAT activities of worms exposed to sediment M were lower than those of reference sediment, the high variability observed probably prevented detection of significant effects.

Table 4. Two-way ANOVA of the effects of sediment and census-days on the studied enzymatic activities. *df*, *F*, *p* are degrees of freedom, fisher’s coefficient and significant value, respectively.

	ChE			**SOD**			**CAT**		
	df	F	p	df	F	p	df	F	p
Census-days	1,91	19,805	<0.01	1,91	21,835	<0.01	1,87	0,426	0,52
Sediment	3,91	15,348	<0.01	3,91	7,586	<0.01	3,87	4,084	<0.01
Interaction	3,91	7,435	<0.01	3,91	5,557	<0.01	3,87	3,154	0,03

4.3.4. Bivariate correlations

Growth followed by daily size-specific egestion rates were significantly ($P<0.1$, $n=4$) correlated with most of the measured contaminant levels, whereas AChE activity only with Cr and Ni (Table 5). The remained enzyme activities showed a poor relationship with measured sediment contaminants.

Table 5. Bivariate Pearson correlation values between measured biological responses and sediment contaminant levels. N = 4, **$P<0.05$, *$0.05<P<0.1$. Egestion refers to daily size-specific egestion rates.

	PCB	**As**	**Cd**	**Cr**	**Cu**	**Zn**	**Hg**	**Pb**	**Ni**	**TOC**
Egestion	-0.67	-0.94*	-0.84	-0.91*	-0.67	-0.73	-0.75	-0.22	-0,954**	-0.15
Growth	-0.92*	-0.99**	-0,975**	-0,980**	-0.91*	-0.93	-0,967*	-0.62	-0.93*	0.24
AChE	-0.72	-0.87	-0.87	-0.90*	-0.73	-0.78	-0.75	-0.34	-0,970**	-0.37
SOD	-0.04	-0.45	-0.21	-0.30	-0.01	-0.06	-0.19	0.34	-0.32	0.23
CAT	-0.33	-0.61	-0.54	-0.62	-0.34	-0.41	-0.37	0.09	-0.77	-0.71

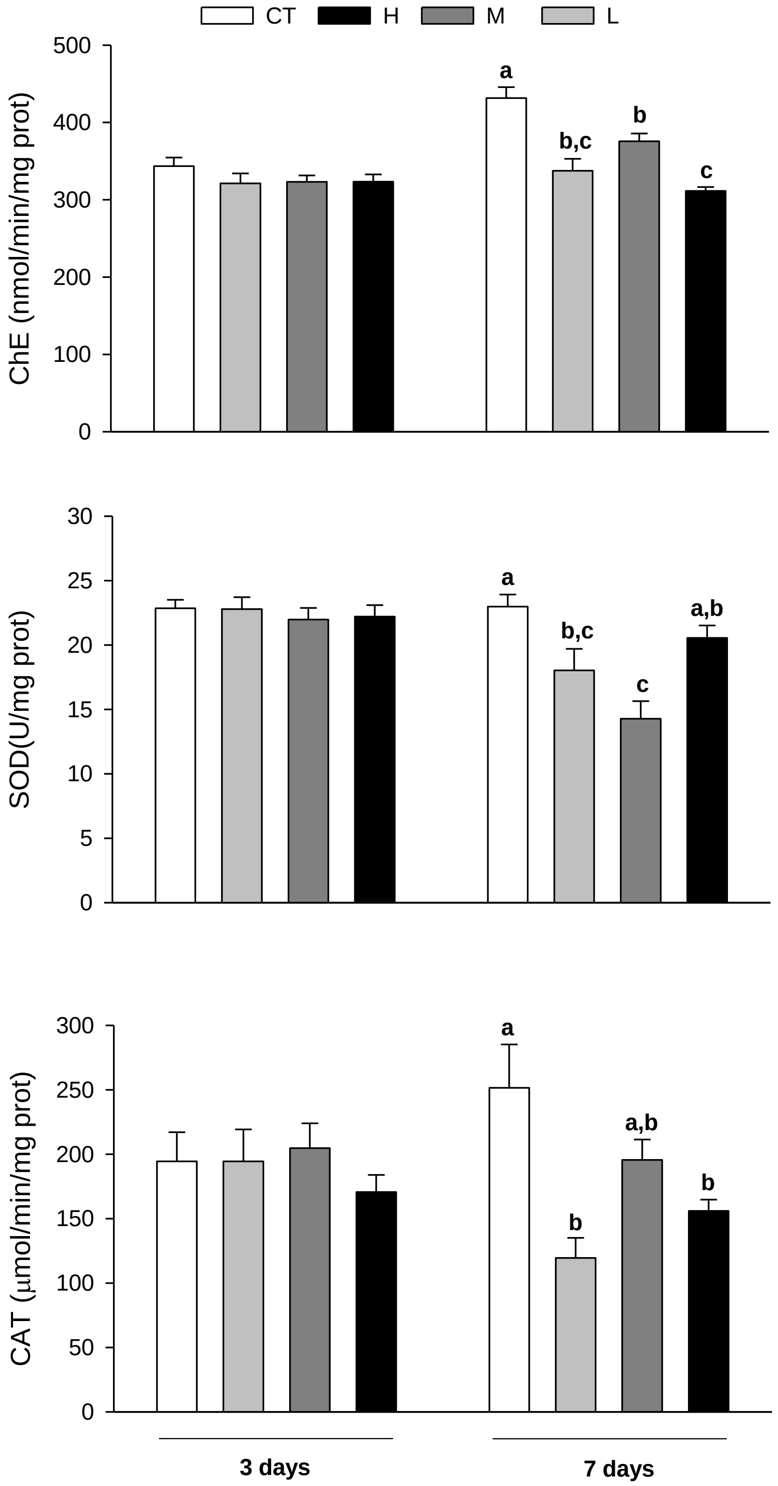

Figure 11. Cholinesterase (ChE, A), superoxide dismutase (SOD, B) and catalase (CAT, C) activities of *Capitella teleta* exposed for 3 and 7 days to reference (REF) and contaminated sediments Southern Mouth (L), Northern Mouth (M) and Port Vell (H). Bars represent Mean ± SEM (pools of 2 organisms, n=12-15). Letters denote homogeneity among treatments ($P<0.05$ following ANOVA and Tukey's post hoc multiple comparison tests).

4.4. Discussion

4.4.1. Chemical analysis

The reference site presents contaminant concentrations far below from the sediments from Barcelona harbor, with values at least 2.5 times fold lower than those of Southern Mouth (the L site, least contaminated area). Concentrations of PCB (#28, #52, #101, #118, #138, #153, #180) detected in the reference site (21.4 (ng/g, dw)) were within range of those found along the Barcelona coastal area (Tolosa et al., 1995), that vary between 3.3 and 46.7 (ng/g, dw) for PCB, thus within the normal scale of the area.

Gibert and co-workers (2009) observed dramatic levels of chemical contamination present in the Barcelona harbor. The chemical analysis showed a clear increasing gradient of contamination from the Southern Mouth to the *Port Vell*. There are two main aspects that contributes for the high contamination level in Port Vell: being this location the innermost part of the harbor, the water flow is smaller when compared with the other two sampling sites (Southern and Northern Mouths) that are located more closer to open waters from the Mediterranean Sea, thus this property added with the exhaustive boat traffic resulting from yachting and recreational boating activities, enhanced the confinement of pollutants in this semi-enclosed area and clearly intensified the pollution (Díez et al., 2006; Martínez-Lladó et al., 2007; Gibert et al., 2009). The Arsenal Basin (Alexandria Harbor, Egypt) is also a semi-enclosed embayment with limited water exchange with the open sea, and an accumulation of persistent contaminants in this basin was also recorded by Barakat and co-workers (2002).

Besides the lesser seawater flushing, the high values of Zn, Cu and Pb compounds are strongly linked to fuelling stations, high traffic conditions, boat repairing and painting facilities (Grifoll et al., 2011). It is important to note that after the prohibition of antifouling paints based on TBT in the 1980s, the Zn/Cu-based antifouling paints have been reported to be commonly used (Paetzel et al., 2003; Sprovieri et al., 2007), leading to their rise and accumulation in marine ecosystems, and justifying the high values detected in the studied sediments (Gibert et al., 2009). The PCB analyzed (congeners #28, #52, #101, #118, #138, #153, #180) have been associated with activities mainly connected to boat repairing facilities, as engine overhaul and maintenance operations, and also to urban development and domestic and industrial wastewaters (Barakat et al., 2002); however, it is difficult to point out a single major source.

Moreover, there are not only heavy metals and PCB in sediments from harbors. Other types of contaminants are certainly present, even in unknown concentrations, due to previous studies on Barcelona harbor: PAH, butyltins (TBT, DBT and MBT) (Martínez-Lladó et al., 2007) and surfactant intermediates (linear alkylbenzenes [LABs], nonylphenols [NPs]) (Díez et al., 2006). Besides, organochlorine pesticides DDTs (and its metabolites) were also present in sediments collected at 2004 (data not published). All this pollutants are commonly found in harbors (Borgmann and Norwood, 1993; van den Hurk et al., 1997; Kilemade et al., 2009; Mamindy-Pajany et al., 2010; Chen et al., 2012; Kerambrun et al., 2012), since both PAH and butyltin compounds are mostly associated to fossil fuels and ship paints, respectively (Islam and Tanaka, 2004), and surfactants and pesticides are consequence of domestic discharges and agricultural activities, respectively, being dumped through urban wastewater outfalls (Díez et al., 2006). So it is important to assume their presence in order to understand the effects on biological responses.

4.4.2. Biological responses

Chemical contaminants are known to adversely affect organisms' physiology, growth, health and behavior (Manyin and Rowe, 2006). Among the different conceivable levels of responses, individual-level responses to chemical contaminants have often been ignored by ecotoxicologists because they are regarded as being too generalized and too difficult to measure routinely (Kerambrun et al., 2012). However, physiological measurements such as growth or feeding activity may provide the key to integrate various molecular and cellular responses in an organism with impaired fitness (Mouneyrac et al., 2010), because these processes contribute to the population's development.

Despite the fact that the measurement of live *Capitella* worms to assess their body parameters is difficult due to contraction and enlargement moves they create (Méndez et al., 2013), these measurements are crucial standardize the size range so this parameter does not influence the results. It is important to refer that length and area measurements are used to calculate body volumes, allowing us to extrapolate the body weight of each polychaete. The strong dependence of feeding activity upon individual body weight in deposit-feeding polychaetes under unstressed conditions has been previously described (Nichols, 1970; Cammen, 1980; Whitlatch and Weinberg, 1982; Rice et al., 1986; Méndez et al., 2001). Indeed the observed relationship between size and specific egestion rates at an individual level allowed discriminating each of the studied sediments.

The reduction or increase of egestion rate by *C. teleta* represents an indirect way to observe effect of contaminants on feeding activity, since it's an indirect measure of ingestion rate; consequently it will affect body weight (growth) (Roast et al., 2000). In this study the three evaluated Barcelona harbor sediments had detrimental effects on growth, egestion rate and daily size-specific egestion rate. There are several factors that could be responsible for the observed detrimental biological effects in *C teleta*: a the developmental stage (Linke-Gamenick et al., 2000), the organic matter content in sediments (Thiyagarajan et al., 2005), the sediment particle size (Whitlatch and Weinberg, 1982) and the presence of contaminants in sediments (Selck et al., 1998; Hu et al., 2003; Horng et al., 2009). In this study, the organisms were of the same developmental stage (adult polychaetes), were fed weekly with an excess of food and all the sediment sized used were below 63 µm, which is the ideal sediment particle size to ensure all the sediment is processed (Méndez et al., 2013) by the deposit-feeder *C. teleta*. Physico-chemical water parameters of pH, salinity, temperature and oxygen also did not varied across treatments and census days, ranging between 8.3 ± 0.7, 30 ± 2 ‰, 20 ± 2 ºC, 7.1 ± 0.5 mg/L, respectively, since the exposures were conducted in laboratory-controlled conditions (data not shown). Therefore differences in contaminant levels across sediment samples and TOC may explain the observed detrimental effects.

Indeed differences in TOC between sediment H and the rest were twofold and those of metals and PCB varied between 3 and 20 fold. Equilibrium partitioning theory establish an inverse relationship between sediment organic carbon and contaminant bio-availability to organisms (Di Toro et al., 1991). Thus despite that sediment H had the greatest levels of contaminants not necessarily may have a much greater toxicity to exposed worms than sediment M. Growth and egestion rate responses of worms exposed to the Barcelona harbor sediments were severely impaired in a concentration related manner, thus mimicking the measured contaminant gradient.

Fichet and co-workers (1998) showed that the larvae of three marine species (*Artemia salina*, *Crassostrea gigas*, *Paracentrotus lividus*) exposed to sediments from two French harbors (La Pallice, an industrial port and Les Sables d'Olonne, a fishing port), contaminated mainly with As, Cd, Cr, Cu, Hg, Ni, Pb, Zn and PCB, had their growth rates inhibited in slightly contaminated sediments and their development severely impaired in highly contaminated sediments. A 10-day bioassay with larval chironomids (*Chironomus tentans*) was used to evaluate sediment samples from four American freshwaters harbors (Michigan City, Indiana; St. Joseph, Michigan; Grand Haven, Michigan; Toledo, Ohio). Three of the four sediment harbors affected significantly *C. tentans* growth, when compared with the reference sites (Call et al., 1999). Unfortunately, in the previous study no data on contaminant levels were reported. Moreira and co-workers (2006) found significant depression of feeding in

Hediste diversicolor (from 30 to 70%) after exposure to sediments from Mira and Sado estuaries (Portugal), which were essentially polluted with heavy metals. Field caging experiments were conducted on two fishes, *Dicentrarchus labrax* and *Scophtalmus maximus*, in the northern French harbor Boulogne sur Mer (Kerambrun et al., 2012). After exposure to sediments polluted with PAH, PCB and heavy metals, a general decrease of fish growth associated with a metal accumulation in gills was documented. Yet, reported contamination levels at Boulogne sur Mer harbor were bellow those measured in Barcelona harbor, having PCB, Zn, Cu residue levels of 10ng/g, 500 µg/g and 135 µg/g, respectively. PCB residue values of 77.8-286 ng/g and heavy metals values of 491.1-1619 µg/g were found in four polluted sediments from three European river basins (Elbe, Scheldt and Llobregat, from Czech Republic, Belgium and Spain, respectively) (Tuikka et al., 2011). Growth inhibition was observed after exposing the nematode *Caenorhabditis elegans* and the oligochaete *Lumbriculus variegatus* to such sediments, when compared to reference sediments exposures. The Baltimore harbor (Maryland, USA) showed similar chemical contamination levels (heavy metals and PCB) to those of sediment M from the Barcelona harbor (Manyin and Rowe, 2006). After exposing a burrowing amphipod (*Leptocheirus plumulosus*) to Baltimore harbor sediments, significant lower growth and reproductive rates were obtained when compared with the reference site (Manyin and Rowe, 2006). Besides, PAH contamination of 10,800 µg/g was found at the Baltimore harbor, and previous studies in the Barcelona harbor (Martínez-Lladó et al., 2007) showed PAH pollution ranging from 580-10,320 µg/g at the inner area of Port Vell. In addition, Martínez-Lladó and co-workers (2007) also found TBT values between 326-4702 and 98-259 ng/g in the inner harbor (Port Vell) and outer harbor. These TBT residue levels are potentially toxic since the lowest observed effect concentration for growth to juveniles of the opheliid polychaete *Armandia brevis* was established at 101 ng/g after 42-day exposure to TBT-contaminated sediments (Meador and Rice, 2001). Furthermore, the sediment concentrations affecting growth in this species are commonly found in urban waterways indicating potentially severe impacts for this and other sensitive species, as concluded by the authors. This means that the severe effects observed on growth and egestion rates in *C. teleta* are likely to be related with the measured (PCB and metals) and not measured residues levels of contaminants such as PAH and TBT.

Biochemical responses of *C. teleta* denoted a clear temporality since effects were only evident after 7 days of exposure. Similar results were obtained by Suriya and co-workers (2012) studying antioxidant responses – CAT, SOD, GST – of *Capitella capitata* exposed to copper, detecting significant changes after 7 days of exposure.

Taking this and their life cycle into account, it is suggested that in laboratory experiments, periods of at least 7 days-exposure on *Capitella* genus are required for significant effects at the subcellular-level.

Measured AChE activity ranges in *C. teleta* (320 and 400 nmol/min/mg protein) were within the top range of other ChE polychaete species. Alla and co-workers (2006) reported ChE values between 30 and 125 nmol/min/mg protein for *Nereis diversicolor*. The ChE activity in body tissues of the polychaete *Arenicola marina* were as high as 59.37 µmol/min/mg protein, being the highest esterase activity recorded in a polychaete worm (Hannam et al. 2008). In this study, there was a significant inhibition of AChE at 7-days in the three contaminated sediments, when compared to the reference site. Given that chemical analysis for organophosphorus compounds and carbamates were not performed in the sampling sediments, it is not possible to directly link this inhibition with the presence of such pollutants. However, these compounds are mostly used in agricultural practice (as pesticides, insecticides, herbicides), and there are reports about their presence in estuaries and harbors due to their entrainment by rain and groundwater (Borgmann and Norwood, 1993; van den Hurk et al., 1997; Barakat et al., 2002; Galanopoulou et al., 2005; Kilemade et al., 2009), thus possibly being present in Barcelona harbor. Even at low concentrations, anticholinergic compounds are known to cause effective damage in organisms (Amitai et al., 1998; Arufe et al., 2007; Čolović et al., 2011; Cacciatore et al., 2012). Inhibition of ChE might also be due to other pollutants, such as detergents from industrial and domestic effluents (Gravato et al., 2010), or yet by PCB that are known to induce cytochrome P450 (phase I) enzymes which in turn increase biotransformation products (including reactive electrophiles) known to inhibit AChE enzyme (Durou et al., 2007). Metals are also known to inhibit cholinesterase activity (Hamza-Chaffai et al., 1998; Dellali et al., 2001; de la Torre et al., 2002; Lionetto et al., 2003, 2011; Gravato et al., 2010; Richetti et al., 2011; Lima et al., 2012). Neverthless, Frasco and co-workers (2005) demonstrated that metal mediated inhibitory effects on ChE are methodological errors related to their interference with DTNB, one of the reagents of the Ellman’s method (Ellman et al., 1961). Still, in this case AChE inhibition in *C. teleta* worms exposed to the harbor sediments may also reflect the weakened condition of the organisms under study. In a filed study aimed to relate biomarker responses of field collected and transplanted invertebrates with the ecological status of polluted rivers, Damásio and co-workers (Damásio et al., 2008, 2011) reported that AChE activity co-varied positively with the ecological status of the invertebrate populations, where high activities of ChE were always associated with samples poorly contaminated (low metals and organic contaminants concentrations), linking the presence of this biomarker with a good environmental status. In summary the observed inhibition of AChE in worms exposed to Barcelona harbor sediments indicated anticholinergic effects that may or not be directly related with measured pollutants but in any case are detrimental. For example AChE inhibition may disrupt worm burrowing behavior and hence food intake and egestion rates, severely impairing growth as it was shown in Figure 10.

The primary defense against oxygen toxicity involves the enzyme SOD (Ameur et al., 2012). The superoxide free radical (O_2^{-}•) is formed by the transfer of a single electron to oxygen; SOD enzyme is the responsible to catalyze the conversion of this free radical to H_2O_2, which needs in turn to be degraded to H_2O and O_2, if mediated by CAT, or reduced to H_2O if mediated by GPx (Wright and Welbourn, 2002; Leslie et al., 2013). In this process, the cleavage of H_2O_2 into the reactive hydroxyl radical (OH^{-}•) is catalyzed by a number of trace metals in their reduced form (e.g., Cu^{+}, Fe^{2+}, Ni^{2+}, and Mn^{2+}), causing damage to the cell (Wright and Welbourn, 2002). Thus, the antioxidant enzymes SOD and CAT can be considered to be the first line of antioxidant enzymatic defense against ROS (Faria et al., 2009) and are expected to increase their activities under oxidative stress. Although not all the contaminated sediments affected the antioxidant defenses in *C. teleta* (sediment H in figure 3B and sediment M in figure 3C, probably due to variability that prevented detection of significant effects), in general, SOD and CAT responses were lower in the contaminated sediments from the Barcelona harbor than in the reference sediment. Previous studies that measured antioxidant enzymes, in estuaries and harbors with equivalent pollution levels as the Barcelona harbor, indicated contrasting variations caused by pollutants with increases (Rodríguez-Ariza et al., 1993; Geracitano et al., 2004; Alla et al., 2006; Moreira et al., 2006; Douhri and Sayah, 2009; Bouraoui et al., 2010; Faria et al., 2010; Gorokhova et al., 2010; Benedetti et al., 2012), decreases (Gravato et al., 2010; Ameur et al., 2012; Oliva, Vicente, et al., 2012) and biphasic responses (Regoli et al., 2004; Sun and Zhou, 2008; Suriya et al., 2012; Won et al., 2012) in enzyme activity. Insofar since the tested sediments were contaminated with heavy metals and PCB and, probably, with PAH, butyltins and other pollutants such as pesticides and surfactants, the observed decrease activities of both antioxidant enzymes could be expected and explained through two main hypotheses: (1) chemicals existing in the sediments, like metals, could directly inhibit enzymes, as stated by Wright and Welbourn (2002) and Viarengo and co-workers (2007); (2) sediment contaminants could indirectly inhibit enzymes when inducing the overproduction of ROS in the cells, leading to an inactivation of enzymes and, consequently, a deficiency of the antioxidant system (Faria et al., 2009). Decreased CAT and SOD activities in glass eels from the Lima estuary, and decreased

CAT activity in yellow eels from the Douro and Lima estuaries, both essentially contaminated with heavy metals and PAH, were related to an insufficient detoxification of ROS (Gravato et al. (2010). Lower CAT and SOD activities in the fishes *Mugil cephalus* and *Dicentrarchus labrax* collected from a polluted coastal lagoon (Bizerte Lagoon, Tunisia) were attributed to a high production of superoxide anion radical, inhibiting antioxidant enzymes, and then leading to a deficiency of the antioxidant system (Ameur et al., 2012). The fact that antioxidant systems may demonstrate increase, decrease or both trends under stress conditions is not contradictory, it depends on several aspects according to the susceptibility of the exposed living species, chemicals (single or mixed contaminants, bioavailability) and intensity of exposure (Regoli et al., 2002, 2003; Bocchetti et al., 2008; Faria et al., 2009). Moreover, since CATs are localized in the peroxisomes of most cells and are involved in fatty acid metabolism, changes in activities may often be difficult to interpret (Oliva, Vicente, et al., 2012). Additionally, the non-specificity of CAT does not allow identifying precisely the origin of enzymatic responses (Alla et al., 2006), and it’s behavior could be explained not by the influence of one particular contaminant, but by a large variety of pollutants (PAH, PCB, heavy metals, etc.) acting as a whole, and producing a decrease in its activity when compared to reference site. Although rarely dose dependent, the utility of antioxidant enzymes lies in their sensitivity in revealing the onset of an oxidative perturbation, even in an early stage or at low levels of chemical stress (Regoli et al., 2004; Benedetti et al., 2012).

In summary the individual-level responses obtained in this study reflect the chemical contamination already stated by Gibert and co-workers (2009). Body weight and daily size-specific egestion rates of *C. teleta* worms showed a gradual reduction from less to more contaminated sediments and were correlated with most measured contaminants. Although this increasing effect was not observed in the studied biomarkers, in 8 out of 9 *sediment x biomarker* combinations worms exposed to harbor sediment had their enzymatic activities impaired relative to those exposed to reference sediments. Considering the overall pattern of our data, detrimental effects on egestion rates of *C. teleta* followed the contaminant gradient measured in our sediments and resulted in lower growth rates. This means that egestion rates and growth were likely related to general stress responses. Alternative the pattern of inhibition of the studied enzymatic activities varied across markers and sediments and were more likely related to specific chemical effects not measured in the present study. Nevertheless, according to Damásio and co-workers (Damásio et al., 2011) biomarker responses varied independently than general responses such as feeding related traits and hence can be used as surrogated assays of specific toxicant effects. In this regards we observed a moderate inhibition of ChE activities across the studied sediments with a slightly greater anticholinesterase activity in the most polluted sediment. Regarding antioxidant responses all three harbor sediments showed equivalent levels of inhibition towards either SOD or CAT.

The impairment in endpoints performed in *C. teleta* in this study showed the high suitability of this organism as ecotoxicological biomonitor, where the evaluated biological responses demonstrated to be potential tools for environmental risk assessment.

Chapter 5. General Discussion and Conclusions

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Harbor environments are endangered to several forms of disturbance related to anthropogenic activities such as transport, shipbuilding, petroleum refinery, urban water treatment, electric power production, among others (Regoli et al., 2002). Since organisms are exposed to mixtures of pollutants, potential combined effects may be hard to interpret. Harbors' ecotoxicological studies are important to understand the effect of such contaminants integrating them at different levels of organization, acting as «a whole», since in nature there is no place where only one contaminant is present. Therefore, harbors can be model environments for developing and validating monitoring procedures on bioaccumulation and biological effects of pollutants.

The traditional approach to assess sediment quality involves chemical analysis (Martín-Díaz et al., 2004). But it is well known that the chemical analysis by itself does not provide information on the potential hazard for the organisms, especially in the case of presence of complex chemical mixtures (Benedetti et al., 2012). To evaluate the effects of pollutants on organisms, various methods have been developed ranging from the sub-cellular to the ecosystem level of biological response. However, measurements at higher levels of biological organization sometimes could be limited because ecologically important effects (e.g. death or impaired organismal function) may have already occurred (Verslycke et al., 2004). In line with this, biochemical responses can be important to fill this gap, since measurements at the biochemical level may detect more quickly and eventually specifically the presence of several toxic compounds, allowing earlier identification of change before deleterious effects reach higher organization levels (Cajaraville et al., 2000; Galloway et al., 2004; Martín-Díaz et al., 2004; Monserrat et al., 2007; Tsangaris et al., 2007). Over the last decades, the integration of different responses resulting from different biological organization levels has been considered to be viable measures of response to stressors. Recently, some attempts have been made to demonstrate the relationship between some biomarkers acting at the sub-cellular level (e.g. acetylcholinesterase, antioxidant responses, damage at the genetic level) and ecologically relevant parameters, such as behavior, growth, reproduction, survival and feeding (Novais et al., 2011).Thus, if responses at different biological organization levels give important data about potential effects on organisms, their combination can reveal much more than when used and assessed separately. The integration of various indicators, such as physicochemical data of waters and/or sediments and physiological and biochemical responses of the organisms in study, is nowadays drawing more attention in environmental monitoring studies (figure 12) (Micheletti et al., 2011).

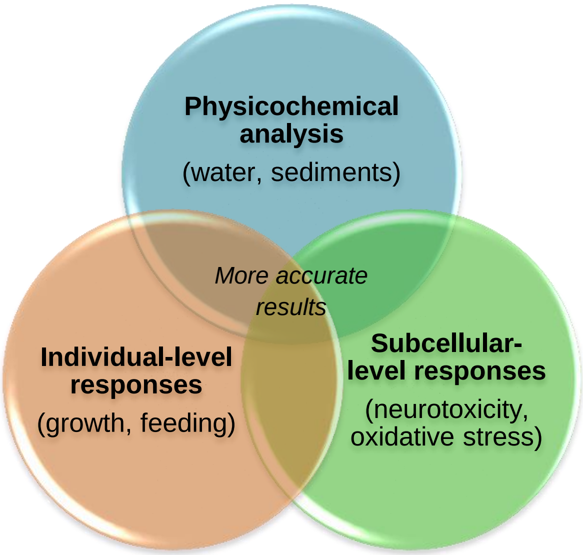

Figure 12. Integration of different variables to assess effects on individuals and relate them to higher levels of biological organization.

In this study, both physiological and biochemical responses were assessed in the polychaetes *C. teleta*, and both revealed to be adequate and potential powerful tools for future ecotoxicological studies with this organism and contamination scenarios, since, in general, responses were significantly different than a reference treatment. Growth and feeding were both inhibited when all the three contaminated sediments were used. This insufficient feeding and growth may eventually lead to reproduction and consequently populations' growth impairment. On the other hand, biomarkers showed a significant decrease of neurological and antioxidant responses, although an inhibition pattern was not visible with the increase of sediment toxicity, yet some of the sediments contain xenobiotics capable of altering *C. teleta* metabolic system. The *C. teleta* antioxidant system showed to be insufficient due to its significant inhibition, probably due to ROS accumulation that can indicate a clear imbalance in the cellular redox status concomitant with the inactivation of the SOD and CAT enzymes involved in the defenses against the oxidative stress (Martín-Díaz et al., 2004; Faria et al., 2009; Novais et al., 2011). Integrating both responses, it could be suggested that a depression in *C. teleta* feeding and growth might be in some way related to anticholinesterase effects and cellular oxidative damage resulting from impairment in organisms' antioxidant defense, with possible reflexes in feeding behavior and growth – as seen – and possibly in and reproduction, among other responses, as stated by other authors (Gravato et al., 2010).

Considering the overall pattern of our data, in sum the following can be concluded:

- This is the first work to provide original data concerning the enzymological characterization of ChE in *C. teleta*: ChE activity is primarily due to AChE.
- A significant decrease of all measured biological responses was found inworms exposed to Barcelona harbor sediments: growth, feeding, daily size-specific egestion rates, and AChE, SOD and CAT enzymes.
- Port Vell was the most contaminated and harmfull area of the harbor to *C. teleta* worms.
- Biological responses presented here are in agreement with studies on community structure in this area, being Port Vell the most toxic area inside the Barcelona harbor (Díez et al., 2006; Martínez-Lladó et al., 2007; Gibert et al., 2009; Grifoll et al., 2011).
- Although it is not possible to point out which contaminant(s) caused effects on *C. teleta*, the use of true-field sediments from Barcelona harbor revealed to be valuable tools for assessing their potential effects on organisms' populations and communities.
- *Capitella teleta* revealed to be good bioindicator and is proposed to be used in further environmental monitoring studies as a "stress screening", especially when assessing sediments' toxicity since they are essentially filter-feeding and burrowing organisms.

In this study, growth, feeding, and biomarkers were assessed, but other parameters such as energy analysis through SFG and Cellular Energy Allocation (CEA) (Verslycke et al., 2004), could also be studied, in order to understand how the organism is dealing and channeling their energy with contaminants' incidence (Novais et al., 2013). Future studies encompassing both issues would be very interesting to get an idea of what is happening metabolically. Additional biological responses at the organism/population level such as avoidance test is currently captivating attention, since its use has been suggested as a first tier to contaminants exposure (Amorim et al., 2008; Novais et al., 2010; Pereira et al., 2013). Other biochemical responses besides neurotoxicity and antioxidant responses, like cytochrome P450 as "phase I enzyme" of detoxification processes and DNA strand breaks (DNA damage), may be used as early warning indicators of stress and hence predict future problems in ecological status (Kilemade et al., 2009; Damásio et al., 2011). «Omics'» methods such genomics, proteomics, transcriptomics applied to ecotoxicology are in remarkable development in the last few years (see Snape et al., 2004; Calzolai et al., 2007; Monsinjon and Knigge, 2007; Lemos et al., 2010), since they offer advantages in terms of time, cost and sample throughout (Martín-Díaz et al., 2004). Thus, these promising biomarkers tools should not be forgotten.

The impairment in endpoints performed in *C. teleta* in this study are potential «early warning tools», meaning that they may indicate potential effects at the polychaete individual level before reaching higher levels of biological organization (population, community, ecosystem), serving as potential tools for environmental risk assessment, allowing to apply remediation or abatement techniques, or others methodologies, in order to avoid worsening of the contamination and its potential effects.

Chapter 6. References

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