

**Is chronic exposure to pollution able to change the
physiological capability of *Corbicula fluminea* to respond
to acute chemical stress?**

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Is chronic exposure to pollution able to change the physiological capability of *Corbicula fluminea* to respond to acute chemical stress?

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Index of abbreviations

A

ATP - Adenosine triphosphate

B

BaP - Benzo[a]pyrene

BChE - Butyrylcholinesterase

BHT - Butylhydroxytoluene

BKF - Benzo[k]fluoranthene

C

CAT - Catalase

CDNB - 1-chloro-2,4-dinitrobenzene

Cu/Zn SOD - Copper/Zinc Superoxide dismutase

CYT P19A1 - Cytochrome P19A1

CYT P19A2 - Cytochrome P19A2

CYT P1A - Cytochrome 1A

CYT b5 - Cytochrome b5

CYT P450 RED - Cytochrome P450

CYT P450 - Cytochromes P450

D

DNA - Deoxyribonucleic acid

DTNB - Dithiobisnitrobenzoate

DTPA - Diethylene-triaminepenta-acetic acid

E

EDTA - Ethylene diaminetetraacetic acid

EPA - Environmental Protection Agency

F

FeSOD - Iron superoxide Dismutase

G

GA - Glucuronic acid

GPx - Glutathione peroxidase

GR - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GST - Glutathione S-transferase

H

H₂O - Water

H₂O₂ - Hydrogen peroxide

I

IDH - Isocitrate dehydrogenase

IUCN - International Union for Conservation of Nature

K

K_{ow} - Octanol-Water partition coefficient

L

LDH - Lactate dehydrogenase

LOOH - Lipid hydroperoxide

LPO - Lipid peroxidation

M

MDA - Malondyaldehyde

MnSOD - Manganese Superoxide Dismutase

MOA - Mode of Action

MO - Microsomal Monooxygenase

N

NAD⁺ - Dinucleotide

NADPH - Dinucleotide phosphate

NAP⁺ - Nicotinamide adenine dinucleotide phosphate

NIS - Non native Invasive Species

NRC - National Research Council

O

O₂⁻ - Superoxide anion

O₂ - Oxygen

OH⁻ - Hydroxide ion

OP - Organo phosphate

•OH - Hydroxyl radical

P

PAHs - Polycyclic aromatic hydrocarbons

PCBs - Polychlorinated biphenyls

PChE - Propionylcholinesterase

PUFA-OOH - Lipid hydroperoxide

R

ROS - Reactive oxygen species

RX - R group (aliphatic, aromatic or heterocyclic) connected to a X group (sulfate, nitrite or halide)

S

SOD - Superoxide dismutase

T

TBARS - Thiobarbituric acid-reactive substances

TBT - Tributyltin

U

UDP-GT - Uridine diphosphate-glucuronyl transferase

W

WFD - Water Framework Directory

WHO - World Health Organization

Resumo

A amêijoia asiática *Corbicula fluminea* (Müller, 1774) é uma espécie invasora que tem-se vindo a estabelecer nos rios de todo o Mundo. É uma espécie invasora não nativa (NIS) em Portugal, que colonizou o Rio Minho (NW Península Ibérica) em 1980, sendo no presente a espécie dominante da comunidade de moluscos. Acredita-se que a invasão deste ecossistema pela *C. fluminea* foi contribuindo significativamente para o declínio de bivalves nativos que enfrentam agora um sério risco de extinção. *C. fluminea* foi-se mostrando capaz de tolerar níveis consideráveis de contaminantes ambientais e esta capacidade pode agir em favor da *C. fluminea* em situações de competição com bivalves nativos menos tolerantes à contaminação química. Aqui, a hipótese de que indivíduos da mesma população de *C. fluminea* mas de locais com níveis distintos de contaminação histórica, respondem de forma diferente a uma exposição aguda foi testada. A lógica por trás da hipótese é que a exposição prolongada à poluição pode levar ao desenvolvimento de tolerância ao stress químico, por exemplo através de um aumento da eficiência dos mecanismos de biotransformação, diminuição da sensibilidade dos alvos moleculares, entre outros. Para testar a hipótese, os animais recolhidos em dois locais do estuário do Minho sob diferentes impactes antropogénicos, após um período de aclimação no laboratório para evitar potenciais efeitos da exposição de campo, foram expostos em dois bioensaios diferentes de 96h a várias concentrações distintas de uma substância modelo, o hidrocarboneto aromático policíclico (PAH) benzo[a]pireno (BaP). No final dos bioensaios, enzimas envolvidas na neurotransmissão, biotransformação, defesa anti-oxidante, produção de energia aeróbia e os níveis de peroxidação lipídica foram usados como biomarcadores. Em ambos os bioensaios nenhum efeito significativo do BaP na actividade da colinesterase foi encontrado. Comparando os resultados obtidos nos grupos de controle, houve uma indução significativa da enzima anti-oxidante catalase (CAT) pelo BaP, sendo a concentração com menor efeito observável (LOEC) de 8 µg/L (cerca de 2,5 vezes maior) nos animais do local mais contaminado (futuramente indicado como local 1) e um LOEC de 2 µg/L (cerca de 3 vezes maior) nos animais do local menos contaminado (futuramente indicado como local 2). Animais do local 1 também mostraram um aumento significativo de outras duas enzimas anti-oxidantes (GR e GPx),

enquanto os do local 2 não mostraram. Nenhum efeito significativo nos níveis de peroxidação lipídica (LPO) foi encontrado em qualquer bioensaio. No entanto, é interessante notar uma redução de LPO nas concentrações mais elevadas testadas coincidindo com uma redução da actividade de glutathione S-transferases (GST), também envolvido na prevenção LPO nos animais do local 1; algum destes efeitos foram observados em moluscos no local 2. Outro achado interessante é a redução significativa de isocitrato desidrogenase (IDH) nos animais do local 2, mas não em animais do local 1; uma vez que a IDH regenera NADPH celular que é um co-fator para a glutathione redutase (GR), estes resultados podem sugerir que o site 2 moluscos não são capazes de induzir GR sob stress BaP devido à falta de NADPH. Portanto, como um todo, as conclusões do estudo indicam que o BaP não é um agente anticolinesterásico de *C. fluminea* e que amêijoas de locais com diferentes níveis de contaminação histórica são capazes de superar o stress oxidativo causado pela exposição aguda ao BaP a 16 mg / L evitando danos oxidativos lipídicos. No entanto, os resultados também sugerem que os moluscos de locais 1 e 2 têm capacidades distintas de lidar com o stress oxidativo provocado pela exposição aguda ao BaP: aqueles do local mais contaminados são capazes de induzir significativamente CAT, GPx e GR, e possivelmente também de usar GST como um redutor de agentes tóxicos sendo capazes de reduzir os seus níveis de LPO basal, aparentemente sem necessidade de aumentar significativamente a produção de energia através da via aeróbica. Pelo contrário, os animais do local menos contaminado não parecem ser capazes de induzir significativamente a GR, possivelmente devido a uma diminuição da capacidade de regeneração NADPH causada pela redução da actividade IDH ao mesmo tempo que parece não utilizar GST como um redutor tóxico, pelo menos na faixa das concentrações testadas. Assim, o presente estudo levanta várias hipóteses que será importante para testar, a fim de ir mais longe sobre os mecanismos de toxicidade e de desintoxicação do BaP em *C. fluminea*, contribuindo também para ir mais longe sobre o papel da contaminação histórica no desenvolvimento da tolerância à poluição nesta espécie.

Palavras chave: *Corbicula fluminea*, tolerance to pollution, oxidative stress, benzo[a]pyrene, acute bioassays, biomarkers

Abstract

The Asian clam *Corbicula fluminea* (Müller, 1774) is an invasive species that has been establishing in rivers from all around the world. It is a non native invasive species (NIS) in Portugal that colonized Minho River (NW Iberian Peninsula) in 1980s, being at the present the dominant species of the community of molluscs. It is believed that the invasion of this ecosystem by *C. fluminea* has been significantly contributing for the decline of native bivalves that are now facing a serious risk of extirpation. *C. fluminea* has been showing to be able to tolerate considerable levels of some environmental contaminants and this capability may act in favour of *C. fluminea* in situations of competition with native bivalves less tolerant to chemical contamination. Here, the hypothesis that individuals from the same *C. fluminea* population but inhabiting sites with distinct levels of historical contamination, respond differently to acute pollution exposure events was tested. The rationale behind the hypotheses is that the long-term exposure to pollution may lead to the development of tolerance to chemical stress, for example through an increase of the efficiency of biotransformation mechanisms, decrease of the sensitivity of molecular targets, among others. To test the hypothesis, animals collected in two sites of the Minho estuary under differential anthropogenic, after a period of acclimation in the lab to avoid potential delayed effects of previous field exposure impact, were exposed in two different bioassays for 96h to distinct concentrations of a model substance, the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (BaP). At the end of the bioassays, enzymes involved in neurotransmission, biotransformation, anti-oxidant defences, aerobic energy production and lipid peroxidation levels were used as biomarkers. In both bioassays no significant effects of BaP on cholinesterase activity were found. In relation to the results obtained in the control groups. A significant induction of the anti-oxidant enzyme catalase (CAT) by BaP was found, with a lowest observed effect concentration (LOEC) of 8 µg/L (about 2.5 fold) in animals from the most contaminated site (thereafter indicated as site 1) and a LOEC of 2 µg/L (about 3 fold difference) in animals from the most contaminated site (thereafter indicated as site 2). Animals from site 1 also showed a significant increase of two other anti-oxidant enzymes (GR and GPx) while those from site 2 did not. No significant effects on lipid peroxidation levels (LPO) were found in any of the bioassays. However, it is interesting to

note a reduction of LPO at the highest concentrations tested coinciding with a reduction of the activity of glutathione S-transferases (GST) also involved in LPO prevention in animals from site 1; any of these effects were observed in clams from site 2. Another interesting finding is the significant reduction of isocitrate dehydrogenase (IDH) in animals from site 2 but not in animals from site 1; since IDH regenerates cellular NADPH which is a co-factor for glutathione reductase (GR), these findings may suggest that site 2 clams are not able to induce GR under BaP stress due to the lack of NADPH. Therefore, as a whole, the findings of the present study indicate that BaP is not an anticholinesterase agent to *C. fluminea* and that clams from sites with different levels of historical contamination are able to overcome the oxidative stress caused by the acute exposure to BaP up to 16 µg/L avoiding lipid oxidative damage. However, the findings also suggest that clams from sites 1 and 2 have distinct capabilities of dealing with acute BaP oxidative stress: those from the most contaminated site are able to induce significantly CAT, GPx and GR, and possibly also to use GST as a toxicant scavenger being able to reduce their basal LPO levels, apparently without need of increasing significantly the production of energy through the aerobic pathway. On the contrary, animals from the less contaminated site seem not be able to significantly induce GR possibly due to a decreased capability of NADPH regeneration caused by the reduction of IDH activity and at same time it seems not to use GST as a toxicant scavenger, at least in the range of concentrations tested. Thus, the present study raises several hypothesis that will be important to test in order to go further on the mechanisms of toxicity and detoxication of BaP in *C. fluminea*, also contributing to go further on the role of historical contamination in the development of tolerance to pollution in this species.

Keywords: *Corbicula fluminea*, tolerance to pollution, oxidative stress, benzo[a]pyrene, acute bioassays, biomarkers

CHAPTER I

GENERAL INTRODUCTION AND OBJECTIVES

General Introduction

1. Non native invasive species

The ecological terminology "non native invasive species" (NIS) refers to a species, which can be a plant or an animal that interacts with the local species, disrupting the normal ecological function of the community in consideration. According to the Federal Laws and Regulation of the United States of America (USA), an invasive species is also an organism which will cause or has a high probability of causing economic, environmental and/or human health damage. In Goodwin *et al.* (1999) the term invasive is a synonym to "nonindigenous", being nonindigenous species one of the most used term in ecology for this kind of behaviour. It is a known fact that a species that is invasive in a particular area is usually non-invasive in its native environment (Colautti and Macisaac, 2004). In fact, the relationship between native and non-native was studied by Alpert *et al.*, (2000) who stated that the NIS grow more quickly than natives when the resources are largely available (low stress); however the capacity of invasive species to outcome the native ones may be reduced when low resources are available (high stress). The success of a species that invades a particular region depends on several aspects, such as short life cycle, occupation of disturbed habitats, presence of clonal organs, among others ecological correlations (Pimentel *et al.*, 2004).

Corbicula fluminea (Müller, 1774) invasive behaviour and its worldwide expansion are due to its rapid growth, earlier sexual maturity, high fecundity and association with human activities, among other factors. These characteristics contribute to the colonization success of this species and have been well documented (e.g. Sousa *et al.*, 2008). One important ecological characteristic is the r- and k- traits. r-strategy seems to fit in the *Corbicula fluminea* case, because the individuals have a relative small size, they produce many offspring and can live in unstable environments (i.e. estuaries). Although it seems to be also a K-strategist, because they can reproduce more than once a year. So apparently *Corbicula fluminea* can have both r- and K-traits, like most of the animals and plants although it's generally considered as an r-strategist organism (Sousa *et al.*, 2008).

The invasive organism needs to possess a certain number of characteristics that favours its invasive behaviour, as well as some environmental/ecological

conditions ideal to the species' establishment in a community (Sousa, 2008). Changes in migration process are relevant, because invasions can occur by human transport (either voluntary or involuntary), such as maritime transport. This migration can benefit the invasive organism as it can occupy a region with no stress (lack of food or lack of natural predators) promoting an easy growth of the population. Environmental changes can also give great conditions for the establishment and expansion of a given species. Environmental changes are global and therefore a different place can gain extremely favourable conditions for an organism to develop such as, changes in temperature or oxygenation conditions, all of this without an adaptation period. Some authors consider the ecological pattern more important to the invasive species' success than a biological pattern. Some studies with *Gammarus* species found a particular ecological profile that was compared with the biological profile, and the last one was not well correlated to its invasive process (Devin and Beisel, 2006). Understanding the mechanism behind invasions is a crucial step towards the scientific knowledge around the ecological damage provoked by these behaviours. Also, Elton's concept of "predator-free space" (the lack of competition, within some communities facilitates the establishment of invading species) may explain the problem in a certain way but the important requirement to the success of an invasion seems to be resources (Byers, 2000). For example, Byers (2000) utter that the mechanism of *Batillaria attramentaria* invasive success (in Padilla Bay, Washington, USA) is due to a superior conversion of resources rather than to the exploitation of a niche without competitiveness. As an example of organisms with high invasive potential are plants, with several species competing each other within the same taxa, such as the competition occurring in Pacific North-western United States between the non-invasive *Rubus ursinus* and invasive *Rubus discolor*. The invasive species diverts less energy from photosynthesis when reproducing than the non-invasive species, so its invasive behaviour is favoured (McDowell and Turner, 2002).

The invasive species represents not only an ecological problem but also an economic one, with costs reaching millions of dollars a year. It was estimated that around eighty-eight species of molluscs established in the USA, including *Corbicula fluminea*, representing a cost-associated damage of about \$1 billion/year (Pimentel *et al.*, 2004). Some ecological models have been built to help policy managers regulate the invasion process traits. The political views

and resources concerning these invasive species are probably not well organized, as they focus on preventing introductions and post-detection activities rather than actual detection. This detection is a valuable asset, contributing not only to the already collected data, but also to bring new insight into policy managers (Mehta *et al.* 2007).

Another characteristic of this invasive behaviour is also the entrainment (e.g. the ballast water of a ship), which is important and crucial because if it doesn't survive to transport there's no invasion. Also, the establishment of the species' may or may not occur in the invaded ecosystem, it depends as well on the resources available (low resources may limit the invasive process), as stated behind, and in this situation, biological and/or ecological conditions determine the spread of the NIS (Kolar and Lodge, 2001).

The concern around invasive species is related to: the economic prejudice it causes and to the common belief that invasive species are the major cause of extinction or population decline of native species. However, data collected on several articles by Gurevitch and Padilla (2004) show us that only 2% (198 of 983) of the species considered are in fact contributing to the population decline in a particular region. Despite all the data collected, Gurevitch (2004) states that population decline and even extinction by invasive species is a realistic concern but invasiveness is a matter that requires more study and objectivity.

1.1. *Corbicula fluminea*

Bivalves, also called lamellibranchs or pelecypods, includes a large group of animals such as clams, mussels, scallops and oysters (Brink, 2001), and the type of reproduction varies between species. It can occur within the water column or inside an organism, such as in *C. fluminea*. Some bivalve species larvae develop in the mantle cavity or attached to external shell surface. In the case of *C. fluminea* the larvae are retained inside the progenitor shell until they are ready to live as juveniles and then are expelled through the exhalant siphon (Brink, 2001).

Corbicula fluminea (Müller, 1776) is found throughout rivers of Asia (Komaru and Konishi, 1999), Europe (Elliott, 2008), South (Bagatini *et al.*, 2007) and North America (Phelps, 1994). They are filter feeding organisms having an inhalant siphon and an exhalant siphon where they collect water with plankton.

It prefers a sandy or rather gravel substrate and it's small, light-colored bivalve with shell ornamented by a distinct, concentric sulcations and with anterior and posterior lateral teeth with many fine serrations. The light-colored shell morph has a yellow-green to light brown periostracum and white to light blue or light purple nacre (Kennedy and Huekelem, 1985). Their size is not larger than 50 mm and they are hermaphroditic been able to reproduce alone and have a life of one to seven years. It is an invasive species, reaching densities above 600 individuals/m² (Nguyen and Pauw, 2002). *Corbicula fluminea* ecological impact is profound but there's also an economic impact, (e.g. it can establish large populations in hydroelectric stations causing serious damages and costs). Most of the studies around *C. fluminea* are of biomonitoring or ecological (Cherry *et al.*, 1980; Diane and Samuel, 1978; Karatayev *et al.*, 2003; Sousa *et al.*, 2008; Wittmann *et al.*, 2008) but there have been some studies with *C. fluminea* that bring new knowledge about the responses to adverse environment and the resistance capacity of this bivalve, making it an interesting model in ecotoxicological studies (Santos *et al.*, 2007; Tatem, 1986; Topping *et al.*, 2004; Vidal and Basse, 2001).

2. Anthropogenic contamination

Incising attention has been put into aquatic environments for several reasons and the contamination of these environments will obviously cause harm to wildlife. Water quality is affected by several activities such as agricultural, industrial and domestic, being agriculture a major source of introduction of chemicals into accessible water (Schwarzenbach *et al.*, 2006). About 300 million tons of synthetic compounds annually used in industrial and consumer products partially find their way into natural waters (Schwarzenbach *et al.*, 2006). Also, oil spills contribute much for water pollution specially for sea coastal pollution (Cairr *et al.*, 2004) causing loss of biodiversity and affecting economic activities depending directly of this ecosystem. The contaminants released to environment can also cause eutrophication, oxygen depletion, toxic algal blooms and hormone disrupting effects, affecting seriously entire populations of ecological and/or economical value (Belfroid *et al.*, 2005; Schwarzenbach *et al.*, 2006). Since the 20th century aquatic environments have become a common disposal site of polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and pesticides increasing year after year the risk

to aquatic organisms. Because of this, several approaches have been developed in an attempt to demonstrate the effects of xenobiotics in environment and their damage (Havelková *et al.*, 2007). Community studies around ecosystems have proved to be a valuable tool to assess contamination of aquatic environments, for example using indices of community structure (Lements and Carlisle, 2003). But biochemical parameters have also been extensively used to complement some flaws in assessment and to elucidate the damages that a particular event caused to ecosystems, as recorded by Tim-Tim *et al.* (2009) in relation to the oil spill from the tanker Prestige. The sediment quality, the myriads of organisms and their interactions, the confluence of one or more influents transporting anthropogenic pollutants and also the importance to economy, makes rivers an interesting object of study and scientific knowledge. Therefore in 2000 (2000/60/EC) was approved the European Water Framework Directive (WFD) to ensure and comprehend better the ecological changes and chemical status in an attempt to protect the environment and, as a consequence, the human itself (Chainho *et al.*, 2008). A lot of work has been done to comprehend rivers and to assemble the ecological relations. Sousa *et al.*, (2007) searched for mollusc distribution and characterization in Minho River leading to a better knowledge of the local biodiversity, concluding that within the several species of mollusc founded, the invasive *C. fluminea* was one of the most representatives of the overall species.

Rivers are a natural receptor of contaminants that has its origin in industry and/or agricultural activities. These contaminants can change the normal functioning of the ecological relationships that exists between the species of a particular place. The contamination is different along the water column. For example, in a study with *Solea senegalensis* and *Pomatoschistus microps* (Fonseca *et al.*, 2011), analysis done reported differences in enzymatic activities of the *S. senegalensis* and *P. microps* probably due to the feeding behaviour and the different water column habitat of these two species. The different behaviour leads to the conclusion that when we are assessing the contamination of a specific local, we have to take in account the living habitat of the species since different species, although living in the same habitat, establish in particular regions of the water body which can have different levels of contamination. Since rivers are a receptor of large quantities of sediment that are transported by adjacent influents, any major source of pollution near these rivers will forcibly affect the health status of the organisms that requires

healthy sediment to live. Impacted sediments can change the individual responses to the normal mechanisms of detoxification which is crucial for an organism to live and respond to changes provoked by anthropogenic activity mainly. Species such as *Hediste diversicolor* are an important organism to evaluate the health of a river in terms of its sediment pollution. Moreira *et al.* (2006) showed changes in oxidative stress of the *H. diversicolor* species as a result of the impacted sediment. This changes the ecological role of this species as an important detritus processor and, therefore, the organic matter decomposition, which is crucial for equilibrium in rivers ecology.

2.1 Polycyclic aromatic hydrocarbons

PAHs are a common anthropogenic type of contaminants which we can find mainly in waters of industrialized areas. They are ubiquitous environmental pollutant and are considered dangerous, being integrated in the WFD (Directive 2000/60/EC) (Wessel *et al.*, 2010). Its source is mostly anthropogenic (biomass combustion, coal burning, cooking oil, oil spills) (Khairy *et al.*, 2009) but can also be by natural causes, such as non-human propagated fires. They are typically organic compounds, with aromatic rings of carbon and hydrogen attached to each other forming a structure that can have 'one- to six-rings'. The toxicity increases with the number of rings, with those with higher rings having a more acute effect than those with fewer rings. However some PAHs with low aromatic rings can have carcinogenic effects (Grueiro-Noche *et al.*, 2010), such as naphthalene (USEPA, 1998). According to Baumard *et al.* (1998), PAHs with heavier molecular weight tend to concentrate more in the finest fraction of the sediment. This is important since fine sediments, because of their size, are generally the particles that are most filtered by burrowing organisms such as bivalves and can bioaccumulate a large amount of contaminants such as PAHs. Although, bivalves can give us valuable information about the level of sediment contamination some data given by the sediment analysis can reveal the opposite (Khairy *et al.*, 2009). Some organisms can biotransform and eliminate most of the metabolites but sometimes, trace levels are found in tissues and can therefore be measured (Wessel *et al.*, 2010). These trace levels can cause DNA damage, as stated by Wessel (2010) that correlated the levels of a mixture of PAHs with genotoxic effect in sole fish. Pichaud *et al.* (2008) stated that immunological system in conjunction with oxidative responses can give us a better knowledge about the damages that a mixture of PAHs or a single PAH

may have. Moreover, one of PAHs primary consequence seems to be the induction of the cytochrome P450 that is responsible for the conversion of PAHs to its metabolites as described by Stagg *et al.* (2000) in *Salmon salar*. The analysis of PAHs that is suspect to damage the organisms, has to take in account the environment itself, because an organism that has a typical burrowing activity will be more exposed to sediment contamination instead of the water body contamination that is highly probable to cause more damage to fishes than to burrowing organisms (Baumard *et al.*, 1998).

The anthropogenic contamination is a major source of PAHs intake into aquatic systems, perturbing and altering the normal ecological relationships between species. Human PAH generating activities can cause chronic exposure of several types of PAHs and this is a problem because we don't find single PAHs alone in the environment with an independent action, we find a mixture of several PAHs and others chemicals compounds that interact with each other and can have synergetic or antagonist effect that may contribute to a declining of a given species, as stated by Blanc *et al.* (2010).

2.2. Benzo[a]pyrene

One of the most worldwide studied PAHs is the benzo[a]pyrene because of its carcinogenicity, being used as a positive control in these types of bioassays (EPA - Environmental protection Agency, 2011). Benzo[a]pyrene has five aromatic rings having a molecular formula of $C_{20}H_{12}$. These five aromatic rings

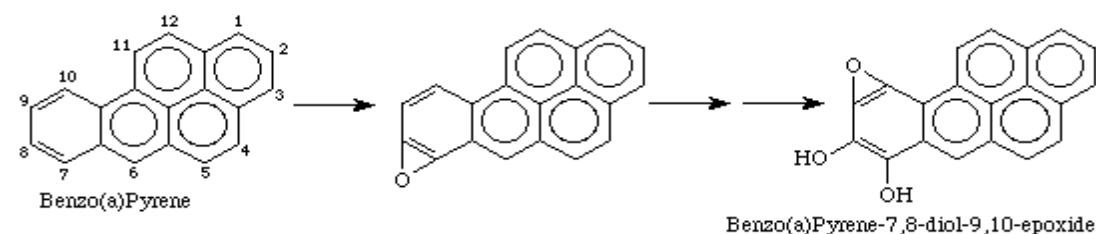


Figure 1– Example of one of the benzo[a]pyrene metabolites. (Conney, 1982).

confer a certain degree of solubility being the less soluble PAHs with higher number of aromatic rings (Meire *et al.*, 2007). It's a hydrophobic compound that has a moderately high K_{ow} (octanol-water partition coefficient) (Table 1) therefore being highly lipophilic and thus being easily absorbed by organisms. Table 1 shows that octanol-water partition coefficient of BaP is relatively high comparing to others common PAHs. This coefficient gives an idea of the

capacity of organisms to bioconcentrate levels of a contaminant within itself and this may change between aquatic vertebrates and invertebrates, although the persistence of a xenobiotic in the organism and the elimination rates of the same metabolites are dependent on the rates of biotransformation of the organism (Livingstone, 1998)

Table 1- Polycyclic aromatic hydrocarbons properties; molecular weight (MW); solubility (S); vapour pressure (VP); Henry's constant (H); Log K_{ow}, octanol-water partition coefficient; no data (n.d.) (adapted from Meire *et al.*(2007)).

PAHs	Number of rings	MW (g/mol)	S (mg/L)	VP (Pa)	H (Pa m ³ /mol)	Log K _{ow}
Naphthalene	2	128	31	10.4	43.01	3.37
Phenanthrene	3	178	1.1	0.02	3.24	4.57
Anthracene	3	178	0.045	0.001	3.96	4.54
Pyrene	4	202	0.132	0.0006	0.92	5.18
Benzo[a]pyrene	5	252	0.0038	7.00x10⁻⁷	0.046	6.04
Indeno(1,2,3-cd)pyrene	6	278	n.d.	n.d.	0.003	n.d.

Benzo[a]pyrene is known to be a hydrophobic contaminant and therefore it associates to sediment particles including suspended and bottom deposits (Guerrero *et al.*, 2003) being an important factor when accessing the contamination processes that occurs in water biota, especially organisms that have a burrowing activity such as clams. This is of major importance since it interferes with bioavailability of xenobiotics and bioaccumulation by the organism, as reported by Guerrero *et al.* (2003). Also important in bioaccumulation and bioavailability of contaminants is the pore water concentration that varies with the type of sediment that is considered and its porosity. The sorption can affect bioaccumulation and bioavailability by reducing the accumulation of contaminants in the sediments and altering the contaminant exposure that a burrowing organism is subjected to (Reible and Lu, 2007).

In ecotoxicology, several studies have been conducted to give us a better knowledge of possible effects of BaP on living organisms in an attempt to preserve and understand possible ecological consequences derived from

anthropogenic sources. Extensive literature describes the effect or potential effect of BaP in wild mussels (*Mytilus edulis* and *Mytilus trossulus*) it has been documented that there's a relationship between the levels of BaP and short as later life gonad development (Hellou and Law, 2003). Also Choy *et al.*, (2006) stated that a failure to eliminate BaP resulted in damage to the reproductive success in pacific oyster *Crassostrea gigas*, meaning that there is an upper limit that, depending on the species, results in an adverse effect in the reproductive success. We know that there is a relationship between the levels of BaP and human carcinogenesis and this is also observed in natural environments. The DNA adduct formation was observed when *Mytilus galloprovincialis* were exposed to BaP and this correlation was supported by the other levels of enzymatic activities such as CAT (catalase), AChE (acetylcholinesterase) (Akcha *et al.*, 2000), although this is not a direct correlation. A similar study done by Banni *et al.*, (2010) showed BaP as a potent phase I and phase II response inducer in *M. galloprovincialis*, inducing DNA adduct formation and activation of some enzymatic pathways of detoxification in both phases. There is also data that suggests BaP might cause changes in reproductive path by decreasing mRNA expression in both CYP19A1 and CYP19A2 genes in *Fundulus heteroclitus* immature oocytes, embryo brains and adult hypothalamus respectively, bringing new insights in the BaP endocrine disruptor behaviour (Dong *et al.*, 2008). In fact, the role of cytochrome P-450 in the detoxification processes is critical to the metabolism of BaP metabolites, being highly activated in bivalves and especially in the digestive gland (Stegeman, 1985). There's a notorious effect between the BaP and its metabolites in the levels of several enzymes. These alterations can affect populations as a bottom-up negative effect, because key structural organisms such as invertebrates can be a target of contamination. They occupy an essential role in ecosystems and if normal ecological function of this community fails this effect can be visible at populations from superior organisms (Galloway and Depledge, 2001). Benzo[a]pyrene immunological changes are still not well studied and frequently are supported by the enzymatic assays that have a solid background and extensive literature. Levels of antioxidant enzymes and lipid peroxidation (LPO) can reflect the damage at organism level. Pan *et al.*, (2006) tested BaP and benzo(k)fluoranthene (BKF) as well as their mixture in *Chlamys ferrari* and found that BaP is more toxic than BKF and the mixture itself, represented by the levels of antioxidant enzymes

and LPO. Also the by-products of BaP metabolism produces reactive oxygen species (ROS) that can alter the cytoskeleton of mussels' haemocytes leading to a loss of the defence function (Gómez-Mendikute *et al.*, 2002).

3. Environmental biomarkers

The effects of pollutants in organisms are of great concern because of its deleterious effects at an individual level, potentially causing risks at population one. In ecotoxicology, the early assessment of chemicals' adverse effects in populations can be determined with analysis of molecular alterations occurring within the organism (Vasseur and Cossu-Leguille, 2006) as resumed in Figure 2. When a xenobiotic enters in the organism it passes to several steps. These steps can enhance its toxicity or they can be excreted. The uptake of a xenobiotic is dependent on chemical characteristics (such as K_{ow}), temperature, turbulence, biochemical factors and others (van der Oost *et al.*, 2003). Although the link between molecular damages and effects at a population level is not a straight relationship, environmental biomarkers are an attempt to enrich this knowledge. According to van der Oost *et al.* (2003) biomarkers "are measurements in body fluids, cells and/or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response".

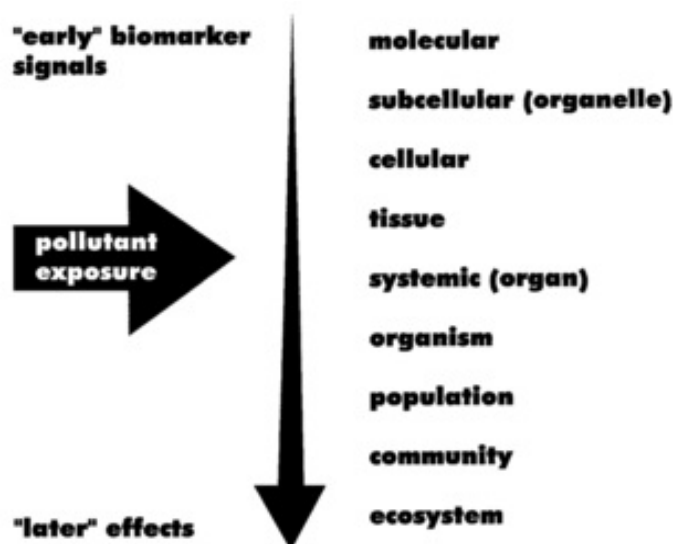


Figure 2 – Scheme of pollutant exposure and the level of effects that can occur (from van der Oost *et al.*, 2003)

The use of biomarkers in aquatic organisms represents a useful tool in environmental health assessments (Valavanidis *et al.*, 2006). Oxidative stress is an example of a process that can be used to assess certain pollutant exposure. The potential damage caused by ROS can range from a neurological level to behavioural changes, including endocrine disruption, genotoxicity, effects on reproduction and others (Vasseur and Cossu-Leguille, 2006). Oxidative stress is relevant because all aerobic life forms will eventually suffer an unbalance between antioxidant defences and prooxidant forces (Winston and Giulioz, 1991) as it's a consequence of natural ageing itself. Some pollutants have the capacity to interfere and enhance toxicity in the organism which can eventually led to repercussions in the ecosystem (Fig. 2). All eukaryotic life forms needs oxygen (O_2) as a key element to acquire energy. The aerobic pathway leading to formation of water (H_2O) produces ROS that can enhance the deleterious effect in cells by oxidative stress (Winston and Giulioz, 1991).

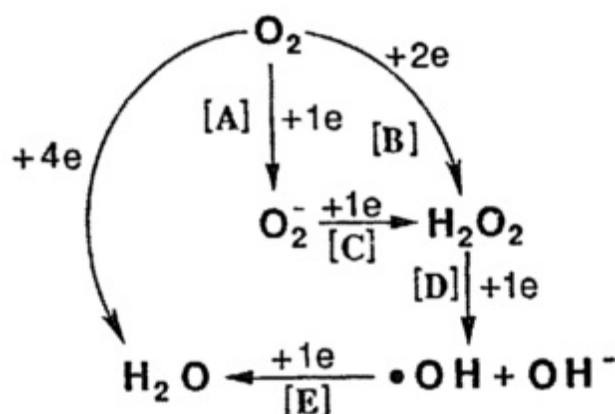


Figure 3 – Oxygen reduction metabolism and the production of reactive oxygen species. The reduction of O_2 to H_2O_2 (hydrogen peroxide – ROS) can have two paths, [B] with the direct reduction of $2e$ or [A] and [C] $1e$ reductions. The hydroxyl radical ($\bullet OH$) is formed by the reduction of $1e$ H_2O_2 [D] which deably binds to OH^- to form a molecule of water with the reduction of $1e$ hydroxyl radical (from Winston and Giulioz, 1991).

An increase on the production of these ROS by successive reductions causes the oxidative stress mentioned above. The reactive oxygen species H_2O_2 , through the Haber-Weiss pathway, can form the hydroxyl radical ($\bullet OH$) which is another powerful ROS with high oxidation activity such as H_2O_2 , although this reaction is not always favourable. Actually the presence of metals, such as iron, through Fenton reaction, seems to be the most effective way of producing

large amounts of $\cdot\text{OH}$, acting as a catalytic (Winston and Giulioz, 1991). The behaviour of these two ROS is extremely important because they are very potent and capable of provoking lipid peroxidation, enzyme inactivation, DNA damages and death (Winston and Giulioz, 1991). However, living organisms are capable to respond to toxicant exposure by inducing anti-oxidant enzymes in order to prevent damage done to DNA, proteins and lipids by inducing antioxidant enzymes to cope these adverse damages regulating the oxidative stress (Valavanidis *et al.*, 2006).

When it enters the organism two types of biotransformation can occur. First it passes through phase I enzymes and then into the phase II enzymes.

3.1. Phase I

The phase I metabolism involves bioactivation or inactivation of the xenobiotic by biochemical process such as oxidation, reduction or hydrolysis turning the molecules more polar and more hydrophilic. This is done by adding reactive functional groups. These reactions are catalyzed by a number of specific enzymes known as microsomal monooxygenase (MO) enzymes. Some of them are the cytochrome P450 (cyt P450), cytochrome b5 (cyt b5) and NADPH cytochrome P450 reductase (P450 RED) (van der Oost *et al.*, 2003). The cyt P450 is an enzyme super-family that is very specific to chemicals, producing a certain type of metabolites but they can be inhibited or induced by the chemical in question (Kane, 2004). In the case of benzo[a]pyrene, the CYP1A acts in the parental compound forming reactive intermediates called epoxides. It can form several metabolites. One of them is the benzo[a]pyrene 4,5-dihydrodiol that is less toxic and rapidly eliminated the other is the benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide that is well known to bind covalently to DNA, a process that may lead to cancer (Kane, 2004). The intermediates formed in these phase most often will be metabolized and detoxified in the phase II reactions.

3.2. Phase II

There are several pathways that can occur in this phase such as glucuronidation, sulfation, methylation, acetylation, glutathione conjugation and others. At this stage, reactive metabolites are conjugated with endogenous molecules (glutathione (GSH) and glucuronic acid (GA)) in an attempt to add

polarity to the intermediate, adding covalent bounds and facilitating the excretion of the chemical (Guo *et al.*, 2011). The first pathway mentioned seems to be the most important in mammals, with the conjugation of GA mediated by uridine diphosphate-glucuronyl transferase (UDP-GT) increasing the hydrophilicity of metabolites (Fernandes, 2005). Every pathway needs a cofactor and in the glutathione conjugation, GSH is the cofactor which will conjugate with the substrate (metabolite) and with the help of GST (glutathione-S-transferase) metabolites becomes polar and are more easily excreted (van der Oost *et al.*, 2003).

3.3. Biomarkers

The term biomarkers have been subjected to several meanings along the years. Some are extensive and others have a more generalized definition but the overall idea is that biomarkers try to establish a connection between biological effects and the potential hazard that a population may be subjected to (Bucheli and Fent, 1995). The impact that a contaminant has in an organism can then be measured by analyzing the responses at a molecular and cellular level. But the term biomarker is also used to express changes at a more complex level of organization. There are examples of biomarkers that can represent the ecosystem status, such as diversity indices, others that can represent the population status, such as age structure and size distribution (Bucheli and Fent, 1995). The term biomarkers is also used as a classification of biological alterations that an organism might suffer or a more complex classification representing as an ecological parameter that describes the ecosystem health status (van der Oost *et al.*, 2003). Conclusively, biomarkers can provide a link between the cause (pollution) and the effect (biological response) covering a gap that sometimes conventional tools, such as chemical analysis don't provide (Bucheli and Fent, 1995). According to the NRC (1987), WHO (1993), biomarkers can be subdivided into three classes:

- Biomarkers of exposure
- Biomarkers of effect
- Biomarkers of susceptibility

Biomarkers of exposure - Gives a measurement of a exogenous substance, metabolites or the products of an interaction that occurred between a

xenobiotic and the target molecule or cell, and this is measured in a compartment within the organism;

Biomarkers of effect - These biomarkers includes the analysis of changes at a biochemical and/or physiological level in tissues or body fluids of an organism that are associated with possible health impairment or disease;

Biomarkers of susceptibility - Biomarkers that respond to changes in the exposure conditions and are inherent to the organism itself. This includes genetic factors and changes in receptors that will eventually lead to an increase of susceptibility of an organism.

This division is made to clarify the way biomarkers are used and not merely by a syntax accuracy. The information given by these biomarkers responses is a biological one and should be considered as a measure of effects of pollutants in an organism. The mechanisms related to biotransformation are often assessed using several enzyme activities and important information about the biochemical and/or physiological condition will be obtained and correlated to toxicant exposure and stress (van der Oost *et al.*, 2003).

Because oxygen is an excellent electron acceptor, the mechanisms surrounding oxidative damage and the production of ROS, mentioned above, are extremely important to know when we are trying to establish a connection between pollution and ecological damages. Enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) have an important role in maintaining the normal health status of an individual (Winston and Giulioz, 1991). Since aerobic respiration produces more energy than anaerobic (38 ATP - adenosine triphosphate - molecules to 2 ATP molecules respectively) it's natural that aerobic path is more used than the anaerobic one. Although this is a clear advantage, it has its own costs. The oxygen consumption occurs in mitochondria and according to Livingstone (2003) 1-3% of this consumption generates ROS. Aquatic animals have also developed enzymatic and non-enzymatic defences to try to fight against this ROS but the direct measure of ROS is very difficult because of its short half-lives and particular technology is needed. The measure of ROS is assessed by redox sensitive dyes, which change accordingly with the enzymatic reactions (Connors, 2004). The measurement of antioxidant system and the level of tissue damage that ROS produces, can be quantified by enzymatic levels of certain enzymes. We can do

this by analyzing antioxidant enzymes, such as SOD, CAT, GPx and glutathione reductase (GR). Other processes like lipid peroxidation (LPO), DNA damage, energetic metabolism alterations measured by activity of enzymes like lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) and also neurotoxic effects that can be assessed with the activity of cholinesterases (ChE), are also used to assess the pollution damage within an organism. The reaction catalysed by CAT is very important because it helps removing the highly reactive H_2O_2 by metabolizing it to H_2O (water) and O_2 (oxygen) and it's an enzyme that is very specific to H_2O_2 (Stegeman *et al.*, 1992).

Table 2- Enzymes involved in biotransformation and the reactions they catalyze. (adapted from Blokhina *et al.*, 2003)

Enzyme	Reaction catalysed
Superoxide dismutase	$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \leftrightarrow 2\text{H}_2\text{O}_2 + \text{O}_2$
Catalase	$2\text{H}_2\text{O}_2 \leftrightarrow \text{O}_2 + 2\text{H}_2\text{O}$
Glutathione peroxidase	$2\text{GSH} + \text{PUFA-OOH} \leftrightarrow \text{GSSG} + \text{PUFA} + 2\text{H}_2\text{O}$
Glutathione S-transferases	$\text{RX} + \text{GSH} \leftrightarrow \text{HX} + \text{R-S-GSH}^*$
Glutathione reductase	$\text{NADPH} + \text{GSSG} \leftrightarrow \text{NADP}^+ + 2\text{GSH}$

* R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrite or halide group

Although it's a largely used biomarker (van der Oost *et al.*, 2003) does not consider it a useful biomarker for environmental risk assessment because it has been observed that induction and inhibition occurs after exposure to environmental contamination, therefore the results from CAT activity should be carefully analyzed.

Glutathione peroxidase (GPx) is a peroxidase type of enzyme that needs a co-factor to transform H_2O_2 to water. This is done by oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). It protects the cells membranes from damage caused by lipid peroxidation (LPO) (van der Oost *et al.*, 2003). Although GPx is not directly involved in the process of detoxification like CAT or SOD, its role is vital for the equilibrium of the reaction (Winston and Giulioz, 1991). Glutathione reductase (GR) also oxidizes the reaction of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP^+ (Blokhina *et al.*, 2003).

Not only the H_2O_2 is highly reactive, superoxide anion (O_2^-) is also very reactive. To detoxify this species, SOD catalysis O_2^- to H_2O_2 that will be later detoxified by CAT and GPx. SOD has a metal cofactor bounded to it and there are three types of this enzyme according with this. We can find FeSOD, MnSOD and

Cu/ZnSOD and the difference between them is the sensitivity to H_2O_2 (Blokina *et al.*, 2003).

When the amount of ROS is significantly higher and antioxidant defenses cannot cope with this, peroxidation of polyunsaturated fatty acids (LPO) can occur and is one of the most important consequence known (Stegeman *et al.*, 1992). The process of LPO is extensive, involving several chain reactions but the essential idea is that there is a formation of lipid radicals that consequently leads to lipid hydroperoxide (LOOH) resulting in a peroxidized membrane, losing permeability and integrity (Valavanidis *et al.*, 2006). This may result in pathological conditions adverse to animals. The LPO is measured by the quantification of thiobarbituric acid reactive substances (TBARS) which is the typical method for LPO.

Another consequence of toxic damage is the energy production that animals have in certain oxygen conditions. In aerobic paths we can assess the isocitrate dehydrogenase (IDH) and in anaerobic paths we can assess the lactate dehydrogenase (LDH), both related to Krebs cycle. LDH is the enzyme responsible for the reversible conversion of pyruvate to lactate (Gravato *et al.*, 2010) and is very important since we can use its value to identify stress conditions under low or no oxygen levels. One example is bivalves, that under the effect of a contaminant they can close the valves and LDH path is sometimes used to sustain the metabolism (Ortmann and Grieshaber, 2003).

IDH can also be used to assess the energetic values from the aerobic path. It catalysis the decarboxylation of isocitrate to 2-oxoglutarate and to do this, NAD^+ or $NADP^+$ is used to produce NADH or NADPH. In the antioxidant system this is important because GR uses NADPH as a cofactor, so the IDH path replenishes this important product in the metabolic reaction of GR (Lima *et al.*, 2007). Cholinesterases (ChE) are a common biomarker of neurotoxic effect and some pollutants are very specific to cholinesterases. These enzymes can be divided in true cholinesterases, such as acetylcholinesterase (AChE), and non-specific esterases or pseudocholinesterases, such as butyrylcholinesterase (BChE) or propionylcholinesterase (PChE) (Mora *et al.*, 1999). They differ from each other by the type of substrate they have more affinity to, translating into different levels of activity depending on the organism and tissue in cause (Mora *et al.*, 1999). AChE is responsible for the deactivation of acetylcholine at nerve endings (van der Oost *et al.*, 2003). An inhibition of this enzyme results in an accumulation of acetylcholine and therefore in an overstimulation of the

sensory and muscular system (in animals that possess these system) provoking nerve firings. Organophosphates are a well known group of AChE inhibitors, proving that AChE is a good and common biomarker when trying to identify these contaminants in water (Basack *et al.* 1998).

4. Objectives

Considering that pollution can influence the competition between NIS and native species and that *C. fluminea* has the capability to tolerate considerable levels of some environmental contaminants, the hypotheses that animals from the same population but inhabiting sites with different contamination levels respond differently to the acute exposure to common environmental contaminants was tested in the present study. The underlying principle behind it is that chronic exposure to pollution may induce general tolerance to chemical stress. The hypothesis was tested by exposing in the laboratory *C. fluminea* specimens from two sites of Minho estuary with different levels of contamination for 96h and assessing biomarkers involved in neurotransmission, biotransformation, antioxidant defences and aerobic pathway of energy production at the end of the assay. LPO levels were also determined as a marker of oxidative damage.

- i. BaP was selected as a model test substance because its mechanisms of toxicity and biotransformation are well known and it is a common environmental contaminant.

5. Thesis Structure

The present thesis is structured in three chapters: the first chapter is a general introduction to the work done and it's an essential part to understand the problem discussed here. Subjects like non invasive species, *Corbicula fluminea*, anthropogenic contamination, biomarkers and objectives are discussed in this chapter; chapter second refers to a paper that includes introduction, material and methods, results, discussion, conclusion and acknowledgements; the final chapter is a general discussion of the work done here. All chapters ends in a references list that supported the chapters idea.

6. References

- Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., & Narbonne, J. (2000). Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*, 49, 269-287.
- Alpert, P., Bone, E., & Holzapfel, C. (2000). Invasiveness, invasibility and the role of environmental stress in the spread of non-native plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3, 52-66.
- Bagatini, Y. M., Higuti, J., & Benedito, E. (2007). Temporal and longitudinal variation of *Corbicula fluminea* (Mollusca, Bivalvia) biomass in the Rosana Reservoir, Brazil. *Acta limnology*, 19, 357-366.
- Banni, M., Negri, a, Dagnino, a, Jebali, J., Ameer, S., & Boussetta, H. (2010). Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. *Ecotoxicology and Environmental safety*, 73, 842-8.
- Basack, S. B., Oneto, M. L., Fuchs, J. S., Wood, E. J., & Kesten, E. M. (1998). Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorus pesticides. *Bulletin of environmental contamination and toxicology*, 61, 569-76.
- Baumard, P., Budzinski, H., Garrigues, P., Sorbe, J. C., Burgeot, T., & Belloq, J. (1998). Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. *Marine Pollution Bulletin*, 36, 951-960.
- Belfroid, C., Vethaak, A. D., Lahr, J., Schrap, S. M., Rijs, G. B. J., Gerritsen, A., Boer, J. D., (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere*, 59, 511-524.
- Beltran, K. S., & Pocsidio, G. N. (2010). Acetylcholinesterase activity in *Corbicula fluminea* Muller, as a biomarker of organophosphate pesticide pollution in Pinacanauan River, Philippines. *Environmental Monitoring and Assessment*, 165, 331-40.
- Blokhina, O., Virolainen, E., & Fagerstedt, K. V. (2003). Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. *Annals of Botany*, 91, 179-194.
- Boltovskoy, D., Izaguirrel, I., & Correa, N. (1995). Feeding selectivity of *Corbicula fluminea* (Bivalvia) on natural phytoplankton. *Hydrobiologia*, (312), 171-182.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72, 248-54.

- Brink, L. A. (2001). Molluscs: Bivalvia. *Identification Guide to Larval Marine Invertebrates of the Pacific Northwest*. Oregon: University of Oregon Libraries (129-149).
- Bucheli, T., & Fent, K. (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, 25, 201-268.
- Byers, J. E. (2000). Competition between two estuarine snails: implications for invasions of exotic species. *Ecology*, 81, 1225.
- Cairrão, E., Couderchet, M., Soares, A M V M, & Guilhermino, L. (2004). Glutathione-S-transferase activity of *Fucus* spp . as a biomarker of environmental contamination. *Aquatic Toxicology*, 70, 277-286.
- Chainho, P., Chaves, M. L., Costa, J. L., Costa, M. J., & Dauer, D. M. (2008). Use of multimetric indices to classify estuaries with different hydromorphological characteristics and different levels of human pressure. *Marine Pollution Bulletin*, 56, 1128-1137.
- Cherry, D. S., Jr, J. H. R., Graney, R. L., & Jr, J. C. (1980). *Dynamics and control of the asiatic clam in the New River, Virginia*. *Dynamics and Control*, 123, 1-43.
- Cheung, C. C. C., Siu, W. H. L., Richardson, B. J., De Luca-Abbott, S. B., & Lam, P. K. S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. *Environmental pollution*, 128, 393-403.
- Choy, E. J., Jo, Q., Moon, H.-B., Kang, C.-K., & Kang, J.-C. (2006). Time-course uptake and elimination of benzo(a)pyrene and its damage to reproduction and ensuing reproductive outputs of Pacific oyster, *Crassostrea gigas*. *Marine Biology*, 151, 157-165.
- Colautti, R. I., & Macisaac, H. J. (2004). A neutral terminology to define “invasive” species. *Diversity and Distributions*, 10, 135-141.
- Conners, D. E. (2004). *Biomarkers of oxidative stress in freshwater clams (Corbicula fluminea) as mechanistic tools to evaluate the impairment of stream ecosystem health by lawn care pesticides*. The International Journal on the Biology of Stress. University of Georgia, Athens, Georgia (USA).
- Cooper, N. L., & Bidwell, J. R. (2006). Cholinesterase inhibition and impacts on behavior of the asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide. *Aquatic toxicology (Amsterdam, Netherlands)*, 76, 258-267.
- Devin, S., & Beisel, J.-N. (2006). Biological and ecological characteristics of invasive species: a gammarid study. *Biological Invasions*, 9, 13-24.
- Diane, L., & Samuel, M. (1978). *The freshwater asian clam Corbicula fluminea as a factor affecting nutrient cycling in the Chowan River, N.C.* *Water Resources Research*.

- Dong, W., Wang, L., Thornton, C., Scheffler, B. E., & Willett, K. L. (2008). Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in *Fundulus heteroclitus*. *Aquatic Toxicology*, 88, 289-300.
- EPA. (2011). Benzo(a)pyrene (BaP) (CASRN 50-32-8). Retrieved from <http://www.epa.gov/iris/subst/0136.htm>
- Elliott, P. (2008). The asian clam (*Corbicula fluminea*) in the River Thames, London, England. *Aquatic Invasions*, 3, 54-60.
- Ellisa, G., & Goldberg, D. M. (1971). An improved manual and semi-automatic assay for NADP-dependent isocitrate dehydrogenase activity, with a description of some kinetic properties of human liver and serum enzyme. *Clinical Biochemistry*, 4, 175-185.
- Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric of acetylcholinesterase determination. *Biochemical Pharmacology*, 7, 88-95.
- Fernandes, F. A. (2005). The use of biomarkers in aquatic toxicology studies. *Revista Portuguesa de Zootecnia*, 12, 67-86.
- Flohé, L., Gunzler, W. A., & Schock, H. H. (1973). Glutathione Peroxidase: A selenoenzyme. *FEBS Letters*, 32, 32-34.
- Foe, C., & Knight, A. (1986). Growth of *Corbicula fluminea* (bivalvia) fed artificial and algal diets. *Hydrobiologia*, (133), 155-164.
- Fonseca, V. F., Franc, S., Serafim, A., Company, R., Lopes, B., Bebianno, M. J., & Cabral, H. N. (2011). Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. *Aquatic Toxicology*, 102, 216-227.
- Frasco, M. F., & Guilhermino, L. (2003). Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. *Fish Physiology and Biochemistry*, 26 149-156.
- Galloway, T. S., & Depledge, M. H. (2001). Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology (London, England)*, 10, 5-23. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11227817>
- Gravato, C., Guimarães, L., Santos, J., Faria, M., Alves, A., & Guilhermino, L. (2010). Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety*, 73, 524-33.
- Grueiro-Noche, G., Andrade, J. M., Muniategui-Lorenzo, S., López-Mahía, P., & Prada-Rodríguez, D. (2010). 3-Way pattern-recognition of PAHs from Galicia (NW Spain) seawater samples after the Prestige's wreck. *Environmental Pollution (Barking, Essex: 1987)*, 158, 207-14.

- Guilhermino, L., Lopes, M. C., Carvalho, A. P., & Soares, Amadeu M.V. (1996). Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia Magna*. *Chemosphere*, 32, 727-738.
- Guo, H., Peng, F., Ma, L., Jiang, Z., & Liu, X. (2011). Hydrolysis technology optimization of phorbol esters by orthogonal experiment. *China journal of Chinese Materia Medica*, 36, 446-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/21617929>
- Gurevitch, J., & Padilla, D. K. (2004). Are invasive species a major cause of extinctions? *Trends in ecology & evolution (Personal edition)*, 19, 470-4.
- Gómez-Mendikute, A., Etxeberria, A., Olabarrieta, I., & Cajaraville, M. P. (2002). Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. *Marine Environmental Research*, 54, 431-6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12408598>
- Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*, 249, 7130-7139.
- Havelková, M., Randák, T., Zlábek, V., Krijt, J., Kroupová, H., Pulkrabová, J., & Svobodová, Z. (2007). Biochemical markers for assessing aquatic contamination. *Sensors*, 7, 2599-2611.
- Kane, A. S. (2004). Applied Toxicology Xenobiotics. *Pathobiology*.
- Karatayev, A., Burlakova, L., Kesterson, T., & Padilla, D. (2003). Dominance of the asiatic clam, *Corbicula fluminea* (Muller), in the benthic community of a reservoir. *Journal of Shellfish Research*, 22, 487-493.
- Kennedy, V. S., & Huekelem, L. V. (1985). Gametogenesis and larval production in a population of the introduced asiatic clam, *Corbicula* sp. (Bivalvia: Corbiculidae), in Maryland. *Biological Bulletin*, 168, 50.
- Khairy, M. A., Kolb, K., Mostafa, A. R., EL-Fiky, A., & Bahadir, M. (2009). Risk assessment of polycyclic aromatic hydrocarbons in a mediterranean semi-enclosed basin affected by human activities (Abu Qir Bay, Egypt). *Journal of Hazardous Materials*, (2008), 1-42.
- Kolar, C. S., & Lodge, D. M. (2001). Progress in invasion biology: predicting invaders. *Trends in ecology & evolution (Personal edition)*, 16, 199-204. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11245943>
- Komaru, A., & Konishi, K. (1999). Non-reductional spermatozoa in three shell color types of the freshwater clam *Corbicula fluminea* in Taiwan. *Zoological Science*, 16, 105-108.
- Lima, I., Moreira, S. M., Rendon-von Osten, J., Soares, A. M., & Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the

- North-western coast of Portugal. *Chemosphere*, 66, 1230-1242. Elsevier. Retrieved from <http://cat.inist.fr/?aModele=afficheN&cpsidt=18461045>
- Laetz, C., Baldwin, D. H., Collier, T. K., Hebert, V., Stark, J. D., & Scholz, N. L. (2009). The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered Pacific salmon. *Environmental Health Perspectives*, 117, 348-353.
- Laura, G., Luís, S. M., Joana, S., Carlos, G., & Lúcia, G. (2009). Yellow eel (*Anguilla anguilla*) development in NW Portuguese estuaries with different contamination levels. *Ecotoxicology*, 18, 385-402.
- Legeay, A., Achard-Joris, M., Baudrimont, M., Massabuau, J.-C., & Bourdineaud, J.-P. (2005). Impact of cadmium contamination and oxygenation levels on biochemical responses in the asiatic clam *Corbicula fluminea*. *Aquatic toxicology (Amsterdam, Netherlands)*, 74, 242-53.
- Lements, W. I. H. C., & Carlisle, D. M. (2003). Growth and secondary production of aquatic insects along a gradient of Zn contamination in Rocky Mountain streams. *North America Benthological Society*, 22, 582-597.
- Livingstone, D. R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 120, 43-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9773498>
- Livingstone, D. R. (2003). Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Revue Medicine Veterinaire*, 6, 427-430.
- Maria, V. L., & Bebianno, M. J. (2011). Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 154, 56-63.
- McDowell, S., & Turner, D. (2002). Reproductive effort in invasive and non-invasive *Rubus*. *Oecologia*, 133, 102-111.
- Mehta, S., Haight, R., Homans, F., Polasky, S., & Venette, R. (2007). Optimal detection and control strategies for invasive species management. *Ecological Economics*, 61, 237-245.
- Meire, R. O., Azeredo, A., Paulo, J., & Torres, M. (2007). Aspectos ecotoxicológicos de hidrocarbonetos policíclicos aromáticos. *Oecologia Brasil*, 11, 188-201.
- Mora, P., Fournier, D., & Narbonne, J.-F. (1999). Cholinesterases from the marine mussels *Mytilus galloprovincialis* and *M. edulis* and from the freshwater bivalve *Corbicula fluminea* Muller. *Comparative Biochemistry and Physiology*, 122, 353 - 361.

- Mora, P., Michel, X., & Narbonne, J.-F. (1999). Cholinesterase activity as potential biomarker in two bivalves. *Environmental Toxicology and Pharmacology*, 7, 253 - 260.
- Netpae, T., & Phalaraksh, C. (2009). Bioaccumulation of copper and lead in asian clam tissues from Bung Boraphet Reservoir , Thailand. *International Journal of Agriculture & Biology*, 11, 783-786.
- Nguyen, L. T., & Pauw, N. D. (2002). The invasive *Corbicula* species (Bivalvia, Corbiculidae) and the sediment quality in Flanders, Belgium. *Sites The Journal Of 20Th Century Contemporary French Studies*, 132, 41-48.
- Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95, 351-358.
- Oilveira, C. A. V. (2010). *Behavioural alterations and biomarker responses of Palaemon serratus exposed to pesticides*. *Biologia*. Aveiro University, Aveiro (Portugal).
- Ortmann, C., & Grieshaber, M. K. (2003). Energy metabolism and valve closure behaviour in the asian clam *Corbicula fluminea*. *Journal of Experimental Biology*, 206, 4167-4178.
- Pan, L. Q., Ren, J., & Liu, J. (2006). Responses of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys farreri*. *Environmental pollution*, 141, 443-51.
- Phelps, H. L. (1994). The asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, D.C. *Estuaries*, 17, 614-621.
- Pichaud, N., Pellerin, J., Fournier, M., Gauthier-Clerc, S., Rioux, P., & Pelletier, E. (2008). Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria*. *Chemistry Central journal*, 2, 23.
- Pimentel, D., Zuniga, R., & Morrison, D. (2004). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics*, 52, 273-288.
- Ramos, R., & García, E. (2007). Induction of mixed-function oxygenase system and antioxidant enzymes in the coral *Montastraea faveolata* on acute exposure to benzo(a)pyrene. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 144, 348-55.
- Reible, D. D., & Lu, X. (2007). Desorption, accumulation and elimination of sediment-associated phenanthrene and benzo(a)pyrene to a freshwater Oligochaete. *Ecotoxicology*, 26, 319-324.
- Santos, H. M., Diniz, M. S., Costa, P. M., Peres, I., Costa, M. H., Alves, S., & Capelo, J. L. (2007). Toxicological effects and bioaccumulation in the freshwater clam (*Corbicula fluminea*) following exposure to trivalent arsenic. *Environmental Toxicology*, 22, 502-509.

- Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U., & Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. *Science (New York, N.Y.)*, 313, 1072-1077.
- Soares, Amadeu M V M. (1998). Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Archives of Environmental Health*, 3, 157-163.
- Sousa, R., Nogueira, A., Gaspar, M., Antunes, C., & Guilhermino, L. (2008). Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. *Estuarine, Coastal and Shelf Science*, 80, 289-295.
- Sousa, R., Antunes, C., & Guilhermino, L. (2008). Ecology of the invasive asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: an overview. *Annales de Limnologie - International Journal of Limnology*, 44, 85-94.
- Sousa, R., Rufino, M., Gaspar, Miguel, & Antunes, C. (2008). Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Muller, 1774) in the River Minho Estuary , Portugal. *October*, 110, 98-110.
- Sousa, R. (2008). *Factors contributing to the invasive success of Corbicula fluminea (Müller, 1774)*.
- Stegeman, J. J. (1985). Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, 89, 21-30.
- Tatem, H. E. (1986). Bioaccumulation of polychlorinated biphenyls and metals from contaminated sediment by freshwater prawns, *Macrobrachium rosenbergii* and clam *Corbicula fluminea*. *Archives Environmental Contamination and Toxicology*, 15, 171-183.
- Tim-Tim, A. L. S., Morgado, F., Moreira, S., Rangel, R., Nogueira, A. J., Soares, A. M., & Guilhermino, L. (2009). Cholinesterase and glutathione S-transferase activities of three mollusc species from the NW Portuguese coast in relation to the "Prestige" oil spill. *Chemosphere*, 77, 1465-1475.
- Topping, B. R., Lopez, C. B., Croteau, M.-N., & Luoma, S. N. (2004). Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve *Corbicula*. *Environmental Science & Technology*, 38, 5002-5009.
- USEPA. (1998). Toxicological review of naphthalene. *System*.
- Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and environmental safety*, 64, 178-89.

- van der Oost, R., Beyer, J., & Vermeulen, N. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13, 57-149.
- Vasseur, P., & Cossu-Leguille, C. (2006). Linking molecular interactions to consequent effects of persistent organic pollutants (POPs) upon populations. *Chemosphere*, 62, 1033-42.
- Verrengia Guerrero, N. R., Taylor, M. G., Wider, E. a., & Simkiss, K. (2003). Influence of particle characteristics and organic matter content on the bioavailability and bioaccumulation of pyrene by clams. *Environmental pollution*, 121, 115-22. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12475068>
- Vidal, M.-laure, & Basse, A. (2001). Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. *Environmental Toxicology and Pharmacology*, 9, 87 - 97.
- Vieira, L. R., Sousa, A., Frasco, M. F., Lima, I., Morgado, F., & Guilhermino, L. (2008). Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps*, 395,87-100.
- Wang, C., Zhao, Y., Zheng, R., Ding, X., Wei, W., Zuo, Z., & Chen, Y. (2006). Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in *Sebastiscus marmoratus*. *Ecotoxicology and environmental safety*, 65, 381-7.
- Wessel, N., Ollivier, H., Goff, J. L., & Burgeot, T. (2010). The toxicity of benzo[a]pyrene on Sole (*Solea solea*) Hepatocytes: Assessment of genotoxic and enzymatic effects. *Polycyclic Aromatic Compounds*, 30, 346-354.
- Wessel, N., Santos, R., Menard, D., Le Menach, K., Buchet, V., Lebayon, N., Loizeau, V., (2010). Relationship between PAH biotransformation as measured by biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (*Solea solea*). *Marine environmental research*, 69, 71-73.
- Winston, G. W., & Giulioz, R. T. D. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic toxicology*, (19), 137-161.
- Wittmann, M., Reuter, J., Schladow, G., Hackley, S., Allen, B., Chandra, S., & Caires, A. (2008). Asian clam (*Corbicula fluminea*) of Lake Tahoe: Preliminary scientific findings in support of a management plan, (December).

CHAPTER II

Is chronic exposure to pollution able to change the physiological capability of *Corbicula fluminea* to respond to acute chemical stress?

Is chronic exposure to pollution able to change the physiological capability of *Corbicula fluminea* to respond to acute chemical stress?

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Abstract

The Asian clam *Corbicula fluminea* (Müller, 1774) is an invasive species that has been establishing in rivers from all around the world and it shows to be able to tolerate considerable levels of some environmental contaminants. This capability may act in favour of *C. fluminea* in situations of competition with native bivalves less tolerant to chemical contamination. Here, the hypothesis that individuals from the same *C. fluminea* population but inhabiting sites with distinct levels of historical contamination, respond differently to acute pollution exposure events. To test the hypothesis, animals collected in two sites of the Minho estuary under differential anthropogenic impact were exposed in two different bioassays for 96h to distinct concentrations of a model substance, the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP), after a period of acclimation in the laboratory to avoid potential delayed effects of previous field exposure. At the end of the bioassays, enzymes involved in neurotransmission, biotransformation, anti-oxidant defences, aerobic energy production and lipid peroxidation levels were used as biomarkers. In both bioassays no significant effects of BaP on cholinesterase activity were found. In relation to the results obtained in the control groups, a significant induction of the anti-oxidant enzyme catalase (CAT) by BaP was found, with a lowest observed effect concentration (LOEC) of 8 µg/L (about 2 fold) in animals from the most contaminated site (thereafter indicated as site 1) and a LOEC of 2 µg/L (about 3 fold) in animals from the less contaminated site (thereafter indicated as site 2). Animals from site 1 also showed a significant increase of two other anti-oxidant enzymes (GR and GPx) while those from site 2 did not. No significant effects on lipid peroxidation levels (LPO) were found in any of the bioassays. However, it is interesting to note a reduction of LPO at the highest concentrations tested coinciding with a reduction of the activity of glutathione S-transferases (GST) also involved in LPO prevention in animals from site 1; any of these effects were observed in clams from site 2. Another interesting finding is the significant reduction of isocitrate dehydrogenase (IDH) in animals from site 2 but not in animals from site 1; since IDH regenerates cellular NADPH which is a co-factor for glutathione reductase (GR), these findings may suggest that site 2 clams are not able to induce GR under BaP stress due to the lack of NADPH. Therefore, as a whole, the findings of the present study indicate that BaP is not an anticholinesterase agent to *C.*

fluminea and that clams from sites with different levels of historical contamination are able to overcome the oxidative stress caused by the acute exposure to BaP up to 16 µg/L avoiding lipid oxidative damage. However, the findings also suggest that clams from sites 1 and 2 have distinct capabilities of dealing with acute BaP oxidative stress: those from the most contaminated site are able to induce significantly CAT, GPx and GR, and possibly also to use GST as a toxicant scavenger being able to reduce their basal LPO levels, apparently without need of increasing significantly the production of energy through the aerobic pathway; on the contrary, animals from the less contaminated site seem not be able to significantly induce GR possibly due to a decreased capability of NADPH regeneration caused by the reduction of IDH activity and seem not use GST as a toxicant scavenger at least in the range of concentrations tested. Thus, the present study raises several hypothesis that will be important to test in order to go further on the mechanisms of toxicity and biotransformation of BaP in *C. fluminea*, also contributing to go further on the role of historical contamination in the development of tolerance to pollution in this species.

Keywords: *Corbicula fluminea*, tolerance to pollution, oxidative stress, benzo[a]pyrene, acute bioassays, biomarkers

1. Introduction

About around 18 318 species around the world have been affected by alien species, causing some of them to enter in the IUCN (International Union for Conservation of Nature) Red List as near extinction, endangered or threatened (Gurevitch and Padilla, 2004). Several reasons are contributing for the global problems caused by non-native invasive species (NIS), including both natural causes and anthropogenic related ones. In some cases, introduction of exotic species is made accidentally as an indirect result of human activities (e.g. ballast waters from ships), while in others NIS are intentionally introduced (e.g. game fish).

The Asian clam *Corbicula fluminea* (Müller, 1774) is an invasive species that has been establishing in rivers from all around the world. It is a NIS in Portugal that has been colonizing the most part of rivers all over the country (Rosa *et al.*, 2011). One of these rivers is the Minho River that is part of the border with Spain in the NW Iberian Peninsula and where *C. fluminea* is present at least

since the 1980s where it was first reported (Araujo *et al.*, 1993). At the present, it is the dominant species of the community of molluscs in the freshwater tidal ecosystem of the Minho River estuary. It is believed that its invasion has been significantly contributing for the decline of native bivalves that are now facing a serious risk of extirpation in this ecosystem (Sousa *et al.*, 2007).

Corbicula fluminea has several characteristics that act in its favour in situations of competition with other bivalves, including rapid growth, earlier sexual maturity, short life span, extensive dispersal capacities (Sousa *et al.*, 2008) that are known for a long time. More recently, it has been found that *C. fluminea* is able to tolerate considerable levels of pollution by metals, sodium sulphate, organophosphate insecticides, PCBs and PAHs (Cooper and Bidwell, 2006; Guimarães and Sígolo, 2008; Jou and Liao, 2006; Sherman *et al.*, 2009; Soucek, 2007). Therefore, this may be also a determinant factor driving its competition with native species and more knowledge on this topic is urgently need to control and mitigate adverse impacts of invasions by this species. The study of mechanisms of toxicity, biotransformation and defence against chemical stress may provide valuable knowledge on how *C. fluminea* is able to tolerate considerable levels of some environmental contaminants. Several biomarkers have been used to investigate the capability of developing resistance to pollution (Damásio *et al.*, 2007; Ross *et al.*, 2002). Among them, enzymes involved in neurotransmission, biotransformation, energy production and oxidative stress defences and damage are among the most used, since they respond to a considerable range of different compounds and the functions they are involved are crucial for the survival and performance of organisms.

In the present study, the hypothesis that individuals from the same *C. fluminea* population but inhabiting sites with distinct levels of historical contamination, respond differently to acute pollution exposure events was tested. The rationale behind the hypothesis is that the long-term exposure to pollution may lead to the development of tolerance to chemical stress, for example through an increase of the efficiency of biotransformation mechanisms, decrease of the sensitivity of molecular targets, among others. To test the hypotheses, animals collected in two sites of Minho estuary under differential anthropogenic impact were exposed in two different bioassays for 96h to distinct concentrations of a model substance, the polycyclic aromatic

hydrocarbon benzo[a]pyrene (BaP), after a period of acclimation in the laboratory to avoid potential delayed effects of previous field exposure. At the end of the bioassays, enzymes involved in neurotransmission, biotransformation, anti-oxidant defences, aerobic energy production and lipid peroxidation levels were used as biomarkers.

2. Material and Methods

2.1 Chemicals

Benzo[a]pyrene (BaP) (CAS no. 50-32-8) was purchased from Sigma-Aldrich Chemical (Germany) with 97% of purity. Chemicals for enzymatic analysis were obtained in Sigma-Aldrich Chemical (Germany), Merck (Germany) and Bio-Rad protein assay (Germany). Ultra-pure water with conductivity of 0.054 μS was used according with the protocols.

2.2 Test organisms

Corbicula fluminea specimens were captured in Minho River, during the winter and at low tide, in two different sites apparently differentially impacted by anthropogenic activities (Fig. 4): site 1 (N 41° 54' 42.00", W 8° 47' 35.30") was located not far from the village of Lanheles, downstream relatively to the towns of Vila Nova de Cerveira and Valença, and the entrance of several Minho River effluents, some of them crossing urban and industrial areas (e.g. Louro River): site 2 (N 42° 03' 05"; W 8° 33' 47") was located upstream in a lower impacted area. Clams from site 1 measured between 25 to 37 mm and those from site 2 between 26 to 36 mm (maximal size in both cases).

Temperature, O₂ dissolved and pH were measured in both sites and it was used a hand sampling net to collect the specimens. They were transported immediately to laboratory using a 32L containers with water from the site. In the laboratory, they were placed in aerated, filtered 130L glass tanks filled with dechlorinated freshwater, with photoperiod of 16h:8h (light:dark) and were acclimatized for a maximum of one week. During this period, clams were feed with 200mL *Chlorella vulgaris* and *Chlamydomonas reinhardtii* combination,

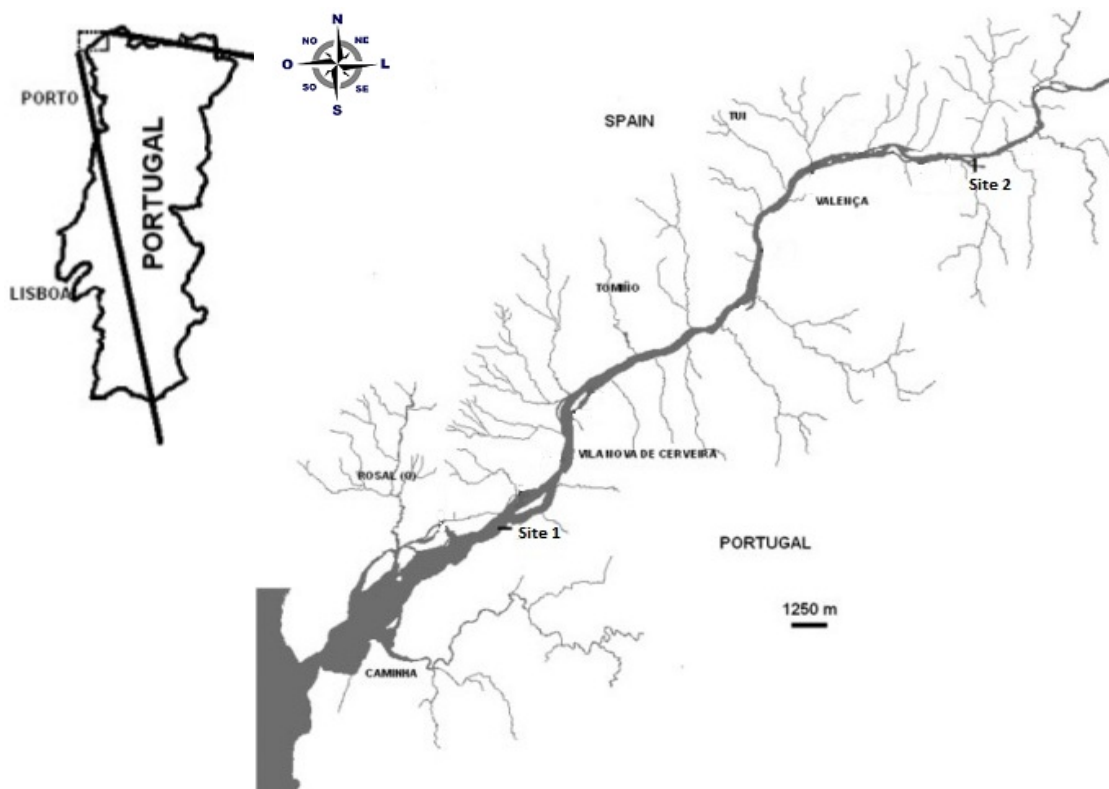


Figure 4 – Sampling sites in the Minho River (adapted from Sousa *et al.*, 2008)

prepared before, since it was the optimal food for a good maintenance of this species in laboratory conditions (Foe and Knight, 1986), and food supply stopped 48h prior to bioassays.

2.3 Laboratory bioassay

In both bioassays a stock solution of BaP was prepared in acetone (32 mg/L) and each test concentration of BaP (0.5; 1; 2; 4; 8; 16 $\mu\text{g/L}$) was obtained by serial dilution of the stock solution in acetone. After the acclimation period, 72 clams from each site (site 1- weight= 6 ± 2 g; shell length= 29 ± 2 mm; site 2- weight= 8 ± 1 g; shell length= 31 ± 2 mm) were placed inside individual glass recipients of 1000 mL with 600 mL of the proper concentration. During the experiment days the animals were not feed and the exposure period was 96h. Nine animals per treatment, exposed individually in glass recipients of 1000 mL with constant aeration, covered and protect from light to prevent photodegradation of BaP. Two controls were used with the same amount of animals, one with tap water and other with tap water+solvent prepared with 1

mL of acetone per 2000 mL of tap water. The abiotic conditions (Table 3 and 4) were monitored every 24 hours during the exposure period for test validation purpose. The water was changed every two days to prevent intoxication from ammonia, which *C. fluminea* is sensible to values of 0.54 mg/L (Sappington, 1987).

Effect criteria were the activity of the enzymes cholinesterases (ChE), involved in cholinergic neurotransmission, isocitrate dehydrogenase involved in the aerobic pathway of energy production and also involved in the anti-oxidant system, the anti-oxidant enzymes catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferases (GST) which is also involved in the biotransformation system, and lipid peroxidation levels (LPO) as marker of oxidative damage

After 96h of exposure, the tissues were removed and separated according to the type of enzyme. Therefore, after weight and shell being measured, gills and foot were separated from the body and the rest of the soft body was discarded. All the tissue isolation process was done on a ice-cold surface to prevent losses of enzymatic activities. Foot was used for IDH and ChE activities and gills for the remaining enzymes. Tissues were isolated, divided in pieces, putted in different eppendorf tubes and frozen at -80C until further preparation.

2.4 Tissue processing and enzymatic analysis

For ChE, samples were homogenized (Ystral GmbH d-7801 Dottingen homogenizer) at 4°C in 500 mL of K-phosphate buffer (pH 7.2; 0.1M) and centrifuged (Sigma, 3K30) at 3300 g for 3 min at 4°C. Supernatant was recovered and used to determine ChE activity as appropriate, by the Ellman's method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). The general procedure for ChE activity involves reacting 0.25 mL of the reaction solution [30 mL of phosphate buffer, 1 mL of the reagent dithiobisnitrobenzoate (DTNB) 10 mM and 0.2 mL of acetylcholine iodide 0.075 M] with 0.05 mL of homogenized tissue (foot). The protein concentration was 0.9 mg/mL (four replicates per sample) in a 96 well microplate. The optical density was measured at 412 nm during 5 min at 25°C.

Using another part of the foot, it was assessed the IDH activity. IDH activity is determined by the measurement of NAPH increase at 340 nm according to

Ellisa and Goldberg (1971) adapted to microplate (Lima *et al.*, 2007). The procedure consists in adding 0.2 mL of the reaction solution containing 40 mL of Tris buffer, 15 mL of Manganese (II) chloride (2 mM) and 15 mL of DL-isocitric acid (7 mM) in ultrapure water with 0.05 mL of homogenized tissue with a content of protein equal to 0.9 mg/mL (four replicates per sample). The reaction occurs when we add 0.05 mL of 0.5 mM NADP and measure immediately at 340 nm during 3 min at 25°C. To the gills eppendorfs is added K-phosphate buffer 0.1 M, pH=7.4 in a proportion of 1:10 (for each 1 g of tissue, 10 mL of buffer is added), homogenized and 250 mL is separated to another eppendorf with 4 µL of BHT (butylhydroxytoluene) 4% and frosted at -80°C. The rest of the homogenate is centrifuged at 10 000g for 20 min. (4°C). The rest of the supernatant is separated to others enzymes (50 µL to CAT, 100 µL to GR, 50 µL to GST and 100 µL to GPx) and also frosted at -80°C.

The measurement of LPO is determined by measuring the thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.*, 1979). Briefly, in a 15mL tube, 1 mL of 12% trichloroacetic acid, 0.8 mL of Tris-HCl (60 mM) pH 7.4 with DTPA 0.1 mM and 1 mL of 0.73% thiobarbituric acid were added to 0.2 mL of homogenate. Then the samples goes to an incubation for about 60min. (100°C) and the 2mL of this is removed and placed on a 2 mL tube and centrifuged at 12 000 g for 5 minutes. LPO levels are then determined reading the absorbance at 535 nm and expressed in nmol TBARS/g fresh weight. The rest of the homogenate is used for antioxidant enzyme measurement. Catalase activity was quantified by the H₂O₂ consumption at 240nm (Claiborne, 1985), where 0.950 mL of phosphate buffer (0.05) M pH 7.0 and 0.5 mL H₂O₂ (30 mM) were added to 0.05 mL of gill sample PMS (post-mitochondrial supernatant) and the enzymatic activity was measured during 30s in kinetic reaction at 240 nm (25°C).

Glutathione reductase (GR) activity is the reduction of GSSG to GSH with consumption of NADPH to NADP⁺ which is measured at 340 nm. It involves the reaction of 0.9 mL of reaction buffer [110 mL k-phosphate buffer 5mM, pH= 7.0 with nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulfide (GSSG) and DTPA] with 0.1 mL of gill supernatant. the reaction is kinetic and is read within 60 sec. at 340 nm.

GST activity is measured by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al.*, 1974) adapted to microplate (Guilhermino *et al.*, 1996). It was used 0.25 mL of reaction solution

[48 mL of phosphate buffer (0.2 M) pH 6.5, 1.521 mL of CDNB (60 mM) in ethanol and 8.78 mL of GSH (10 mM) in ultra-pure water] and added to 0.05 mL of gill supernatant, with protein concentrations of 0.9 mg mL⁻¹. The kinetic reaction was measured at 340 nm, every 21 sec. and during 5 min. and 16 seconds at 25°C.

To measure the activity of GPx, it was assessed the decrease in NADPH at 340 nm using H₂O₂ as substrate (Flohé *et al.*, 1973). This enzyme is measure in a indirect way, since it uses the glutathione reductase (GR) to measure the reduction of GSSG to GSH that was previously produced by GPx. The procedure used involves adding 0.8 mL of phosphate buffer 0.05 mM pH 7.0 with 1 mM EDTA, 1 mM sodium azide and 1 U/mL GR; 0.05 mL of GSH 4 mM; 0.05 mL of NADPH 0.8 mM; 0.01 mL of 0.5 mM H₂O₂ to 0.09 mL of gill supernatant. The kinetic reaction is then read at 340 nm for 1 min. A spectrophotometer (SpectraMax M2^e) was used to determine all enzymes activities (including microplate enzymes) and also LPO levels. All microplate enzymes were done with the quantification of protein as described by Bradford (1976) and adapted to microplate (Guilhermino *et al.*, 1996). Bovine γ-globulins (Sigma-Aldrich, USA) were used as standard and the readings were done at 600 nm. The dying was prepared with 0.25 mL of Bradford reagent (1 mL of Bradford reagent to 4 mL of ultra pure water) and added to 0.01 mL of clams protein sample. The readings were done after 15 min. of agitation and protect from light.

3. Results

3.1 Data analysis

All data analysis were performed using SPSS Statistics 17.0[®] software package. Test of homogeneity of variances (Levene Statistic) was performed to assess the homogeneity of variances. For each bioassay, one-way analysis of variance (ANOVA) was performed to check differences between the tested concentrations. The Dunnett test was used to assess differences between control+solvent and each of the BaP concentrations and to determine the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of BaP for each of the biomarkers.

3.2 Abiotic parameters

The results of the abiotic parameters measured along the bioassay are shown in tables 3 and 4. In both bioassays, the water dissolved oxygen was always above 9 mg/L, the pH variations was lower than 1 unit and the water temperature variation was lower than 1°C. Furthermore, no mortality occurred in any of the controls. Therefore, both bioassays were considered valid.

Table 3- Abiotic parameters from clams site 1 during four days of exposure to benzo[a]pyrene

	Benzo[a]pyrene concentrations (µg/L)							
	0	0'	0,5	1	2	4	8	16
Dissolved Oxygen (mg/L)	9,72 ± 0,01	9,71 ± 0,05	9,70 ± 0,03	9,71 ± 0,04	9,75 ± 0,02	9,74 ± 0,03	9,73 ± 0,03	9,72 ± 0,04
pH	8,40 ± 0,01	8,39 ± 0,01	8,41 ± 0,01	8,41 ± 0,01	8,40 ± 0,01	8,41 ± 0,01	8,41 ± 0,01	8,42 ± 0,01
Temperature (°C)	16,10 ± 0,1	16,22 ± 0,14	16,16 ± 0,18	16,20 ± 0,18	16,19 ± 0,17	16,11 ± 0,10	16,23 ± 0,07	16,05 ± 0,22

Table 4- Abiotic parameters from clams site 2 during four days of exposure to benzo[a]pyrene

	Benzo[a]pyrene concentrations (µg/L)							
	0	0'	0,5	1	2	4	8	16
Dissolved Oxygen (mg/L)	10,32 ± 0,01	10,40 ± 0,01	10,46 ± 0,02	10,45 ± 0,04	10,52 ± 0,01	10,53 ± 0,01	10,55 ± 0,01	10,51 ± 0,03
pH	8,57 ± 0,01	8,56 ± 0,01	8,57 ± 0,01	8,56 ± 0,01	8,56 ± 0,01	8,57 ± 0,01	8,57 ± 0,01	8,57 ± 0,01
Temperature (°C)	16,96 ± 0,30	16,88 ± 0,32	16,75 ± 0,33	16,74 ± 0,35	16,62 ± 0,28	16,54 ± 0,28	16,51 ± 0,30	16,73 ± 0,36

3.3 Biological effects.

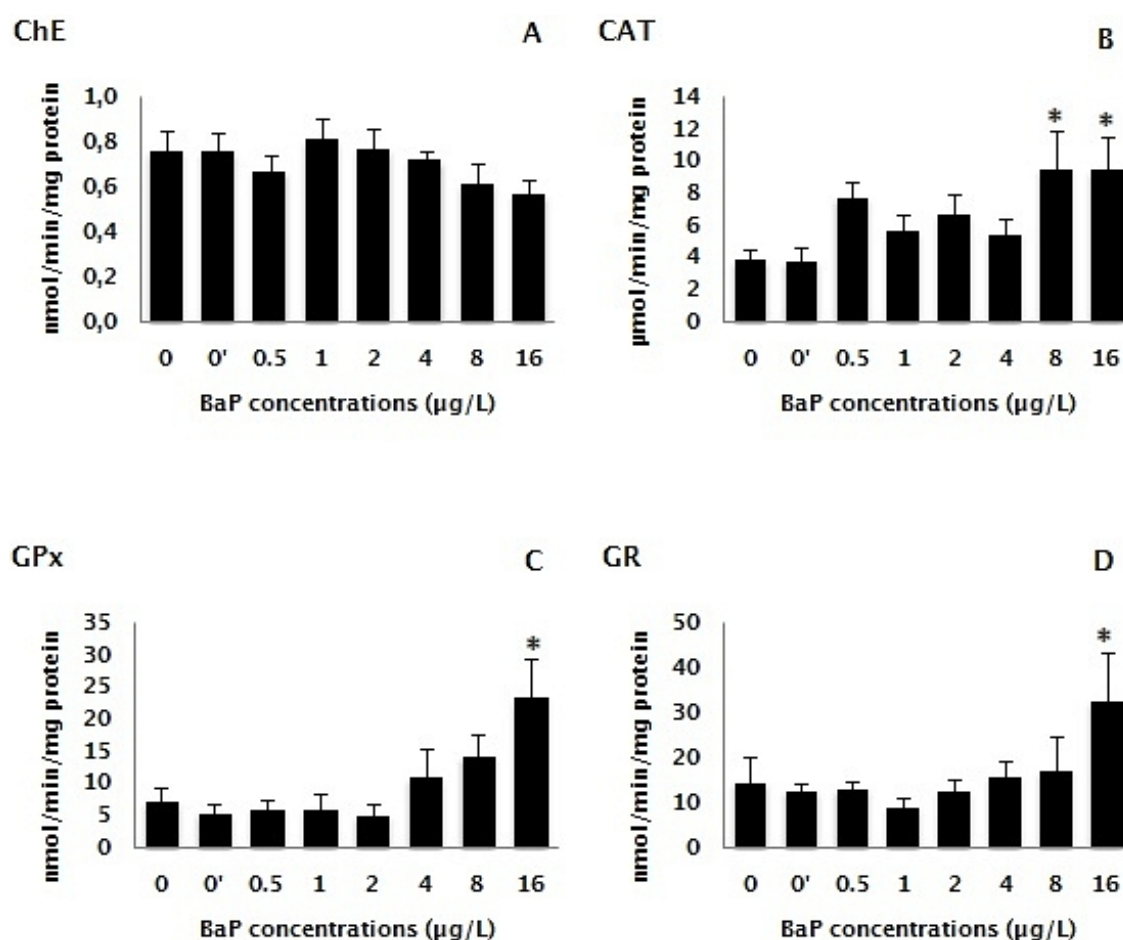
In the bioassay with animals from site 1, no mortality occurred in any of the treatments and no significant differences for any of the tested parameters were found between the control and the solvent-control groups as indicated by the one-way ANOVA comparing all the treatments done for each biomarker. Therefore, all the comparisons made to determine NOEC and LOEC values were done against the solvent-control group.

3.3.1 Effects of benzo[a]pyrene in animals from site 1

No significant differences in ChE activity were found between different treatments ($F_{(7, 71)} = 1.223$, $p = 0.303$) (Fig. 5A). Significant differences among

treatments were found for CAT activity ($F_{(7, 69)} = 3.002$, $p = 0.009$), GPx activity ($F_{(7, 66)} = 3.937$, $p = 0.001$) and GR activity ($F_{(7, 64)} = 2.223$, $p = 0.045$). The activity of CAT (Fig. 5B) was increased by BaP exposure with NOEC and LOEC values of 4 and 8 $\mu\text{g/L}$, respectively, and about 2,5 fold increase at the two highest concentrations tested. The activity of GPx (Fig. 5C) and GR (Fig. 5D) were also increased by BaP exposure with NOECs and LOECs of 8 and 16 $\mu\text{g/L}$, respectively, for both enzymes. The maximal induction of GPx was 4,2 folds, while that of GR was 2,5 folds.

No statistically significant effects were found for GST activity ($F_{(7, 59)} = 1,932$, $p = 0.083$) probably due to a considerable variability in the determinations but a reduction of activity is apparent at the two highest concentrations tested. (Fig. 5E). Lipid peroxidation (LPO) was not significantly affected by BaP ($F_{(7, 71)} = 2,050$, $p = 0.062$) (Fig. 5G), despite a reduction found in animals exposed to the highest concentrations. No significant differences in IDH activity were found among treatments ($F_{(7, 68)} = 1.089$, $p = 0.381$) (Fig. 5F).



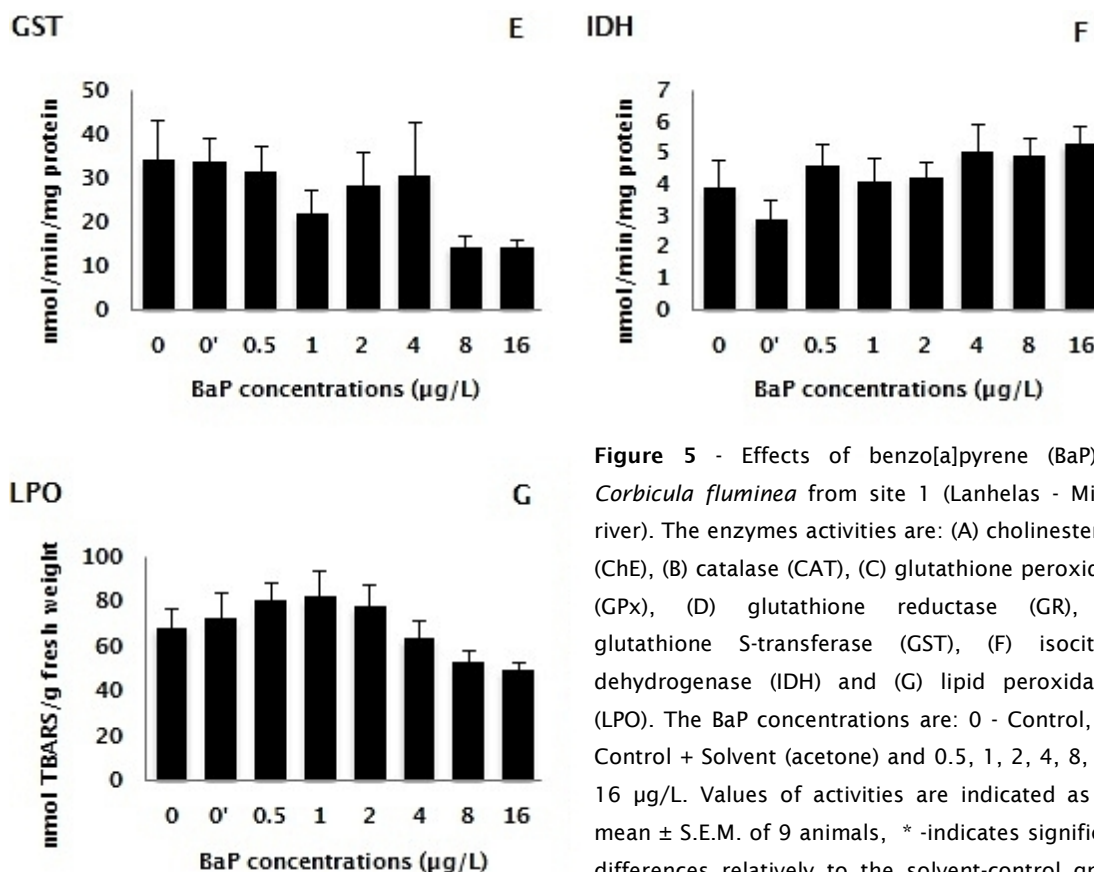


Figure 5 - Effects of benzo[a]pyrene (BaP) in *Corbicula fluminea* from site 1 (Lanhelas - Minho river). The enzymes activities are: (A) cholinesterase (ChE), (B) catalase (CAT), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR), (E) glutathione S-transferase (GST), (F) isocitrate dehydrogenase (IDH) and (G) lipid peroxidation (LPO). The BaP concentrations are: 0 - Control, 0' - Control + Solvent (acetone) and 0.5, 1, 2, 4, 8, and 16 µg/L. Values of activities are indicated as the mean $\pm$ S.E.M. of 9 animals, * -indicates significant differences relatively to the solvent-control group (0') ($p \leq 0.05$ Dunnett test)

3.3.2 Effects of benzo[a]pyrene in animals from site 2

No significant differences in ChE activity were found between different treatments ($F_{(7, 71)} = 0,470$, $p = 0.852$) (Fig. 6A). Significant differences among treatments were found for CAT activity ($F_{(7, 68)} = 6,674$, $p = 0.000$) (Fig. 6B) but no significant differences were found in GPx activity ($F_{(7, 66)} = 4,189$, $p = 0.001$) (Fig. 6C) and GR activity ($F_{(7, 65)} = 1,664$, $p = 0.136$) (Fig. 6D). The activity of CAT was increased by BaP exposure with NOEC and LOEC values of 1 and 2 µg/L

respectively, and about 3,8 fold increase at concentration of 4 µg/L, which has the highest activity value. No statistically significant effects were found for GST activity ($F_{(7, 67)} = 0,693$, $p = 0.678$) (Fig. 6E) but IDH ($F_{(7, 64)} = 4,111$, $p = 0.001$) suffer a statistically significant difference in all BaP treatments, with the NOEC and LOEC being 0' and 2 µg/L respectively (Fig. 6F). Lipid peroxidation (LPO) was not significantly affected by BaP ($F_{(7, 70)} = 1,744$, $p = 0.115$) (Fig. 6G).

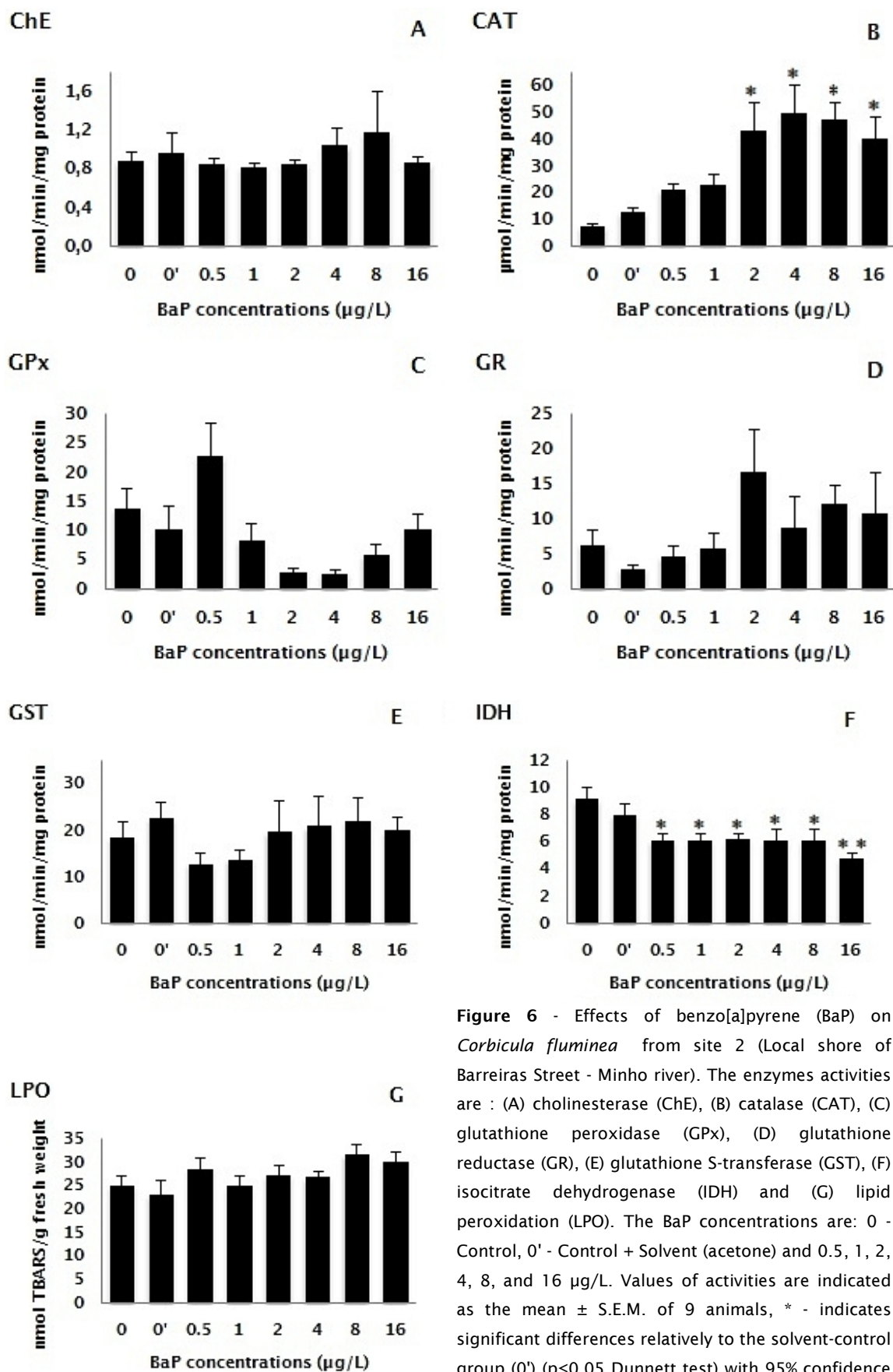


Figure 6 - Effects of benzo[a]pyrene (BaP) on *Corbicula fluminea* from site 2 (Local shore of Barreiras Street - Minho river). The enzymes activities are : (A) cholinesterase (ChE), (B) catalase (CAT), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR), (E) glutathione S-transferase (GST), (F) isocitrate dehydrogenase (IDH) and (G) lipid peroxidation (LPO). The BaP concentrations are: 0 - Control, 0' - Control + Solvent (acetone) and 0.5, 1, 2, 4, 8, and 16 µg/L. Values of activities are indicated as the mean $\pm$ S.E.M. of 9 animals, * - indicates significant differences relatively to the solvent-control group (0') ($p \leq 0.05$ Dunnett test) with 95% confidence interval and ** - means significant differences observed from control group (0') ($p \leq 0.01$ Dunnett test)

4. Discussion

The concentrations tested on the present study may be considered ecologically relevant since they have been found in sediments, water column and estuaries contaminated with petrochemical products (Vieira *et al.*, 2008).

In both bioassays, no significant effects of BaP on ChE activity of *C. fluminea* were found indicating that the toxicant is not an anticholinesterase agent to this species. This result is in good agreement with the findings of previous works on other species (Akcha *et al.*, 2000; Pan, Ren, & J. Liu, 2006; Pichaud *et al.*, 2008; R. Ramos & García, 2007; Wessel, Ollivier, *et al.*, 2010). However, significant inhibition of BaP has been also reported in the literature. For example, a significant inhibition on *Pomatochistus microps* ChE activity was found at concentrations similar to those tested here (Vieira *et al.*, 2008). Species differences in ChE sensitivity or in metabolites formed, among other factors, may contribute to explain these apparent contradictory effects.

In clams from both sites 1 and 2, BaP induced oxidative stress as indicated by the induction of CAT activity but animals seem to have been able to overcome the situation since no increase of LPO was found indicating no increased lipid oxidative damage after exposure to the toxicant. However, a careful analysis of the results indicates differences in responses to oxidative stress between animals from site 1 and site 2. Site 1 clams were able to significantly induce CAT, GPx and GR, and possibly to use GST as a toxicant scavenger by binding to the enzyme at the highest concentrations tested as the reduction of enzymatic activity seems to suggest; the decrease of LPO to levels lower than those found in controls seems to support this hypothesis. Clams of site 2 clearly respond to toxicant exposure by increasing CAT activity even at lower concentrations (LOEC= 2 µg/L) than those needed to cause a significant induction of CAT in animals from site 1 (LOEC= 8 µg/L); however they seem not be able to induce GPx and GR, at least so much as clams from site 1, they show no evidences of GST activity decrease. However, the variability of the results makes difficult the interpretation. An interesting difference between the two groups of animals is on the response of IDH activity. No significant differences among treatments were found in the bioassay with animals from site 1 suggesting that they did not need to significantly increase the aerobic pathway of energy production to face chemical stress. However, animals from site 2 show a significant reduction of the enzyme activity at all the

concentrations tested that is not concentration-dependent. Since IDH regenerates cellular NADPH which is a co-factor for glutathione reductase (GR), these findings may suggest that site 2 clams are not able to induce GR under BaP stress due to the lack of NADPH. Therefore, although both groups of animals manage to overcome oxidative stress in the concentrations tested, they seem to respond differently to it. Additional studies are necessary to test the hypothesis raised by the present findings.

In general the results of the present study are in good agreement with the effects of BaP in several species that have been reported in other laboratorial studies. For example, induction of CAT activity after exposure to BaP was found in the subtropical coral *Montastraea faveolata* (Ramos e Garcia, 2007) and in common goby *Pomatoschistus microps* (Vieira *et al.*, 2008). As in clams from site 1, inductions of GR and GPx activities were found in japanese scallop *Chlamys farreri* and rockfish *Sebastiscus marmoratus* (Pan *et al.*, 2006; C. Wang *et al.*, 2006). Also in Vieira *et al.* (2008) there's a significantly induction of anti-oxidant enzymes by BaP were CAT activity decreases at concentrations of 4 µg/L.

GST is involved in phase II of the metabolism process. It catalyses the conjugation of endogenous substances and xenobiotics with (glutathione) GSH. The GSH plays an important role on preventing damage from ROS to cells. Induction of GST has been observed in *P. microps* exposed to BaP suggesting that GSH conjugation is involved in BaP removal (Vieira *et al.*, 2008). Moreover, Maria and Bebianno (2011) stated that the absence of GST activity is due to GR inhibition that recycles the GSH. If there's no GR, GSH is not produced and GST activity is indirectly inhibited. Also, in *Sebastiscus marmoratus* fish, BaP did not induce or inhibit GST (Wang *et al.*, 2006), which is also in accordance with the results of the present work for both populations. Therefore, the mechanism of GST induction or inhibition does not respond in the same way in various animals. Although there's a tendency to an inhibitory effect of GST in population from site 1, this effect is not significant when compared to control. BaP has five rings and it seems to be a clear connection between GST activity and the numbers of rings that a PAH has (Ramos and García, 2007). Although BaP had no significant effect on GST activity, GR was statistically significantly affected at concentrations of 16 µg/L in population from site 1 but not from site 2. An increase of GR activities is normal when there's an evident effect of BaP toxicity (Maria and Bebianno, 2011; Vieira *et al.*, 2008) even though in the

present work, we can't say for sure that this is case. In the same way of GR, which interferes with the prevention of oxiradical formation, GPx also acts as a defence system that inhibits the formation of these compounds. This enzyme catalyses the metabolism of H_2O_2 to water and at the same time it oxidizes the GSH to GSSG (van der Oost *et al.*, 2003). Statistically significant levels of GPx activities and BaP were found in population from site 1 at concentration of 16 $\mu\text{g/L}$ and this was also verified in another study where BaP induce the activity of GPx (Pan *et al.*, 2006; Wang *et al.*, 2006) at the same concentrations exposed in the present work. The activity of GPx seems to be affected by seasons, meaning that high temperatures affect the activity by inhibiting it and at low temperatures it has no effect in activity (Laura *et al.*, 2009). The values obtained can also be related to levels of GR because when the activity of GR is low or inhibited, the recycling of GSH is not done and the levels of GPx are depleted (Maria and Bebianno, 2011).

The neurotransmitter AChE, is known to be highly sensitive to organophosphate (OP) and carbamate pesticides has several studies indicate (Beltran and Pocsidio, 2010; Guilhermino *et al.*, 1996; Mora *et al.*, 1999; Oliveira, 2010; Soares, 1998) but when considering the effects that PAHs have in AChE activity, the literature is somewhat confusing with articles showing inhibition Vieira *et al.* (2008). In the present work, only ChE activity was assessed and it shows that there's no inhibition from BaP in ChE activity in either populations or even a relevant tendency to such effect.

The levels of LPO remain practically unchanged in every concentration and in both populations studied. Despite the levels of antioxidant enzymes changed, lipid membrane damages were not significant. A major function of GPx is protecting membranes from damage due to LPO (van der Oost *et al.*, 2003) and roughly analyzing the data from both GPx and LPO levels in population from site 1, we can see that when there is an increase in GPx activity there is a decrease in LPO levels, although in population from site 2 this is not clear.

IDH activity was the enzyme that was most affected, with a slightly increase in population from site 1 and with a significant decrease in population from site 2 to all concentrations. This enzyme catalyses oxidative decarboxylation of isocitrate to 2-oxoglutarate requiring NAD^+ or $NADP^+$. It regenerates NADPH that is a key component of GR to maintain the cellular redox state (Lima *et al.*, 2007). An increase of IDH activity was also observed by Lima *et al.* (2007) in an exposure of *M. galloprovincialis* to petrochemical compounds in the field.

Table 5- Enzymatic activities from BaP exposure (d.w. - dry weight). Levels presented are those with statistical significance at lowest concentration.

Enzymatic activities of some organisms exposed to BaP								
	AChE	CAT	GST	IDH	GPx	GR	LPO	References
<i>Mytilus galloprovincialis</i>	<u>Concentration</u> 1.1 ng/kg d. w. <u>Level</u> 5 (nmol/min/mg protein)	<u>Concentration</u> 1.1 ng/kg d. w. <u>Level</u> 42 (nmol/min/mg protein)	<u>Concentration</u> 1.1 ng/kg d. w. <u>Level</u> 158 (nmol/min/mg protein)	_____	_____	_____	_____	(Akcha <i>et al.</i> , 2000)
	<u>Concentration</u> 19 µg/L <u>Level</u> 0.015 (µmol/min/mg protein)	<u>Concentration</u> 19 µg/L <u>Level</u> 0.015 (µmol/min/mg protein)	<u>Concentration</u> 19 µg/L <u>Level</u> 250 (µmol/min/mg protein)	_____	_____	_____	_____	(Banni <i>et al.</i> , 2010)
<i>Chlamys ferrari</i>	_____	_____	_____	_____	<u>Concentration</u> 0.5 µg/L <u>Level</u> 13 (nmol/min/mg protein)	_____	<u>Concentration</u> 10 µg/L <u>Level</u> 0.6 MDA contents nmol/min/mg	(Pan, Ren, and Liu, 2006)
<i>Mytilus galloprovincialis</i>	_____	<u>Concentration</u> 10 µg/L <u>Level</u> 90 (µmol/min/mg protein)	<u>Concentration</u> 10 µg/L <u>Level</u> 50 (µmol/min/mg protein)	_____	<u>Concentration</u> 10 µg/L <u>Level</u> 90 (µmol/min/mg protein)	<u>Concentration</u> 10 µg/L <u>Level</u> 115 (µmol/min/mg protein)	_____	(Maria and Bebianno, 2011)
<i>Montastraea faveolata</i>	_____	<u>Concentration</u> 0.1 ppm <u>Level</u> 7 (µmol/min/mg protein)	<u>Concentration</u> 0.1 ppm <u>Level</u> 1.7 (µmol/min/mg protein)	_____	_____	_____	_____	(Ramos and García, 2007)
<i>Sebastiscus marmoratus</i>	_____	_____	_____	_____	<u>Concentration</u> 0.5mg/Kg <u>Level</u> 1.6 (µmol/min/mg protein)	_____	_____	

5. Conclusion

In conclusion, the results of this study indicate that BaP is not an anticholinesterase agent to *C. fluminea*, that this PAH is able to cause oxidative stress in this species at concentrations in the low $\mu\text{g/L}$ range, but not significant lipid peroxidation damage up to 16 $\mu\text{g/L}$. The comparison of animals from two different levels of historical contamination suggest that although both groups are able to overcome oxidative stress with no lipid peroxidation damage in the range of concentrations tested, suggests that clams from sites 1 and 2 have distinct capabilities of dealing with acute BaP oxidative stress: those from the most contaminated site (site 1 - near Lanhelas town) are able to induce significantly CAT, GPx and GR, and possibly also to use GST as a toxicant scavenger being able to reduce their basal LPO levels, apparently without need of increasing significantly the production of energy through the aerobic pathway; on the contrary, animals from the less contaminated site (site 2 - near Barreiras street) seem not be able to significantly induce GR possibly due to a decreased capability of NADPH regeneration caused by the reduction of IDH activity and seem not use GST as a toxicant scavenger at least in the range of concentrations tested. Thus, the present study raises several hypothesis that will be important to test in order to go further on the mechanisms of toxicity and biotransformation of BaP in *C. fluminea*, also contributing to go further on the role of historical contamination in the development of tolerance to pollution in this species.

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References

- Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., & Narbonne, J. (2000). Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*, 49, 269-287.
- Alpert, P., Bone, E., & Holzapfel, C. (2000). Invasiveness, invasibility and the role of environmental stress in the spread of non-native plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3, 52-66.
- Bagatini, Y. M., Higuti, J., & Benedito, E. (2007). Temporal and longitudinal variation of *Corbicula fluminea* (Mollusca, Bivalvia) biomass in the Rosana Reservoir, Brazil. *Acta limnology*, 19, 357-366.
- Banni, M., Negri, a, Dagnino, a, Jebali, J., Ameer, S., & Boussetta, H. (2010). Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. *Ecotoxicology and Environmental safety*, 73, 842-8.
- Basack, S. B., Oneto, M. L., Fuchs, J. S., Wood, E. J., & Kesten, E. M. (1998). Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorus pesticides. *Bulletin of environmental contamination and toxicology*, 61, 569-76.
- Baumard, P., Budzinski, H., Garrigues, P., Sorbe, J. C., Burgeot, T., & Belloq, J. (1998). Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. *Marine Pollution Bulletin*, 36, 951-960.
- Belfroid, C., Vethaak, A. D., Lahr, J., Schrap, S. M., Rijs, G. B. J., Gerritsen, A., Boer, J. D., (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere*, 59, 511-524.
- Beltran, K. S., & Pocsidio, G. N. (2010). Acetylcholinesterase activity in *Corbicula fluminea* Muller, as a biomarker of organophosphate pesticide pollution in Pinacanauan River, Philippines. *Environmental Monitoring and Assessment*, 165, 331-40.
- Blokhina, O., Virolainen, E., & Fagerstedt, K. V. (2003). Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. *Annals of Botany*, 91, 179-194.
- Boltovskoy, D., Izaguirrel, I., & Correa, N. (1995). Feeding selectivity of *Corbicula fluminea* (Bivalvia) on natural phytoplankton. *Hydrobiologia*, 312, 171-182.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72, 248-54.

- Brink, L. A. (2001). Molluscs: Bivalvia. *Identification Guide to Larval Marine Invertebrates of the Pacific Northwest*. Oregon: University of Oregon Libraries (129-149).
- Bucheli, T., & Fent, K. (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, 25, 201-268.
- Byers, J. E. (2000). Competition between two estuarine snails: implications for invasions of exotic species. *Ecology*, 81, 1225.
- Cairrão, E., Couderchet, M., Soares, A M V M, & Guilhermino, L. (2004). Glutathione-S-transferase activity of *Fucus* spp . as a biomarker of environmental contamination. *Aquatic Toxicology*, 70, 277-286.
- Chainho, P., Chaves, M. L., Costa, J. L., Costa, M. J., & Dauer, D. M. (2008). Use of multimetric indices to classify estuaries with different hydromorphological characteristics and different levels of human pressure. *Marine Pollution Bulletin*, 56, 1128-1137.
- Cherry, D. S., Jr, J. H. R., Graney, R. L., & Jr, J. C. (1980). *Dynamics and control of the asiatic clam in the New River, Virginia*. *Dynamics and Control*, 123, 1-43.
- Cheung, C. C. C., Siu, W. H. L., Richardson, B. J., De Luca-Abbott, S. B., & Lam, P. K. S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. *Environmental pollution*, 128, 393-403.
- Choy, E. J., Jo, Q., Moon, H.-B., Kang, C.-K., & Kang, J.-C. (2006). Time-course uptake and elimination of benzo(a)pyrene and its damage to reproduction and ensuing reproductive outputs of Pacific oyster, *Crassostrea gigas*. *Marine Biology*, 151, 157-165.
- Colautti, R. I., & Macisaac, H. J. (2004). A neutral terminology to define “invasive” species. *Diversity and Distributions*, 10, 135-141.
- Connors, D. E. (2004). *Biomarkers of oxidative stress in freshwater clams (Corbicula fluminea) as mechanistic tools to evaluate the impairment of stream ecosystem health by lawn care pesticides*. The International Journal on the Biology of Stress. University of Georgia, Athens, Georgia (USA).
- Cooper, N. L., & Bidwell, J. R. (2006). Cholinesterase inhibition and impacts on behavior of the asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide. *Aquatic toxicology (Amsterdam, Netherlands)*, 76, 258-267.
- Devin, S., & Beisel, J.-N. (2006). Biological and ecological characteristics of invasive species: a gammarid study. *Biological Invasions*, 9, 13-24.
- Diane, L., & Samuel, M. (1978). *The freshwater asian clam Corbicula fluminea as a factor affecting nutrient cycling in the Chowan River, N.C*. *Water Resources Research*.

- Dong, W., Wang, L., Thornton, C., Scheffler, B. E., & Willett, K. L. (2008). Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in *Fundulus heteroclitus*. *Aquatic Toxicology*, 88, 289-300.
- EPA. (2011). Benzo(a)pyrene (BaP) (CASRN 50-32-8). Retrieved from <http://www.epa.gov/iris/subst/0136.htm>
- Elliott, P. (2008). The asian clam (*Corbicula fluminea*) in the River Thames, London, England. *Aquatic Invasions*, 3, 54-60.
- Ellisa, G., & Goldberg, D. M. (1971). An improved manual and semi-automatic assay for NADP-dependent isocitrate dehydrogenase activity, with a description of some kinetic properties of human liver and serum enzyme. *Clinical Biochemistry*, 4, 175-185.
- Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric of acetylcholinesterase determination. *Biochemical Pharmacology*, 7, 88-95.
- Fernandes, F. A. (2005). The use of biomarkers in aquatic toxicology studies. *Revista Portuguesa de Zootecnia*, 12, 67-86.
- Flohé, L., Gunzler, W. A., & Schock, H. H. (1973). Glutathione Peroxidase: A selenoenzyme. *FEBS Letters*, 32, 32-34.
- Foe, C., & Knight, A. (1986). Growth of *Corbicula fluminea* (bivalvia) fed artificial and algal diets. *Hydrobiologia*, 133, 155-164.
- Fonseca, V. F., Franc, S., Serafim, A., Company, R., Lopes, B., Bebianno, M. J., & Cabral, H. N. (2011). Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. *Aquatic Toxicology*, 102, 216-227.
- Frasco, M. F., & Guilhermino, L. (2003). Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. *Fish Physiology and Biochemistry*, 26 149-156.
- Galloway, T. S., & Depledge, M. H. (2001). Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology (London, England)*, 10, 5-23. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11227817>
- Gravato, C., Guimarães, L., Santos, J., Faria, M., Alves, A., & Guilhermino, L. (2010). Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety*, 73, 524-33.
- Grueiro-Noche, G., Andrade, J. M., Muniategui-Lorenzo, S., López-Mahía, P., & Prada-Rodríguez, D. (2010). 3-Way pattern-recognition of PAHs from Galicia (NW Spain) seawater samples after the Prestige's wreck. *Environmental Pollution (Barking, Essex: 1987)*, 158, 207-14.

- Guilhermino, L., Lopes, M. C., Carvalho, A. P., & Soares, Amadeu M.V. (1996). Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia Magna*. *Chemosphere*, 32, 727-738.
- Guo, H., Peng, F., Ma, L., Jiang, Z., & Liu, X. (2011). Hydrolysis technology optimization of phorbol esters by orthogonal experiment. *China journal of Chinese Materia Medica*, 36, 446-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/21617929>
- Gurevitch, J., & Padilla, D. K. (2004). Are invasive species a major cause of extinctions? *Trends in ecology & evolution (Personal edition)*, 19, 470-4.
- Gómez-Mendikute, A., Etxeberria, A., Olabarrieta, I., & Cajaraville, M. P. (2002). Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. *Marine Environmental Research*, 54, 431-6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12408598>
- Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*, 249, 7130-7139.
- Havelková, M., Randák, T., Zlábek, V., Krijt, J., Kroupová, H., Pulkrabová, J., & Svobodová, Z. (2007). Biochemical markers for assessing aquatic contamination. *Sensors*, 7, 2599-2611.
- Kane, A. S. (2004). Applied Toxicology Xenobiotics. *Pathobiology*.
- Karatayev, A., Burlakova, L., Kesterson, T., & Padilla, D. (2003). Dominance of the asiatic clam, *Corbicula fluminea* (Muller), in the benthic community of a reservoir. *Journal of Shellfish Research*, 22, 487-493.
- Kennedy, V. S., & Huekelem, L. V. (1985). Gametogenesis and larval production in a population of the introduced asiatic clam, *Corbicula* sp. (Bivalvia: Corbiculidae), in Maryland. *Biological Bulletin*, 168, 50.
- Khairy, M. A., Kolb, K., Mostafa, A. R., EL-Fiky, A., & Bahadir, M. (2009). Risk assessment of polycyclic aromatic hydrocarbons in a mediterranean semi-enclosed basin affected by human activities (Abu Qir Bay, Egypt). *Journal of Hazardous Materials*, 1-42.
- Kolar, C. S., & Lodge, D. M. (2001). Progress in invasion biology: predicting invaders. *Trends in ecology & evolution (Personal edition)*, 16, 199-204. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11245943>
- Komaru, A., & Konishi, K. (1999). Non-reductional spermatozoa in three shell color types of the freshwater clam *Corbicula fluminea* in Taiwan. *Zoological Science*, 16, 105-108.
- Lima, I., Moreira, S. M., Rendon-von Osten, J., Soares, A. M., & Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere*, 66, 1230-1242. Elsevier. Retrieved from <http://cat.inist.fr/?aModele=afficheN&cpsidt=18461045>

- Laetz, C., Baldwin, D. H., Collier, T. K., Hebert, V., Stark, J. D., & Scholz, N. L. (2009). The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered Pacific salmon. *Environmental Health Perspectives*, 117, 348-353.
- Laura, G., Luís, S. M., Joana, S., Carlos, G., & Lúcia, G. (2009). Yellow eel (*Anguilla anguilla*) development in NW Portuguese estuaries with different contamination levels. *Ecotoxicology*, 18, 385-402.
- Legeay, A., Achard-Joris, M., Baudrimont, M., Massabuau, J.-C., & Bourdineaud, J.-P. (2005). Impact of cadmium contamination and oxygenation levels on biochemical responses in the asiatic clam *Corbicula fluminea*. *Aquatic toxicology (Amsterdam, Netherlands)*, 74, 242-53.
- Lements, W. I. H. C., & Carlisle, D. M. (2003). Growth and secondary production of aquatic insects along a gradient of Zn contamination in Rocky Mountain streams. *North America Benthological Society*, 22, 582-597.
- Livingstone, D. R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 120, 43-9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9773498>
- Livingstone, D. R. (2003). Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Revue Medicine Veterinaire*, 6, 427-430.
- Maria, V. L., & Bebianno, M. J. (2011). Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 154, 56-63.
- McDowell, S., & Turner, D. (2002). Reproductive effort in invasive and non-invasive *Rubus*. *Oecologia*, 133, 102-111.
- Mehta, S., Haight, R., Homans, F., Polasky, S., & Venette, R. (2007). Optimal detection and control strategies for invasive species management. *Ecological Economics*, 61, 237-245.
- Meire, R. O., Azeredo, A., Paulo, J., & Torres, M. (2007). Aspectos ecotoxicológicos de hidrocarbonetos policíclicos aromáticos. *Oecologia Brasil*, 11, 188-201.
- Mora, P., Fournier, D., & Narbonne, J.-F. (1999). Cholinesterases from the marine mussels *Mytilus galloprovincialis* and *M. edulis* and from the freshwater bivalve *Corbicula fluminea* Muller. *Comparative Biochemistry and Physiology*, 122, 353 - 361.
- Mora, P., Michel, X., & Narbonne, J.-F. (1999). Cholinesterase activity as potential biomarker in two bivalves. *Environmental Toxicology and Pharmacology*, 7, 253 - 260.

- Netpae, T., & Phalaraksh, C. (2009). Bioaccumulation of copper and lead in asian clam tissues from Bung Boraphet Reservoir , Thailand. *International Journal of Agriculture & Biology*, 11, 783-786.
- Nguyen, L. T., & Pauw, N. D. (2002). The invasive *Corbicula* species (Bivalvia, Corbiculidae) and the sediment quality in Flanders, Belgium. *Sites The Journal Of 20Th Century Contemporary French Studies*, 132, 41-48.
- Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95, 351-358.
- Oilveira, C. A. V. (2010). *Behavioural alterations and biomarker responses of Palaemon serratus exposed to pesticides*. Biologia. Aveiro University, Aveiro (Portugal).
- Ortmann, C., & Grieshaber, M. K. (2003). Energy metabolism and valve closure behaviour in the asian clam *Corbicula fluminea*. *Journal of Experimental Biology*, 206, 4167-4178.
- Pan, L. Q., Ren, J., & Liu, J. (2006). Responses of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys ferrari*. *Environmental pollution*, 141, 443-51.
- Phelps, H. L. (1994). The asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, D.C. *Estuaries*, 17, 614-621.
- Pichaud, N., Pellerin, J., Fournier, M., Gauthier-Clerc, S., Rioux, P., & Pelletier, E. (2008). Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria*. *Chemistry Central journal*, 2, 23.
- Pimentel, D., Zuniga, R., & Morrison, D. (2004). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics*, 52, 273-288.
- Ramos, R., & García, E. (2007). Induction of mixed-function oxygenase system and antioxidant enzymes in the coral *Montastraea faveolata* on acute exposure to benzo(a)pyrene. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 144, 348-55.
- Reible, D. D., & Lu, X. (2007). Desorption, accumulation and elimination of sediment-associated phenanthrene and benzo(a)pyrene to a freshwater Oligochaete. *Ecotoxicology*, 26, 319-324.
- Santos, H. M., Diniz, M. S., Costa, P. M., Peres, I., Costa, M. H., Alves, S., & Capelo, J. L. (2007). Toxicological effects and bioaccumulation in the freshwater clam (*Corbicula fluminea*) following exposure to trivalent arsenic. *Environmental Toxicology*, 22, 502-509.
- Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U., & Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. *Science (New York, N.Y.)*, 313, 1072-1077.

- Soares, Amadeu M V M. (1998). Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Archives of Environmental Health*, 3, 157-163.
- Sousa, R, Nogueira, A., Gaspar, M, Antunes, C, & Guilhermino, L. (2008). Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. *Estuarine, Coastal and Shelf Science*, 80, 289-295.
- Sousa, R., Antunes, C., & Guilhermino, L. (2008). Ecology of the invasive asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: an overview. *Annales de Limnologie - International Journal of Limnology*, 44, 85-94.
- Sousa, R., Rufino, M., Gaspar, Miguel, & Antunes, C. (2008). Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Muller, 1774) in the River Minho Estuary , Portugal. *October*, 110, 98-110.
- Sousa, R. (2008). *Factors contributing to the invasive success of Corbicula fluminea* (Müller, 1774).
- Stegeman, J. J. (1985). Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, 89, 21-30.
- Tatem, H. E. (1986). Bioaccumulation of polychlorinated biphenyls and metals from contaminated sediment by freshwater prawns, *Macrobrachium rosenbergii* and clam *Corbicula fluminea*. *Archives Environmental Contamination and Toxicology*, 15, 171-183.
- Tim-Tim, A. L. S., Morgado, F., Moreira, S., Rangel, R., Nogueira, A. J., Soares, A. M., & Guilhermino, L. (2009). Cholinesterase and glutathione S-transferase activities of three mollusc species from the NW Portuguese coast in relation to the “Prestige” oil spill. *Chemosphere*, 77, 1465-1475.
- Topping, B. R., Lopez, C. B., Croteau, M.-N., & Luoma, S. N. (2004). Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve *Corbicula*. *Environmental Science & Technology*, 38, 5002-5009.
- USEPA. (1998). Toxicological review of naphthalene. *System*.
- Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and environmental safety*, 64, 178-89.
- Vanderoost, R., Beyer, J., & Vermeulen, N. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13, 57-149.
- Vasseur, P., & Cossu-Leguille, C. (2006). Linking molecular interactions to consequent effects of persistent organic pollutants (POPs) upon populations. *Chemosphere*, 62, 1033-42.

- Verrengia Guerrero, N. R., Taylor, M. G., Wider, E. a, & Simkiss, K. (2003). Influence of particle characteristics and organic matter content on the bioavailability and bioaccumulation of pyrene by clams. *Environmental pollution*, 121, 115-22. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12475068>
- Vidal, M.-laure, & Basse, A. (2001). Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. *Environmental Toxicology and Pharmacology*, 9, 87 - 97.
- Vieira, L. R., Sousa, A., Frasco, M. F., Lima, I., Morgado, F, & Guilhermino, L. (2008). Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps*, 395,87-100.
- Wang, C., Zhao, Y., Zheng, R., Ding, X., Wei, W., Zuo, Z., & Chen, Y. (2006). Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in *Sebastiscus marmoratus*. *Ecotoxicology and environmental safety*, 65, 381-7.
- Wessel, N., Ollivier, H., Goff, J. L., & Burgeot, T. (2010). The toxicity of benzo[a]pyrene on Sole (*Solea solea*) Hepatocytes: Assessment of genotoxic and enzymatic effects. *Polycyclic Aromatic Compounds*, 30, 346-354.
- Wessel, N., Santos, R., Menard, D., Le Menach, K., Buchet, V., Lebayon, N., Loizeau, V., (2010). Relationship between PAH biotransformation as measured by biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (*Solea solea*). *Marine environmental research*, 69, 71-73.
- Winston, G. W., & Giulioz, R. T. D. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic toxicology*, 19, 137-161.
- Wittmann, M., Reuter, J., Schladow, G., Hackley, S., Allen, B., Chandra, S., & Caires, A. (2008). Asian clam (*Corbicula fluminea*) of Lake Tahoe: Preliminary scientific findings in support of a management plan, (December), 1-26

CHAPTER III

GENERAL DISCUSSION

1. General discussion

Emerging concern has been put into aquatic environments and how pollution affects water quality and especially aquatic biota. Several human activities introduce chemicals into aquatic environments that will cause at short/long period, prejudice to life (Schwarzenbach *et al.*, 2006) being this is harshly dangerous, since numerous activities depend directly from a healthy ecosystem and what it can produce. Rivers are a common disposal site of several sources of contamination, such as agricultural, industrial, domestic activities and numerous approaches have been conducted to understand and develop better knowledge around the subject. It's usual to find anthropogenic contaminants in river such as polycyclic aromatic hydrocarbons (PAHs), specially around industrialized areas. Their effects in aquatic biota have been studied since they are considered dangerous being integrated in the European Water Framework Directive (WFD) (Wessel *et al.*, 2010). One of the most known and also must studied PAHs is benzo[a]pyrene (BaP). It's known to change levels of some enzymes such as glutathione S-transferase (GST) , catalase (CAT) in mussels (Banni *et al.*, 2010) at concentrations reaching 19 µg/L and in some species it increases activity of glutathione peroxidase (GPx) and causes lipid damage (Pan *et al.*, 2006). In addition we can find in literature that concentrations of 3 µg/L of BaP are well correlated with LPO damage and anti-oxidant enzymatic activities of *Perna viridis* mussel (Cheung *et al.*, 2004). Organisms are capable of metabolize BaP into its metabolites that can be more harmful to the organism. Although, these metabolites are known to cause several damages including oxidative damage, they might deplete enzymatic levels of antioxidant defence system leading for example to DNA damage, a common effect of BaP exposure (Wessel *et al.*, 2010). In the present work the acute effects of (BaP) on two different groups of *Corbicula fluminea* from distinct sites of Minho River exposed to different historical contamination were assessed using biomarkers involved in different physiological functions, such as neurotransmission enzyme cholinesterase (ChE), detoxification enzymes such as GST, CAT, glutathione reductase (GR) and GPx, aerobic energy production enzyme, isocitrate dehydrogenase (IDH) and lipid peroxidation levels (LPO).

The values obtained in individuals from the most contaminated site (site 1) show statistically significant changes in CAT levels, with a lowest observed effect concentration (LOEC) of 8 µg/L, 2 fold relatively to control+solvent and also

showed a significant increase of GPx and GR at highest concentrations. LPO levels were not statistically significant, but it's interesting to see a reduction at highest concentration that coincides with the levels of GST that is also involved in the protection of cells from lipid damage.

Individuals from the less contaminated site (site 2) also showed no significant changes in cholinesterase levels but showed statically significant changes in CAT levels, with a LOEC of 2 µg/L, about 3 fold relatively to control+solvent, lesser than individuals from site 1. This indicates that chronic contamination may change how animals respond to pollution stress, because site 1 is the most contaminated site and animals have a higher LOEC than those from site 2. Also, individuals from site 2 present significant changes in levels of IDH at all BaP treatments, which is a curious effect because IDH regenerates cellular nicotinamide adenine dinucleotide phosphate reduced form (NADPH) that will be used by GR in the detoxification path and consequently affecting GST activity.

Corbicula fluminea is probably one of the most studied non native invasive species in freshwater ecosystems with several approaches around its invasive behaviour and how it occurs (Sousa, 2008). Although ecological studies around invasive process and its characteristics are important, ecotoxicological studies can be a crucial tool that might bring new insights into the matter, given that not only invasive behaviour depends on ecological processes but also on biological mechanisms that reacts to adverse environmental conditions (e.g. anthropogenic pollution).

These results may suggest that chronic exposure possibly changes the way how *Corbicula fluminea* reacts to polluted environments and that this may be considered as an advantage in its invasive behaviour. Further analysis and approaches in this subject should be considered in order to develop a better understanding on *Corbicula fluminea* biological responses when inhabiting sites that were or are been a target of environmental pressure.

References

- Banni, M., Negri, a, Dagnino, a, Jebali, J., Ameer, S., & Boussetta, H. (2010). Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. *Ecotoxicology and Environmental safety*, 73, 842-8.
- Cheung, C. C. C., Siu, W. H. L., Richardson, B. J., De Luca-Abbott, S. B., & Lam, P. K. S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. *Environmental pollution*, 128, 393-403.
- Pan, L. Q., Ren, J., & Liu, J. (2006). Responses of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys ferrari*. *Environmental pollution*, 141, 443-51.
- Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U., & Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. *Science (New York, N.Y.)*, 313, 1072-1077.
- Sousa, Ronaldo. (2008). *Factors contributing to the invasive success of Corbicula fluminea (Müller, 1774)*.
- Wessel, N., Ollivier, H., Goff, J. L., & Burgeot, T. (2010). The toxicity of benzo[a]pyrene on Sole (*Solea solea*) Hepatocytes: Assessment of genotoxic and enzymatic effects. *Polycyclic Aromatic Compounds*, 30, 346-354.