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Effects of environmental contaminants and natural stressors on feral *Carcinus maenas*: influence of the exposure history

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Aos meus “passarinhos” e à Avó.

“Fairy tales are more than true: not because they tell us that dragons exist, but because they tell us that dragons can be beaten.”

Coraline,
Neil Gaiman

“The statistics on sanity are that one out of every four people is suffering from a mental illness. Look at your 3 best friends. If they're ok, then it's you.”

Rita Mae Brown

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Author's declaration

In agreement with the Portuguese law though the article 4th of the "Regulamento Geral dos Terceiros Ciclos de Estudos na Universidade do Porto" of January 4th (GR.04/01/2014), the author states devotion in a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published or under publication articles included in this Thesis and presented below.

Publications

The following published or under publication papers were prepared under the scope of this thesis:

Rodrigues AP, Oliveira P, Guilhermino L, Guimarães L. 2012. Effects of salinity stress on neurotransmission, energy metabolism, and anti-oxidant biomarkers *of Carcinus maenas* from two estuaries of the NW Iberian Peninsula. *Marine Biology* 159:2061–74.

Rodrigues AP, Lehtonen KK, Guilhermino L, Guimarães L. 2013. Exposure of *Carcinus maenas* to waterborne fluoranthene: Accumulation and multibiomarker responses. *Science of The Total Environment* 443:454–463.

Rodrigues AP, Gravato C, Guimarães L. 2013. Involvement of the antioxidant system in differential sensitivity of *Carcinus maenas* to fenitrothion exposure. *Environmental Science: Processes & Impacts* 15:1938–48.

Rodrigues AP, Santos LH, Ramalhosa MJ, Delerue-Matos C, Guimarães L. Sertraline accumulation and effects in an estuarine decapod: importance of the history of exposure to chemical stress. *Submitted to Journal of Hazardous Materials*.

Rodrigues AP, Santos LH, Oliva-Teles MT, Delerue-Matos C, Guimarães L. Joint effects of salinity and the antidepressant sertraline in an estuarine decapod. *Submitted to Environmental Pollution.*

Rodrigues AP, Oliva-Teles MT, Mesquita SR, Delerue-Matos C, Guimarães L. Spatial and temporal variation in integrated biomarker responses of a decapod in estuaries with varying levels of abiotic stress and heavy metal contamination. *In preparation.*

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Effects of environmental contaminants and natural stressors on feral *Carcinus maenas*: influence of the exposure history

Abstract

Over the years several environmental protective measures have been discussed and applied to maintain or improve the quality of aquatic ecosystems and to regulate environmental discharges of pollutants. Nevertheless both classical and new compounds are daily produced, reaching at a given moment environmental compartments. Estuaries are unique ecosystems, with high biological productivity and richness, and extreme natural fluctuations due to their transitional characteristics. Frequently, estuaries exhibit intense urban and industrial occupation, with consequent environmental degradation. Thus, estuarine organisms are often subjected to environmental contaminants and abiotic fluctuations that may induce specific biological responses, which depend among others on characteristics of affected sites and species. However, available data concerning biological effects of chemical and natural stressors on invertebrates is still scarce considering this is the most numerous species group. Due to their ability to cause severe detrimental effects in biota many classical contaminants have been included in priority lists and knowledge on their impact in estuarine invertebrates, and temporal and spatial contamination trends, is much required. Regarding emerging compounds, their ecotoxicological effects are poorly understood and there is a lack of empirical data to improve prediction of environmental risk.

Biomarkers-based environmental monitoring provides an integrated assessment of the effects of the mixture of contaminants present in affected sites on the health and functioning of sentinel species. Moreover, biomarkers provide early-warning signs of detrimental effects useful to anticipate protective measures and prevent the impact of contaminants at higher levels of organisation when changes are often irreversible and lethal. However, this requires understanding on two important issues. One is the susceptibility of the biomarkers employed to abiotic variation. The

other is the possible differential sensitivity and adaptation to pollution of the populations under study. Organisms chronically exposed to pollution may develop adaptive responses to deal with the chemical challenge. Such responses are vital to the sustainability of populations, communities and ecosystems, but may difficult interpretation of monitoring data. Further, increased sensitivity to new stressors often occurs in tolerant organisms. These situations may influence data interpretation and decision about the need to implement remediation measures. They may also complicate the environmental risk assessment of hazardous substances to the aquatic environment.

The central objective of this Thesis was to investigate responses and susceptibility of the key crustacean *Carcinus maenas* to environmental contaminants and to salinity (as abiotic stressor), in relation to its previous history of exposure to contamination. To reach the central objective, green crabs from Rivers Minho (low impacted) and Lima (with a history of moderate pollution levels) with different levels of contamination and anthropogenic pressure were used as experimental organisms. Laboratory experiments lasting for seven days, and seasonal and inter-annual chemical-biological effects monitoring, were performed using a battery of environmental biomarkers related to the mode of action of the test substances and suitable to investigate differential sensitivity to contamination, as well as temporal and spatial trends in health status.

Exposure to waterborne fluoranthene was first carried out with crabs collected at the Minho estuary. Biomarkers assessing bioaccumulation, cell damage, energy production and availability, and pathways of neurotransmission, detoxification and oxidative stress were employed. Relevant bioaccumulation was observed after the short exposure. Adding to this, increases in detoxification and enzymatic anti-oxidant defences in the digestive gland, as well as neurotoxic effects in the muscle, translated in inhibition of acetylcholinesterase activity, were observed at environmentally relevant concentrations. The study further provided new knowledge on exposure and assessment and effects of this priority

contaminant. It also allowed derivation of a useful battery of sensitive biomarkers for use in subsequent investigations.

Biomarkers of biotransformation and oxidative stress, as well as of neuromuscular transmission were next shown to be influenced by salinity, contributing with original knowledge on the effects of this natural stressor on physiological responses of *C. maenas* and alerting for the need to address this when using such parameters in environmental monitoring programmes. Exposure to salinity levels ranging from 4 to 45 psu of crabs from the Minho and the Lima (with a history of moderate contamination by heavy metals and polycyclic aromatic hydrocarbons among others) estuaries revealed that depending on the biomarkers and the site of origin of the crabs both hypo- and hypersalinity could influence the responses observed. Salinity stress also had more negative effects in crabs originating from the moderately polluted estuary.

Differential sensitivity of crabs from these estuaries to contamination was subsequently confirmed in exposures to the model organophosphate fenitrothion. Here crabs collected at the Lima estuary were found to be more tolerant of fenitrothion exposure showing lower lethality and inhibition of acetylcholinesterase activity. New insight was provided on the possible mechanisms of enhanced tolerance in decapods. Differential sensitivity appeared to occur by way of enhanced activity of the glutathione redox system. The data were consistent with the occurrence of physiological acclimation rather than genetic adaptation. The work raised further questions on how would Lima crabs respond to exposure to emerging contaminants of concern and whether differences observed relative to Minho crabs would be important in terms of environmental risk assessment. Because in the near future depression is predicted to be one of the three leading causes of burden of disease worldwide, the effects of sertraline in *C. maenas* were then investigated.

The experiments carried out brought up important new insight on sertraline effects and its interaction with salinity in a key decapod, with impact for environmental risk assessment. Sertraline is a widely prescribed antidepressant, which bioaccumulation potential and effects on estuarine

invertebrates are sparsely known. Exposure of crabs from both estuaries revealed that sertraline was able to bioaccumulate and elicit relevant alterations in biomarkers of neurotransmission (related to ventilatory and locomotory functions), anti-oxidant defences and oxidative damage, indicating their usefulness to assess environmental contamination by this psychopharmaceutical. However, crabs from the polluted estuary were much more sensitive than those from the low impacted one (by four orders of magnitude) and than the most sensitive freshwater species tested up–to–date using reproduction as endpoint (by about three orders of magnitude). Moreover, combined antagonistic and synergistic effects of sertraline and salinity were observed. For some biomarkers these were dependent on the concentration level tested and also influenced by the estuary of origin of the crabs. Interestingly, from the results obtained, sertraline appeared to modulate cholinergic neurotransmission in *C. maenas*. This modulation may possibly occur through the Na^+/K^+-ATPase α subunit, which is also involved in *C. maenas* osmoregulatory processes.

Finally, a monitoring study was carried out to elucidate about spatial and temporal variation in abiotic stressors and heavy metal contamination in sediments and biota, provide baseline data on these estuaries, and compare with the findings of laboratory experiments. The data indicated a decrease in metal levels in sediments and crabs' tissues from 2010 to 2012, suggesting an improvement of the quality of the polluted estuary. Despite the enhanced tolerance to fenitrothion detected previously, decreased health status of crabs from the contaminated estuary were found, consistent with their higher sensitivity to salinity stress and the emerging compound tested.

Overall, the present investigations indicated that chronic exposure in even moderately contaminated sites may elicit differential sensitivity to contamination in this decapod, even though the species has a high ability to adapt to different environments, which favours its well known invasive behaviour. Acclimation processes leading to both enhanced tolerance or sensitivity appeared to occur by way of altered responses of biotransformation and anti-oxidant defences, including the glutathione

redox system. These should be taken into consideration to improve site-specific monitoring and environmental risk assessment. Routine monitoring of the estuaries investigated in this thesis should be carried out in the future to confirm whether there is a sound improvement of environmental quality in the Lima estuary or just a transient amelioration. Finally, to improve the ecological relevance of risk estimations, ecotoxicological testing for purposes of environmental risk assessment should be broadened to include assessments of feral organisms from different backgrounds, from local representative taxonomic groups, and more sensitive biomarkers related to their mode of action, in site-specific programmes.

Efeitos de contaminantes ambientais e stressores naturais em *Carcinus maenas* selvagem: influência da história de exposição

Resumo

Ao longo dos anos várias medidas de proteção ambiental têm sido discutidas e aplicadas para manter ou melhorar a qualidade dos ecossistemas aquáticos e para regulamentar as descargas de poluentes no ambiente. No entanto, compostos clássicos e novos são produzidos diariamente, atingindo em algum momento os ecossistemas aquáticos. Os estuários são ecossistemas únicos, com elevada produtividade e riqueza biológicas, e flutuações naturais extremas devido às suas características de sistemas de transição. Frequentemente, os estuários apresentam intensa ocupação urbana e industrial, com consequente degradação ambiental. Desta forma, os organismos estuarinos estão frequentemente expostos a contaminantes ambientais e flutuações abióticas capazes de induzir respostas biológicas específicas, dependentes entre outras das características locais e dos organismos afetados. No entanto, os dados disponíveis sobre os efeitos biológicos de stressores químicos e naturais em invertebrados são ainda escassos tendo em conta que se trata do grupo de espécies mais numeroso. Devido à sua capacidade de causar efeitos nefastos no biota, vários contaminantes clássicos estão incluídos na lista de compostos prioritários, sendo necessário o estudo do seu impacte em invertebrados estuarinos, e de tendências temporais e espaciais de contaminação. No que diz respeito aos compostos emergentes, os seus efeitos ecotoxicológicos são ainda pouco conhecidos, havendo falta de dados empíricos para melhorar a previsão de avaliação de risco.

A monitorização ambiental baseada em biomarcadores fornece uma avaliação integrada dos efeitos da mistura de contaminantes na saúde e funcionamento de espécies-sentinela de locais afetados. Além disso, os biomarcadores fornecem sinais de alerta precoces e úteis de efeitos

prejudiciais, de modo a antecipar medidas de proteção e prevenir o impacte de contaminantes a níveis mais elevados de organização, onde as alterações são muitas vezes irreversíveis e letais. No entanto, a monitorização requer a compreensão de dois tópicos importantes. O primeiro referente à suscetibilidade a fatores abióticos dos biomarcadores usados. O segundo diz respeito à eventual sensibilidade diferencial e adaptação à poluição das populações em estudo. Os organismos cronicamente expostos à poluição podem desenvolver respostas adaptativas para lidar com o stresse químico. Essas respostas são fundamentais para a sustentabilidade das populações, comunidades e ecossistemas, mas podem dificultar a interpretação dos dados de monitorização. Além disso, o aumento da sensibilidade a novos stressores ocorre frequentemente em organismos tolerantes. Estas situações podem influenciar a interpretação dos dados e a decisão sobre a necessidade de implementar medidas de remediação, e complicar as avaliações de risco ambiental de substâncias perigosas para o ambiente aquático.

O objetivo central da presente Tese foi investigar as respostas e a suscetibilidade do crustáceo-chave *Carcinus maenas* a contaminantes ambientais e à salinidade (enquanto stressore abiótico), em relação à sua história prévia de exposição à contaminação. Para atingir o objetivo central, caranguejos provenientes dos estuários dos Rios Minho (pouco impactado) e Lima (com níveis moderados de poluição) foram usados como organismos experimentais. Exposições laboratoriais com a duração de sete dias e monitorização sazonal e interanual de efeitos químico-biológicos foram realizadas, utilizando uma bateria de biomarcadores ambientais relacionados com o modo de ação das substâncias testadas e adequado para investigar a sensibilidade diferencial à contaminação, bem como as tendências temporais e espaciais no estado de saúde.

A exposição a fluoranteno foi realizada com os caranguejos provenientes do estuário do Rio Minho. Foram determinados biomarcadores para avaliar a bioacumulação, o dano celular, a produção e disponibilidade de energia, e ainda a neurotransmissão, detoxificação e stresse oxidativo. Observou-se bioacumulação relevante de fluoranteno

após o período de exposição. Adicionalmente, o aumento da detoxificação e defesas antioxidativas enzimáticas na glândula digestiva ocorreram a concentrações ambientalmente relevantes, bem como efeitos neurotóxicos no músculo, ilustrados pela inibição da atividade da acetilcolinesterase. O estudo forneceu novos conhecimentos sobre a exposição e efeitos deste tóxico prioritário. Também possibilitou a derivação de um conjunto de biomarcadores útil e sensível para utilizar em investigações futuras.

Em seguida, verificou-se que a salinidade influenciou os biomarcadores de biotransformação e stresse oxidativo, bem como de transmissão neuromuscular, contribuindo com novos conhecimentos sobre os efeitos deste stressore natural em respostas fisiológicas de *C. maenas* e alertando para a necessidade de o considerar aquando do uso destes parâmetros biológicos em programas de monitorização ambiental. A exposição de caranguejos dos Rios Minho e Lima a níveis de salinidade entre 4 e 45 psu revelou que, dependendo dos biomarcadores e do local de origem dos organismos, as respostas biológicas poderiam ser influenciadas por condições de hipo- e hipersalinidade. O stresse salino teve mais efeitos nefastos em caranguejos provenientes do estuário moderadamente poluído.

A sensibilidade diferencial dos caranguejos destes estuários foi posteriormente confirmada em exposições ao organofosforado-modelo fenitrotião. Os caranguejos capturados no estuário do Rio Lima foram mais tolerantes à exposição a fenitrotião, exibindo menor mortalidade e inibição da atividade da acetilcolinesterase. Obteve-se uma nova perceção sobre os possíveis mecanismos que aumentam a tolerância em decápodes. A sensibilidade diferencial parece ocorrer devido ao aumento da atividade do sistema redox da glutationa. Os dados obtidos são consistentes com a ocorrência de aclimatação fisiológica em vez de adaptação genética. O trabalho levantou mais questões sobre como os caranguejos do Rio Lima responderiam à exposição a contaminantes emergentes de interesse e se as diferenças observadas em relação aos caranguejos do Minho seriam importantes em termos de avaliação de risco ambiental. Dado que se prevê que no futuro próximo a depressão constitua uma das três principais

causas de doença a nível mundial, os efeitos da sertralina em *C. maenas* foram então investigados.

As experiências realizadas contribuíram com novos e importantes conhecimentos sobre os efeitos da sertraline e da sua interação com a salinidade num decápode chave, com impacte na avaliação de risco ambiental. A sertralina é um antidepressivo amplamente prescrito, cujo potencial de bioacumulação e efeitos em invertebrados estuarinos são pouco conhecidos. A exposição de caranguejos provenientes dos dois estuários em estudo revelou que a sertralina bioacumulou e que foi capaz de induzir alterações relevantes nos biomarcadores de neurotransmissão (relacionada com ventilação e locomoção), defesas antioxidativas e danos oxidativos, indicando a sua utilidade para avaliar a contaminação ambiental por este psicofármaco. No entanto, os caranguejos do estuário poluído foram bastante mais sensíveis do que os do estuário pouco impactado (por quatro ordens de magnitude) e do que as espécies de água doce mais sensíveis utilizando o parâmetro reprodução (por cerca de três ordens de magnitude). Além disso, foram observados efeitos sinergísticos e antagonísticos resultantes da combinação da sertralina e salinidade. Para alguns biomarcadores os efeitos foram dependentes da concentração testada e também influenciados pelo estuário de origem dos caranguejos. Curiosamente, dos resultados obtidos, a sertralina pareceu modular a neurotransmissão colinérgica em *C. maenas*. Esta modulação pode possivelmente ocorrer através da subunidade α da Na^+/K^+-ATPase, que também está envolvida nos processos de osmorregulação em *C. maenas.*

Finalmente, um estudo de monitorização foi realizado para elucidar sobre a variação espacial e temporal de stressores abióticos e de contaminação por metais pesados em sedimentos e biota, fornecer dados basais destes estuários e comparar com os resultados provenientes das exposições laboratoriais. Os dados indicaram uma diminuição nos níveis de metais em sedimentos e tecidos dos caranguejos do ano 2010 para 2012, sugerindo uma melhoria da qualidade do estuário poluído. Apesar da maior tolerância ao fenitrotião verificada anteriormente, a diminuição do estado de saúde dos caranguejos do estuário contaminado foi

detetada, consistente com o aumento da sensibilidade ao stresse salino e ao composto emergente testado.

No geral, os presentes estudos indicaram que a exposição crónica, mesmo em locais moderadamente contaminados, pode provocar sensibilidade diferencial à contaminação neste decápode, apesar da sua ampla capacidade de adaptação a diferentes ambientes, o que favorece o seu reconhecido comportamento invasor. Os processos de aclimatação que conduzem ao aumento da tolerância ou sensibilidade parecem ocorrer por meio de alteração das respostas de biotransformação e defesas antioxidativas, incluindo o sistema redox da glutationa. Estes devem ser levados em consideração para melhorar a monitorização e a avaliação de risco ambiental. Devem-se realizar no futuro monitorizações de rotina nos estuários investigados na presente Tese, para confirmar se se trata de uma melhoria consistente da qualidade ambiental no estuário do Lima ou apenas de uma melhoria transitória. Finalmente, para melhorar a relevância ecológica de estimativas de risco, os testes ecotoxicológicos para fins de avaliação de risco ambiental devem ser alargados para incluir avaliações de organismos selvagens de diferentes origens, de grupos taxonómicos representativos locais e biomarcadores mais sensíveis relacionadas com o seu modo de ação, em programas específicos para os locais.

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Acronyms and abbreviations

5-HT	Serotonin
ABC proteins	ATP binding cassette proteins
AChE	Acetylcholinesterase
AChEg	Ganglion acetylcholinesterase
AChEm	Muscle acetylcholinesterase
ANOVA	Analysis of variance
APHA	American Public Health Association
ATCh	Acetylthiocholine
BChE	Butyrylcholinesterase
BTCh	Butyrylthiocholine
BW	Brackish water
BW284C51	1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide
CAT	Catalase
CBD	Convention of Biological Diversity
CbE	Carboxylesterase
CDNB	1-chloro-2,4-dinitrobenzene
CFCs	Chlorofluorocarbons
ChE	Cholinesterase
CLC	Civil Liability Convention
CNS	Central nervous system
CYP 450	Cytochrome P450
DO	Dissolved oxygen
DTNB	5,5'-dithio-bis-2-nitrobenzoate
dw	Dry weight
Ea	Energy available
EAP	Environmental Action Program
ECs	Emerging contaminants
ECPD	Environmental and Consumer Protection Directorate
ERL	Effects range-low
ERM	Effects range-median
ESI	Electrospray ionisation source
EU	European Union
FEN	Fenitrothion

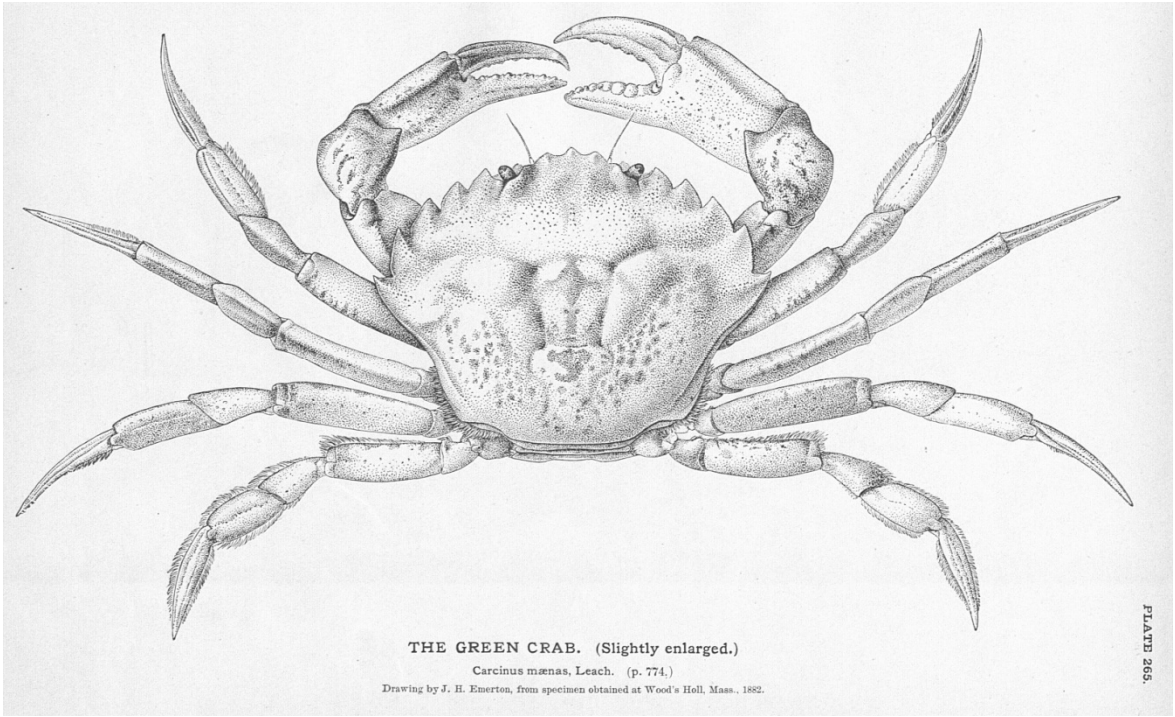
FF	Fixed wavelength fluorescence
FLU	Fluoranthene
GC–MS	Gas chromatography–mass spectrometry
GHG	Greenhouse gases
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione *S*–transferases
HighS	High salinity
HMW	High molecular weight
HPLC	High–performance liquid chromatography
IA	Independent action
IBR	Integrated biomarker responses
IC	Inhibition concentration
IDH	$NADP^+$–dependent isocitrate dehydrogenase
IMO	International Marine Organisation
IPCC	Intergovernamental Panel on Climate Change
iso-OMPA	Tetramonoisopropyl pyrophosphortetramide
K_m	Michaelis-Menten constant
LC	Lethal concentration
LDH	Lactate dehydrogenase
LMS	Lysosomal membrane stability
LMW	Low molecular weight
LOD	Limit of detection
LowS	Low salinity
LPO	Lipid peroxidation
MDL	Method detection limit
MOA	Mode of action
MRM	Multiple reaction monitoring
MSFD	Marine Strategy Framework Directive
NORS	Norsertraline
NR	Neutral red dye
NRR	Neutral red retention
OECD	Organisation for Economic Co–operation and Development
OP	Organophosphate

OSPAR Convention	Oslo and Paris Conventions for the Protection of the Marine Environment of the North-East Atlantic
PCA	Principal Component Analysis
PAHs	Polycyclic aromatic hydrocarbons
PC	Principal component
PChE	Pseudocholinesterase
PDMS	Polydimethylsiloxane
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
PPCPs	Pharmaceuticals and personal care products
PrChE	Propionylcholinesterase
PSS	Physiological saline solution
PTCh	Propionylthiocholine
ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SERT	Sertraline
SeSa	Sertraline × Salinity
SOD	Superoxide dismutase
SPE	Solid phase extraction
SSD	Species sensitivity distribution
SSRI	Selective serotonin reuptake inhibitor
SW	Saltwater
T	Temperature
TBARS	Thiobarbituric acid reactive substances
TG	Total glutathione
UDP	Uridine 5'–diphospho–glucuronosyltransferase
UHPLC-MS/MS	Ultra high-performance liquid chromatography-tandem mass spectrometry
UNCCD	United Nations Convention to Combat Desertification
UNCED	United Nations Conference on Environment and Development
UNEP	United Nations Environmental Programmes
UNFCCC	United Nations Framework Convention on Climate Change
USEPA	United States Environmental Protection Agency
UV	Ultraviolet

V_{max}	Maximal velocity
WCED	World Commission on Environment and Development
WFD	Water Framework Directive
wt	Wet tissue

Chapter I

Introduction



THE GREEN CRAB. (Slightly enlarged.)

Carcinus mænas, Leach. (p. 774.)

Drawing by J. H. Emerton, from specimen obtained at Wood's Holl, Mass., 1882.

PLATE 265.

Introduction

1. Sustainable development

In 1972 the Stockholm Declaration (1972) stated the need for common orientations to conserve and enhance the human environment, and the right to a healthy environment. This summit represented a milestone in what concerns environmental protection. A cascade of other actions was subsequently taken to address a number of preoccupations that had been raised mostly by the publication of the book “Silent Spring” by Rachel Carson (1962). The book presented many case studies related to harmful effects of chemical pesticides on the environment. The controversy and awareness instilled created the conditions that led to the 1972 agreement. From this point forward, the environment has been considered a major issue for which coordinated legislation was required to ensure a sustainable development. Consequently, in 1973 the European Union (EU) founded the Environmental and Consumer Protection Directorate (ECPD) and the first Environmental Action Program (EAP) was instituted. Later, the principles of the Stockholm Declaration constituted the basis of a new report “Our Common Future” from the United Nations World Commission on Environment and Development (WCED, 1987). Also known as Brundtland Report in honour of the WCDE chair, the Norwegian Prime Minister Gro Harlem Brundtland, it defined sustainable development as the “development that meets the needs of the present without compromising the ability of future generations to meet their own needs” (WCED, 1987). Since then, it has been recognised that sustainable development requires the integration of interrelated domains like environmental, economic, social, and political sustainability. Following these initiatives, and worrying acknowledgement of increasing environmental degradation, a crucial meeting occurred in 1992, during the United Nations Conference on Environment and Development (UNCED). This summit was held in Rio de Janeiro (Brazil), where Agenda 21 was adopted (UNCED, 1992).

Reduction of toxic compounds and harmful wastes produced replacement of fossil fuels by alternative sources of energy, reduction of vehicle/traffic emissions to limit atmospheric pollution, and water scarcity were some of the points included in Agenda 21 (UNCED, 1992). Furthermore, the greatest challenges for sustainable development were pointed out and other agreements prepared to face climate change, loss of biodiversity and desertification: the United Nations Framework Convention on Climate Change (UNFCCC), the Convention of Biological Diversity (CBD) and the United Nations Convention to Combat Desertification (UNCCD). The UNFCCC aim was to stabilise the atmospheric concentrations of greenhouse gases (GHG) at levels below the dangerous interference with the climate systems and was followed by the Kyoto Protocol (1997). The latter established legal obligations for develop countries to reduce GHG emissions. The CBD aimed to protect the biodiversity and promote its sustainable use, as well as the equal division of its benefits. The UNCCD focused on particular regions of the planet (arid, semiarid, and dry sub–humid areas) with the most vulnerable ecosystems and peoples. Since then, many programs have been implemented in order to promote sustainable development and environmental protection.

At the same time the specific concerns raised about environmental contamination gave place to important developments over the years. Indeed, effective measures to protect environmental quality and sustainability emerged relatively recently. However, before and still after them, many contaminants originating from both natural and anthropogenic sources have been introduced or occur in the natural environment in continuously increasing levels. Moreover, water covers approximately 70% of Earth's surface (Drinkwater et al., 2009). Thus, aquatic ecosystems are the final destination for most contaminants. Contaminants may reach these systems intentionally or accidentally, through direct discharges (*e.g.,* industrial and domestic effluents), surface runoff (*e.g.,* agriculture landfills), atmospheric deposition (*e.g.,* ashes from forest fires), and spills of oils (*e.g.,* Prestige and Erika oil spills) and hazardous and noxious substances (Neuparth et al., 2011), among others

(Schiedek et al., 2007). Depending on their physico–chemical characteristics, contaminants may either stay in the water column or accumulate in sediments and food webs.

Hence, following the increase in awareness and knowledge about chemical contaminants, as well as their properties and behaviour, many organisations started working to protect aquatic systems and the marine environment (*e.g.,* the International Marine Organisation – IMO, the United Nations Environmental Programmes – UNEP). In Europe, the *Torrey Canyon* accident (1967) on the SW coast of United Kingdom, which released over 100,000 tonnes of oil with heavy consequences for the environment, marked the beginning of international cooperation against marine pollution (Table I.1.). International regulations such as the Civil Liability Convention (CLC) and the Agreement for Cooperation in Dealing with Pollution of the North Sea by Oil and Other Harmful Substances (the "Bonn Agreement"), signed in 1969, represented the turning point regarding the international cooperation on marine pollution. Later, in the sequence of the intensively media-covered incident with the Dutch ship *Stella Maris* (1971) that tried to dump chlorinated wastes in the North Sea, several countries signed in 1972 the Convention for the Prevention of Marine Pollution by Dumping from Ships and Aircraft (the "Oslo Convention"). Concern was extended to terrestrial discharges of harmful substances discharged and in 1974 the Convention for the Prevention of Marine Pollution from Land–Based Sources (the "Paris Convention") was signed. In 1992, the Convention for the Protection of the Marine Environment of the North–East Atlantic joined the Oslo and Paris Commissions (the "OSPAR Convention"). Within the "OSPAR Convention" additional annexes were prepared to protect and conserve the maritime area of the convention, its ecosystems and biodiversity (Table I.1.). Other protection programs covering more restricted areas were also created, like the "Helsinki Convention" (1974), the "Barcelona Convention" (1976) and the "Bucharest Convention" (1992), to protect the Baltic Sea, the Mediterranean Sea and the Black Sea, which were convened to achieve coordinated management of source catchments and receiving marine areas. Within the international

agreements to achieve environmental sustainability, a proposal for a European Directive on the Ecological Quality of Surface Waters was also made. This proposal led to adoption by many countries of monitoring schemes and environmental quality objectives and standards for inland and coastal waters (Hering et al., 2010). The proposal itself was never adopted but eventually resulted in the draft document of the Water Framework Directive (WFD, Directive 2000/60/EC) (Hering et al., 2010).

Table I.1. Conventions established to protect the aquatic ecosystems, the participating countries, and their main objectives.

Convention name (year)	Participating countries	Main objectives
Oslo Convention (1972)	Belgium, Denmark, France, Finland, Germany, Iceland, Ireland, The Netherlands, Norway, Portugal, Spain, Sweden, United Kingdom	Prevention of marine pollution by dumping from ships and aircrafts
Paris Convention (1974)	Austria, Belgium, Denmark, France, Germany, Iceland, Luxembourg, The Netherlands, Norway, Portugal, Spain, Sweden, Switzerland, United Kingdom	Prevention of marine pollution from land–based sources
HELCOM (1974)	Denmark, Estonia, EU, Finland, Germany, Latvia, Lithuania, Poland, Russia, Sweden	Protect the marine environment of the Baltic Sea from all sources of pollution
The Regional Seas Programme (1974)	Black Sea, East Asian Seas, Eastern Africa, Mediterranean, Pacific, Red Sea, Gulf of Aden, ROPME Sea Area, South Asian Seas, South-East Pacific, Western Africa, Wider Caribbean	Sustainable management and use of the marine and coastal environment
Barcelona Convention (1976)	Albania, Algeria, Bosnia–Herzegovina, European Community, Croatia, Cyprus, Egypt, Israel, Lebanon, Libya, Malta, Morocco, Serbia and Montenegro, Slovenia, Syria, Tunisia, Turkey	Protect the Mediterranean Sea against pollution
OSPAR Commission (1992)	Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, The Netherlands, Norway, Portugal, Spain, Sweden, Switzerland, United Kingdom	Protect the marine environment of the North–East Atlantic
Bucharest Convention (1992)	Bulgaria, Georgia, Romania, Russia, Turkey, Ukraine	Protect the Black Sea against pollution
WFD (2000)	European Commission countries	Achieve the good qualitative and quantitative status of all water bodies
MSFD (2008)	European Commission countries	Achieve the good environmental status of EU's marine waters

The WFD and the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) were adopted more recently to establish a common regulatory framework that was transposed to national legislations by member countries. The WFD is intended to achieve a good chemical and ecological status for Rivers, lakes, groundwater and coastal waters, protecting the aquatic ecology and habitats, the drinking water resources and the recreational waters.

The MSFD aims to protect the marine environment by achieving a good environmental status and thus complements the WFD. However, despite the implementation of these directives, daily new and old compounds continue to reach the aquatic environment and may cause adverse effects on ecosystems. Of these, priority toxicants such as polycyclic aromatic hydrocarbons (PAHs), heavy metals or pesticides, and emerging contaminants (ECs) of concern like pharmaceuticals and personal care products (PPCPs) are among those posing higher risks to aquatic organisms and requiring further investigation. Additionally, many aquatic ecosystems are intensively explored (*e.g.,* fisheries, commercial and tourist routes, recreational uses) becoming seriously threatened by contamination and requiring the application of monitoring and management practices targeted to sustainable development. Coastal and estuarine systems are especially prone to suffer the impact of human pressure and environmental contamination.

2. Estuarine contamination

Estuaries are transitional ecosystems where freshwater mixes with saltwater. As areas of very high productivity, estuaries provide large food supply, good dissolved oxygen conditions and shelter to many species. They also constitute essential reproductive and nursery grounds for several species with high commercial and ecological value, including shorebirds, marine fishes, shellfish, and crustaceans (Day et al., 1989). Their privileged locations contribute to the establishment of demographic centres and many industrial and agricultural activities. This high human

activity has been triggering environmental changes with adverse impact in the health of estuarine organisms, compromising local biodiversity and ecosystems' functioning. Thus, due to decades of anthropogenic pressures, many estuaries are impacted, among others, by excessive levels of pollutants, nutrients, and organic matter (Kennish, 2002). After issuing of the WFD, various restoration measures have been taken to allow for recovery of affected systems. Amelioration of water quality was subsequently observed in several ecosystems (Eertman et al., 2002; Van den Bergh et al., 2005; Borja et al., 2006). Nevertheless, much is still to be done to manage affected systems. Among others, there is the need to improve fundamental knowledge about the effects of these environmental contaminants in estuarine organisms and implement specific parameters and assessment methods.

2.1. Environmental contaminants

Good examples of contaminants raising highest concern in estuarine systems are heavy metals, such as mercury, lead, cadmium, zinc, and the natural or synthetic organic chemicals mentioned earlier (*i.e.* PAHs, pesticides, nitrates and phosphates, PPCPs) (Schiedek et al., 2007).

PAHs are frequently found in water, sediments, soils, and biota (Dissanayake et al., 2010; Patrolecco et al., 2010; Uno et al., 2010; Mzoughi and Chouba, 2011). They have a natural origin but are also formed during various human activities. The huge amount of PAHs found in the environment is mostly of anthropogenic origin and can be divided in pyrogenic (from the combustion of fossil fuels, wood, coal) or petrogenic (associated with petroleum). As mentioned, PAHs reach aquatic ecosystems through direct discharges (*e.g.,* petroleum spillage, domestic, and industrial wastes), or surface runoff and atmospheric deposition. Structurally, PAHs are composed by two or more fused aromatic rings (Fig. I.1.). Their physico–chemical properties are dependent on the number of aromatic rings and, consequently, on their molecular weight; low molecular weight (LMW) PAHs have up to three rings, whereas high molecular weight (HMW) PAHs have four or five rings (CCME, 1999). In the

environment, LMW PAHs can be found in the water column, where they tend to adsorb to suspended particulate matter, or in sediments. HMW PAHs are mainly associated to sediments (Mzoughi and Chouba, 2011). Another important characteristic of PAHs is the possibility to be photoactivated by ultraviolet (UV) radiation (Okay and Karacik, 2008). Concern about PAHs and their potential detrimental effects is also related to their persistence in the environment. Since they are lipophilic compounds, PAHs are rapidly absorbed by biota and may bioaccumulate (Valavanidis et al., 2008). Additionally, from the metabolism of the parent compounds several metabolites can be originated, which in some cases are more toxic than the parent PAHs. Some compounds and/or their metabolites are classified as carcinogenic, mutagenic, and teratogenic, and many other biological responses were registered after PAHs exposure of organisms from different taxa (from invertebrates to mammals) (CCME, 1999). For these reasons, the United States Environmental Protection Agency (USEPA) and the EU established lists of priority PAHs that should be investigated and regularly monitored (ATSDR, 1995). Though the impact of PAHs has been widely studied in a number of species, knowledge on their effects and measurement in estuarine and coastal invertebrates is still required.

Metals are natural inorganic compounds, which in the solid state evidence crystal structure. Among their properties stand out their high reflectivity, and electric and thermal conductivity, as well as their particular mechanical properties (*e.g.,* strength and ductility) (Nordberg et al., 2007). These characteristics enhanced the industrial applicability and interest by metals. However, in aqueous solution metals may be present as metal ions and react with other ions. The solubility of metals thus depends on the pH, the presence of others ions in the solution, their oxidation state, and the oxidation–reduction conversion (Nordberg et al., 2007). Metals can reach the aquatic environment by natural processes (*e.g.,* erosion of metal deposits) or due to anthropogenic activities (*e.g.,* mining, smelting, fossil fuel combustion). Some are essential for many biological functions (*e.g.,* as structural elements and components of several proteins), while for others

no biological importance or requirement is known. In any case, elevated concentrations of metals may induce harmful effects on living organisms (Nordberg et al., 2007). Cadmium, mercury and lead are considered some of the most toxic metals. The adverse effects that can be induced by metals include damage to the central nervous system (CNS) (Kumar and Gill, 2009), carcinogenesis (*e.g.,* chromium and nickel) (Nordberg et al., 2007), embryonic and/or larval malformation and mortality, alterations of the synthesis and activity of several enzymes (Nordberg et al., 2007; Jezierska et al., 2009).

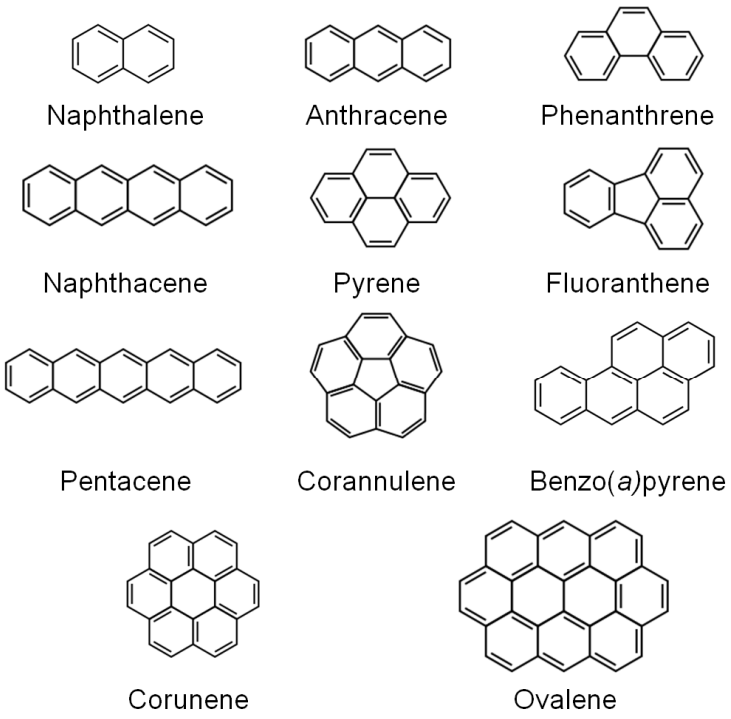


Fig. I.1. Structure of low and high molecular weight polycyclic aromatic hydrocarbons.

Natural pesticides (*e.g.,* nicotine) were used for many years, but were eventually replaced by more powerful, specific, and inexpensive synthetic organic compounds. These are used to control pests (*e.g.,* insects, weeds, fungi) in primary production and disease control (Connell and Miller, 1984). However, once in the environment, they can exert effects on species other than the predicted target. Moreover, following leaching and

run-off they enter into aquatic ecosystems where their resistance to environmental degradation contributes to long–term effects elicited on non-target species. There are many classes of pesticides (*e.g.,* organophosphate, carbamate, organochlorine, and pyrethroid) with different physico–chemical properties, persistence, mode of action (MOA), and toxic potential. Some are lipophilic, can bioaccumulate in living organisms and biomagnify through food chains (*e.g.,* organochlorine), while others are hydrophilic and exhibit low persistence in the environment (Connell and Miller, 1984). Concerning their toxicity, after metabolisation, some pesticides may also originate sub–products that may be more toxic to aquatic organisms than the parent compounds (*e.g.,* organophosphates).

The enrichment of aquatic ecosystems by nitrates and phosphates, as a result of human activity (*e.g.,* surface runoff, domestic effluents, animal wastes), deteriorates water quality. Eutrophication is defined by the Organisation for Economic Co–operation and Development (OECD) as “the increase in the rate of production and accumulation of organic carbon in excess”. Many adverse consequences may occur due to eutrophication (Connell and Miller, 1984): increased production of algae and macrophytes, reducing the penetration of light; shifts in the composition of phytoplankton and benthic communities due to the imbalance of nutrients, favouring occurrence of toxic algal blooms and reduction of biodiversity; reduction of the amount of dissolved oxygen in the water caused by the increased primary production, which originates hypoxic zones that endanger aquatic resources. At high concentrations inorganic nitrogen may also be toxic to estuarine invertebrates (Camargo and Alonso, 2006).

In recent years increased attention has been given to EC. These may be new chemicals but also substances that have been present in the environment for long (Brack et al., 2012). However, in any case, their impact and importance is only starting to be addressed. In many instances this is due to the lack of adequate detection and measurement methods. Available data is also very limited, so that their fate, behaviour, and

ecotoxicological effects are often unrecognised. Moreover, they are not currently included in routine European monitoring programmes. All this poses a major difficulty for regulatory bodies in their decision-making and indicates the need for research on such substances. Among these, the diverse and large group of PPCPs includes all the products used for health or cosmetic purposes and those used in animal health treatments, as well. PPCPs include human and veterinary pharmaceuticals of various classes (*e.g.,* painkillers, antibiotics, contraceptives, lipid–regulators, tranquilisers, antidepressants, hormones) and a huge diversity of personal care products (*e.g.*, skin, dental, and hair care products, soaps, sunscreen agents, fragrances, preservatives). Personal care products can reach the aquatic environment following their direct use. Pharmaceuticals, however, can reach aquatic systems due to inappropriate disposal or be released after medical treatment as parent compounds and/or their metabolites in human excreta. Several works also indicated that wastewater treatment plants may not be fully efficient in the removal of many of the pharmaceuticals that reach the sewage (Lajeunesse et al., 2011; Brozinski et al., 2012; Lahti et al., 2012; Yuan et al., 2013). Nevertheless, the detection and quantification of pharmaceuticals in environmental samples is mainly dependent on analytical procedures adapted to the low concentrations present and the characteristics of the different matrices. Hence, methodological difficulties and the high costs of chemical quantification still limit environmental monitoring of pharmaceuticals (Petrovic et al., 2011), despite the improvements that took place over the last decade and led to easier and more accurate measurements (Huerta et al., 2012). Works reporting the occurrence of pharmaceuticals in waters, sediments and biota, however, are becoming more and more frequent (Brooks et al., 2005; Richardson et al., 2005; Ramirez et al., 2009; Corcoran et al., 2010; Houtman, 2010; Pal et al., 2010; Santos et al., 2010; Richards et al., 2011; Fang et al., 2012; Gelsleichter and Szabo, 2013; Klosterhaus et al., 2013). Also, because the concentrations detected in the aquatic environment are comparatively lower than those found for conventional contaminants, pharmaceuticals were initially not expected to

be life threatening to aquatic organisms. Despite this, effects in non-target vertebrates and invertebrates, ranging from sub-individual to population levels, and affecting vital functions such as neuroendocrine regulation, and survival, growth or reproduction have been described (Kreke and Dietrich, 2008; Mesquita et al., 2011; Aguirre-Martínez et al., 2013a; Aguirre-Martínez et al., 2013b; Martins et al., 2013). Particular concern is raised by the fact that pharmaceuticals are designed to perform a biological effect before degradation and their potential to affect wildlife since main targeted receptors are phylogenetically well conserved (Gunnarsson et al., 2008). For many highly prescribed pharmaceuticals, information on their bioaccumulation in and effects on estuarine and coastal species is still scarce. Data is especially limited for invertebrate species. This is unexpected when considering that invertebrates represent more than 95% of the known animal species (Schulte-Oehlmann et al., 2004). Moreover, they provide species with key roles in the structuring of estuarine food webs and supporting important ecosystem functions.

2.2. Natural stressors

For their natural characteristics, estuaries also exhibit high variability in important abiotic factors as water temperature, pH, dissolved oxygen, and salinity. These are particularly relevant since variations of such parameters outside the optimal species' ranges can act as severe environmental stressors for aquatic organisms affecting their survival and maintenance (Anger et al., 1998; Heugens et al., 2001). Many of these are also able to influence the toxicity of several compounds, with synergistic and antagonistic effects reported for different combinations of natural and toxicant stressors in various species (Holmstrup et al., 2010). For these reasons and, therefore, the ability to act as confounding factors, they need to be addressed in environmental monitoring and assessment programmes. Further, it has been increasingly recognised that they are also influenced by the ongoing global climate changes.

Since the Industrial Revolution, the increase in GHG emissions (carbon dioxide, methane, nitrous oxide, and aerosols like chlorofluorocarbons –

CFCs) has been causing a global warming of the Earth's surface. Increasing temperature has been linked to changes on the global hydrological cycle (Allen and Ingram, 2002; Held and Soden, 2006; Anadón et al., 2007) and the cryosphere (Anadón et al., 2007; IPCC, 2007). A cascade of events follows the increase in CFCs and temperature: the depletion of the ozone layer facilitates the penetration of UV radiation; carbon dioxide leads to ocean acidification promoting decalcification of various organisms and their shells; the ice in the Arctic and Antarctica melts leading to sea levels rising; increased atmospheric pressure and wind fields intensify upwelling events; altered atmospheric circulation changes the precipitation patterns and promotes extreme weather events (*e.g.,* storms, droughts, floods), which are becoming more frequent and severe (Anadón et al., 2007; IPCC, 2007; IPCC, 2014). All these are shifting the distribution and abundance of many species and increasing the frequency and intensity of harmful algal blooms, among others. They also influence oceans and coasts, their productivity, communities' structure, phenology, and migration patterns of several species (Anadón et al., 2007). Changes in water temperature, dissolved oxygen, UV radiation or salinity may affect resident organisms, food webs, community interactions, and essential ecosystems' processes and functions (Jochum et al., 2012). These changes affect different regions in different ways and intensity, depending on the type of ecosystem. Additionally, they may facilitate the spread of invasive species due to their broader range of tolerance to disturbance (IPCC, 2007). Ecosystem responses may be slow when changes occur at large spatial scales, or occur in shorter periods for changes at regional scales (IPCC, 2007).

At the regional level, extreme weather events are of particular relevance. Drought and flood are dangerous and devastating natural events that cause alterations in habitat structure and availability, and salinity shifts that influence the distribution, abundance, and physiology of aquatic organisms. Due to the observed hydrological alterations, they are becoming more frequent and intense. According to the latest Intergovernamental Panel on Climate Change (IPCC) report, extreme sea level events have been increasing, and are previewed to increment the risk

in the near future of coastal floods particularly in Atlantic and Southern European regions (IPCC, 2014). The frequency and intensity of heat waves are also very likely to increase in these areas. These observations support previous predictions of salinity shifts induced by global climate changes (Bindoff et al., 2007; Hosoda et al., 2009). Indeed, following precipitation and evaporation patterns, long term trends indicated the occurrence of global water freshening in subpolar and tropical regions and a salinification of shallower parts of the subtropical oceans (IPCC, 2007; Hooper et al., 2013). The increased drought frequency and sea-level rise were forecasted to cause salinity changes in several estuarine and coastal waters.

These alterations deserve special attention as salinity is able to act on physiological processes (Whiteley et al., 2001; Vargas-Chacoff et al., 2009) and the life history (Oltra and Todoli, 1997; Martin et al., 2009) of species, altering their geographical distribution, and the structure and dynamics of aquatic communities (Colburn, 1988; Williams et al., 1990; Anger et al., 1998; Heugens et al., 2001; Boix et al., 2008). Besides affecting community composition, salinity may also act as an ecological barrier to animals' movement influencing the genetic diversity of vertebrate (Stockwell and Mulvey, 1998) and invertebrate (Browne and Hoopes, 1990) populations. Moreover, interactions between salinity changes and toxicity of chemical contaminants are also expected to occur. For example, increased metal toxicity is usually reported at low salinity levels (reviewed in Hall and Anderson, 1995 and Heugens et al., 2001). The higher toxicity has been related to decrease metal speciation, and increased bioavailability and accumulation that occur at lower salinity levels (Bjerregaard and Depledge, 1994; Hall and Anderson, 1995; Heugens et al., 2001). On the other hand, organic contaminants appear to be more bioavailable in saltwater than in freshwater (*e.g.,* pesticides) (Hall and Anderson, 1995). Toxicity of organophosphate (OP) pesticides also appears to increase with salinity possibly due to increased bioaccumulation (Heugens et al., 2001).

Overall, interactions between salinity and contaminants are complex because salinity can influence both the contaminant itself and the physiology of organisms. Their effects are thus difficult to predict from single exposure to toxicants (Noyes et al., 2009). Hence research on levels and impact of contaminants occurring in estuarine systems and their relation to natural environmental stressors is highly needed to assess their status, predict risks to local ecological receptors, and eventually delineate mitigation and protection measures.

3. Environmental biomarkers, monitoring, and risk assessment

3.1. Environmental biomarkers

Truhaut (1977) defined ecotoxicology as “a branch of toxicology concerned with the study of toxic effects, caused by natural and synthetic pollutants, to the constituents of ecosystems (animals including human, vegetable and microbial), in an integrated context”. The toxicity of a contaminant towards an organism is always dependent on the dose of contaminant and the duration of the exposure (Moriarty, 1999). Paracelsus stated that “all things are poisons (...). It is only the dose which makes a thing poison” (Klaassen, 2008). This is an important point in toxicological and ecotoxicological sciences. However, while toxicology focuses specifically on mechanisms of toxicity and effects of pollutants in the individual, ecotoxicology is also interested in their ecological effects. Not only contaminants rarely occur in isolation, but also, as mentioned earlier, other environmental factors will modulate their availability to aquatic organisms and effects in ecosystems (Hylland, 2006b). In the scope of ecotoxicology, tools and methodologies have been developed to detect and assess the effects of environmental contaminants. Investigation of sub-lethal effects is the main goal as it reflects actual field scenarios in which organisms are mostly exposed to low concentrations of pollutants over long periods. Depending on the pollutant and its mode of action, the exposure may entail gene expression, biochemical, histological, and physiological alterations that may have repercussions on survival, growth,

and reproduction, thus affecting populations, communities and ecosystems (Newman, 1998; van der Oost et al., 2003; Guimarães et al., 2009; Martins et al., 2013). The investigation and assessment of ecotoxicological effects can, thus, span various levels of biological organisation, from molecular to ecosystem levels. Given their ecological relevance, population and community-level endpoints are often desirable to assess harmful effects of environmental contamination. However, these responses are unspecific and deleterious effects at these levels of organisation are only detected when there is already considerable environmental degradation (Hylland, 2006a). Moreover, such effects are often irreversible preventing successful application of remediation actions or risk reduction measures. There is, therefore, a pressing requirement for early-warning biological responses, of easy application and cost-effective, that may assist in detecting effects in biota and providing association with the presence of contaminants in the ecosystems (Galloway et al., 2004). On this regard, it is important to note that effects at lower levels of biological organisation (molecular to individual) occur more rapidly than those at higher levels, thus showing higher potential to the timely anticipation of adverse effects induced by toxicant exposure. They also provide more specific responses of contaminant exposure, related to their MOA or mechanisms of toxicity (Galloway et al., 2004). Such approaches usually take advantage of the so–called biomarkers. Biomarkers are measurements in body fluids, cells or tissues at the biochemical, cellular, and physiological levels, which indicate alterations in mechanisms, processes, structures, and behaviours within an organism due to contaminant's exposure (Livingstone, 1993). Biomarkers have been successfully applied as sensitive diagnostic and prognostic early–warning tools in aquatic ecosystems (Livingstone, 1993; van der Oost et al., 2003). They often provide insight into the potential mechanisms of contaminant effects and important links between laboratory toxicity and field assessments. Recently, they have also been recognised as providing a valuable contribution to gauge the good ecological status enshrined in the WFD (Hagger et al., 2008) and the MSFD (Fossi et al., 2012), reflecting the

combined effects of all the stressors to which the organisms are subjected. For their involvement in the metabolism of toxicants, biomarkers related to detoxification mechanisms are widely used in laboratory and field studies (Filho et al., 2001; van der Oost et al., 2003; Maria et al., 2009; Pereira et al., 2009; Almeida et al., 2012; Oliveira et al., 2013).

Several processes occur since contaminant uptake up to elimination and/or accumulation. All of them are dependent on the contaminant (*e.g.,* physico–chemical characteristics, concentration reaching the target sites/receptors, duration of the exposure) and the living organism (*e.g.,* affected species, genetic background, life cycle stage, nutritional and reproductive status). However, many biological processes are transversal to contaminants and organisms. The general route of contaminants in an organism starts with their uptake from the environment, passes through several detoxification phases, and finally undergoes excretion and/or bioaccumulation. The uptake of contaminants into an organism can occur through the dermis, gills, lungs and/or digestive tract. Through circulation, contaminants may be distributed to all the organism's body and enter into the cells, where they reach their target site(s), by crossing membranes (lipophilic contaminants), diffusion (polar molecules) and endocytosis (hydrophilic contaminants) (Newman, 1998; Klaassen, 2008).

Pollutant metabolism involves four important phases. In phase 0, numerous energy-dependent non-specific transporters help reducing the accumulation of toxicant by actively removing them out of the cell before they actually reach intracellular compartments (Ferreira et al., 2013). Those toxicants that enter into the cytoplasm undergo phases I and II of biotransformation (van der Oost et al., 2003; Klaassen, 2008). Phase III involves the energy-dependent transport of multiple structurally and functionally different products resulting from biotransformation across a variety of cellular membranes (Ferreira et al., 2013; van der Oost et al., 2013). Phase III transport proteins prevent the retention of already detoxified molecules inside cells and tissues and are, therefore, complementary to biotransformation reactions. The proteins acting on phase 0 and phase III are multixenobiotic resistance transporters, which

belong to the evolutionarily conserved super–family of the ATP binding cassette (ABC) proteins, present in almost all living organisms from prokaryotes to mammals (Ferreira et al., 2013).

Phase I and II biotransformation lead to the formation of hydrophilic conjugated xenobiotics (Klaassen, 2008). Their reactions are catalysed by several enzyme families, grouped in four classes according to the reaction catalysed (hydrolysis, reduction, oxidation, conjugation). These enzymes exhibit higher activity levels in tissues involved in food processing like the liver in vertebrates, and the digestive gland or pyloric caeca in invertebrates. The hydrolysis, reduction and oxidation reactions introduce or expose a functional group in the parent compound (*e.g.,* –OH, –SH, –NH_2) (Klaassen, 2008). They compose the phase I reactions and are performed by enzymes such as carboxylesterases (CbEs), peroxidases, and the cytochrome P450 (CYP 450). Phase II reactions comprise the conjugation of phase I metabolites with polar molecules (*e.g.,* glutathione, glucuronic acid) by enzymes such as glutathione *S*–transferases (GST) and uridine 5'–diphospho–glucuronosyltransferase (UDP–glucuronosyltransferase) (van der Oost et al., 2003; Klaassen, 2008). In some cases, biotransformation activates the parent compounds originating more reactive metabolites (*e.g.,* the oxon metabolites originated from the biotransformation of OP pesticides). The balance between detoxification and activation determines the toxicity of a contaminant and the sensitivity exhibited by different species (Klaassen, 2008). Reactive oxygen species (ROS) are sub–products of biotransformation reactions and comprise radical and non-radical molecules: superoxide anion radical, hydroxide radical, alkaloid radical, hydrogen peroxide, among others. These are cytotoxic through oxidative damage to DNA, proteins, and lipids (Klaassen, 2008). Anti-oxidant enzymes and molecules such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), or vitamin E are protective mechanisms against oxidative damages promoted by ROS (Lushchak, 2011).

Some compounds are then excreted (in faeces and urine), while others can be stored in specialised organelles (*e.g.* lysosomes) or bound to

proteins (*e.g.* metallothioneins) or pigments (*e.g.* melanin) (Klaassen, 2008). Bioaccumulation consists in the accumulation of the contaminants in an organism tissue and it is the balance between their uptake and their elimination (Livingstone, 1993). For lipophilic contaminants, it varies with the lipid content of the organism. The molecular shape and size of contaminants additionally influence bioaccumulation. Biomagnification occurs with some persistent lipophilic compounds, which concentration may increase throughout the food chain due to tissue accumulation.

3.2. Environmental monitoring and risk assessment

Biomarkers related to other physiological mechanisms responsive to environmental contamination may also be used, as for instance enzymes associated to osmoregulatory processes or energy production, immunological parameters or molecules involved in neuroendocrine regulation (Long et al., 2003; Dissanayake et al., 2008; Kreke and Dietrich, 2008; Guimarães et al., 2009). Exposure to toxicants usually triggers a cascade of biological responses from which useful biomarkers may be devised. But individually biomarkers provide limited evidence to the diagnosis of MOA, health status of organisms, and assessment of environmental contamination. The use of a knowledge-based battery of biomarkers is therefore adequate for integration in monitoring programmes to assess environmental contamination and help characterising natural variation and the potential confounding influence of other environmental factors (Guimarães et al., 2009; Guimarães et al., 2012). Such approaches can be routinely used to elucidate about the status of an ecosystem and, when carried out over several years, they provide appraisal of their evolving conditions and the effectiveness of the actions aimed at environmental protection. On the other hand, they allow gathering baseline data useful to assess hazards from specific punctual incidents like accidental spillages or unexpected discharges to the aquatic environment.

Chemical analyses in abiotic and biotic compartments, *per se*, give important information on the levels of environmental contamination, and

on bioavailability to aquatic organisms, but no information on the deleterious effects that those levels may cause on biota. Comparison of the chemical levels determined with reference values is oftentimes limited because for many contaminants definition of reference values is still not available (especially for ECs) and different species and populations may exhibit differential sensitivity to environmental contamination. Moreover, they give information on the contaminants thought to be more relevant in the study sites, although other substances may also be contributing to the overall toxic effects. Hence, integrated chemical and biological effects assessments are recommended (Cajaraville et al., 2000; Hylland, 2006a; Thain et al., 2008). As biological effects tools, biomarkers are thus widely employed in environmental monitoring and risk assessment of aquatic pollutants. They may also elucidate about the different routes of exposure when applied to species from diverse habitats and/or trophic levels (Livingstone, 1993). However, many of these parameters may vary also in relation to age, gender, and reproductive stage of the species, as well as with seasonality, temperature or salinity, among other factors (Hylland, 2006a). Monitoring studies should thus address and control for a possible influence of such factors when assessing the effects of contaminant exposure. Abiotic parameters as water temperature, salinity, hardness, and nutrient levels should be measured at the same time as samples are collected for chemical analysis and biomarker determinations. Age, size, gender, and reproductive stage of study organisms should be taken into consideration prior to sampling. Sampling periods and frequency depend mostly on the purposes of the monitoring. But simultaneous sampling in low impacted areas, which may be taken as reference for expected natural variability, should be considered to improve interpretation of the data gathered and infer about pollutant effects and health status of contaminated sites of interest.

Environmental monitoring allows quantification of the integrated effects of the mixture of contaminants present in affected sites and their interaction with natural stressors on the functioning and health of sentinel species. Nevertheless, it requires understanding on potential differential

sensitivity and adaptation to pollution of the populations under study. Organisms under chronic exposure to environmental contamination often develop adaptive responses to deal with the chemical challenge and achieve a new physiological steady state allowing for homeostasis maintenance (Mouneyrac et al., 2011). These adaptive responses (*i.e*. molecular, cellular, physiological or behavioural responses that increase fitness of organisms) are vital to the sustainability of populations, communities, and ecosystems, but may difficult interpretation of monitoring data. Further, increased sensitivity to new stressors often occurs in tolerant organisms (Mouneyrac et al., 2011).

Tolerance to environmental pollution may occur through either genetic adaptation or physiological acclimation (Klerks and Weis, 1987; Posthuma and Van Straalen, 1993). Adaptation is related to mutation and selection of genes modifying physiological functions. Molecular mechanisms of adaptation may involve constitutive up- or downregulation of tolerance genes and proteins, alternation of a target or receptor gene or an inducible change in gene regulation. Acclimation may be achieved by physiological adjustments of existing cellular and enzymatic processes without altering the genetic makeup and may thus occur readily (Mouneyrac et al., 2011). Increased upregulation of detoxifying proteins involved in the metabolism of metals and xenobiotics (*e.g.,* metallothioneins, heat shock proteins, GST, CYP 450 monooxigenases, CbE) usually occurs in acclimation (Claudianos et al., 2006). Therefore, acclimation often involves additional metabolic costs required for detoxification and stress-response processes.

Nonetheless, these processes may influence data interpretation and decision about the need to implement remediation measures in affected ecosystems, as well as, inference on the impact of future environmental degradation on non-exposed species. Differential sensitivity to contamination also complicates the environmental risk assessment of hazardous substances to the aquatic environment. Risk calculation of environmental contaminants to aquatic systems is mostly based on chemical-specific hazard data obtained in laboratory toxicity assays using standard species, from a limited number of taxonomic groups, reared

under controlled conditions or species originating from low impacted (or more pristine) sites (EUR 20418 EN12). Frequently, due to limited available data, risk estimation to the estuarine and marine environment employs data obtained with freshwater species. Adaptive responses caused by previous exposure to contamination and other stressors in ecosystems hampers the extrapolation of these results to predict effects of chemicals on natural populations in the field and may lead to under- or overestimation of risk, consequently affecting management and protection of estuarine systems. Overall, organisms living in naturally changing environments, such as estuaries, and moderately disturbed conditions, may survive due to a suite of adaptation and defence mechanisms allowing to cope with contamination and fluctuating factors (*e.g.*, salinity). However, compared to naïve animals, they are seldom used to assess effects and risk of toxicants and improve predictions to natural estuarine environment.

4. The NW Portuguese estuaries of the Minho and Lima Rivers

The estuaries of the Minho and Lima Rivers are located in the Northwest of the Iberian Peninsula (Fig. I.2.). This region is under the influence of the wet Atlantic climate, having an average annual precipitation of 1,300 mm (Fatela et al., 2007). A similar mesotidal stratified typology was described for these estuaries (Ferreira et al., 2003). The Minho River is under relatively low human pressure and limited inputs of urban, agriculture, and industrial activities (Ferreira et al., 2003). Available data indicate low contamination levels in sediment samples from freshwater wetlands and the mouth of the estuary (Sousa et al., 2008; Reis et al., 2009; Guimarães et al., 2012). It is also classified as a NATURA 2000 site. Moreover, it has been used as reference site in several ecotoxicological studies (Moreira et al., 2006; Quintaneiro et al., 2006; Guimarães et al., 2012).

The estuary of Lima River is located approximately 20 km to the south of the Minho estuary. It is under moderate human pressure, receiving chemical inputs from untreated effluents of urban and industrial origin (Ferreira et al., 2003). It has a large shipyard, an important commercial

seaport, a marina and a fishing harbour at its mouth. Over the years the River mouth has undergone a reshaping with elimination of a sand-spit (Ramos et al., 2006). This estuary is also under the influence of other sources of disturbance, such as a cellulose factory in the upper reaches, input of agricultural runoff and urban and industrial sewage discharges transporting nutrients and other substances into the estuarine area (Ramos et al., 2006). Chemical analyses in sediments showed moderate levels of heavy metals and PAHs at the mouth of the estuary and the levels of nitrate, nitrite, and phosphate suggest a poorer water quality, compared to Minho estuary (Guimarães et al., 2012; Azevedo et al., 2013). Dredging of the navigational channel (Costa-Dias et al., 2010) is also expected to induce resuspension of contaminants eventually contained in the sediments. Upstream, the estuary presents a much less disturbed state, with shallow salt marshes and tidal sandy islands (Ramos et al., 2006).

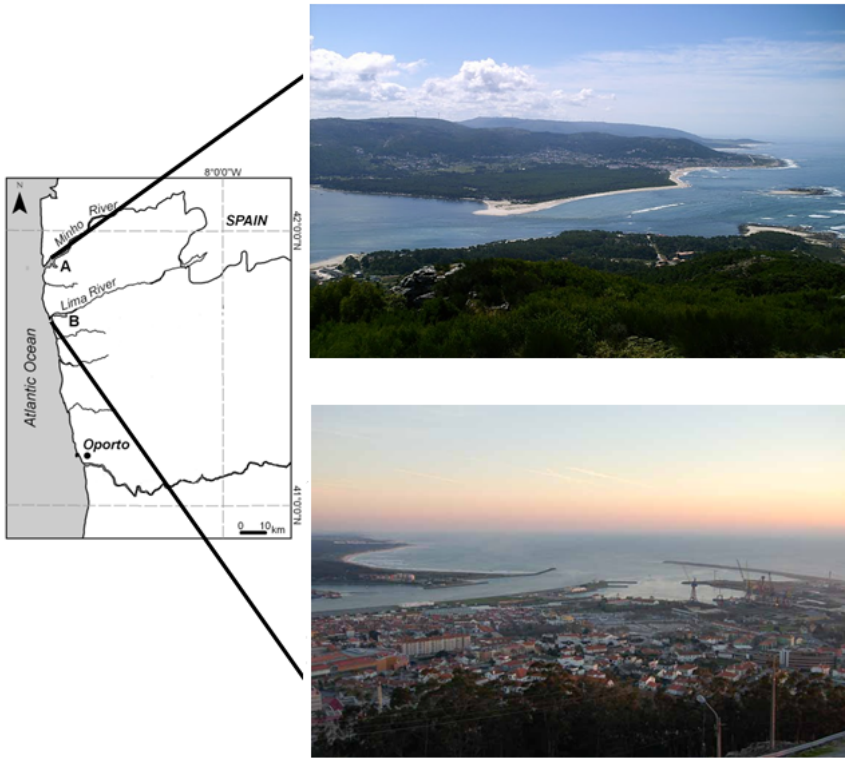


Fig. I.2. Geographical localisation of Minho and Lima Rivers in the Northwest of the Iberian Peninsula.

Previous studies employing batteries of early-warning biomarkers and integrated monitoring approaches indicated reduced health status associated to heavy metal contamination of the populations of glass and yellow eels, and common goby, from the Lima estuary compared to those from the Minho estuary (Guimarães et al., 2009; Gravato et al., 2010; Guimarães et al., 2012). These results raised the need for further investigation of sediments contamination by heavy metals and their effects in local key structuring invertebrate species, such as the green crab *Carcinus maenas*. This species may, however, show some tolerance to heavy metal contamination (Bryan and Gibbs, 1983). Also, former studies based on laboratory experiments with crabs from these estuaries showed that they exhibited different responses to cadmium exposure (Mesquita et al., *submitted*). This set a requirement for further investigation on potential differential sensitivity to contamination elicited by chronic exposure to the moderate contamination levels in Lima estuary.

5. The green crab *Carcinus maenas*

Carcinus maenas (Decapode, Portunidae) (Fig. I.3.) is an epibenthic crustacean, commonly found in estuarine and coastal areas, hiding under rocks and vegetation and burying in the substrate. It is known by different designations such as green crab, shore crab and also European green crab and European shore crab. It is omnivorous, eating live preys, carrion and algae. Its predatory behaviour has been considered as a structuring characteristic of marine and estuarine benthic communities (Raffaelli et al., 1989). The life span of *C. maenas* ranges between 4–7 years. This species tolerates a wide range of salinity (euryhaline), temperature (eurythermic), oxygen and habitat conditions (Klassen and Locke, 2007).

C. maenas has a wide native range, from the Baltic Sea to north-east Atlantic Ocean and North Africa (Fig. I.4.) (Crothers, 1967). However, *C. maenas* has colonised several places worldwide (reviewed in Klassen and Locke, 2007) and is included in the list of the 100 world's worst invasive

species due to its aggressive competitor and predatory behaviour which interferes with the native communities (Klassen and Locke, 2007).

5.1. Morphology

C. maenas has the dorsoventrally flattened body covered by a resistant carapace impregnated with calcium salts. The carapace colours of adult *C. maenas* may vary from green to orange and brown, depending on the inter–moult duration (Fig. I.3.) (Reid et al., 1997). The green becomes red due to the photodegradation of pigments present in the exoskeleton, during a long inter–moult (Reid et al., 1997). Carapace colour has been related with physiological and behaviour characteristics (Reid et al., 1997 and references therein). Red crabs evidenced lower tolerance to hypoxia and low salinities, but carapaces are structurally stronger so that males are more successful in mating and retaining females (reviewed by Reid et al., 1997). Genders are easily identified by external characteristics: females exhibited a broad and rounded abdomen with five segments, while males have a triangular form and three segments (Crothers, 1967) (Fig. I.3.).

An endoskeleton – apodemes – is projected from the exoskeleton, which takes form as tendons where muscles are attached or hard skeletal parts like the endophragmal shelf (that besides attaching muscles, protects the ventral ganglion and joints articulations) (Saxena, 2005). The first pair of appendages, called chelae, is specialised in defence and feeding, while the fifth is used in swimming. Autotomy of appendages is very common in crabs as a consequence of direct conflict between individuals or as a protective instinct to escape from predators (Crothers, 1967; Abello et al., 1994).

To grow *C. maenas* has to moult periodically his carapace. The ecdysis is regulated by hormones (ecdysteroids) produced by the dorsal ganglion, the y–organ (in the maxilla) and the x–organ/sinus gland complex (in the eyestalk) (Crothers, 1967). All cuticle structures are changed: the entire animal exits by the fissure along the backside of the body, leaving the exoskeleton empty (exuviae). Freed from the old carapace, the crab absorbs large amount of water, swelling his body. Characteristically, the

crabs exhibit a soft body and, to avoid predators, they remain buried in the sediments. Calcium is remobilised to the new skeleton, increasing its rigidity and strength. The percentage of growth and the frequency of moulting decrease over the years. *C. maenas* may moult 12 times in the first year, followed by only one or two times a year for the rest of its life cycle (Saxena, 2005). Factors such as food availability and light and temperature conditions are known to influence the moulting frequency (Saxena, 2005).

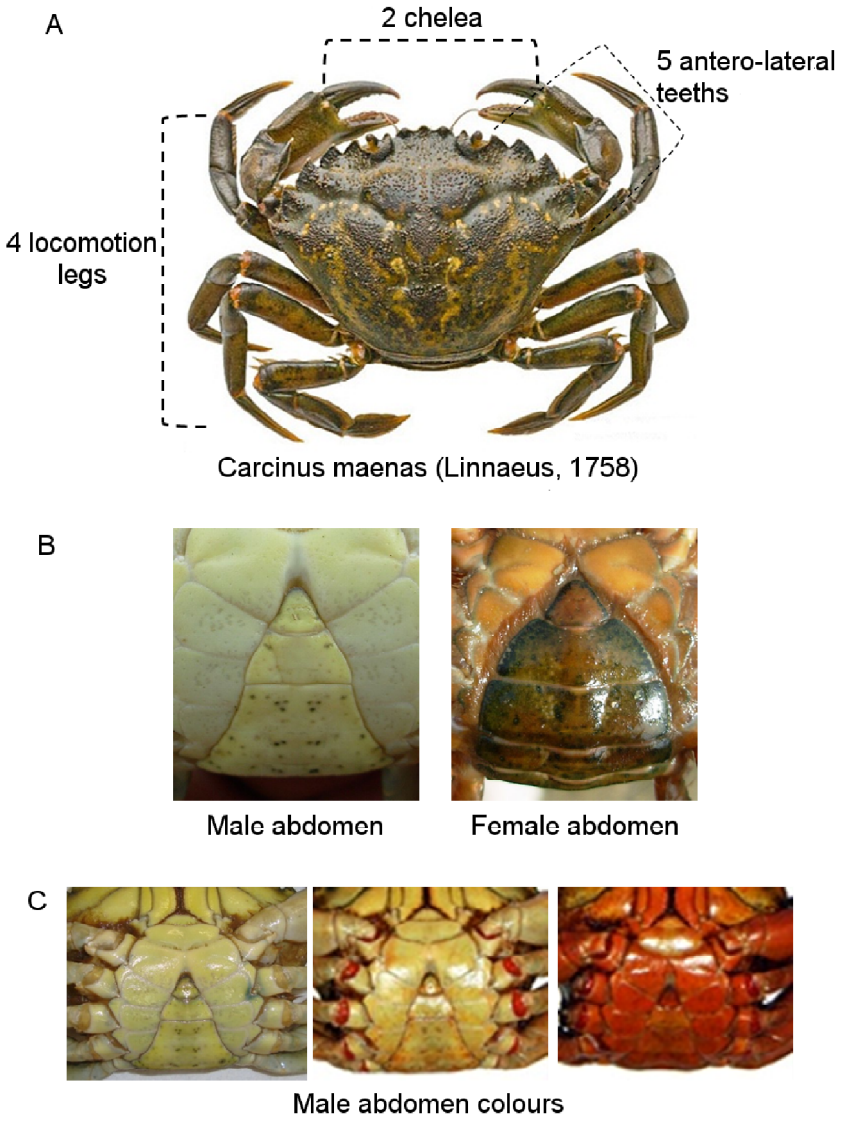


Fig. I.3. External morphology of *Carcinus maenas*. (A) Dorsal view of the carapace and appendages. (B) Differences between male and female abdomen. (C) Abdomen colours from green to red.

5.2. Physiology

C. maenas has nine pairs of gills accommodated inside the carapace in chambers located on each side of the body (Crothers, 1967). Under normal conditions, the water gets in the gill chambers by the openings under the walking appendages, leaving by the mouth opening and producing the commonly observed bubbling (Crothers, 1967). However, when crabs are buried, the water flow is reversed, entering by the mouth opening and leaving by the chelae opening (Crothers, 1967). Oxygen is transported by haemocyanin, a copper–containing protein, present in haemolymph.

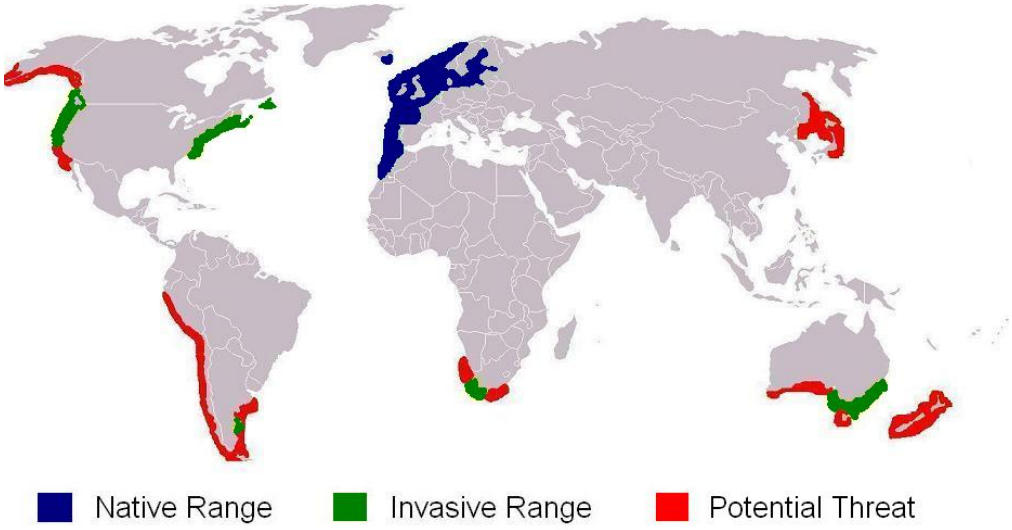


Fig. I.4. World distribution of *Carcinus maenas,* the native and invasive ranges. Source: Carnivora.

Crustaceans have an open circulatory system. Haemolymph is the circulatory fluid with functions identical to vertebrate blood. Circulation is achieved by the heart (a single–chambered organ) with a complementary support promoted by branchial hearts (Crothers, 1967). *C. maenas* is an efficient hyperosmoregulator, able to regulate the volume and osmolality of its body fluids (Whiteley et al., 2001). The osmolality of haemolymph may be achieved by active and passive uptake of specific ions from the water, by morphological and structural adaptations, and by production and transport of organic osmolytes (*e.g.*, amino acids) (Gilles and Pequeux,

1986; Gilles, 1998; Whiteley et al., 2001; Cieluch et al., 2004). The posterior gills (7–9) are directly involved on ionic transport in *C. maenas*, exhibiting increased total metabolic and Na^+–K^+–ATPase activity (Siebers et al., 1982; Whiteley et al., 2001). Osmoregulation involves energetic costs to animals and induces physiological and behavioural responses (Crothers, 1967; Urbina et al., 2010). When exposed to low salinities, *C. maenas* locomotor activity increases (*halokinesis*[1]), as well as the heart rate, the cardiac output, and the gill blood flow (Hume and Berlind, 1976; Ameyaw-Akumfi and Naylor, 1987; Mcgaw et al., 1999).

The digestive system of *C. maenas* is very simple (Crothers, 1967); secretion of enzymes, digestion, and absorption occurs mainly in the digestive gland. The principal waste product is ammonia, but amines, urea, and uric acid are also produced (Crothers, 1967). They can be excreted by urine, gill and gut diffusion, and deposition in the cuticle. Despite quite simple, the nervous system of *C. maenas* allows producing simple and complex reflexes. The complex reflexes cover righting, copulation, feeding and escape from predators (Crothers, 1967). *C. maenas* is able to detect vibrations in sediments and water, alterations of water flows, distanced objects (Crothers, 1967). Some of the success of this species may be related with its sense of touch, smell/taste and sight. The tactile hairs on the body surface, the mechanoreceptors and chemoreceptors in the limbs and antennae, and the photoreceptors in the eyes maintain *C. maenas* informed about what goes around him (Crothers, 1967).

5.3. Life cycle

During its life cycle, *C. maenas* goes through planktonic larval stages and a benthic adult (Fig. I.5.). Females can only copulate after moulting. After internal fertilization, females carry the eggs on her pleopods on a brood chamber. Several months later, they release the first planktonic zoea stage at the mouth of the estuary, during the night in ebbing neap tides; vertical migrations enhance the transport of larvae to coastal areas

[1] Halokinesis – The term derives from the Greek words *hals* “salt” and *kinei* “to move”.

(Queiroga et al., 1997). Gender is determined genetically; the sexual characteristics are determined by the androgenic glands and the eyestalk hormone in male and female, respectively (Crothers, 1967). Except for the first zoea and megalopa, larval development occurs offshore and lasts between 50 and 80 days (Klassen and Locke, 2007). After the forth zoea stage, a metamorphic moult occurs, originating a semi–pelagic megalopa. Under appropriate environment, megalopa moults into a juvenile crab.

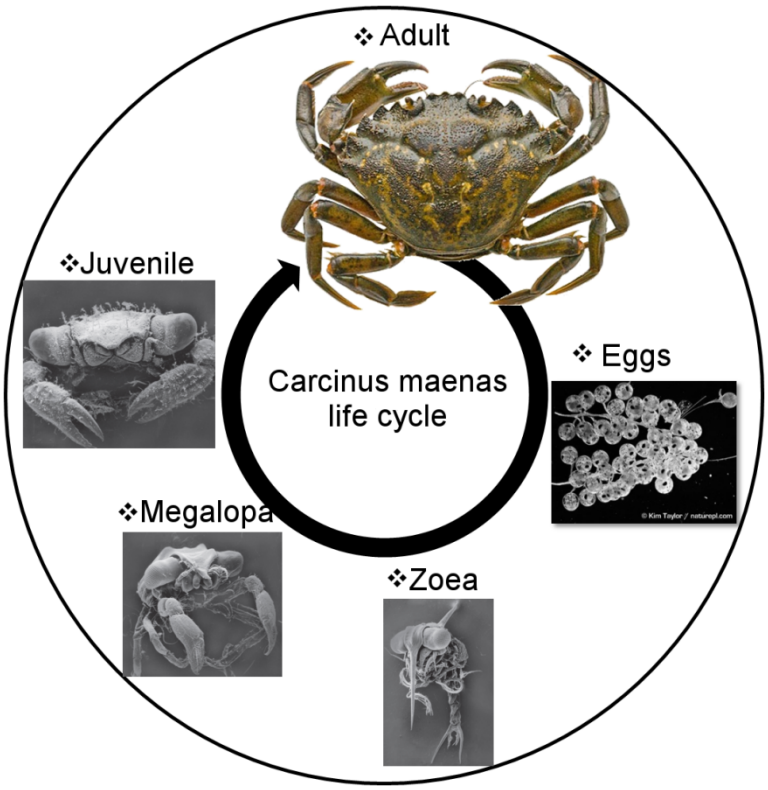


Fig. I.5. Life cycle of *Carcinus maenas* (Crothers, 1967). Scanning electron micrographs of zoea IV larvae, megalopa larvae and first instars juvenile crab (adapted from Ekerholm, 2005).

5.4. *C. maenas* – model organism in ecotoxicological studies

C. maenas has been used in ecotoxicological studies as model organism due to its widely geographical distribution, habitat, well known biology and behaviour (Naylor, 1958; Crothers, 1967; Sorenson, 1973; Hume and Berlind, 1976; Depledge, 1984; Dawirs, 1985; Skaggs and Henry, 2002; Bessa et al., 2010), and because it is relatively easy to capture and maintain in the laboratory. These studies were performed to

evaluate the toxicity of common contaminants such as pesticides, monoaromatic hydrocarbons, PAHs, metals, contaminated effluents (Thurberg et al., 1973; Depledge, 1984; Bjerregaard and Vislie, 1985; Bjerregaard and Vislie, 1986; Weeks et al., 1993; Bamber and Depledge, 1997b; Lundebye et al., 1997; Pedersen et al., 1997; Watson et al., 2004a; Bjerregaard et al., 2005; Nørum et al., 2005; Martín-Díaz et al., 2008a; Ghedira et al., 2009; Scarlett et al., 2009; Dissanayake et al., 2010). Recently, *C. maenas* has also been used to assess the effects of ECs such as pharmaceuticals (*e.g.*, fluoxetine, caffeine, carbamazepine, ibuprofen, novobiocin) (Mesquita et al., 2011; Aguirre–Martínez et al., 2013a; Aguirre–Martínez et al., 2013b), to prove the occurrence of trophic transfer of microplastic (Farrell and Nelson, 2013), as well as the effects of nanomaterials on nerve functions (Windeatt and Handy, 2013).

Numerous field studies were performed with *C. maenas*, allowing to distinguish among contaminated and control sites and reflecting a pollution gradient when applicable (Capuzzo and Leavitt, 1988; Aagaard, 1996; Bamber and Depledge, 1997a; Wedderburn et al., 1998; Astley et al., 1999; Dissanayake and Galloway, 2004; Watson et al., 2004b; Locatello et al., 2009; Pereira et al., 2009; Dissanayake and Bamber, 2010; Jebali et al., 2011; Pereira et al., 2011; Caçador et al., 2012; Pereira et al., 2012; Ben-Khedher et al., 2013). *C. maenas* was also used to investigate sediment quality in *in situ* studies (Moreira et al., 2006; Martín-Díaz et al., 2008b; Martín-Díaz et al., 2009; Dissanayake and Bamber, 2010; Buratti et al., 2012) and in cross transplantation exposures between a contaminated and a reference site (Maria et al., 2009).

Among the most frequently evaluated parameters employed stand out the use of:

- Biochemical effects (biomarkers of neurotoxicity, energy metabolism, anti-oxidant defences, oxidative damages, reproduction);
- Uptake, assimilation, bioaccumulation (of parent compounds and/or their metabolites in tissues, haemolymph and/or urine), trophic transfer;

- Cellular responses (cell viability, immune function, LMS);
- Physiological alterations (heart rate, ventilation, respiration, osmoregulatory ability, oxygen consumption);
- Behavioural endpoints.

For this reasons, *C. maenas* has been identified as a good biological indicator for assessing the health status of estuarine and coastal ecosystems. Although this is also a key species of Minho and Lima estuaries, and has been recommended for inclusion in Portuguese monitoring programmes within the WFD (Picado et al., 2007), it has seldom been used as bioindicator in these systems (Moreira et al., 2006).

6. Study biomarkers

Among biomarkers commonly used, several related to tissue accumulation, cell viability, energy production and availability, neurotransmission, biotransformation, anti–oxidant defences, and oxidative damage have been proved useful to investigate toxic effects of contaminants in aquatic invertebrates and successfully incorporated in integrated chemical and biological effects monitoring (Cajaraville et al., 2000; Martín-Díaz et al., 2005; Neuparth et al., 2005; Viarengo et al., 2007; Cravo et al., 2009; Douhri and Sayah, 2009; Maria et al., 2009; Mesquita et al., 2011; Pereira et al., 2011). For this and their involvement in modes of action of the compounds investigated, they were selected for the work presented in this Thesis.

When assessing PAHs in particular, detecting the presence of PAH-type metabolites or compounds in body fluids or tissues is helpful as screening tool indicative of exposure to these compounds. Classical analytical methods (*e.g.*, high-performance liquid chromatography, *HPLC*, gas chromatography – mass spectrometry, *GC-MS*) have been successfully replaced by rapid, accurate and cost-effective methods (*e.g.*, fixed wavelength fluorescence, FF) to detect PAHs and their metabolites in fish bile and soft tissues (liver, muscle, brain) (Lin et al., 1996; Fernandes et al., 2007; Balk et al., 2011; Almeida et al., 2012). These were later adapted to

determinations in urine and haemolymph of crab species (Dissanayake and Galloway, 2004; Watson et al., 2004a; Dissanayake and Bamber, 2010; Dissanayake et al., 2011). In organisms with low rates of metabolisation and/or excretion like invertebrates, prompting accumulation, determination of both parent compounds and metabolites is tissues may also be more effective than in urine samples (Watson et al., 2004a).

Lysosomes are capable to store a wide range of contaminants. These substances may threaten the integrity and fluidity of lysosomal membranes due to lipid unsaturation/hydrophobicity and generation of ROS able to damage cellular structures (Svendsen and Weeks, 1995; Grundy et al., 1996). Loss of lysosomal membrane stability (LMS) is therefore a useful indication of cell viability and health status of mollusc and crustacean species. Hence, it has been applied in laboratory and field studies, including in tiered biomonitoring approaches to assess the level of pollutant-induced stress syndrome in sentinel organisms (Grundy et al., 1996; Galloway et al., 2004; Lowe et al., 2006; Viarengo et al., 2007; Martínez-Gómez et al., 2010; Sheir and Handy, 2010; Buratti et al., 2012).

Cholinesterase (ChE) enzymes are serine-hydrolases with important functions in the organism. Of those, the activity of acetylcholinesterase (AChE) has been used as environmental biomarker to detect neurotoxic effects in aquatic organisms. AChE contributes to the normal function of the sensory and neuromuscular systems, catalysing the degradation of acetylcholine into choline and acetate allowing its removal from the synaptic cleft (Payne et al., 1996). Post-synaptic cells may thus recover their resting potential and continue normal cholinergic transmission. AChE inhibition causes acetylcholine to accumulate in the synaptic cleft. This leads to overstimulation originating neuromuscular paralysis (*ex*: interminable muscle contractions) throughout the body and, eventually, death by asphyxiation. Several compounds are recognised inhibitors of AChE activity. AChE inhibition is the primary MOA of OP and carbamate pesticides (Varò et al., 2003; Almeida et al., 2010). But PAHs (Vieira et al., 2008), metals (Frasco et al., 2005), and natural toxins (Kankaanpää et al., 2007) were also shown to inhibit the enzyme activity. Other ChEs such as

butyrylcholinesterase (BChE) and propionylcholinesterase (PrChE), which have higher affinity to butyrylcholine and propionylcholine, respectively, are non-specific esterases or pseudocholinesterases (PChE) (Payne et al., 1996). These enzymes are thought to have a role in detoxification of some compounds (Solé et al., 2008).

Lactate dehydrogenase (LDH) and $NADP^+$–dependent isocitrate dehydrogenase (IDH) are assessed for their role in the anaerobic and aerobic energy production pathways. LDH is involved in glycolysis and gluconeogenesis (Walsh and Henry, 1990; Cristescu et al., 2008). Several studies demonstrated that organisms under chemical stress (*e.g.*, exposure to mercury, zinc, PAHs, hypoxia) may exhibit altered enzyme activity (Wu and Lam, 1997; Long et al., 2003). Mitochondrial IDH acts on the regeneration of $NADP^+$ providing NADPH to be used by glutathione reductase (GR) during the recycling of oxidised glutathione (GSSG) into its reduced form (GSH). Hence it contributes to the cellular defence against oxidative damage caused by ROS generated during biotransformation reactions (Jo et al., 2001). The induction of IDH activity was shown to suppress oxidative stress and its damages (Jo et al., 2001). Dealing with chemical stress may also impose metabolic costs that will lead to depletion of available energy reserves which are diverted from growth. Energy reserves are quantified as glycogen, protein, and lipid content of the test organism and have thus been used as biochemical endpoint in field and laboratory studies to evaluate contaminant exposure (Smolders et al., 2004; Verslycke et al., 2004; Mouneyrac et al., 2008).

When organisms are exposed to contaminants their biotransformation and anti–oxidant defences are activated (Lushchak, 2011). In crustacean, several enzymes and other molecules acting in these processes provide good indications of exposure and health status of organisms (Maria et al., 2009; Pereira et al., 2009; Oliveira et al., 2013). The anti–oxidant defence system includes low molecular weight free radical scavengers (*e.g.,* carotenoids, vitamins, GSH) and high molecular weight proteins (*e.g.,* GST, CAT, GPx, GR) (Livingstone, 2001). GSH is an anti–oxidant and free radical scavenger, and also a cofactor in biotransformation reactions, during

which it becomes oxidised (GSSG) (Livingstone, 2001; Lushchak, 2011). GST are involved in phase II biotransformation, conjugating the lipophilic compounds to facilitate their excretion from the cells; GPx catalyses the conversion of hydrogen peroxide into oxygen and water (Livingstone, 2001). Both, GST and GPx used GSH as cofactor and GR is responsible for the reduction of GSSG into GSH. Detection of oxidative damage to cellular macromolecules is common in organisms exposed to toxicants. It occurs when ROS production exceeds the capacity of the anti–oxidant defences, causing cellular damage and modifying regulatory cascades (Livingstone, 2001; Lushchak, 2011). Among others, lipid peroxidation (LPO) provides a useful measure of the extent of such damage.

7. Thesis objectives and outline

The central objective of this thesis was to investigate effects of environmental contaminants and salinity, as crucial abiotic factor, on a key crustacean of NW Iberian estuaries in relation to the previous exposure history of the organisms. To address these effects a set of non–specific and specific parameters related to contaminants' MOA was selected. This multibiomarker approach was employed in laboratory exposure experiments and field studies to evaluate responses to priority and EC and salinity as well. The working hypotheses were that: *i)* laboratorial exposure to priority and EC would cause alterations in selected biomarkers with potential consequences to the individual and the population; *ii)* these alterations would depend on the site of origin of the crabs suggesting an influence of the previous exposure history in the Lima estuary; and *iii)* biomonitoring studies would reveal alterations in health status reflecting dynamic changes in the levels of environmental contamination and abiotic factors in the study sites.

To tackle this main objective, and following the introductory chapter, the thesis was structured in 7 chapters presenting the results of the work developed (Chapters II to VII) and a general discussion (Chapter VIII).

In Chapter II a seven-day laboratory exposure to waterborne fluoranthene (FLU), a priority PAH previously detected in the Lima estuary, was carried out. The aims were to develop a multibiomarker approach to be used subsequently and to evaluate early warning toxicity of this environmental contaminant towards *C. maenas*. Crabs were collected at the Minho estuary. The biomarkers selected were related to key physiological functions and covered bioaccumulation, cell damage, energy production, and availability, and pathways of neurotransmission, detoxification, and oxidative stress. Based on these results, and on knowledge available on the MOA of toxicants to be further investigated, a battery of biomarkers was chosen for application in the next studies.

Chapters III to V are focused on the potential influence of the history of exposure to contamination on the crabs' response to salinity, and to a model contaminant and an EC. In Chapter III, laboratory exposures to various salinity levels were performed with crabs collected at the mouth of Minho and Lima estuaries. This study intended to investigate a potential influence of salinity on selected *C. maenas* biomarkers, as possible confounding factor in monitoring studies, and contribute with knowledge on additional effects of salinity changes in invertebrates already living under environmental stress, as occurs in polluted estuaries. The results were in support of possible differential sensitivity to contamination of crabs from these estuaries.

To further clarify this issue, in Chapter IV, the objective was to investigate potential differences in sensitivity to the model OP fenitrothion (FEN) of *C. maenas* from a low impacted and a moderately contaminated estuary. The contribution of anti–oxidant biomarkers to FEN metabolism in invertebrates and their involvement in enhanced tolerance to the toxicant was also addressed. FEN was chosen due to its recognised primary MOA (through inhibition of B–esterases activity) and wide use in the control of pests in forests and crops, stored grains, poultry sheds, and in public health programs. Differential responses of AChE to FEN exposure were assessed *in vitro* and *in vivo*.

In Chapter V, the aim was to investigate differential sensitivity to a new compound and bridge the gap of information on the effects of pharmaceuticals in estuarine invertebrates, which limits prediction of their impact to field scenarios and risk calculations envisaging decisions on the need for protection of contaminated ecosystems. Exposure experiments were carried out with SERT, a selective serotonin reuptake inhibitor (SSRI) evermore detected in the aquatic environment, and crabs from both sites. Bioaccumulation and biomarker responses related to SERT MOA were evaluated following subacute exposure to levels detected in the environment and high exposure levels.

The study presented in Chapter VI concerned an investigation on potential interactions between SERT and salinity, in a context of multiple stressors effects, and their relation to the history of exposure to moderate contamination. Concurrent exposure to man–made and natural stressors has become a norm for the estuarine environment. But limited information is available on the combined effects of emerging pharmaceuticals and natural stressors in estuarine organisms. The objective was, therefore, to improve knowledge gathered on the study of Chapter V and gain a better understanding on the differential responses obtained.

Chapter VII presents the results of an integrated chemical and biological effects monitoring study. The work was carried out over two alternate years to assess temporal and spatial variability in contamination by heavy metals, abiotic conditions and health status of *C. maenas* in the study estuaries. Two sampling sites were established in each estuary, one at the mouth and one upstream, in relation to crabs' life cycle.

Chapter VIII comprises a general discussion of the studies performed and provides final concluding remarks and future perspectives.

8. References

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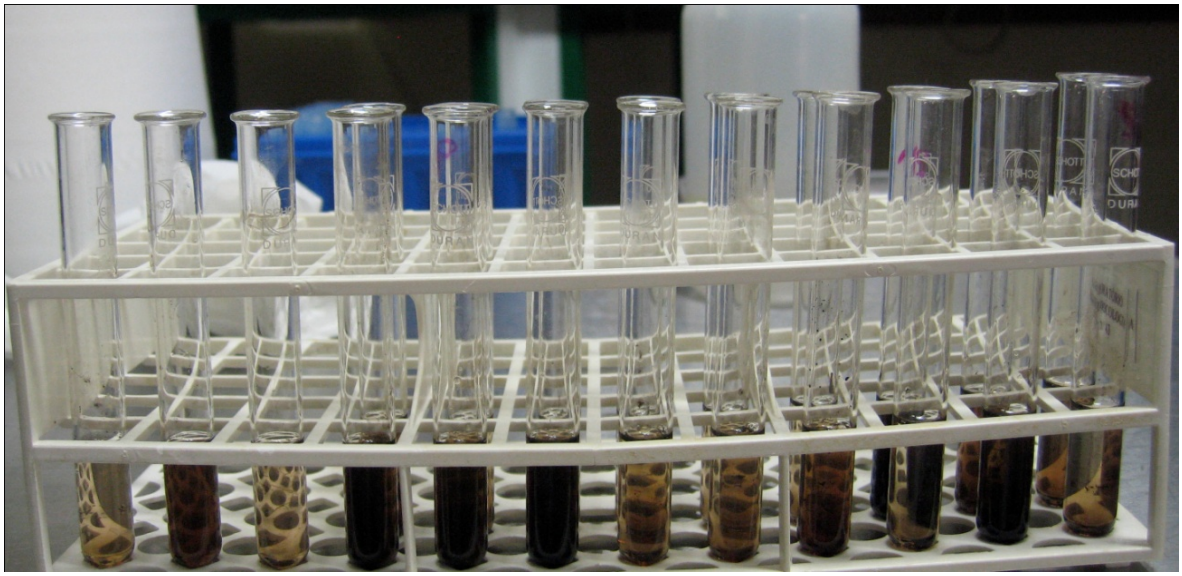
Chapter II

Exposure of *Carcinus maenas* to waterborne fluoranthene: accumulation and multibiomarker responses



Exposure of *Carcinus maenas* to waterborne fluoranthene: accumulation and multibiomarker responses

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Abstract

Fluoranthene (FLU) is a priority polycyclic aromatic hydrocarbon (PAH) commonly detected in estuarine sediments, water and biota. Despite this, information on FLU detection, accumulation and effects on marine crustaceans is scarce. This work investigated the accumulation of FLU in *Carcinus maenas* and the responses of several early–warning biomarkers after a 7–day laboratory exposure to five FLU concentrations (2.56 to 100 μg L^{-1}). After exposure to FLU, sub–samples of the crabs' digestive gland and muscle were collected for biomarker determinations. The remaining digestive gland and muscle, together with the rest of the whole-body soft tissues, were analysed for FLU residues by gas chromatography–mass spectrometry (GC–MS). The biomarkers assessed were: *i)* the quantification of FLU–type compounds by fixed wavelength fluorescence (FF); *ii)* the activities of glutathione *S*–transferases (GST) and glutathione reductase (GR), and the levels of total glutathione (GT) and lipid peroxidation (LPO) for oxidative stress; *iii)* the activity of acetylcholinesterase (AChE) for neurotoxicity; *iv)* the activities of isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH) enzymes, and total protein, glycogen and lipids as indicators of changes in energy metabolism and storage; and *v)* the lysosomal membrane stability (LMS) as a measure of cell damage. The results showed strong (R^2>0.95) concentration–dependent accumulation of FLU residues (as measured by GC–MS) in the remaining whole–body soft tissues and of FLU–type compounds (as measured by FF) in the digestive gland and muscle. A strong positive linear relationship (R^2=0.91) between FLU residues and FLU–type compounds was also found. Comparing to

controls, activities of GST and GR were significantly higher in crabs exposed to ≥16 and ≥40 µg L^{-1} FLU, respectively. TG levels and IDH activity showed a significant trend to increase with FLU concentrations whereas AChE activity exhibited the opposite trend. FF measurements in the digestive gland and muscle proved to be an expeditious cost–effective method to assess the uptake and availability of FLU and its metabolites in *C. maenas*. The results suggest that under continuous environmental FLU may enhance detoxification and anti–oxidant defences, and cause alterations in the aerobic energy pathway, as well as neuromuscular toxic effects that may increase *C. maenas* risk of predation.

1. Introduction

During the last decades concern about contamination of aquatic ecosystems by polycyclic aromatic hydrocarbons (PAHs) has grown steadily. These broadly distributed organic contaminants are released into the aquatic environment from natural (*e.g.*, volcanic eruptions, forest fires) and anthropogenic sources. The latter include municipal and industrial discharges, incomplete combustion of fossil fuels and waste incineration, discharge of drilling waste and produced water from offshore oil production, and spillage of petrochemical products from oil drilling, refinery, and transport activities (Albers and Loughlin, 2003; Balk et al., 2011). Despite awareness and efforts to limit discharges into the environment (*e.g.*, through the OSPAR Convention), these inputs continue to cause contamination of marine and coastal areas, therefore, highlighting an ever growing need to detect PAHs in aquatic organisms and assess their adverse effects.

Among aquatic organisms, fish readily metabolise PAHs so that measured body residues may often be low (van der Oost et al., 2003). Nevertheless, severe effects of PAH exposure have been demonstrated in fish species, such as increased mortality, decreased reproduction, hepatic and carcinogenic lesions, developmental abnormalities, and reduced swimming performance (Collier and Varanasi, 1991; Hawkins et al., 1991;

Billiard et al., 2008). Similarly to fish, some invertebrates, including crustaceans, are capable of efficiently metabolise and eliminate PAHs, although at slower rates (James et al., 1995; Livingstone, 1998). In agreement with this, previous studies demonstrated that bound residues and conjugated metabolites of PAHs, including fluoranthene (FLU), can accumulate in crustaceans and cause various effects, including at the population level (James et al., 1995; Lotufo, 1998; Barata et al., 2002; Bell et al., 2004; Schuler et al., 2004).

FLU is included in the lists of priority substances in the field of water policy of the European Commission and the United States Environmental Protection Agency. It is commonly found in the aquatic environment and is one of the most concentrated PAH compound observed in sediments, particulate matter, and water (Baumard et al., 1998; van Hattum et al., 1998). High water concentrations of FLU have been detected in systems around the world, ranging from ≤45 µg L^{-1} in unspecific waste water (IARC, 1983) to ≤23.8 µg L^{-1} in estuarine pore water (Maskaoui et al., 2002), and ≤1.4 µg L^{-1} in seawater (Law et al., 1997). It has a persistent lipophilic character, comparing with other compounds of the same group, and it is among the most abundant PAHs accumulated in molluscs and crustaceans (Baumard et al., 1998; Ricciardi et al., 2010; Bouzas et al., 2011). Moreover, waterborne exposures to FLU were shown to cause severe toxic effects in some crustaceans (Suedel and Rodgers, 1996; Lotufo, 1998; Spehar et al., 1999; Barata et al., 2002; Sepic et al., 2003; Bell et al., 2004; Schuler et al., 2004). However, data on FLU accumulation, detection and effects are still limited; the lack of such data holds true also for crab species, such as the shore crab (*Carcinus maenas*), which is a dominant crab in European estuarine habitats and shallow coastal areas (Almeida et al., 2008). Because of its broad distribution area, response to environmental contamination and easy laboratory maintenance, the species is commonly used as a suitable bioindicator and test organism (Dissanayake and Galloway, 2004; Galloway et al., 2004; Maria et al., 2009; Pereira et al., 2009; Mesquita et al., 2011; Buratti et al., 2012). Moreover, as a valuable food source for human consumption, it is exploited in some

regions by seafood industries for the preparation of shellfish pastes, empanadas, and patties, thus having economic importance.

The present study investigated the relationships between exposure, accumulation and effects of FLU in *C. maenas* exposed for seven days to this PAH. Exposure and uptake of FLU were assessed by gas chromatography–mass spectrometry (GC–MS) and by fixed wavelength fluorescence (FF). During the past two decades determination of PAH metabolites in fish bile and soft tissues (*i.e*. liver, muscle, brain) by FF has been successfully used to assess exposure to PAHs partly replacing the time–consuming and expensive classical analytical methods (*e.g*., HPLC, GC–MS) (Lin et al., 1996; Fernandes et al., 2007; Balk et al., 2011; Almeida et al., 2012). This technique has also been adapted and used to measure PAH metabolites in urine and haemolymph of crab species (Dissanayake and Galloway, 2004; Watson et al., 2004). However, in organisms such as crabs that show slow rates of PAH metabolisation and excretion, leading to retention of PAH–type compounds for prolonged periods (James et al., 1995), their quantification in tissues (including those involved in biotransformation) may provide other desirable indications of PAH exposure, and suitable data to relate with the effects observed. Indeed, studies on accumulation, distribution, and biotransformation of naphthalene and benzo(a)pyrene performed in the decapods *Hemigrapsus nudus* and *Homarus americanus*, respectively, have shown that these compounds and their metabolites are largely retained in the digestive gland and in the muscle for long post–administration periods (Laurén and Rice, 1985; James et al., 1995). Therefore, in the present study sub–samples of the digestive gland and muscle were collected at the end of the exposure experiments and analysed by FF for the determination of FLU–type compounds. The remaining digestive gland and muscle together with the rest of the whole–body soft tissues were subsequently analysed for FLU residues by GC–MS.

The use of multibiomarker approaches that measure changes at the biochemical, cellular, and physiological levels are highly advisable and has been proven valuable in both laboratory and field studies (Galloway et al.,

2004; Maria et al., 2009; Guimarães et al., 2012). Moreover, it has been recognised that biomarkers, when used in a battery, provide a comprehensive assessment of pollutant–induced stress offering suitable tools to help define the Good Environmental Status (qualitative descriptor 8: "concentrations of contaminants are at levels not giving rise to pollution effects") required by the Marine Strategy Framework Directive (Lyons et al., 2010). Hence, in addition to the determination of FLU–type compounds, several other biomarkers often applied to assess the effects of environmental pollutants in *C. maenas* (Maria et al., 2009; Pereira et al., 2009; Ricciardi et al., 2010; Mesquita et al., 2011; Buratti et al., 2012) were employed in this study. Lysosomal membrane stability (LMS) of haemolymph cells was assessed as a measure of cell damage. Biomarkers of phase II biotransformation (activity of glutathione *S*–transferases [GST]), anti–oxidative defences (activity of enzymes glutathione peroxidase [GPx] and glutathione reductase [GR], and levels of total glutathione [TG]) and oxidative damage (lipid peroxidation [LPO]) were determined in sub-samples of digestive gland. Biomarkers of neurotoxicity (acetylcholinesterase activity [AChE]) and energy metabolism (lactate dehydrogenase activity [LDH] and $NADP^+$–dependent isocitrate dehydrogenase activity [IDH], which is also involved in anti–oxidant responses) were quantified in muscle sub–samples. Total energy available (*Ea*) was determined in both tissues by quantifying the contents in glycogen, lipids, and proteins.

2. Material and methods

2.1. Crab sampling and acclimation

C. maenas individuals were collected in the Minho estuary using baited hand nets. This estuary is under relatively low anthropogenic pressure, showing low contamination levels, and has been used as reference estuary in several ecotoxicological studies (Menezes et al., 2006; Reis et al., 2009; Guimarães et al., 2012). Intermoult male crabs with complete appendices

were selected (3.9 ± 0.07 cm carapace width, mean ± SD) and immediately transported to the laboratory.

Once in the laboratory, the organisms were placed individually in 2 L capacity glass beakers containing filtered seawater at salinity near that measured locally at the time of collection (*i.e.* 15). Temperature was set at 15°C. The beakers were covered with appropriate lids and continuous aeration was provided. The crabs were allowed to acclimatise to these conditions for seven days. They were fed with TETRAFAUNA (*Gammarus*) every 2 days, 2h before medium renewal.

2.2. Chemicals

All the reagents used were of analytical grade. FLU (99% purity) and reagents used in the biochemical determinations were purchased from Sigma–Aldrich Chemical (Steinheim, Germany), except the Bio–Rad protein assay dye reagent that was purchased from Bio–Rad Laboratories, Inc.

2.3. Exposure experiments

The crabs were individually exposed to five FLU concentrations (2.6, 6.4, 16.0, 40.0 and 100.0 µg L^{-1}) for a period of seven days. Stock solutions were prepared in analytical grade acetone for each tested concentration. Test solutions were prepared by dilution of 100 µL of the respective stock solution in 2 L of filtered seawater (salinity 15).

Two control treatments were included: a control group prepared with filtered seawater only and a control+solvent group prepared by dilution of 100 µL acetone in 2 L of filtered seawater. Nine crabs were exposed per treatment; the exposure conditions were the same as described for the acclimation period. Medium renewal was performed every 24h. Phototoxicity of FLU by ultra–violet (UV) radiation was considered as negligible since fluorescent light has low UV emission (Barata and Baird, 2000). pH, dissolved oxygen, and temperature were measured both in the old and the freshly prepared test media using a portable multiparametric sensor (WTW multi 340i) with the appropriate probes (pH Sen Tix 41 and

Tetracon 325). During the test, the oxygen levels were always within 90% of saturation levels. Mean salinity was 15 ± 0.1 (standard error, SE) and mean pH 8.1 ± 0.1; differences between freshly prepared and old test solutions never exceeded 0.20 and 0.15 units, respectively. No mortality was recorded during the experiment in any of the test concentrations.

At the end of the exposure period, haemolymph samples were collected and immediately used to assess LMS through the neutral red retention (NRR) assay. Sub–samples of digestive gland and muscle from each crab were subsequently collected into microtubes and stored at –80°C until further analysis. These sub–samples were used for the measurement of FLU–type compounds by FF, and the determination of biochemical biomarkers and energy content. Seven to nine samples/crabs per experimental condition were analysed for each biomarker. The remaining digestive gland (~50%) and muscle (~80%) together with the rest of the crabs' soft tissues, hereafter referred to as whole–body soft tissues analysed for simplicity, were collected into glass vials and used for the quantification of FLU by GC–MS. FLU determination by GC–MS was performed in one pool of crabs' tissues per test concentration. Each pool contained the soft tissues of nine experimental crabs and was analysed in duplicate.

2.4. Determination of FLU in soft tissues of crabs and water samples

FLU was extracted from the whole–body soft tissues using a microwave-assisted extraction method described by Aguinaga et al. (2008). The samples were then processed by solid phase microextraction and GC–MS according to the protocol developed by Rocha et al. (2011) and the results expressed in ng g^{-1} dry weight (dw). Briefly, the solid phase microextraction device and the fibres, coated with polydimethylsiloxane (PDMS) with a 100 µm film thickness, were from Supelco (Bellefonte, PA). The fibres were immersed in 15 mL sample solutions in amber glass vials capped with PTFE–coated septa. GC–MS analysis was performed using a gas chromatograph (Varian CP-3800) coupled with an ion trap mass spectrometer (Varian Saturn 2200). Identification of FLU was based on the

comparison of its GC–retention time and mass spectrum with an appropriate standard (Rocha et al., 2011). The certified reference material used for quality control was SRM 2977 (National Institute of Standards and Technology, USA); recovery rate was 99%. The detection limit for FLU residues was 0.1 ng g^{-1} dw.

Water samples (50 mL) of freshly prepared (n=3) and old (n=3) experimental media were collected from test vessels at 40.0 and 100.0 µg L^{-1} for chemical analysis. These samples underwent a solid phase microextraction using 100 µm PDMS fibres and subsequent desorption in the GC–MS. Determination of FLU in water samples was validated by spiking some water samples with known concentrations of the compound and calculating recovery rate. Recovery was always above 94%.

2.5. Measurement of biomarkers

2.5.1. Haemolymph sampling and NRR assay

The NRR assay was performed according to the protocol developed by Moore et al. (2004). Briefly, a neutral red (NR) stock solution (0.028 g of NR dye in 1 mL of dimethyl sulphoxide) was used to prepare a working solution by diluting 0.010 mL of stock solution with 5 mL physiological saline solution (PSS) adjusted to the experimental salinity (PSS; 0.5 M NaCl, 0.011 M KCl, 0.012 M $CaCl_2 \cdot 6H_2O$, 0.026 M $MgCl_2 \cdot 6H_2O$, 0.045 M $Na_2HPO_4 \cdot 12H_2O$, 0.045 M Trizma-Base, 1 M HCl; pH 7.4). Haemolymph samples (0.1 mL) were withdrawn from the arthrodial membrane at the base of the third walking leg with a hypodermic needle (28 g×1″) containing 0.1 mL PSS. The cell suspensions were transferred into microtubes kept cooled on ice water for max. 10 min. 50 µL of the sample were then pipetted onto glass microscope slides and allowed to adhere in a light-proof humidity chamber for 15 min. The NR working solution was applied onto each slide and a coverslip was placed. The slides were examined under a light microscope (400× magnification in a Leica DM 2000) at 15, 30, 45, 90, 120, 160, and 180 min. Colour intensification in the cytoplasm of blood cells, indicating leakage of the accumulated dye

from the lysosomes, changes in the size and colour of lysosomes (formation of secondary lysosomes) and changes in cell shape were investigated. When at least 50% of the cells recorded (*ca.* 100 single cells observed during each examination) showed some of the alterations described above, the sample was considered as having passed the assessment threshold status and this time-point (in min) was recorded as the result parameter.

2.5.2. FLU–type compounds in muscle and digestive gland

Samples of muscle and digestive gland were homogenised (1:10 wt v^{-1}) in ice–cold phosphate buffer (pH 7.4, 0.1 M) and used to quantify FLU and its metabolites, hereafter indicated as FLU–type compounds (Almeida et al., 2012). The homogenates were diluted (1:200) with 50% methanol. Fluorescence readings (Jasco FP-6200 spectrofluorimeter) were made at 359 and 462 nm as excitation and emission wavelengths, respectively, using a fluorescence cuvette (109.004F–QS) from Hellma Analytics. The amount of FLU–type compounds was determined using an eight-point calibration curve (R^2=0.99) of FLU. The results were expressed as ng per mg tissue protein (see below).

2.5.3. Biotransformation, anti–oxidant defences, and oxidative damage

The digestive gland was homogenised (1:10 wt v^{-1}) in phosphate buffer (pH 7.4, 0.1 M). Part of the homogenate was used to determine the endogenous LPO by measuring spectrophotometrically (at 535 nm) thiobarbituric acid reactive substances (TBARS), as adapted by Filho et al. (2001). LPO levels were expressed in nmol of TBARS per g of wet tissue. The remaining homogenate was centrifuged at 10,000 ×*g* for 20 min at 4°C. The post–mitochondrial supernatant was used to determine GST, GR and GPx activities, and TG levels. Determination of GST activity was based on the method of Habig et al. (1974). The conjugation of reduced glutathione (GSH) with 1–chloro–2,4–dinitrobenzene (CDNB) was followed in a microplate reader (at 340 nm) as previously described in Rodrigues et

al. (2012). GST activity was expressed in nmol of substrate conjugated per min per mg of protein. GR activity was measured by assessing the decrease of NADPH levels caused by the reduction of oxidised glutathione (GSSG) to GSH (at 340 nm) (Cribb et al., 1989). GR activity was expressed in nmol of oxidated $NADP^+$ per min per mg of protein. GPx activity was determined by measuring the decrease in NADPH (at 340 nm) using hydrogen peroxide as substrate, according to Mohandas et al. (1984). GPx activity was expressed in nmol per min per mg of protein. TG levels were determined as described in Tietze (1969) by following the change in absorbance at 412 nm due to the recycling reaction of GSH with 5,5'-dithio-bis-2-nitrobenzoate (DTNB) in the presence of GR. TG levels were expressed as nmol of recycled GSH per min per mg of protein.

2.5.4. Neurotransmission and energetic metabolism

Determination of AChE activity was based on the method of Ellman et al. (1961). The increase of absorbance due to the reaction of thiocholine with DTNB was followed at 412 nm in a microplate reader as previously described (Rodrigues et al., 2012). AChE activity was expressed in nmol of substrate hydrolised per min per mg of protein. While it is known that different cholinesterase isoforms may be present in muscle tissue, recent experiments have shown that AChE is the main form present in *C. maenas* muscle (Rodrigues et al., *unpublished data*). LDH activity was assayed by the method of Vassault (1983). The decrease in absorbance due to NADH oxidation resulting from the conversion of pyruvate to lactate was measured at 340 nm in a microplate reader as described in Rodrigues et al. (2012). LDH activity was expressed in nmol per min per mg of protein. IDH activity was determined through the method developed by Ellis and Goldberg (1971). The increase in absorbance due to the reduction of $NADP^+$, mediated by IDH, was monitored at 340 nm using a microplate reader. IDH activity was expressed in nmol of NADPH substrate regenerated per min per mg of protein. Protein concentrations in the

samples were determined by the Bradford method (1976), using bovine γ–globuline as protein standard.

2.5.5. Energy available (Ea)

Protein, glycogen, and lipid contents were measured in sub–samples of muscle and digestive gland. Samples were homogenised in ice-cold buffer (0.1 M Tris–HCl pH 8.5, 15% [w v^{-1}] poly vinyl pyrrolidone, 153 µM $MgSO_4$, 0.2% [w v^{-1}] Triton X-100) and the homogenates were used to determine the principal biochemical energy reserve fractions. To determine the protein content 0.01 N NaOH was added to the homogenates. A 30–min incubation at 60°C followed. Total protein content was determined by the method of Bradford (1976), using bovine γ–globuline as standard. Glycogen content was quantified with the Anthrone reagent using the protocol established by Roe and Dailey (1966) and oyster glycogen as standard. Lipids were extracted according to the protocol established by Bligh and Dyer (1959), with some modifications. Chloroform and methanol were added to the homogenate. After centrifugation the bottom layer was transferred into glass tubes, followed by the addition of sulfuric acid into each tube. The tubes were subsequently heated for 15 min at 200°C. After cooling down and adequate dilution of the samples, the absorbance was read at 340 nm, using tripalmitin as standard. For the calculation of *Ea*, the biochemical fractions were transformed into energetic equivalents using their respective combustion energy (24 000 mJ mg^{-1} protein, 17 500 mJ mg^{-1} glycogen, 39 500 mJ mg^{-1} lipid) (Gnaiger, 1983) and summed up.

All cuvette absorbance assays were performed using a Jasco 6405 UV/VIS spectrophotometer. Microplate determinations were carried out with a Bio Tek Power Wave 340 microplate reader. FLU–type compounds were measured using a Jasco 6200 spectrofluorometer.

2.6. Data analysis

Assumptions of normality and variance homogeneity were checked using the Shapiro–Wilks and the Levene tests, respectively. Possible

differences between the control and the control + solvent groups were sought for all the parameters analysed using the *t*–Student test. Because no significant differences between the two groups were found they were pooled together for the purposes of graphical presentation of the results and data analysis. Simple linear regression was used to assess the relationships between FLU exposure concentrations, FLU in the whole–body soft tissues analysed, and FLU–type compounds in muscle and the digestive gland. For GC–MS determinations of FLU, half of the detection limit was taken as the control value. Data pertaining to LMS, GST, GPx, GR, LPO, *Ea* and contents in proteins, glycogen and lipids were analysed by one–way analysis of variance (ANOVA), followed by the Dunnett's test. Data concerning TG, AChE and IDH were analysed by linear contrast analysis of variance. Results are presented as means and respective SE, except when indicated otherwise. The significance level was set at $p<0.05$ for all tests performed. All statistical analyses were carried out with IBM SPSS version 19.0.

3. Results

3.1. FLU content in water samples and whole-body soft tissues

Regarding water chemistry, no differences were found between nominal and measured concentrations of FLU in freshly prepared experimental media; measured concentrations were 39.96 ± 1.78 (mean ± SD) and 100.02 ± 6.76 µg L^{-1} for nominal concentrations of 40 and 100 µg L^{-1}, respectively. Thus, the different FLU exposure levels are hereafter indicated as nominal concentrations. In samples taken from the old experimental media, prior to the daily water renewal, measured FLU concentrations were 19.97 ± 0.86 and 49.39 ± 4.27 µg L^{-1} for nominal concentrations of 40 and 100 µg L^{-1}, respectively, differing by *ca.* 50% from the freshly prepared ones. At the end of the seven–day exposure, the concentrations of FLU in the whole–body soft tissues analysed ranged from 1.48 ± 0.04 (mean ± SE, control group) to 9.70 ± 0.52 ng g^{-1} dw (group exposed to the highest FLU concentration). The results also indicated a

significant concentration–dependent accumulation of FLU residues in the whole–body soft tissues analysed (R^2=0.986, Fig. II.1.A).

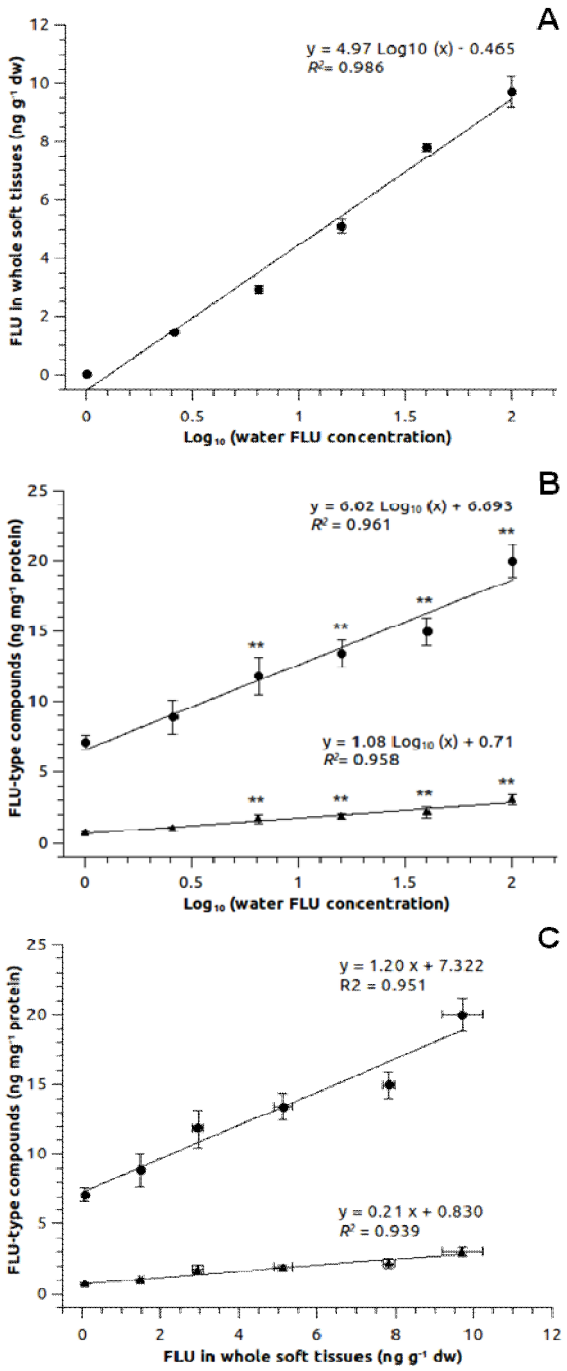

Fig. II.1. Fluoranthene (FLU) concentrations in *C. maenas* at the end of 7 days of exposure. FLU residues (GC–MS) in the whole soft tissues analysed as a function of water concentrations (A). FLU–type compounds (fixed wavelength fluorescence) in digestive gland (●) and muscle (▲) as a function of water concentrations (B). Relationship between FLU–type

compounds in digestive gland (●) and muscle (▲) and FLU residues in the whole soft tissues analysed (C). Values represent the mean with the corresponding standard error. **Indicates statistical significance as indicated at $p<0.01$.

3.2. FLU-type compounds

At the end of the seven–day exposure, FLU–type compounds determined by FF ranged on average from 7.16 ± 0.49 to 20.00 ± 1.16 ng mg^{-1} protein in the digestive gland and from 0.78 ± 0.07 to 3.06 ± 0.36 ng mg^{-1} protein in the muscle tissue. A concentration–dependent increase of FLU–type compounds with FLU exposure concentration was found in both cases (digestive gland: $R^2=0.961$; muscle: $R^2=0.958$; Fig. II.1.B). Significant differences in the concentration of FLU–type compounds relative to the control group, indicating accumulation of the parent compound and its metabolites, were found in crabs exposed to FLU concentrations ≥6.4 µg L^{-1} for the digestive gland (one-way ANOVA: $F_{(5,56)}=23.65$, $p<0.0001$; Dunnett's test: $p<0.05$) and the muscle tissue ($F_{(5,56)}=12.74$, $p<0.0001$; Dunnett's test: $p<0.001$). Strong significant relationships were also found between FLU–type compounds and FLU residues in whole soft tissues (digestive gland: $R^2=0.951$; muscle: $R^2=0.939$; Fig. II.1.C).

3.3. Lysosomal membrane stability

Concerning LMS of haemocytes, no significant differences in NRR times were found between experimental groups ($F_{(5,49)}=0.12$, $p=0.988$), indicating no apparent changes in cellular viability (Fig. II.2.A).

3.4. Biotransformation, anti–oxidant defences, and oxidative damage

Phase II biotransformation was induced by FLU exposure, as indicated by the significant increase (2.5 to 4–fold) in GST activity observed at concentrations ≥16 µg L^{-1}, compared to the control group (one–way

ANOVA; $F_{(5,45)}$=9.90, p<0.0001; Dunnett's test, p<0.001) (Fig. II.2.B). No significant differences among the treatments were observed in GPx activity ($F_{(5,44)}$=1.31, p=0.278) (Fig. II.2.C).

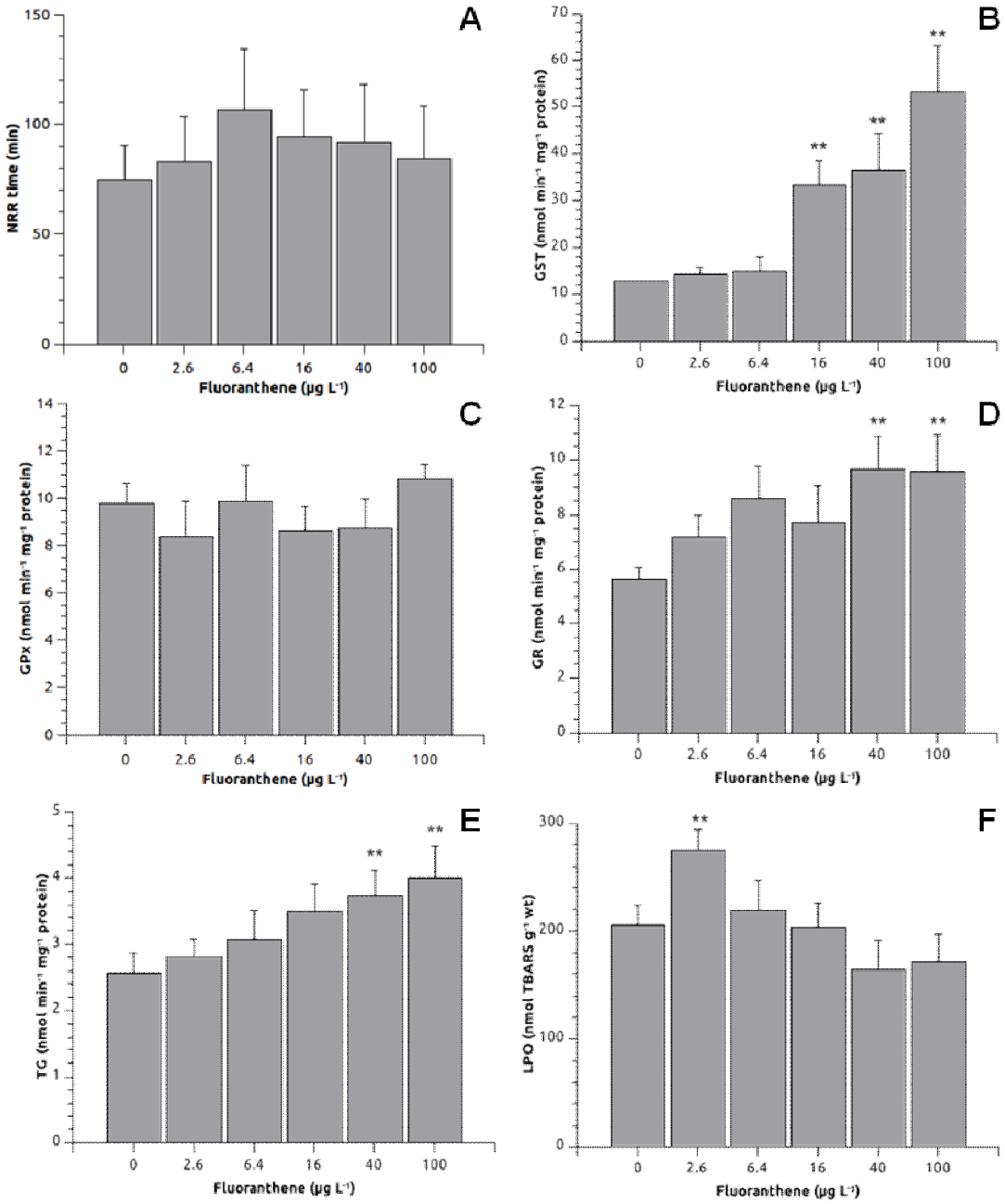

Fig. II.2. Biomarkers of cell damage, anti–oxidant defences, and oxidative stress assessed in crabs exposed for seven days to waterborne fluoranthene. Lysosomal membrane stability, assessed through the neutral red retention assay (NRR, A). Activity of the enzymes glutathione *S*–transferases (GST, B), glutathione peroxidase (GPx, C), and glutathione

reductase (GR, D). Levels of total glutathione (TG, E) and lipidic peroxidation (LPO, F). Values represent the mean with the corresponding standard error. **Indicates statistical significance as indicated at $p<0.01$ either by the Dunnett's test or linear contrast analysis.

However, the activity of the anti–oxidant enzyme GR was significantly induced (+70%) in crabs exposed to concentrations ≥40 μg L^{-1} ($F_{(5,45)}=3.39$, $p=0.011$; Dunnett's test, $p<0.05$) (Fig. II.2.D). A significant increasing trend of TG levels with the FLU exposure concentration was also observed (one–way ANOVA with linear contrast analysis: $F_{(1,5)}=10.37$, $p=0.002$); crabs exposed to the highest test concentration showed an elevation of *ca*. 50% relative to the control group (Fig. II.2.E).

In addition, LPO levels were significantly higher in crabs exposed to 2.6 μg L^{-1} compared to controls ($F_{(5,56)}=2.57$, $p=0.037$; Dunnett's test: $p<0.05$), indicating oxidative damage of lipid macromolecules in these organisms. However, in higher FLU concentrations the mean LPO levels were similar to control levels (Fig. II.2.F).

3.5. Neurotoxicity

A significant decreasing trend of muscle AChE activity with elevating FLU concentration was observed ($F_{(1,52)}=9.40$, $p=0.003$), indicating a neurotoxic effect. The crabs exposed to the highest test concentrations showed about 40% inhibition of the enzyme activity relative to the control group (Fig. II.3.A).

3.6. Energy metabolism

IDH activity showed a significant trend to increase with the FLU concentration ($F_{(1,55)}=7.41$, $p=0.009$), suggesting an increase in the aerobic pathway of energy production (Fig. II.3.B). In crabs exposed to the highest FLU concentration the IDH activity was 30% higher than in controls. In contrast, no significant differences in LDH activity were found among the experimental treatments ($F_{(5,55)}=0.38$, $p=0.862$) (Fig. II.3.C).

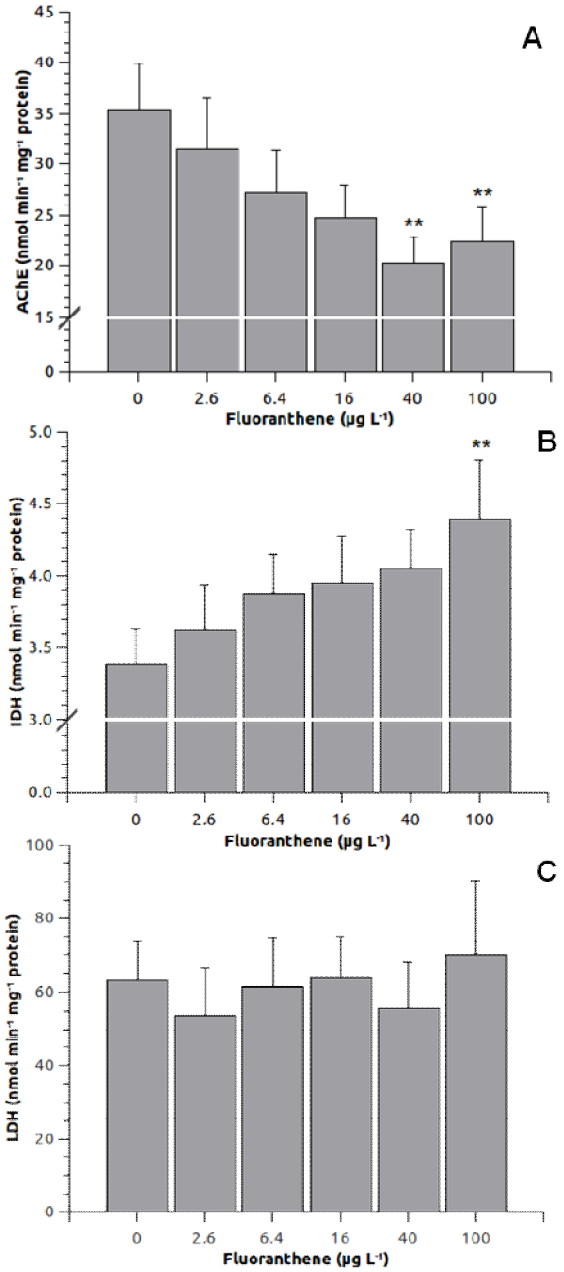

Fig. II.3. Biomarkers of neurotoxicity and energy metabolism assessed in crabs exposed for seven days to waterborne fluoranthene. Activity of the enzymes acetylcholinesterase (AChE, A), $NADP^+$–dependent isocitrate dehydrogenase (IDH, B) and lactate dehydrogenase activity (LDH, C). Values represent the mean with the corresponding standard error. **Indicates statistical significance as indicated at $p<0.01$ either by the Dunnett's test or linear contrast analysis.

3.7. Energy storage

The *Ea* parameters determined in *C. maenas* muscle and digestive gland tissues after seven days of exposure to different concentrations of FLU are shown in Table II.1. Protein and glycogen reserves were similar in the two tissues but the amount of total lipid was 3–5 folds higher in the digestive gland than in the muscle.

Ea reflected this pattern, ranging on average between 10.03 ± 1.45 (minimum) and 13.51 ± 1.52 (maximum) mJ mg^{-1} protein in the digestive gland, and between 3.40 ± 0.50 (minimum) and 4.56 ± 0.39 (maximum) mJ mg^{-1} protein in muscle tissue. Regarding the effects of exposure to FLU, no significant differences among treatments were observed for any of these parameters (digestive gland: protein, $F_{(5,56)}=1.08$, $p=0.379$; glycogen, $F_{(5,56)}=0.24$, $p=0.944$; lipids, $F_{(5,56)}=0.51$, $p=0.768$; *Ea*, $F_{(5,56)}= 0.406$, $p=0.842$; muscle: protein, $F_{(5,57)}=1.22$, $p=0.313$; glycogen, $F_{(5,57)}=1.18$, $p=0.331$; lipids, $F_{(5,57)}=0.48$, $p=0.791$; *Ea*, $F_{(5,57)}=2.46$, $p=0.063$).

4. Discussion

4.1. FLU content in water samples and whole–body soft tissues

FLU concentrations in the experimental media were approximately 50% of nominal values at the end of 24h of exposure, *i.e*. before the renewal of test solutions in the experimental vials. These losses are likely to reflect uptake of FLU by crabs as well as adsorption to glass and to the carapace of the crabs.

Volatilisation was considered as negligible here given that the test vessels were covered. In this regard, previous studies concerning the toxicity of FLU to *Chironomus tentans* have shown a high concentration of FLU residues in exuviae after 96h of exposure, demonstrating that a substantial portion of the body burden was associated with this structure (Bell et al., 2004). According to these authors, the chitinous components of the exoskeleton may serve as a partitioning phase for FLU, thereby decreasing body burdens of the compound during the transition from

larvae to adult. The chitinous nature of *C. maenas* carapace may also favour this process, thus contributing to the marked losses detected prior to the daily water renewals.

Table II.1. Energy reserves available in *C. maenas* exposed for 7 days to waterborne fluoranthene. Values represent the mean ± standard error (within parentheses) of protein (mJ mg^{-1}), glycogen (mJ mg^{-1}), and lipid (mJ mg^{-1}) contents, as well as total energy available (*Ea*, mJ mg^{-1}).

		Fluoranthene (µg L^{-1})					
Parameter		0	2.6	6.4	16	40	100
Digestive gland	Proteins	1.34	1.49	1.41	1.39	1.43	1.34
		(0.07)	(0.04)	(0.05)	(0.04)	(0.02)	(0.05)
	Glycogen	0.33	0.34	0.30	0.31	0.31	0.32
		(0.01)	(0.02)	(0.02)	(0.02)	(0.03)	(0.04)
	Lipids	10.66	11.68	9.66	9.45	8.30	9.53
		(1.44)	(1.47)	(1.23)	(1.88)	(1.43)	(1.78)
	Ea	10.88	13.51	11.38	11.15	10.03	11.18
		(1.62)	(1.52)	(1.28)	(1.92)	(1.45)	(1.82)
Muscle	Proteins	1.38	1.11	1.18	1.02	1.41	1.42
		(0.13)	(0.18)	(0.15)	(0.13)	(0.15)	(0.17)
	Glycogen	0.26	0.29	0.32	0.29	0.25	0.29
		(0.02)	(0.02)	(0.04)	(0.03)	(0.03)	(0.02)
	Lipids	2.92	2.62	2.91	2.52	3.16	2.86
		(0.26)	(0.29)	(0.34)	(0.34)	(0.28)	(0.38)
	Ea	4.56	3.55	4.56	3.40	4.83	4.56
		(0.29)	(0.36)	(0.39)	(0.50)	(0.28)	(0.36)

Quantification of FLU residues by GC–MS indicated uptake by the test organisms and a concentration–dependent accumulation in whole soft tissues. These results are in good agreement with previous findings for other arthropod species. Bioaccumulation of FLU has been previously reported to occur in midges, amphipods, and copepods during lethal and sublethal toxicity tests (Lotufo, 1998; Bell et al., 2004; Schuler et al., 2004). Moreover, the results suggest that in natural ecosystems

contaminated by FLU, dietary transfer to *C. maenas* predators, including humans, may occur.

4.2. FLU–type compounds

The levels of FLU–type compounds determined in muscle and digestive gland were also concentration–dependent and showed high relationships (R^2>95%) with FLU residues in the whole–body soft tissues analysed. The significant accumulation found in both tissues at exposure concentrations ≥6.4 µg L^{-1} suggests that distribution of FLU through circulation occurred in the exposed crabs (James et al., 1995).

Thus, fluorescence measurements proved as a useful tool to detect uptake and accumulation of FLU–type compounds in *C. maenas* tissues. The technique has also been successfully employed earlier in *C. maenas* haemolymph to study exposure to pyrene and its metabolites (Watson et al., 2004). This approach is advantageous in the sense that both the parent compound and its metabolites are measured.

This is particularly important in the case of FLU since it has been previously shown that some FLU metabolites may be at least as toxic to some species as the parent compound, contributing to the overall effects observed (Sepic et al., 2003; Schuler et al., 2004). Compared to GC–MS the method also requires a considerably smaller amount of sample and allows a cost–effective determination of PAH compounds in individual organisms. Furthermore, in species that show slow elimination rates, PAH metabolites may represent a significant part of tissue burdens, *e.g.*, as found for naphthalene and benzo(a)pyrene accumulation in muscle and the digestive gland of other decapod species (Laurén and Rice, 1985; James et al., 1995). In such situations, the assessment of the parent substance alone may lead to underestimated measurements, overlooking the risks posed by the potentially toxic metabolites produced from metabolism and biotransformation (Livingstone, 1998).

4.3. Lysosomal membrane stability

Concerning LMS, control values obtained in this study for the NRR time are in agreement with those determined for *C. maenas* by other authors both in field and laboratory studies (Galloway et al., 2004; Buratti et al., 2012). However, in spite of the FLU accumulation found in *C. maenas* tissues, no differences in LMS were found between experimental treatments, suggesting that for this exposure period FLU effects were not yet apparent at this higher level of biological complexity. Lysosomes are able to store a wide range of contaminants including PAHs, which can influence their membrane permeability and the acid hydrolase content (Svendsen and Weeks, 1995; Grundy et al., 1996).

Studies on molluscs have shown that PAHs, including FLU, cause loss of lysosomal membrane integrity of haemolymph cells (Grundy et al., 1996; Lowe et al., 2006). This effect was thought to be due to altered membrane fluidity resulting from lipid unsaturation and hydrophobicity and/or the generation of oxyradicals during PAH metabolism that can cause oxidative damage to cellular structures (Grundy et al., 1996).

Nevertheless, those studies employed either longer exposure periods or concentrations at least two–fold higher than the concentrations tested in the present work. These differences are likely to account for the no–effect results on LMS obtained here. It is also probable that, under our exposure conditions, the significantly induced biotransformation and anti–oxidant defences may have contributed, through detoxification and oxyradicals scavenging, to prevent the loss of lysosomal membrane integrity possibly caused by exposure to FLU.

4.4. Biotransformation, anti–oxidant defences, and oxidative damage

Exposure to FLU significantly increased the levels of GST (at exposure concentrations ≥16 μg L^{-1}), and GR and TG (at concentrations ≥40 μg L^{-1}) biomarkers. GST are phase II biotransformation enzymes with critical roles in the detoxification of xenobiotics and defence against oxidative damage, and peroxidative products of DNA and lipids. In detoxification, GST

catalyse the conjugation of phase I metabolites with reduced glutathione (GSH), to facilitate excretion from the organism. GSH is also involved in the direct scavenging of oxyradicals formed during detoxification and acts as a cofactor for GPx activity in the conversion of hydrogen peroxide into oxygen and water.

During this process GSH becomes oxidised and may be subsequently recycled in reactions involving GR activity. These parameters may be altered by a number of xenobiotics, hence their use as biomarkers in fish, molluscs, and crustacean species (Filho et al., 2001; van der Oost et al., 2003 Maria et al., 2009; Pereira et al., 2009; Mesquita et al., 2011; Almeida et al., 2012).

The increased GST activity found in the digestive gland of crabs exposed to ≥16 µg L^{-1} suggests that this enzyme is involved in the detoxification of FLU. This result is well supported by the significant accumulation of FLU–type compounds observed in the digestive gland.

Also, the elevated TG levels observed at concentrations ≥40 µg L^{-1} and the fact that no significant alterations of GPx activity were found after exposure to FLU further suggest that GSH is involved in the detoxification and direct scavenging of oxyradicals, rather than acting as a GPx co–factor. The pattern of GR alteration found in crabs exposed to FLU closely follows that of TG, indicating that GSH recycling is taking place, most probably to cope with the detoxification requirements imposed by FLU exposure. Interestingly, LPO was significantly increased only in crabs exposed to 2.6 µg L^{-1} and near to control values in crabs exposed to higher test concentrations. This result is consistent with the elevations in GST (at concentrations ≥16 µg L^{-1}) and biomarkers of anti–oxidant defences (at concentrations ≥40 µg L^{-1}). Moreover, it suggests that the enhancement in anti–oxidant defences is preventing LPO in organisms exposed to higher test concentrations by keeping the oxyradicals generated through metabolisation reactions at low levels (Livingstone, 1998).

4.5. Neurotoxicity

In this work, muscle AChE activity was inhibited by approximately 40% at FLU concentrations ≥40 μg L^{-1}, indicating exposure to an anticholinergic agent (Ludke et al., 1975). AChE activity, involved in cholinergic transmission, has been widely applied in laboratory assays and monitoring studies with vertebrate and invertebrate species to evaluate neurotoxic effects. Inhibition of AChE activity was initially applied as an indication of exposure to organophosphates and carbamates but was later shown to be sensitive to a variety of environmental contaminants (*e.g.*, metals, detergents, surfactants, PAHs) and also to natural toxins (Payne et al., 1996; Kang and Fang, 1997; Guilhermino et al., 2000; Kankaanpää et al., 2007). Concerning PAHs, as lipophilic compounds that can penetrate phospholipid monolayers increasing membrane unsaturation and hydrophobicity, they may possibly influence membrane–bound enzymes such as AChE. However, the effects of PAH exposure on AChE activity are somewhat contradictory. *In vitro* studies have shown inhibition of AChE activity by FLU and other PAHs in the electric eel (Kang and Fang, 1997). The results obtained led the authors to hypothesize that PAHs with three or more aromatic rings are more potent AChE inhibitors than those with a lower number of rings. *In vivo* inhibition of AChE activity by pyrene was reported in *Pomatoschistus microps* by Oliveira et al. (2011). However, exposure of *Lateolabrax japonicus* and *Dicentrarchus labrax* to benzo(a)pyrene and pyrene, respectively, elicited no relevant alterations in AChE activity (Jifa et al., 2006; Almeida et al., 2012). While species differences in biotransformation mechanisms and AChE sensitivity to PAHs may explain these findings, further research is needed to clarify this issue. It is also of note that moderate levels of AChE inhibition, such as those found in the present study, have been associated with a variety of effects in vertebrates and invertebrate species, including depressed locomotory behaviour (Jensen et al., 1997; Oliveira et al., 2011). Such alterations may render the organisms more easily targeted by predators. In support of this, it is also worth mentioning that not only cholinergic transmission was shown to be involved in crab's locomotion (Sorenson, 1973), but also a

significant correlation between AChE activity and *C. maenas* locomotory behaviour was found previously (Mesquita et al., 2011). The present results thus suggest that on chronic exposure to FLU in the wild, the shore crab may be at increased difficulty to find food and escape from its predators.

4.6. Energy metabolism

In the present study, muscle IDH activity increased with elevated FLU concentration, probably in an attempt to cope with energetic requirements resulting from oxidative stress triggered by the exposure. In consequence of the catabolic action by IDH, the $NADP^+$ molecule is recycled becoming available to maintaining sustained cellular anti-oxidant defences (Lee et al., 2002). Indeed, regeneration of GSH by GR occurs at the expense of NADPH (Lee et al., 2002), and inverse relations have been found between IDH induction and the generation and presence of oxyradicals, as well as the extent of damage to DNA and lipids (Jo et al., 2001). These results are in good agreement with the levels of FLU–type compounds found in muscle tissue. The results presented herein also suggest no changes in the anaerobic pathway of energy production after a seven–day exposure of *C. maenas* to waterborne FLU at concentrations as high as 100 µg L^{-1}, as shown by the lack of differences in LDH activity between control and exposed crabs. Induction of LDH is thought to rapidly provide additional energy to cope with chemical stress (Diamantino et al., 2001).

Nevertheless, studies assessing the effects of PAHs on invertebrate LDH are scarce. Vijayavel and Balasubramanian (2006) recorded a decrease in LDH activity in digestive gland, gills, and haemolymph of the crab *Scylla serrata* after exposure to naphthalene. In contrast, exposure of the oyster *Crassostrea virginica* to sediments contaminated with an involved mixture of metal and PAHs did not affect plasma LDH levels (Chu et al., 2002). This result may indicate differences in the effects caused by single PAHs or their mixtures or otherwise be a consequence of distinct relative proportions and roles of LDH and other pyruvate reductases (*i.e.* opine dehydrogenases) in these species (Urich, 1994).

4.7. Energy storage

Depletion of organisms' energy reserves (*e.g.*, total protein, carbohydrate, and lipid content) has been shown to reflect metabolic costs of dealing with chemical stress (De Coen and Janssen, 1997). For this, the energetic value derived from tissue gross biochemical composition has been used as a biochemical endpoint in field and laboratory studies (Smolders et al., 2004; Verslycke et al., 2004; Mouneyrac et al., 2008). In this work, similar levels of glycogen and proteins were found in *C. maenas* muscle and digestive gland. Lipid levels, and hence the total energy reserves available, were three– to five–fold higher in the digestive gland than in the muscle tissue. In agreement with this, the digestive gland is considered to be the major storage organ of energy reserves in decapod crustaceans (Chang, 1995). As to the effects of FLU, no differences were found between control and exposed crabs. These results are in accordance with the lack of differences between experimental groups concerning LDH activity. They also suggest that possible additional energetic requirements imposed by toxicant stress were fully met through the amount and quality of food provided to the experimental animals during the seven–day exposure period.

In summary, the results presented herein showed that after seven days of exposure to FLU significant accumulation of the compound was measured in the tissues of *C. maenas*. Fluorescence measurements indicated FLU accumulation of FLU–type compounds in both the digestive gland and muscle tissues at exposure concentrations ≥6.4 µg L^{-1}, showing high correlations with the exposure concentrations and the FLU–content in the whole–body soft tissues analysed by GC–MS. FF provided a cost–effective, expeditious method to assess the uptake and availability of FLU and its metabolites for potential dietary transfer to higher trophic levels. Moreover, determination of FLU–type compounds was observed to be the most sensitive endpoint of exposure to FLU. The results presented herein suggest that measurement of FLU–type compounds in feral *C. maenas*, in addition to the suite of anti–oxidant biomarkers used herein, may be

potentially useful in environmental monitoring programmes aiming at assessing PAH contamination. Besides contributing with bioaccumulation data, if combined with the detection of other PAH–type compounds, such as those of naphthalene, pyrene and benzo(a)pyrene, it may possibly provide a rapid and less expensive alternative to determine the origin of PAH contamination (*i.e*. petrogenic, pyrogenic). To address this hypothesis, future studies should focus on the measurement of PAH–type compounds, as well as PAH–type metabolites in urine and haemolymph samples for validity purposes, in ecosystems with different levels of PAH contamination. The bioaccumulation observed was accompanied by increases in detoxification (at concentrations ≥16 µg L^{-1}) and enzymatic anti–oxidant defences (at ≥40 µg L^{-1}) in the digestive gland, as well as neurotoxic effects in the muscle (at ≥40 µg L^{-1}). Altogether, the results suggest that under continuous exposure in natural environments polluted by FLU, *C. maenas* may experience increased oxidative stress, changes in the aerobic energy pathway, as well as neuromuscular effects that may increase the risk of predation. Furthermore, under natural conditions, the organisms are not exposed to a single PAH, but to a complex mixture of these and other contaminants. In this context, the present results raise further concern. Indeed, previous studies evaluating the adverse effects of mixtures of PAHs (Barata et al., 2005) or mixtures of PAHs with other contaminants (*e.g*., metals) (Millward et al., 2004) showed that when in mixture PAH adverse effects can be additive or even more severe than the sum of the individual effects.

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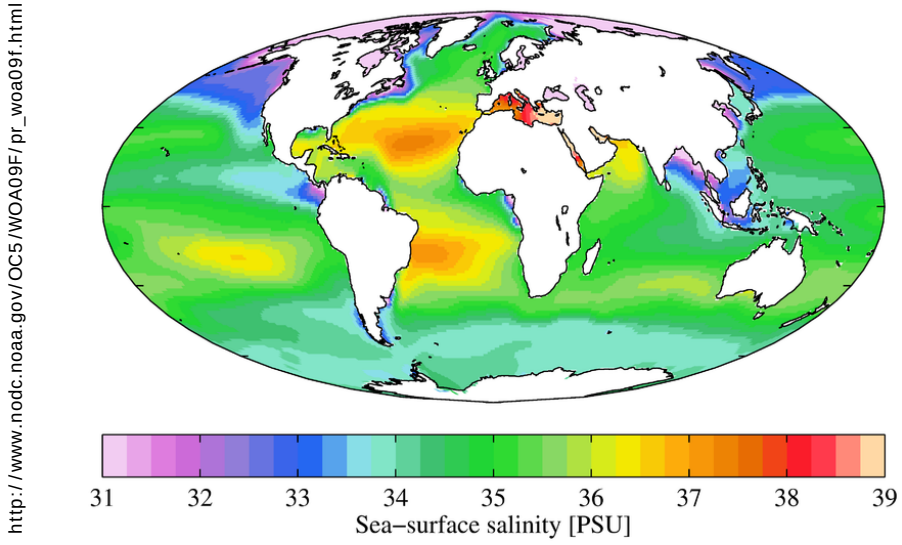
Chapter III

Effects of salinity stress on neurotransmission, energy metabolism, and anti-oxidant biomarkers of *Carcinus maenas* from two estuaries of the NW Iberian Peninsula



Effects of salinity stress on neurotransmission, energy metabolism, and anti-oxidant biomarkers of *Carcinus maenas* from two estuaries of the NW Iberian Peninsula

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Abstract

This study investigated the effects of salinity on biomarkers of oxidative stress, energy metabolism, and neurotransmission of *Carcinus maenas* from an estuary low impacted by pollution and from an estuary under chemical stress in the NW Iberian Peninsula. Crabs were collected in the field and, following an acclimation period, they were exposed for 7 days to five salinity levels ranging from 4 to 45 psu. At the end of the exposure period, stress biomarkers were determined in samples of muscle and digestive gland. The biomarkers assessed in the muscle were the activity of the enzymes cholinesterases (ChE), of which acetylcholinesterase (AChE) is involved in neurotransmission, and lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) that are involved in energy metabolism. The biomarkers assessed in the digestive gland were (1) the activity of the enzymes glutathione *S*–transferases (GST), glutathione reductase (GR), and glutathione peroxidase (GPx), involved in phase II biotransformation and the anti–oxidant defence system; (2) the levels of total glutathiones (TG), also belonging to the anti–oxidant system; and (3) the levels of lipid peroxidation (LPO) as a measure of oxidative damage. The results showed a significant influence of salinity on neurotransmission, energy metabolism, anti–oxidant status, and oxidative damage of *C. maenas*. For some biomarkers, this influence was dependent on whether the crabs were collected at the low–polluted estuary or at the contaminated estuary. In particular, crabs collected at the low–polluted estuary showed altered neurotransmission and anti-oxidant defences (GR). Crabs collected at the impacted estuary showed alterations in neurotransmission, energy

metabolism (IDH and LDH), biotransformation, and anti–oxidant defences (GST, GR, GPx, and TG), as well as in oxidative damage, indicating that salinity change superimposes higher stress on these organisms. For ChE, IDH, and TG, altered responses were induced by both hypo– and hypersalinity.

1. Introduction

Variations of water temperature, pH, and salinity outside the optimal species' ranges can act as environmental stressors for aquatic organisms, affecting their survival and maintenance (Anger et al., 1998; Heugens et al., 2001). In particular, salinity may influence physiological processes (Whiteley et al., 2001; Vargas-Chacoff et al., 2009) and the life history (Oltra and Todoli, 1997; Martin et al., 2009) of species, altering their geographical distribution, and the structure and dynamics of aquatic communities (Williams et al., 1990; Anger et al., 1998; Heugens et al., 2001; Calosi et al., 2007; Boix et al., 2008; Breen and Metaxas, 2009). At the physiological level, salinity was shown to alter vital processes such as osmoregulation, rates of protein synthesis and oxygen uptake, scope for growth, and extracellular acid–base balance (Guerin and Stickle, 1997; Whiteley et al., 2001; Henry et al., 2003; Intanai et al., 2009).

Interactions between salinity changes and toxicity of chemical contaminants may also occur (Heugens et al., 2001). For example, increased metal toxicity is usually found at low salinity levels, whereas toxicity of organophosphate insecticides appears to increase when salinity rises (reviewed in Hall and Anderson, 1995; Heugens et al., 2001). Adding to this, in recent years, concern on the impact of salinity on aquatic organisms was further intensified because the analysis of temporal trends indicates the occurrence of global water freshening in subpolar and tropical regions and a salinification of shallower parts of the subtropical oceans (Bindoff et al., 2007; Hosoda et al., 2009). Moreover, increased drought frequency and sea–level rise are also predicted to lead to salinity

changes in many estuarine ecosystems around the world (Bindoff et al., 2007).

Estuaries are transitional areas of high productivity, usually providing large food supply, good dissolved oxygen conditions, and shelter to residential species. Owing to these characteristics, they constitute essential reproductive and nursery grounds for a variety of species with high commercial and ecological value, including shorebirds, marine fishes, shellfish, and crustaceans (Day et al., 1989). However, due to decades of continuous human activities, many estuaries are impacted, among others, by excessive levels of pollutants, nutrients, and organic matter (Kennish, 2002). In numerous situations, these anthropogenic changes additionally entail modifications of the abiotic environment resulting in the exposure of estuarine organisms to multiple natural and anthropogenic stressors.

Biomarker responses measured at the biochemical, cellular, and physiological levels have been widely used to assess aquatic contamination in marine and estuarine areas. In particular, biochemical biomarkers involved in neurotransmission, biotransformation, and oxidative stress are routinely employed to investigate the toxic effects of contaminants in coastal and estuarine invertebrates (Martín-Díaz et al., 2005; Elumalai et al., 2007; Mesquita et al., 2011) and evaluate environmental quality (Cajaraville et al., 2000; Cravo et al., 2009; Locatello et al., 2009; Maria et al., 2009). Good examples of this are the activity of several enzymes and the levels of non–enzymatic cofactors, such as acetylcholinesterase (AChE), involved in the cholinergic transmission; $NADP^+$–dependent isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH), involved in aerobic and anaerobic metabolism, respectively; glutathione *S*–transferases (GST), involved in phase II biotransformation but also with an anti–oxidant role; glutathione peroxidase (GPx), glutathione reductase (GR), and total glutathione (TG) all belonging to the anti–oxidant system. Whereas these biomarkers are sensitive to chemical contamination, their responses may also be influenced and/or modulated by abiotic factors, including salinity, in several estuarine species (Pfeifer et al., 2005; Menezes et al., 2006; Seo et al., 2006; Cailleaud et al., 2007; Liu et al., 2007; Paital and Chainy,

2010; Freire et al., 2011). However, studies addressing salinity effects on these biomarkers and its possible contributions to intersite variability commonly use test organisms collected in low–impacted reference sites or reared under optimal laboratorial conditions. More research is therefore needed on the additional effects of salinity changes in invertebrates already living under environmental stress, as occurs in polluted estuaries.

The green crab *Carcinus maenas* is a key invertebrate, common in estuarine and coastal ecosystems from Europe and North Africa. It is also a successful invasive species in other geographical regions, as for instance in coastal areas of South Africa, Australia, Japan, Canada, and United States. This euryhaline species has the ability to efficiently hyperosmoregulate in habitats with low and/or variable salinity (Siebers et al., 1982). It tolerates salinities ranging from 40 psu to as low as 4 psu without harm (Crothers, 1967; McGaw and Naylor, 1992); this advantage contributes to the wide ranging distribution patterns of this species. The ability to adapt to low and varying salinities is partly due to the ionocytes (*i.e.* cells specialised in ionic exchanges) and involves the regulation of their cellular volume and of the haemolymph osmotic concentration (osmolality). The regulation of haemolymph osmolality is achieved by distinct mechanisms, namely active uptake of Na^+ and Cl^- from the surrounding environment, morphological/structural adaptations, and production and transport of organic osmolytes, such as non-essential amino acids (Gilles and Pequeux, 1986; Gilles, 1998; Whiteley et al., 2001; Cieluch et al., 2004). Salinity changes rapidly activate these mechanisms, in such a way that haemolymph osmotic and ionic (*i.e.* Na^+ and Cl^-) concentrations achieve a new equilibrium status within 6–12h following a salinity reduction (Towle, 1997; Henry et al., 2003).

Due to its ecological role, wide geographical distribution, abundance, and sensitivity to contamination, *C. maenas* is frequently used in ecotoxicological evaluations and biomonitoring studies where a variety of biomarkers (Bamber and Depledge, 1997; Wedderburn et al., 1998; Galloway et al., 2004) also involving neurotransmission, biotransformation, and oxidative stress responses (Martín-Díaz et al., 2005; Maria et al.,

2009; Pereira et al., 2009; Mesquita et al., 2011; Pereira et al., 2011) are measured. Interestingly, recent studies showed that depending on the crustacean species, and its natural habitat, deviations from optimal salinity, either increases or decreases, may influence the generation of reactive oxygen species (ROS) and the activity and/or expression of anti-oxidant enzymes (Cailleaud et al., 2007; Freire et al., 2011; Liu et al., 2007; Paital and Chainy, 2010; Van Horn et al., 2010). Though, for certain species, hypersalinity may be more stressful to the organisms than hyposalinity, entraining stronger changes in the activity of some anti–oxidant enzymes (Freire et al., 2011). Despite this, studies on the effects of salinity on *C. maenas* biomarkers are scarce (Martín-Díaz et al., 2004) and none of them focused on possible effects on biotransformation and oxidative stress.

The aim of the present study was, therefore, to investigate the effects of salinity changes on stress biomarkers of *C. maenas* from two NW Iberian estuaries with different levels of pollution. The Minho estuary is relatively low impacted by pollution, while the Lima estuary is polluted by heavy metals and polycyclic aromatic hydrocarbons (PAHs) and has levels of nitrites, nitrates, and phosphates indicative of poor water quality (Gravato et al., 2010; Guimarães et al., 2012). Moreover, a recent study on the population genetic structure of *C. maenas*, encompassing 14 different locations found no significant genetic differentiation among sites separated by several 100 s of km along a 1,200 km stretch of the Iberian Peninsula coast (Domingues et al., 2010). This suggests that the patterns of physiological variation of *C. maenas* from the Minho and the Lima estuaries are likely to reflect environmental differences between these two systems. In this study, crabs collected at the mouth of the Minho and Lima estuaries were thus exposed to five salinity levels ranging from 4 to 45 psu. At the end of the bioassays, the following biomarkers were determined: (1) the activity in the muscle of the enzymes cholinesterase (ChE), IDH, and LDH; (2) the activity in the digestive gland of the enzymes GST, GPx, and GR; and (3) the levels in the digestive gland of TG and of

lipid peroxidation (LPO) as measure of oxidative damage to membrane lipids.

2. Materials and methods

2.1. Study sites

Test organisms were collected at the mouth of the Minho and the Lima estuaries, located in the Northwest of the Iberian Peninsula (Fig. III.1.), where the wet Atlantic climate prevails with an average annual precipitation of 1,300 mm (Fatela et al., 2007). The Minho estuary is located approximately 20 km to the north of the Lima estuary. These are both mesotidal stratified estuaries of similar typology (Ferreira et al., 2003). The Minho River has relatively low human pressure, showing characteristics of low urban, agricultural, and industrial contamination (Ferreira et al., 2003). Its estuary is classified as a NATURA 2000 site, and available chemical analyses of metals and PAHs in sediments show very low levels of these contaminants at the mouth of the estuary (Reis et al., 2009; Gravato et al., 2010; Guimarães et al., 2012). Moreover, it has been used as reference site in several ecotoxicological studies (Elumalai et al., 2007; Gravato et al., 2010; Mesquita et al., 2011; Guimarães et al., 2012). The Lima estuary receives chemical inputs from untreated effluents of urban and industrial origin. It is under the influence of a harbour and a shipyard (near which the sampling site was located), and a paper mill. Chemical analyses in sediments showed moderate levels of heavy metals and PAHs at the mouth of the estuary (Gravato et al., 2010; Guimarães et al., 2012). Further, the levels of nitrate, nitrite, and phosphate indicate a poor water quality (Guimarães et al., 2012). These estuaries were selected to study the influence of salinity on stress biomarkers of organisms from a low–polluted site and of organisms living under conditions of chemical stress.

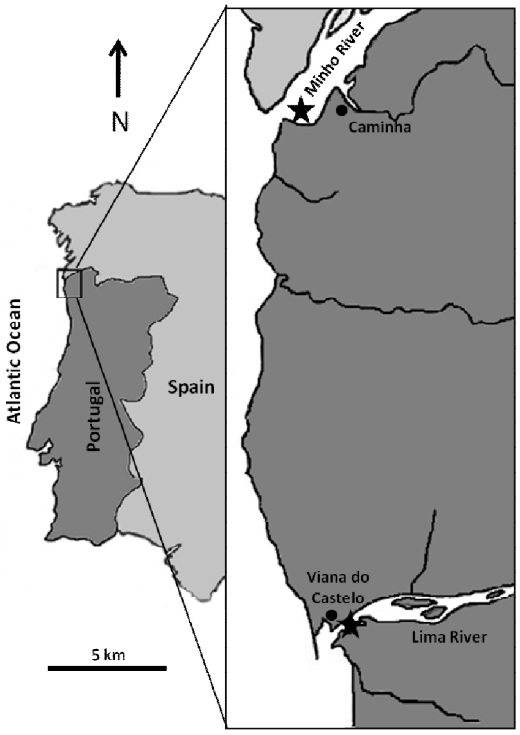

Fig. III.1. Location of the estuaries of Rivers Minho and Lima, in the Northwest Iberian coast. The sampling sites are indicated by stars.

2.2. Crab sampling and acclimation

Intermoult male crabs were collected in November 2010 using trawl nets, at similar points at the mouth of the Minho (4.2 ± 0.09 cm carapace width; mean ± standard deviation, SD, n = 50) and the Lima (4.0 ± 0.18 cm, n = 50) estuaries (Fig. III.1.). Crabs with complete appendages were selected and immediately transported to the laboratory. Water temperature, salinity, dissolved oxygen, and pH were measured, in triplicate, during each sampling, using a multiparametric sea gauge WTW multi 340i with the appropriate probes (pH Sen Tix 41 and Tetracon 325). The mean values, and corresponding SD, obtained for these parameters were similar for the two sampling sites (Table III.1.) and mostly in the range previously obtained for these same locations (Sousa et al., 2006; Sousa et al., 2008; Guimarães et al., 2009; Guimarães et al., 2012). Once in the laboratory, the organisms were placed in tanks (length: 240 cm, width: 120 cm, height: 72 cm, water volume: 300 L) for 10 days, separated by the estuary of origin. The tanks contained filtered seawater of 14 psu

(close to the salinity at the time of capture) and were continuously aerated; temperature was kept at 15 ± 0.6ºC and the photoperiod was 16:8h day/night. After this initial period, the crabs were transferred to individual 2 L flat beakers. The test vessels were covered and continuous aeration was provided. The crabs were then allowed to gradually acclimate to the test salinities, to avoid osmotic shock. This acclimation was performed according to the schematic representation of Fig. III.2., and previous studies in *C. maenas* (Towle, 1997; Henry et al., 2003). Accordingly, after salinity transfer from full strength seawater to 10 psu, haemolymph osmotic and ionic concentrations achieved a new equilibrium within 6–12h. Sodium chloride is considered the major osmolyte in *C. maenas*, and hence the regulation of the fluxes and permeabilities of Na^+ and Cl^- is essential to attain osmotic equilibrium (Towle, 1997; Henry et al., 2003). Crabs were fed with TETRAFAUNA (*Gammarus*), every 2 days, 2h before medium renewal.

Table III.1. Water physico–chemical parameters (mean ± SD) measured (in triplicate) in each site during crab sampling.

Parameters	Minho	Lima
T (ºC)	14.45 ± 0.12	13.62 ± 0.16
Sal (psu)	13.26 ± 6.53	12.10 ± 4.97
DO (mg l^{-1})	10.42 ± 0.17	10.57 ± 0.46
pH	7.35 ± 0.18	7.91 ± 0.17

2.3. Experimental design

Crabs from both sampling sites were exposed to five different salinity levels (4, 8, 14, 25, and 45 psu) for 7 days. Test media of 4–25 psu were prepared by dilution of filtered seawater with distilled water; test solutions of 45 psu were obtained by dissolution of marine salts (Tropic Marine, Germany) in the filtered seawater. Ten crabs were used per treatment, individually exposed in 2 L flat beakers. The test medium was renewed

every 72 h. The values of pH, dissolved oxygen, and conductivity were measured in the old and the freshly prepared test solutions. Test conditions were the same described for the acclimation period.

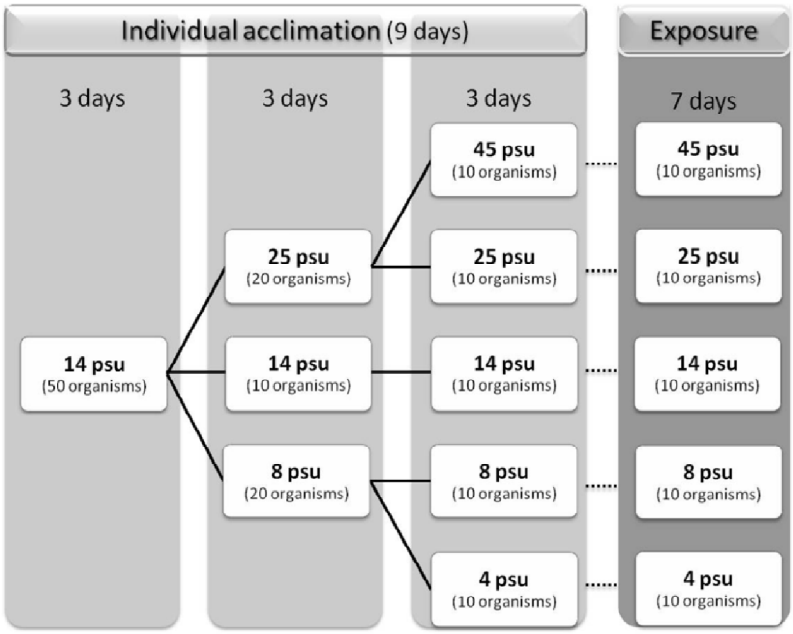

Fig. III.2. Schematic representation of the strategy adopted for salinity acclimation and exposure periods of crabs from the cohorts of the Minho and the Lima estuaries.

2.4. Chemicals

All the reagents used were of analytical grade and purchased from Sigma–Aldrich Chemical (Steinheim, Germany), except the Bio–Rad protein assay dye reagent that was purchased from Bio–Rad Laboratories, Inc.

2.5. Tissue sampling and the determination of stress biomarkers

At the end of the exposure period, animals were anaesthetised on ice, weighed, and measured. Three sub-samples of muscle of the first right walking leg were isolated and stored at –80ºC until analysis. These sub–samples were used to determine the activities of the enzymes ChE, IDH, and LDH. Muscle tissue was selected because of its high IDH and LDH activity (Walsh and Henry, 1990).

A sub–sample of digestive gland was isolated and kept at –80ºC until analysis. The biomarkers analysed in this tissue were the activities of the enzymes GST, GR, and GPx, and the levels of TG and LPO. The digestive gland was selected because of its main role on the detoxification of xenobiotics and hence high levels of biotransformation and anti–oxidant enzymes (Livingstone, 1998).

Determination of biomarkers in the muscle was performed as follows. One muscle sub-sample was homogenised in 1 mL of ice–cold phosphate buffer (pH 7.2, 0.1 M) and centrifuged at 6,000 ×*g* for 5 min at 4ºC. The supernatant was used to determine ChE activity according to Ellman's method (Ellman et al., 1961) modified for measurement in saltwater crustaceans using a microplate reader (Menezes et al., 2006). Briefly, the increase in absorbance due to the colour increase resulting from the reaction of thiocholine with 5,5'–dithio–bis–2-nitrobenzoate (DTNB) was followed at 412 nm. The enzymatic activity was expressed in nmol of substrate hydrolysed per min per mg of protein.

Another muscle sub–sample was homogenised in 1 mL of ice–cold Tris/NaCl buffer (pH 7.2, 50 mM), followed by centrifugation at 15,000 ×*g*, during 15 min at 4ºC. LDH activity was measured in this supernatant following the method of Vassault (1983) modified for measurement in saltwater crustaceans using a microplate reader (Menezes et al., 2006). The amount of pyruvate consumed was determined by continuously monitoring the decrease in absorbance (at 340 nm) due to the oxidation of NADH. The enzyme activity was expressed in nmol per min per mg of protein. The third sub–sample of muscle was homogenised in 1 mL of ice–cold Tris/NaCl buffer (pH 7.8, 50 mM), followed by centrifugation at 15,000 ×*g*, during 15 min at 4ºC. IDH activity was assayed according to the method described by Ellis and Goldberg (1971) modified for measurement in saltwater crustaceans using a microplate reader (Mesquita et al., 2011). This method is based on the monitoring at 340 nm of the increase in absorbance due to the reduction of $NADP^+$ by the enzyme. The enzymatic activity was expressed in nmol of NADPH regenerated per min per mg of protein.

For the determination of biotransformation and oxidative stress biomarkers, the digestive gland was homogenised (1:10 wt v^{-1}) in phosphate buffer (pH 7.4, 0.1 M). Part of the homogenate was used to determine the endogenous LPO by measuring at 535 nm the thiobarbituric acid reactive substances (TBARS) formed, as performed by Ohkawa et al. (1979) and Bird and Draper (1984). LPO levels were expressed as nmol of TBARS per g of tissue. The remaining homogenate was centrifuge at 10,000 ×*g* for 20min, at 4ºC. The homogenate obtained after sedimentation of the mitochondrial fraction by centrifugation (post–mitochondrial supernatant fraction) was isolated and used for the determination of GST, GR, and GPx activities and TG levels.

GST activity was determined according to the method developed by Habig et al. (1974) modified for measurement in saltwater crustaceans using a microplate reader (Menezes et al., 2006). The conjugation of reduced glutathione (GSH) with 1–chloro–2,4–dinitrobenzene (CDNB) was followed at 340 nm. The enzymatic activity was expressed in nmol of substrate conjugated per min per mg of protein. GR activity, which catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) with the concomitant oxidation of NADPH to $NADP^+$, was measured by following the decrease in NADPH levels, at 340 nm, as previously described (Cribb et al. 1989). The enzyme activity was expressed in nmol of oxidised $NADP^+$ per min per mg of protein. GPx activity was assayed by measuring the decrease in NADPH at 340 nm, using hydrogen peroxide as substrate, according to the method developed by Mohandas et al. (1984). The enzymatic activity was expressed in nmol per min per mg of protein. TG was assayed as described by Tietze (1969) and Baker et al. (1990), by following the change in absorbance at 412 nm through a recycling reaction of GSH with DTNB in the presence of GR. TG levels were expressed as nmol of recycled GSH per min per mg of protein.

Protein concentration in the samples was determined according to the Bradford's method (Bradford, 1976) modified for measurement in saltwater crustaceans using a microplate reader (Menezes et al., 2006). Bovine γ–globulin was used as protein standard.

All cuvette absorbance assays were performed in a Jasco 6405 UV/VIS spectrophotometer; all microplate determinations were carried out in a Bio Tek Power Wave 340 microplate reader.

2.6. Data analysis

Results are presented as mean ± standard error of the mean (SEM). For each endpoint, data were analysed by two way analysis of variance (ANOVA). Salinity and the site of origin were taken as sources of variation in a full–factorial model with interaction. When significant differences were found, their origin was identified using either the Sidak test (when a significant main effect of salinity was found) or a combination of planned contrasts and the Sidak test (when a significant effect of the interaction term was found). The assumptions of normality of data distributions and homogeneity of variances were checked using the Shapiro–Wilk and the Levene's tests, respectively. Values more than 2.5 standard deviations above their group mean were considered as outliers and removed from the analyses, for they were producing departures from normality and/or lack of variances homogeneity. The significance level was set to $p<0.05$ for all tests performed.

3. Results

3.1. Effects of salinity on biomarkers of neurotransmission and energy metabolism

Exposure of *C. maenas* collected at the mouth of the Minho and the Lima estuaries to different salinity levels elicited significant changes in muscle activities of ChE, LDH, and IDH enzymes (Figs. III.3. and III.4.).

Significant differences among salinity levels and a significant interaction between salinity and the sampling site were found for ChE activity (Table III.2.). This indicates that crabs from the Minho and the Lima estuaries were affected differently by salinity exposure. In crabs collected from the Minho estuary, ChE activity was significantly higher in organisms exposed to 8 psu than in those exposed to the remaining salinities (Sidak test,

p<0.05), suggesting that neuromuscular transmission was increased in these animals (Fig. III.3.). In crabs collected from the Lima estuary, ChE activity was significantly higher at 45 psu and remained unchanged between 4 and 25 psu (Sidak test, p<0.05) (Fig. III.3.). Within each salinity level, crabs from the Minho estuary showed significantly higher ChE activity at 8 and14 psu (approximately +30% and +27%, respectively), and lower ChE activity at 45 psu, than those from the Lima estuary exposed to the same salinities (Planned pairwise comparisons, p<0.05).

LDH activity was significantly affected by salinity and by the interaction Salinity × Sampling site (Table III.2.). No significant differences among salinity levels were found for LDH activity of crabs collected from the Minho estuary (Sidak test, p>0.05) (Fig. III.4.). However, crabs from the Lima estuary exposed to 8 psu showed a significant increase in the anaerobic energy metabolism (between +40% and +73%) than those exposed to all other salinity levels (Sidak test, p<0.05). Comparing sampling sites, LDH activity was 50% higher in crabs from the Lima estuary exposed to 8 psu than in those from the Minho estuary exposed to the same salinity (Planned pairwise comparisons, p<0.05).

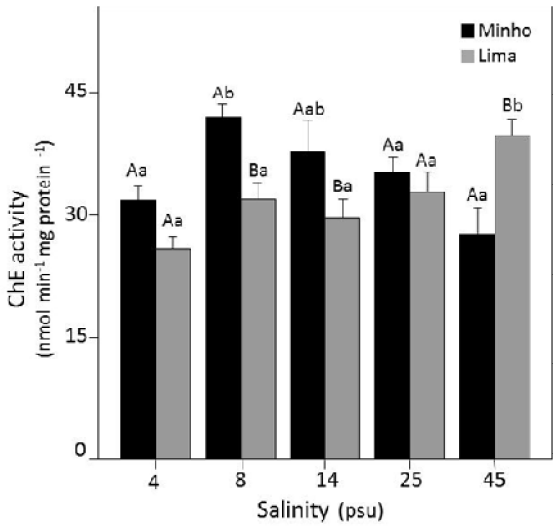

Fig. III.3. Mean and corresponding standard error of cholinesterase (ChE) activity determined in the muscle of crabs from the Minho (low pollution) and the Lima (contaminated) cohorts exposed for 7 days to different salinity levels. For each sampling site, 10 crabs were exposed per salinity level, in a total of 100 animals analysed. Significant differences between

sampling sites within each salinity level are identified by different capital letters (two-way ANOVA with planned pairwise comparisons, $p<0.05$); significant differences among salinity levels within each estuary are identified by different small letters (two-way ANOVA and Sidak test, $p<0.05$).

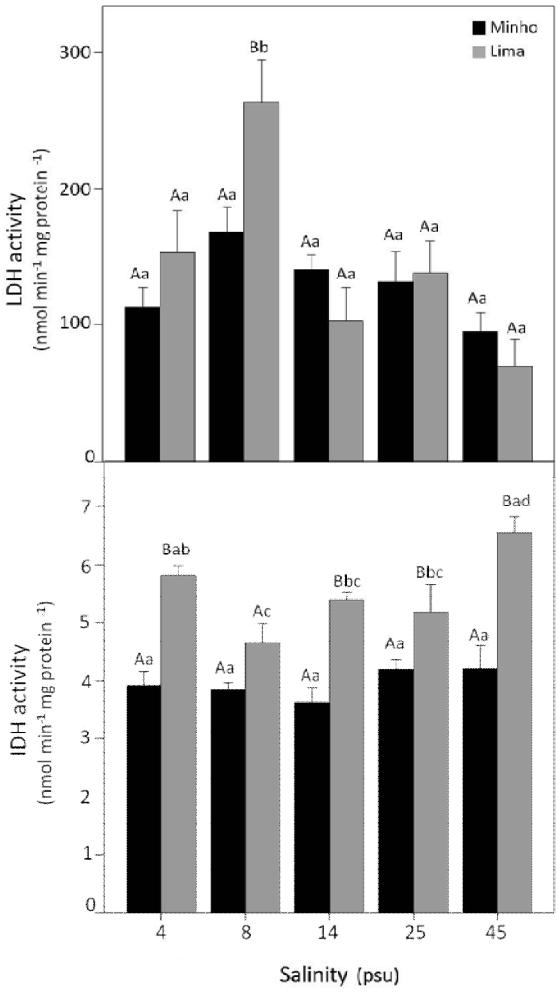

Fig. III.4. Mean and corresponding standard error of lactate dehydrogenase (LDH) and $NADP^+$–dependent isocitrate dehydrogenase (IDH) activities determined in the muscle of crabs from the Minho (low pollution) and the Lima (contaminated) cohorts exposed for 7 days to different salinity levels. For each sampling site, 10 crabs were exposed per salinity level, in a total of 100 animals analysed. Significant differences between sampling sites within each salinity level are identified by different capital letters (two–way ANOVA with planned pairwise comparisons, $p<0.05$); significant differences among salinity levels within each estuary are identified by different small letters (two–way ANOVA and Sidak test, $p<0.05$).

Table III.2. Results of the fullfactorial two-way ANOVA performed to investigate the effects of salinity and the sampling site on the biomarkers analysed.

Parameter	Source of Variation	df	F	P
Neurotransmission and energy metabolism				
ChE	Salinity	4, 88	3.02	0.022
	Sampling site	1, 88	3.64	0.060
	Salinity x Sampling site	4, 88	6.75	< 0.001
LDH	Salinity	4, 87	9.51	< 0.001
	Sampling site	1, 87	1.35	0.249
	Salinity x Sampling site	4, 87	2.93	0.025
IDH	Salinity	4, 86	4.65	0.002
	Sampling site	1, 86	75.0	< 0.001
	Salinity x Sampling site	4, 86	2.64	0.039
Biotransformation and anti-oxidant defences				
GST	Salinity	4, 86	1.69	0.159
	Sampling site	1, 86	4.56	0.036
	Salinity x Sampling site	4, 86	1.00	0.413
GPx	Salinity	4, 83	2.38	0.059
	Sampling site	1, 83	7.29	0.008
	Salinity x Sampling site	4, 83	1.40	0.242
GR	Salinity	4, 87	5.28	0.001
	Sampling site	1, 87	1.89	0.173
	Salinity x Sampling site	4, 87	1.84	0.129
TG	Salinity	4, 85	7.48	< 0.001
	Sampling site	1, 85	59.1	< 0.001
	Salinity x Sampling site	4, 85	1.97	0.106
Oxidative damage				
LPO	Salinity	4, 88	0.91	0.464
	Sampling site	1, 88	31.2	< 0.001
	Salinity x Sampling site	4, 88	2.72	0.035

ChE, cholinesterase; *LDH*, lactate dehydrogenase; *IDH*, $NADP^+$–dependent isocitrate dehydrogenase; *GST*, glutathione *S*–transferases, *GPx*, glutathione peroxidase; *GR*, glutathione reductase; *TG*, total glutathione; *LPO*, lipid peroxidation.

Salinity, sampling site, and the interaction between these factors significantly affected IDH activity (Table III.2.). Again, no significant differences among salinity levels were found for crabs collected from the

Minho estuary (Fig. III.4.). In Lima crabs, significantly higher levels of IDH activity were observed in crabs exposed to extreme salinities (*i.e.* 4 and 45 psu), indicating a higher rate of NADPH recycling in these organisms (Sidak test, $p<0.05$). IDH activity remained unchanged between 8 and 25 psu.

3.2. Effects of salinity on biomarkers of biotransformation and anti–oxidant defences

Mean activities of biotransformation and anti–oxidant defence biomarkers determined in the digestive gland of *C. maenas* are presented in Fig. III.5. For GST and GPx activities, only a significant effect of the sampling site was found (Table III.2.). Globally, significantly higher GST (+54%) and GPx (+23%) activities were found in crabs collected from the Lima estuary relatively to those collected from the Minho estuary, indicating increased biotransformation and anti–oxidant activity in these animals (Fig. III.5.). For GR activity, though crabs from the two sampling sites appeared to display different response patterns (Fig. III.5.), due to high interindividual variability only significant differences among salinity levels could be depicted (Table III.2.). On average, the highest GR activities were found in crabs exposed to 8 and 25 psu.

TG levels were significantly affected by salinity and the sampling site (Table III.2.). As previously found for LDH, IDH, GST, and GPx, no significant differences among salinity levels were found for crabs collected from the Minho estuary (Sidak test, $p>0.05$) (Fig. III.5.). In contrast, TG levels were greatly increased in Lima crabs exposed to 4, 8, and 45 psu (by +140%, +82%, and +143%, respectively), compared to those exposed to 14 psu (Sidak test, $p<0.01$), indicating an enhancement of anti-oxidant defences. Additionally, TG levels were on average approximately 50% higher in Lima crabs than in Minho crabs (Planned pairwise comparisons, $p<0.01$).

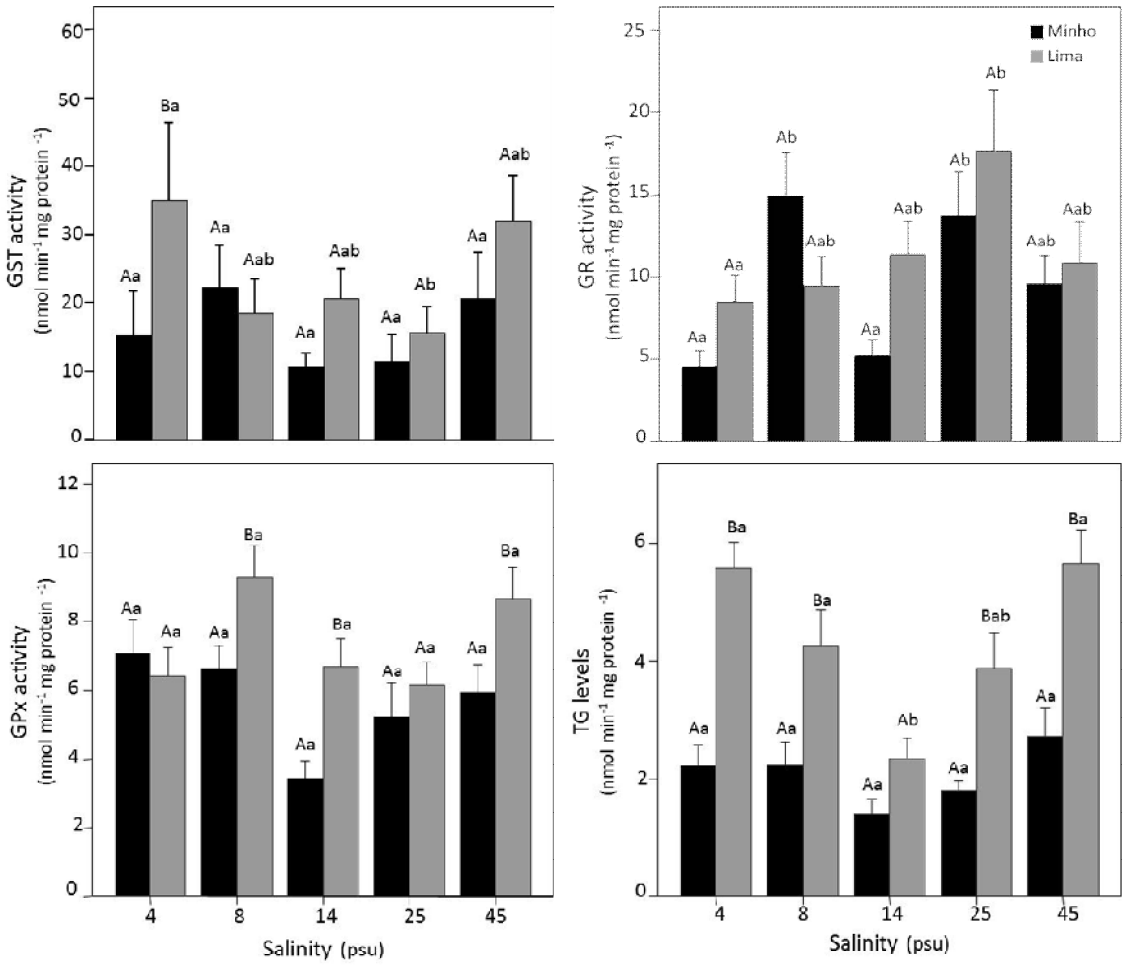

Fig. III.5. Glutathione *S*–transferases (GST), glutathione reductase (GR) and peroxidase (GPx) activity, and levels of total glutathione (TG), determined in the digestive gland of crabs from the Minho (low pollution) and the Lima (contaminated) cohorts exposed for 7 days to different salinity levels. Values represent the mean with the corresponding standard error. For each sampling site, 10 crabs were exposed per salinity level, in a total of 100 animals analysed. Different capital letters identify significant differences between sampling sites within each salinity level (two–way ANOVA with planned pairwise comparisons, $p<0.05$); different small letters identify significant differences among salinity levels within each estuary (two–way ANOVA and Sidak test, $p<0.05$). For GST and GPx, only a significant effect of the estuary of origin on the enzymatic activities could be depicted. GR activity was significantly affected by the salinity level only.

3.3. Effects of salinity on oxidative damage

The sampling site and the interaction Salinity × Sampling site significantly affected LPO levels (Table III.2.). Once more, no significant differences of LPO levels were recorded in Minho crabs exposed to different salinities (Fig. III.6.). In Lima crabs, the highest LPO levels were recorded in crabs exposed to 8 psu and were followed by a decreasing trend in crabs exposed to salinities ranging from 14 to 45 psu.

In particular, oxidative damage of lipid macromolecules was significantly higher in crabs exposed to 8 psu (+35%) than in those exposed to 45 psu (Sidak test, $p<0.05$). In addition, within each salinity level, significantly lower LPO levels were measured in crabs from the Lima estuary exposed to 14 (–25%), 25 (–30%), and 45 psu (–45%), compared to those from the Minho estuary (Planned pairwise comparisons, $p<0.01$).

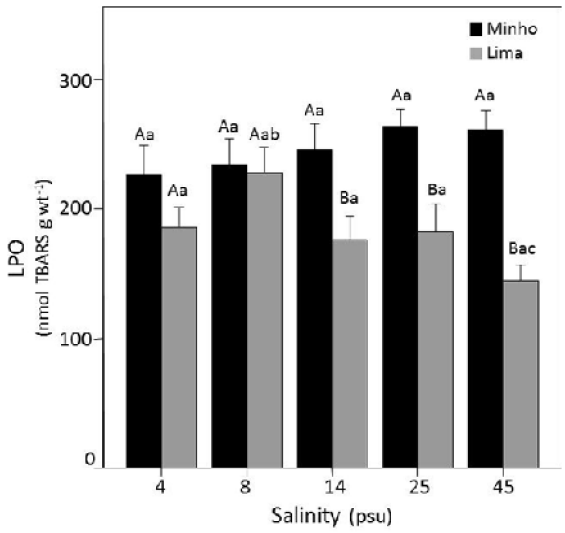

Fig. III.6. Lipid peroxidation (LPO) levels determined in the digestive gland of crabs from the Minho (low pollution) and the Lima (contaminated) cohorts after a 7–day exposure to different salinity levels. Values represent the mean with the corresponding standard error bars. For each sampling, 10 crabs were exposed per salinity level, in a total of 100 animals analysed. Significant differences between sampling site within each salinity level are identified by different capital letters (two–way ANOVA with planned pairwise comparisons, $p<0.05$); significant differences among salinity levels within each estuary are identified by different small letters (two–way ANOVA and Sidak test, $p<0.05$).

4. Discussion

4.1. Neurotransmission and energy metabolism

ChE are a family of enzymes, of which AChE is involved in cholinergic transmission (Payne et al., 1996). Because of its sensitivity to various types of chemical contaminants (*e.g.*, organophosphate and carbamate pesticides, heavy metals, surfactant agents), invertebrate ChE activity has long been used as biomarker of neurotoxicity in both laboratory and field studies (Day and Scott, 1990; Lundebye et al., 1997; Cajaraville et al., 2000; Guilhermino et al., 2000; Monserrat et al., 2007; Locatello et al., 2009). Recent works have shown, however, that ChE activity of several invertebrate organisms (*i.e.* ragworms, mussels, brown shrimps, and copepods) may also respond to salinity stress (Scaps and Borot, 2000; Pfeifer et al., 2005; Menezes et al., 2006; Cailleaud et al., 2007). In the present work, salinity change also triggered important alterations of ChE activity in *C. maenas*. Crabs collected at the mouth of the Minho estuary showed higher ChE activity at low salinity (8 psu) whereas those from the Lima estuary showed higher ChE activity at 45 psu than at the remaining salinities. Different patterns of ChE response to salinity stress were previously observed in other species. A decrease in gill AChE activity with increasing salinity was found in *Mytilus* sp. (Pfeifer et al., 2005). The authors interpreted this decrease as being possibly related to the variation in intra– and extracellular concentrations of inorganic ions that results from osmotic adaptation. Transient increases in AChE activity with increasing salinity were reported for the ragworm *Nereis diversicolor* (Scaps and Borot, 2000). In contrast, no significant effects of salinity on the enzyme activity were found in the brown shrimp (*Crangon crangon*) (Menezes et al., 2006). Also, a recent study with the copepod *Eurytemora affinis* indicated that these organisms tend to show optimum AChE activity at the salinity range to which they are adapted (Cailleaud et al., 2007). It is important to note that when *C. maenas* is exposed to salinities below 25.5–27 psu, it starts to hyperosmoregulate, by actively taking up salts through the gills (primarily Na^+ and Cl^-) from the ambient medium, in order

to maintain its haemolymph osmotic and ionic concentrations above those in the external water (Towle, 1997; Henry et al., 2003; Cieluch et al., 2004). As previously suggested (Pfeifer et al., 2005), this altered ionic balance may have triggered an increase in ChE activity of Minho crabs. The higher ChE activity of these crabs may also result from augmented locomotory activity. *C. maenas* was previously shown to respond to low salinity (25 and 50% seawater) with increased locomotion, a function in which cholinergic transmission is known to be involved (Sorenson, 1973). The results further suggest that previous exposure to chemical stress in the Lima estuary influenced ChE response to salinity. The Lima estuary is polluted by metals and PAHs and has increased levels of nutrients relative to the Minho estuary (Gravato et al., 2010; Guimarães et al., 2012). Exposure to hyposalinity superimposed to the effects resulting from living under such a stressful environment may have triggered the different ChE response of Lima crabs.

LDH and IDH are enzymes involved in the cellular respiration, in the anaerobic and aerobic pathways, respectively. LDH participates in important metabolic processes, such as the glycolysis and the gluconeogenesis (Walsh and Henry, 1990; Cristescu et al., 2008). Recently, measurements of gluconeogenesis capacity and of the activity and expression of phosphoenolpyruvate carboxykinase, indicated that in the estuarine crab *Chasmagnathus granulate*, muscle gluconeogenesis might be one of the pathways implicated in the metabolic adjustment of amino acids during hypo– and hyperosmotic stress (Schein et al., 2005). Moreover, increased LDH activity has been reported in response to chemical and natural stress, such as exposure to mercury, zinc, petroleum hydrocarbons, and hypoxia and salinity challenge (Wu and Lam, 1997; Diamantino et al., 2001; Long et al., 2003; Elumalai et al., 2007). In the present study, only crabs collected at the mouth of the Lima estuary showed altered LDH activity. Interestingly, studies have shown that exposure to heavy metals, which are relevant contaminants found in the Lima estuary (Guimarães et al., 2012), may reduce the ability of *C. maenas* to regulate haemolymph inorganic osmolytes. Laboratory exposures of *C.*

maenas to copper and mercury at low salinity (~40% seawater) led to decreased serum concentrations of Na^+, K^-, and Cl^- and/or osmoregulatory ability, indicating a synergistic effect between salinity and metal toxicity (Thurberg et al., 1973; Bjerregaard and Vislie, 1985; Bjerregaard and Vislie, 1986). Exposure of *Gammarus duebeni* to zinc and low salinity (10 psu) was also associated with depression of haemolymph osmolality due to reduced Na^+ levels (Johnson and Jones, 1990). A field study conducted by Bamber and Depledge (1997b) additionally revealed lower osmoregulatory capacity of *C. maenas* collected from sites contaminated by heavy metals and organic pollutants. In the present study, the higher LDH activity exhibited by *C. maenas* exposed to lower salinities, particularly 8 psu, may provide additional energy to osmoregulatory needs. If crabs are to maintain successfully under salinity stress, adjustment of organic osmolytes may provide an important pathway to cope with possible decreases in haemolymph ionic concentrations resulting from a synergistic interaction between previous exposure to contamination in Lima estuary and salinity stress. Other studies have also demonstrated alterations of LDH activity or metabolic changes in association with salinity stress. Exposure of the brown shrimp *C. crangon* to low salinity elicited an increase in LDH activity (Menezes et al., 2006). Hulathduwa et al. (2007) showed that in two species of hyperosmoregulator mud crabs (*Eurypanopeus depressus* and *Panopeus simpsoni*), low salinity increased energy expenditure and reduced food consumption and energy absorption, leading to lower scope for growth. In juveniles of the tropical prawn *Macrobrachium rosenbergii*, it was observed that freshwater and high salinity (30 psu) depressed whole animal rates of protein synthesis probably to direct energy towards osmoregulation (Intanai et al., 2009).

IDH is a mitochondrial enzyme that also has a key role in cellular defence against oxidative damage caused by ROS, supplying NADPH needed for the regeneration of GSSG into GSH by GR (Jo et al., 2001). Formation of GSSG is known to occur during the conversion of hydrogen peroxide into water, mediated by GPx, and the GST conjugation of electrophilic compounds with GSH (Livingstone, 2001; Lushchak, 2011).

Recently, Jo and colleagues (2001) have shown that elevation of mitochondrial NADPH and GSH by IDH enzyme suppresses oxidative stress and concomitant ROS–mediated damage. In the present study, salinity had no effects on IDH activity of Minho crabs, but was increased in Lima crabs exposed to extreme salinities, that is, 4 and 45 psu. Moreover, in Lima crabs, LDH and IDH showed reverse patterns of activity. The pattern of variation found appears to suggest that in the Lima cohort the IDH pathway of energy metabolism is relevant under conditions of strong osmotic stress (hypo– or hyperosmotic). Both low and high salinities appear to affect the redox balance probably due to the generation of ROS. Previous works reported an influence of salinity in ROS generation, and the activity and/or expression of anti–oxidant enzymes, in estuarine and intertidal invertebrates (Seo et al., 2006; Liu et al., 2007; An and Choi, 2010; Freire et al., 2011). For example, up–regulation of GR expression was found after exposure of the intertidal copepod *Tigriopus japonicus* to 24 and 40 psu, though down–regulation was observed after exposure to 0–12 psu (Seo et al., 2006). Exposure to 25 and 45 psu caused oxidative stress in the ark shell *Scapharca broughtonii* and increased expression and activity of the anti-oxidant enzymes catalase (CAT) and superoxide dismutase (SOD); the effects were more pronounced upon exposure to low salinity (An and Choi, 2010). In this study, the different patterns of activity displayed by the Minho and the Lima cohorts also suggest an influence of the crabs pre–exposure to chemical stress in the Lima estuary on the responses to salinity stress. In this regard, it is noteworthy that Lima crabs also appear to have higher IDH constitutive levels as suggested by the higher IDH activity of animals exposed to 14 psu, as compared to those from the Minho estuary.

4.2. Biotransformation and anti–oxidant defences

Biotransformation and oxidative stress biomarkers have been widely used as indicators of exposure to pollutants. Nevertheless, the development of oxidative stress is considered to be a common response to any substantial stress, including osmotic challenge (Lushchak, 2011).

Under normal conditions, the metabolism of ROS is controlled by the anti–oxidant systems, which establish equilibrium between pro–oxidant and anti–oxidant processes (Livingstone, 2001; Lushchak, 2011). In aquatic organisms, the anti–oxidant systems comprise low molecular weight free radical scavengers (*e.g.*, carotenoids, vitamins A, C, and E, GSH; the latter also acting as a cofactor for GR and GST) and high molecular weight enzymes and proteins (*e.g.*, GST, CAT, SOD, GPx, GR, glucose–6–phosphate dehydrogenase, metallothioneins, ferritin) (Livingstone 2001). In the present study, GR activity in the digestive gland varied only in relation to salinity exposure. In general, activity induction tended to be higher in crabs exposed to high salinities, indicating regeneration of GSSG into GSH due to oxidative stress. As previously mentioned, exposure of *T. japonicus* to low and high salinities elicited down– and up–regulation of the GR gene, respectively (Seo et al., 2006). Some authors referred, however, that GR activation is dependent on tissue specificities and the duration of the exposure (Paital and Chainy, 2010; Yin et al., 2011). In contrast, here changes in GST and GPx levels were only found in relation to the sampling site. Overall, Lima crabs showed enhanced activities, compared to Minho crabs. Besides their anti-oxidant role, GST are involved in phase II biotransformation, catalysing the conjugation of lipophilic compounds with the sulfhydryl groups of GSH facilitating their excretion from the cells (Livingstone, 2001). These results thus suggest an effect of chemical contamination on Lima crabs. Furthermore, although at the end of the exposure experiments crabs from both cohorts were already in clean water for approximately 1 month, one cannot exclude a possible induction effect of contaminants still accumulated in the tissues of Lima crabs. In particular, hypo– and hyperosmotic stress may have originated a mobilisation of lipid reserves as source of energy. Such mobilisation could lead to the release of stored contaminants into the circulation that would cause the enzyme inductions. Long–term exposure to pollutants in the Lima estuary may also explain the elevated activities of these biomarkers. Depending on seasonal influences, higher constitutive levels of these and other important physiological biomarkers were reported in *C. maenas*

chronically exposed to moderate and high contamination levels, as a means to cope with exposure to toxicant stress (Pereira et al., 2009; Dissanayake et al., 2011). In addition, biomonitoring studies performed in fish species collected from these estuaries also showed seasonal higher GST activity in organisms from the Lima than in those from the Minho estuary (Guimarães et al., 2009; Guimarães et al., 2012). Following salinity exposure, significantly higher TG levels were found in crabs from the Lima estuary, compared to those from Minho. Also, the pattern of variation resembled that observed for IDH activity, with higher levels measured in organisms exposed to extreme salinities. Hypo– and hypersalinity thus appear to cause the generation of ROS in Lima crabs, altering the cellular redox status. GSH is produced in the cytosol and transported into the mitochondria (Jo et al., 2001). Here it acts as an anti–oxidant and free radical scavenger, and participates as cofactor in biotransformation reactions (Livingstone, 2001; Lushchak, 2011), becoming oxidised during these processes. Other works have shown that salinity may influence ROS generation and alter free radical processes, in many instances causing oxidative damage. For example, in the shrimp *Litopenaeus vannamei* exposed to acute salinity change, increased CAT, SOD, and GPx activities were related to increased ROS metabolism responsible for oxidative damages (Liu et al., 2007). In the digestive gland of *Scylla serrata*, the activity of CAT, SOD, and GPx anti–oxidant enzymes decreased with exposure to low salinities (Paital and Chainy, 2010).

4.3. Oxidative damage

A steady–state level of anti–oxidants is provided by the balance between the generation and elimination of ROS (Livingstone, 2001; Lushchak, 2011). Despite this, under normal conditions some ROS escape the defence mechanisms and will cause damage to cellular components (*e.g.*, macromolecules), inducing and modifying regulatory cascades, among others (Lushchak, 2011). Furthermore, when the protection system fails to overcome the effects of toxicant–induced ROS, oxidative damage

will accumulate in the cells. In this study LPO levels were influenced by salinity in crabs collected at the mouth of the Lima estuary. LPO was significantly higher in crabs exposed to 8 psu and lower in crabs exposed to 45 psu. The lower LPO levels found in response to hypersalinity are possibly due to a combined compensatory effect of the increased TG levels also found in these organisms. The oxidative damage found in response to hyposalinity may result from increased oxidative stress that could not be compensated by concomitant changes in TG levels. Interestingly, a recent study has shown that exposure of ark shells to low salinity (25 psu) led to an increase in the concentration of hydrogen peroxide and LPO (An and Choi, 2010). The elevated concentration of this ROS induced a sustained increase in SOD expression but a transient increase in CAT expression. This apparently resulted in a failure of CAT to completely remove the hydrogen peroxide, also contributed by SOD activity, and hence in increased LPO (An and Choi, 2010).

In conclusion, exposure to salinities ranging from 4 to 45 psu significantly influenced *C. maenas* biomarkers of neurotransmission (ChE), energy metabolism (LDH and IDH), anti–oxidant stress (GR and TG), and oxidative damage (LPO). For ChE, LDH, IDH, and LPO, this influence was dependent on the sampling site. In crabs from the Minho estuary (low pollution), salinity change only induced significant alterations in ChE and GR. In those from the Lima estuary, salinity had more negative effects, significantly influencing ChE, LDH, IDH, GR, TG, and LPO. These results suggest that long–term exposure of the Lima cohort to pollution and abiotic stress may have affected the response of these crabs to salinity change. Salinity appears to represent an additional stress possibly increasing ROS generation in Lima crabs. In addition, depending on the studied cohort, both hypo– and hypersalinity were found to influence biomarkers of neurotransmission, aerobic metabolism, and anti–oxidant defences. These results add to the understanding of how physiological processes are modulated by salinity in a key estuarine crustacean. They further support the need to account for salinity as a modifying factor when

using these biomarkers in biomonitoring studies with *C. maenas* to assess pollution and environmental quality.

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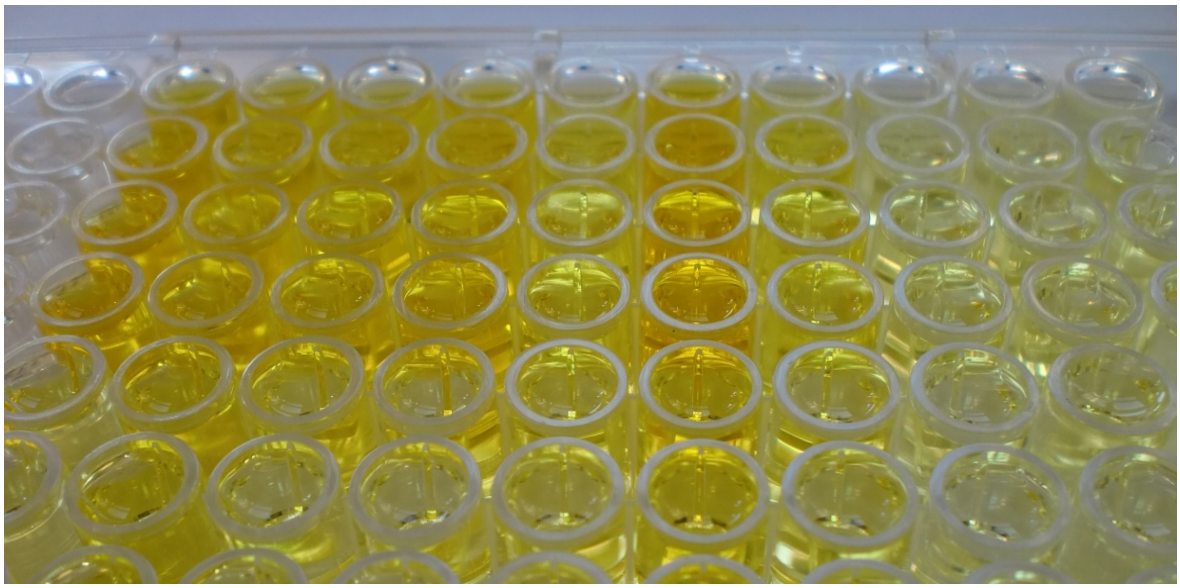
Chapter IV

Involvement of the anti-oxidant system in differential sensitivity of *Carcinus maenas* to fenitrothion exposure



Involvement of the anti-oxidant system in differential sensitivity of *Carcinus maenas* to fenitrothion exposure

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Abstract

Carcinus maenas is an invertebrate with worldwide distribution and high ability to adapt to different environments, which is frequently used in environmental monitoring. Despite this, it is not clear how historical exposure to moderate contamination may influence sensitivity to further chemical stress in this important decapod species. This study investigated differential responses to organophosphate fenitrothion of *C. maenas* from a moderately contaminated estuary and a low impacted one, using *in vitro* and *in vivo* biomarker assays. To clarify potential differences in sensitivity, a biochemical characterisation of muscle cholinesterases was first performed. The results indicated acetylcholinesterase (AChE) as the main form present in *C. maenas* muscle. Exposure assays revealed that crabs from the moderately contaminated site were less sensitive to fenitrothion showing lower AChE inhibition than those from the low impacted site. Other biomarker changes detected in these animals were: increased anaerobic metabolism (muscle lactate dehydrogenase), enhanced phase II biotransformation (glutathione *S*–transferases in the digestive gland) and anti-oxidant defences (*i.e.* activities of glutathione reductase, glutathione peroxidase and catalase, and levels of total glutathiones in the digestive gland). Altogether, the results pointed out a role for the glutathione redox system towards tolerance to fenitrothion exposure.

1. Introduction

Estuaries are areas of high economic, societal, and ecological value, which provide numerous resources, benefits and services to mankind. They harbour a great variety of aquatic species, such as shorebirds, marine fishes, shellfish, and crustaceans, serving as key reproduction and nursery grounds for many of them (Day et al., 1989). Worldwide, urbanisation, agriculture, and industrialization have jeopardised the ecological integrity of estuaries, their commercial and recreational uses and fishery resources (Kennish, 2002). Estuarine organisms are often subject to multiple stress factors, among which prevail excessive levels of organic matter, nutrients, and pollutants (Kennish, 2002). In many instances, populations historically exposed in these areas may exhibit different sensitivity to contamination compared to populations of the same species originating from low impacted sites (Bryan and Gibbs, 1983; Ait Alla et al., 2006). This may have serious implications for the sustainability of estuarine biodiversity and cause increased challenges to monitoring and risk assessment programmes. Hence, investigating such differential sensitivity, and toxicity mechanisms by which it may occur, is of great relevance to assess environmental status and plan remediation actions anticipating a thoughtful follow–up under amelioration of water quality. Furthermore, it provides an important understanding favouring an effective interpretation of biological responses in monitoring programs of affected estuaries. This is even more important in estuaries subjected to moderate contamination levels, where subtle biological responses may be elicited and confounding factors may thus be more influential (Pereira et al., 2011).

Given their ecological relevance, population and community level endpoints are often desirable to assess detrimental effects of environmental contamination. Nevertheless, their interest as early warning indicators of potential impact is limited, seldom contributing to the management of affected systems before serious damage occurs. Also, it is recognised that timely detection of effects requires assessment and monitoring at lower levels of organization (Moore, 1998; Martínez-Gómez

et al., 2010). Biomarkers have been broadly proposed as tools to detect effects on organisms and ecosystems before they attain significant ecological relevance. Recently, they have been recognised as providing a valuable contribution to gauge the good ecological status enshrined in the Marine Strategy Framework Directive (Directive 2008/56/EC) and the Water Framework Directive (Directive 2000/60/EC), reflecting the effects of all the stressors to which the organisms are subjected (Fossi et al., 2012). These tools have also been successfully used to identify populations showing different sensitivity to contamination. Recently, Rodrigues et al. (2012) have found that exposure of shore crabs (*Carcinus maenas*) from low impacted and moderately polluted NW Iberian estuaries to salinity stress caused significant alterations in neurotoxic, detoxification, and anti-oxidant biomarkers that were dependent on the estuary of origin of the organisms. The results suggested that crabs from these estuaries may show different responses to environmental stress, making this a relevant case study to address how historical exposure to moderate contamination may influence differences in sensitivity to further chemical stress and account for possible effects on common monitoring biomarkers (Maria et al., 2009; Martín-Díaz et al., 2009; Pereira et al., 2011).

C. maenas is a dominant ravenous feeder, whose predatory behaviour is thought to be a structuring feature of marine and estuarine benthic communities (Raffaelli et al., 1989). Because of its ecologically important role, widespread geographic distribution, and sensitivity to contamination it is often used in laboratory and field studies aiming at assessment and monitoring of effects (Maria et al., 2009; Martín-Díaz et al., 2009; Dissanayake et al., 2011; Rodrigues et al., 2012). Yet, it is not clear whether historical exposure to moderate pollution levels may influence sensitivity to contamination in this important decapod species. Moreover, investigation of mechanisms involved in tolerance to contamination has seldom been addressed in this species.

The present work thus aimed at investigating potential differences in sensitivity to the organophosphate (OP) fenitrothion (FEN) of *C. maenas* from a low impacted (Minho) estuary and a moderately contaminated

(Lima) estuary of NW Iberian Peninsula using sub–individual biomarker responses. FEN was chosen as a model compound because of its definite primary mode of action (MOA) (through inhibition of B–esterases activity) and wide use in the control of pests in forests and crops, stored grains, poultry sheds, and in public health programs (EPA, 1995; NRAAVC, 1999). FEN has not been detected previously in the Lima estuary, which appears to be contaminated by organochlorine pesticides (Villaverde et al., 2008). However, few studies on the presence of pesticides in Lima River are available in the literature. Also, previous studies concluded that FEN is highly toxic to aquatic invertebrates and birds, and moderately to very highly toxic to estuarine organisms (EPA, 1995), but its effects on *C. maenas* are still poorly understood. Muscle cholinesterase (ChE) activity was used as the endpoint because of its sensitivity to OP pesticides (reviewed by Fulton and Key, 2001), involvement in cholinergic transmission in crab's locomotion (Sorenson, 1973), and its interest to environmental assessments. Energy metabolism was ascertained by measuring muscle activity of lactate dehydrogenase (LDH) and $NADP^+$–dependent isocitrate dehydrogenase (IDH). Biomarkers for FEN detoxification (Jokanović et al., 1996) and the defence against oxidative stress were assessed in the digestive gland. These were the activity of glutathione *S*–transferases (GST), catalase (CAT), glutathiones reductase (GR) and peroxidase (GPx) and the levels of total glutathione (TG). Components of the glutathione redox cycle, such as the glutathione molecule, were suggested to play a role in mediating the toxicity of OPs (Jokanovíc et al., 1996 and references therein). However, their contribution, as well as that of other anti-oxidant biomarkers, to FEN metabolism of invertebrate populations remains unclear. Finally, oxidative damage to cell membranes was assessed through the levels of lipid peroxidation (LPO). In an integrative approach, these biochemical parameters are thought to elucidate about the exposure, differential responses and susceptibility to contaminants (Timbrell, 1998).

2. Materials and methods

2.1. Study sites

The work was carried out with crabs collected in two selected NW Iberian estuaries; a low impacted (Minho estuary) and a moderately contaminated estuary (Lima). These are transitional systems with different urban and industrial patterns of activity. The Minho estuary, included in the Natura 2000 network, shows low susceptibility to human influence (Ferreira et al., 2003) and low levels of environmental contamination (Reis et al., 2009; Guimarães et al., 2012), despite the presence of a few localised sources of pollution. It has previously been used as a reference site in several ecotoxicology studies (Guerreiro et al., 2002; Moreira et al., 2006; Quintaneiro et al., 2006; Rodrigues et al., 2006; Monteiro et al., 2007; Gravato et al., 2010). The Lima estuary, with several industries and high population density, receives wastewaters of industrial, soil leaching, livestock and urban origin, evidencing high susceptibility to human influence (Ferreira et al., 2003). It supports a pulp and paper mill, and has an industrial harbour and a shipyard, showing moderate sediment concentrations of metals and polycyclic aromatic hydrocarbons when compared with the Minho and other European estuaries (Gravato et al., 2010; Guimarães et al., 2012).

Sampling and all animal experiments were conducted in compliance with the ethical guidelines of the European Union Council (Directive 2010/63/EU of 22nd September) for the protection of animals used for experimental and other scientific purposes.

2.2. Crab sampling and acclimation

Intermoult male crabs, with complete appendages, were collected using trawl nets at the mouth of Minho (4.6 ± 0.04 cm carapace width; mean ± standard deviation [SD], n = 70) and Lima estuaries (4.1 ± 0.04 cm, n = 70). The detailed locations of the sampling sites are provided elsewhere (Rodrigues et al. 2012). Water physico–chemical parameters (*i.e.* temperature, pH, dissolved oxygen, and salinity) were measured in

triplicate during each collection campaign. A multiparametric sea gauge WTW multi 340i with the appropriate probes (pH Sen Tix 41 and Tetracon 325) was used. The measurements obtained are presented in Table IV.1. Once in the laboratory, the crabs were placed in tanks (300 L) for 20 days, separated by the estuary of origin. The tanks were filled with filtered seawater (14 psu) and continuously aerated.

Temperature was kept at 15 ± 0.6ºC and the photoperiod was 16/8h day/night. Some of these animals were used in ChE characterisation and *in vitro* exposure to FEN. The remaining animals were used in *in vivo* exposure assays. These crabs were placed individually in flat glass beakers containing 2 L of filtered seawater, covered with appropriate lids. Salinity, temperature and photoperiod were maintained as described previously. The crabs were allowed to acclimate to these conditions for another seven days. During the acclimation period, the medium was totally renewed every 2 days and crabs were fed with Tetrafauna (*Gammarus*) before medium renewal.

2.3. Chemicals

The Bio–Rad protein assay dye reagent was purchased from Bio–Rad Laboratories, Inc. All the other reagents used, including FEN, were of analytical grade and were purchased from Sigma–Aldrich Chemical (Steinheim, Germany).

2.4. Biochemical characterisation of muscle ChEs

To better understand differential responses to FEN, a characterization of muscle ChE forms was first performed using specific substrates and inhibitors. Acetylcholinesterase (AChE) activity was subsequently assessed both *in vitro* and *in vivo* following exposure to FEN. ChEs can be divided into AChE, pseudocholinesterase (PChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PrChE). Characterisation of the forms present in a tissue relies on activity measurements using different substrates and specific inhibitors. Affinity of AChE is higher for acetylthiocholine (ATCh)

than that for butyrylthiocholine (BTCh) or propionylthiocholine (PTCh). Eserine sulphate inhibits ChEs providing a clear indication of the contribution of non–specific esterases to the measured activity (Eto, 1974). True AChE is strongly inhibited by 1,5–bis–(4–allyldimethyl–ammoniumphenyl)–pentan–3–one dibromide (BW284C51) at concentrations in the mM range. Tetramonoisopropyl pyrophosphortetramide (iso–OMPA) inhibits BChE (Eto, 1974).

Table IV.1. Water temperature (T), pH, dissolved oxygen (DO), and salinity (S) measured (in triplicate) in Minho and Lima estuaries during crab sampling. Values represent the mean ± standard deviation (SD).

Parameters	Minho	Lima
T (ºC)	14.45 ± 0.12	13.62 ± 0.16
H	7.35 ± 0.18	7.91 ± 0.17
DO (mg L^{-1})	10.42 ± 0.17	10.57 ± 0.46
Sal (psu)	13.26 ± 6.53	12.10 ± 4.97

2.4.1. Sample preparation

Fifteen crabs from each estuary were used in the biochemical characterisation of muscle ChEs. A piece of leg muscle tissue was collected from each crab, immediately frozen in liquid nitrogen, and stored at –80ºC until further analysis. The samples were subsequently homogenised in ice-cold phosphate buffer (pH 7.2, 0.1 M), and centrifuged at 3,300 ×*g* for 5 min at 4ºC. The supernatants were recovered and used to prepare three pools per estuary that were used to test the different substrates and inhibitors. The pools were prepared with a volume of supernatant containing an equal amount of protein from each crab.

2.4.2. Substrates

The hydrolytic affinity of the enzymatic isoforms towards specific substrates was determined by assaying ChE activity in the presence of ATCh, BTCh or PTCh. In each case ten concentrations were used ranging

from 0.024 to 25.00 mM. Blank reactions were specifically made for each substrate concentration using the same volume of homogenization buffer (0.1 M, pH 7.2) instead of the sample.

2.4.3. Inhibitors

Eserine sulphate, BW284C51, and iso–OMPA were the selected inhibitors tested. Nine concentrations of each inhibitor were prepared, ranging from 1.56 to 400 mM for eserine sulphate and BW284C51, and from 0.016 to 4 mM for iso–OMPA. Eserine sulphate and BW284C51 were dissolved in ultra–pure water; iso–OMPA was dissolved in ethanol. The different concentrations of inhibitors were obtained by dilution of the concentrated stock solutions. For each concentration of inhibitor tested a specific reaction blank was made, where the sample was replaced by phosphate buffer (0.1 M, pH 7.2). For the iso–OMPA assays an extra condition containing ethanol (in a final concentration of 1%) was tested. Controls not containing the inhibitors were also prepared. In these tests, ChE activity was assayed for 10 min using 1 mM ATCh as the substrate.

2.4.4. Determination of ChE activity

Enzymatic activity was determined by the Ellman's method (Ellman et al., 1961). Briefly, thiocholine formed through hydrolysis promoted by samples' ChE reacts with 5,5–dithio–bis–2-nitrobenzoate (DTNB) causing an increase in absorbance that was followed at 412 nm in a microplate reader as previously described (Rodrigues et al., 2012). Protein concentrations in the samples and pools were determined by the Bradford method (Bradford, 1976), using bovine γ–globulin as the standard. All determinations were made in triplicate. ChE activity was expressed in nmol of substrate hydrolysed per min per mg of protein.

2.5. *In vitro* effects of FEN on muscle AChE activity

Three pools of muscle supernatant were prepared for each estuary as described above. The pools were used to investigate the *in vitro* effects of

FEN on AChE inhibition. For this, a stock solution of FEN was made with acetone, and mixed with the AChE reaction buffer to obtain eight test concentrations, ranging from 3.1 to 400.0 µM. A control and a solvent–control (with a final acetone concentration of 1%) were included in the assays. Specific reaction blanks were prepared for each test condition by replacing the sample by the same volume of phosphate buffer (0.1 M, pH 7.2). AChE activity was measured for 10 min using 1 mM ATCh as the substrate.

2.6. Biomarker responses of *C. maenas* to FEN

2.6.1. In vivo *assays*

In vivo assays were carried out with crabs from the two studied estuaries to investigate *C. maenas* responses to FEN exposure. In these assays five FEN concentrations were tested (1.6, 3.2, 6.4, 12.8, and 25.6 nM). Stock solutions of FEN were made in analytical grade acetone. Test solutions were prepared by diluting 100 mL of the respective stock solution in 2 L of filtered seawater. Two control treatments were included: a control group prepared with filtered seawater only and a control + solvent group prepared by dilution of 100 mL acetone in 2 L filtered seawater. In each assay, ten crabs were tested per treatment; the crabs were exposed individually for seven days. The exposure conditions were the same described for the acclimation period. During the exposure, the crabs were kept under low light levels, in conditions as close to natural as possible. Stress to the animals was minimised by keeping the surroundings peaceful and by careful handling of the crabs. Test solutions were completely renewed every 24h. Temperature, pH and dissolved oxygen were measured at 0h and 24h (before medium renewal) in the test beakers. Mortality was registered daily. At the end of the exposure period, subsamples of the digestive gland and leg muscle from each surviving crab were collected, frozen and stored at –80ºC until determination of the biomarkers selected.

2.6.2. Biomarkers determination

Muscle AChE activity and protein concentrations were assayed as described above. ATCh was used as the substrate for AChE determinations. For the determination of LDH activity, a sub–sample of muscle was homogenised in ice–cold Tris/NaCl buffer (pH 7.2, 50 mM). The homogenate was centrifuged at 3300 ×*g*, for 3 min at 4ºC; the supernatant recovered was used to determine the enzyme activity by the method of Vassault (1983). Briefly, the conversion of pyruvate to lactate conduces to NADH oxidation, which is measured by following the concomitant decrease in absorbance at 340 nm in a microplate reader as described in Rodrigues et al. (2012). The enzyme activity was expressed in nmol per min per mg of protein. To assess IDH activity, a sub–sample of muscle was homogenised in ice–cold Tris/NaCl buffer (pH 7.8, 50 mM). The homogenate was centrifuged at 3300 ×*g*, for 15 min at 4ºC. IDH activity was determined in the supernatant recovered, through the method developed by Ellis and Goldberg (1971). The reduction of $NADP^+$, mediated by IDH, was monitored at 340 nm using a microplate reader. IDH activity was expressed in nmol of the NADPH substrate regenerated per min per mg of protein.

Determination of biomarkers of biotransformation, anti-oxidant defences, and oxidative damage was done in the digestive gland. For this, the digestive gland was homogenised (1:10 w/v) in phosphate buffer (pH 7.4, 0.1 M). Part of the homogenate was used to determine the endogenous LPO by measuring spectrophotometrically (at 535 nm) thiobarbituric acid reactive substances (TBARS), as adapted by Filho et al (2001). LPO levels were expressed in nmol of TBARS per g of wet tissue. The remaining homogenate was centrifuged at 14 000 ×*g* for 20 min at 4ºC. The post–mitochondrial supernatant was used to measure GST, GPx, GR, and CAT activity, as well as TG levels. GST activity was determined according to the method of Habig et al. (1974). Briefly, the formation of GSH conjugates with 1–chloro–2,4–dinitrobenzene (CDNB) was followed in a microplate reader (at 340 nm) as previously described in Rodrigues et al. (2012). GST activity was expressed in nmol of the substrate conjugated per

min per mg of protein. GPx activity was determined by measuring the decrease in NADPH using hydrogen peroxide as the substrate, according to Mohandas et al. (1984). Measurements were done at 340 nm using a cuvette spectrophotometer. GPx activity was expressed in nmol per min per mg of protein. GR activity was measured by assessing the decrease of NADPH levels due to the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH), at 340 nm using a cuvette spectrophotometer (Cribb et al., 1989). GR activity was expressed in nmol of oxidated $NADP^+$ per min per mg of protein. CAT activity was determined according to Clairborne (1985) and represents the consumption of hydrogen peroxide as measured at 240 nm in a cuvette spectrophotometer. The enzyme activity was expressed in mmol of substrate converted per min per mg of protein. TG levels were determined as described by Tietze (1969) by following the change in absorbance at 412 nm due to the recycling reaction of GSH with DTNB in the presence of GR. TG levels were expressed as nmol of recycled GSH per min per mg of protein.

All cuvette assays were performed using a Jasco 6405 UV/VIS spectrophotometer. Microplate determinations were carried out with a Bio Tek Power Wave 340 microplate reader. All biochemical analyses were performed at 25ºC.

2.7. Data analysis

ChE catalytic efficiency was determined for each tested substrate by calculating the following kinetic parameters: maximal velocity (V_{max}), Michaelis–Menten constant (K_m) and their ratio (V_{max}/K_m). For this, experimental curves based on the Michaelis–Menten equation were fitted to substrate affinity data. Significant differences in the kinetic parameters between crabs from the two study sites were assessed by comparison of the regression slopes and intercepts of the kinetic curves (Zar, 1999). Data pertaining to specific inhibitors and *in vitro* exposure assays were analysed using the non-parametric Friedman test. Concentrations causing 50% inhibition of muscle ChE activity (IC_{50}), as well as those causing 50% lethality (LC_{50}) in *in vivo* assays, were determined by Probit analysis (Finney,

1971). Significant differences in the biochemical biomarkers, assessed *in vivo*, in relation to exposure concentrations of FEN and the estuary of origin of the crabs were analysed by two-way analysis of variance (ANOVA) with interaction followed by contrast analysis. ANOVA assumptions were tested using the Shapiro–Wilk and the Levene's test. Significant differences were accepted for $p<0.05$ in all tests performed. The Fisher 2 × *K* Exact test was performed with the PEPI package (Abramson, 2004). All other statistical analyses were carried out in SPSS IBM v19.0.

3. Results

3.1. Biochemical characterisation of muscle ChEs

ChE activity increased with the concentration of each tested substrate and no inhibition by excess of the substrate was ever observed in the range of concentrations tested. Rates of substrate hydrolysis were the highest for ATCh and the lowest for BTCh and indicated similar substrate preferences (ATCh > PTCh > BTCh) in crabs from both study sites (Table IV.2.). The catalytic efficiency for ATCh was, however, significantly higher in crabs from the Lima estuary than in those from the Minho, as indicated by comparison of the regression coefficients of the kinetic curves obtained ($p<0.001$). Incubation with eserine sulphate caused a significant inhibition of ChE activity in crabs from both study sites (Friedman test, $p<0.05$). At the lowest test concentration (1.56 µM) inhibition by eserine was already higher than 45% (Fig. IV.1.). At the highest concentration tested (400 µM) eserine caused an almost complete inhibition (92% and 94% in crabs from the Minho and the Lima estuaries, respectively) of ChE activity. A strong inhibition of ChE activity (over 90% at 1.56 µM and over 95% at 400 µM) was also observed after incubation with BW284C51 (Fig. IV.1.) in crabs from both sampling sites (Friedman test, $p<0.05$), indicating AChE as the main form present in *C. maenas* muscle tissue. Incubation with the selective inhibitor iso–OMPA caused no significant decrease in ChE activity, further supporting the previous finding (Fig. IV.1.). In addition, no

significant inhibition of ChE activity by 4 µM iso–OMPA was observed when using BTCh or PTCh as substrates (data not shown).

Table IV.2. Kinetic parameters (Michaelis-Menten constant, K_m; maximal velocity, V_{max}; catalytic efficiency, ratio V_{max}/K_m) obtained by assaying cholinesterase activity, with each tested substrate, from *Carcinus maenas* collected at the Minho and the Lima estuaries. Values represent the mean ± standard error. Different letters indicate statistical significance at $p<0.05$.

Substrate	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1} prot)	V_{max}/K_m (µl min^{-1} mg^{-1} prot)
Minho			
Acetylthiocholine	0.120 ± 0.010[a]	33.17 ± 3.48	275.77 ± 9.01[a]
Propionylthiocholine	0.095 ± 0.012	11.16 ± 1.12	121.03 ± 5.86
Butyrylthiocholine	0.011 ± 0.002	1.14 ± 0.072	109.60 ± 15.66
Lima			
Acetylthiocholine	0.084 ± 0.006[b]	39.45 ± 3.29	470.14 ± 27.14[b]
Propionylthiocholine	0.124 ± 0.007	17.40 ± 1.51	140.22 ± 6.59
Butyrylthiocholine	0.021 ± 0.007	1.11 ± 0.086	74.86 ± 25.39

3.2. *In vitro* effects of FEN on muscle AChE activity

Incubation of muscle supernatants with FEN caused a significant inhibition of AChE activity at concentrations ≥50 µM (Friedman test, $p<0.05$) in Minho crabs and ≥25 µM in Lima crabs (Friedman test, $p<0.05$) (Fig. IV.2.). Nevertheless, total AChE inhibition was never observed. For concentrations ≥25 µM AChE inhibition was significantly higher in crabs from the Lima than in those from the Minho estuary (Friedman test, $p<0.05$). The IC_{50} values obtained were 254.2 µM (95% confidence interval: 195.4–365.5) for crabs collected from the Minho estuary and 44.6 µM (95% confidence interval: 20.1–69.4) for crabs collected from the Lima estuary.

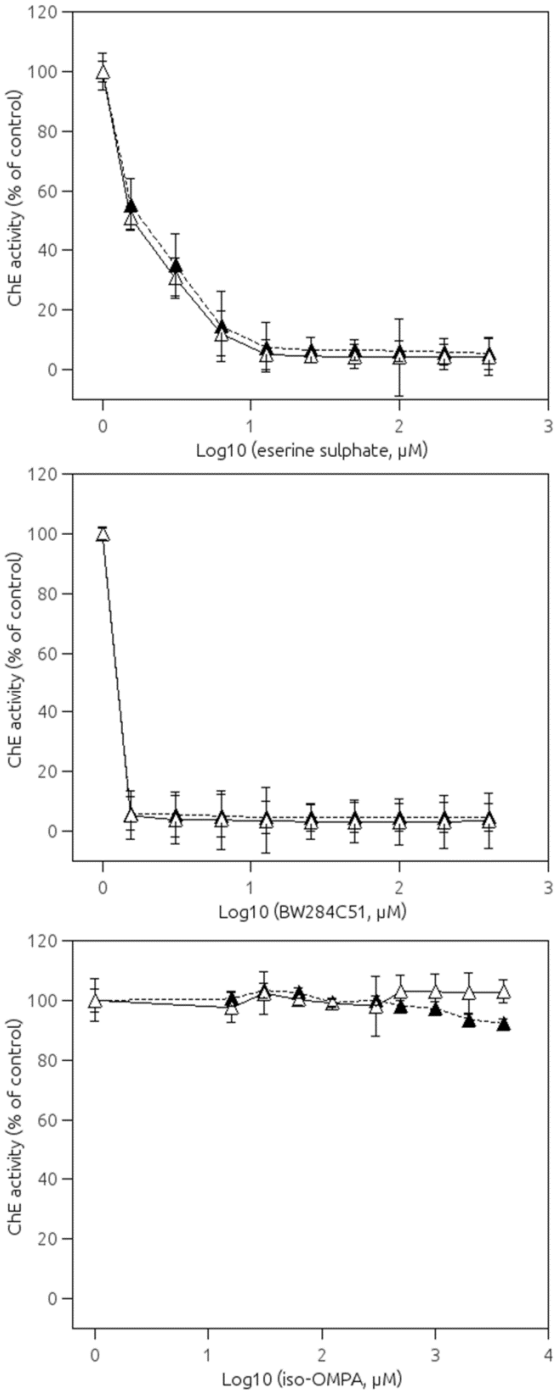

Fig. IV.1. Effects of the specific inhibitors, eserine sulphate (top), BW284C51 (middle), iso–OMPA (bottom), on cholinesterase (ChE) activity (mean ± standard error in % of control) of *C. maenas* from the Minho (black triangles) and the Lima (white triangles) estuaries using acetylthiocholine as the substrate. For iso–OMPA the control and the control–solvent were considered together as no significant differences were found between them.

3.3. Biomarker responses of *C. maenas* to FEN

The highest concentrations of FEN tested were found to be in the lethal range. In particular, exposure to 25.6 nM FEN caused about 40% and 70% mortality in crabs from the Lima and the Minho estuaries, respectively.

Crabs from the Minho estuary exposed to ≤3.2 nM FEN showed significantly higher AChE activity (by about 30%) than those from the Lima estuary exposed to the same conditions (planned contrast analysis, $p<0.05$) (Fig. IV.3.).

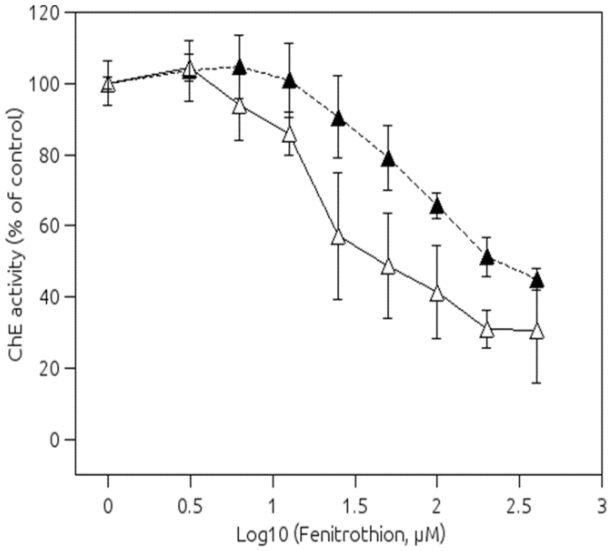

Fig. IV.2. Acetylcholinesterase (AChE) activity (mean ± standard error in % of control) after *in vitro* exposure to fenitrothion of supernatants prepared with *C. maenas* collected from the Minho (black triangles) and the Lima (white triangles) estuaries, using acetylthiocholine as the substrate.

Significant AChE inhibition relative to controls was detected at ≥12.8 nM FEN in crabs from both sampling sites. However, at 25.6 nM FEN AChE inhibition in Minho crabs was much stronger (–70%) than that found for Lima crabs (–30%) (planned contrast analysis, $p<0.05$). For all the other biomarkers studied there was a significant interaction between FEN exposure and the site of origin of the crabs (two–way ANOVA, Table IV.3.), indicating that crabs from the two study cohorts exhibited different response patterns to the tested toxicant.

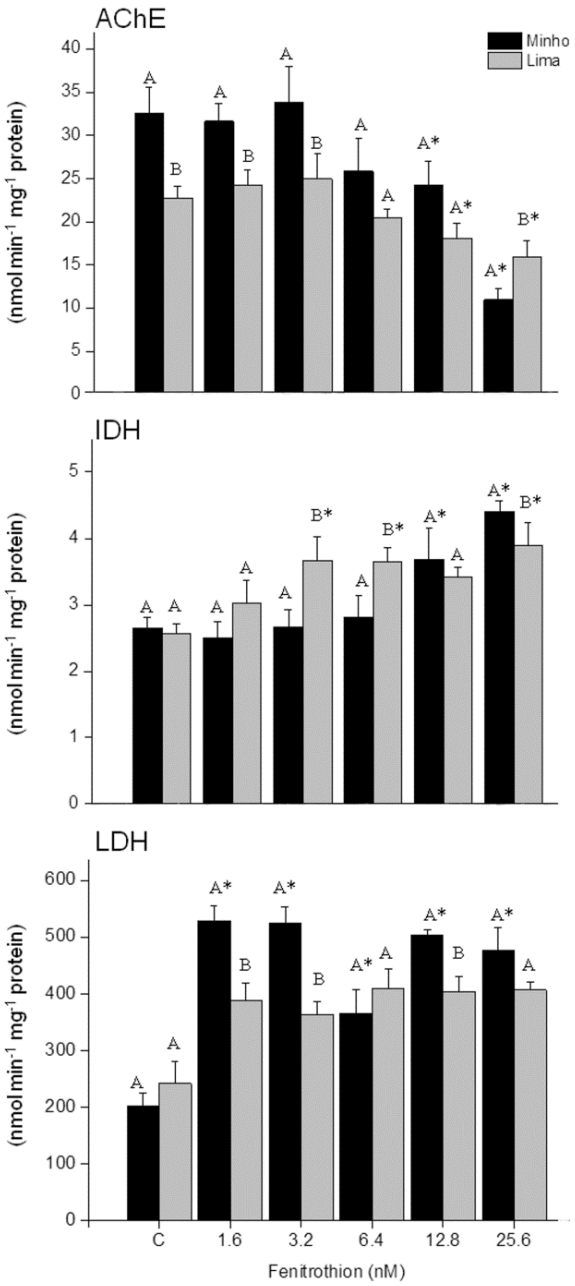

Fig. IV.3. Mean and corresponding standard error of acetylcholinesterase (AChE), $NADP^+$–dependent isocitrate dehydrogenase (IDH), and lactate dehydrogenase (LDH) activity determined in the muscle of crabs from the Minho and the Lima estuaries exposed for 7 days to fenitrothion. Significant differences between sampling sites within each condition are identified by different capital letters (two–way ANOVA with planned pairwise comparisons, $p<0.05$). The asterisks indicate significant differences within each estuary compared to the respective control group (two–way ANOVA followed by contrast analysis, $p<0.05$).

Muscle IDH activity was significantly increased in Minho crabs exposed to ≥12.8 nM FEN, relative to controls (planned contrasts analysis, $p<0.05$) (Fig. IV.3.). Conversely, in Lima crabs IDH activity was significantly increased by ~30% at 3.2, 6.4 and 25.6 nM FEN, compared to controls. Significant differences (ranging from 30–35%) between crabs from the Minho and the Lima estuaries were found for FEN concentrations of 3.2, 6.4 and 25.6 nM (planned contrasts analysis, $p<0.05$). With respect to LDH, Minho crabs exhibited increased (about 2.5–fold) enzyme activity in all the test treatments, relative to the respective controls (planned contrasts analysis, $p<0.05$) (Fig. IV.3.). The response pattern was opposite to that of IDH with higher activities recorded at lower FEN concentrations (1.6 and 3.2 nM) and slightly lower activities recorded at higher FEN concentrations (12.8 and 25.6 nM). In contrast, no significant differences among treatments were found in crabs from the Lima estuary.

Regarding the anti-oxidant defence biomarkers (TG, GR, GPx, and CAT), in Minho crabs, the treatment caused no significant differences in TG levels relative to controls (planned contrasts analysis, $p<0.05$) (Fig. IV.4.). In Lima crabs exposure to FEN significantly decreased TG levels at ≥6.4 nM, compared to controls. GR, GPx and CAT showed bell–shaped responses in Minho crabs exposed to FEN. Significant inductions were found at lower FEN concentrations, followed by decrease to control levels (GPx and CAT), or even inhibition of activity, at ≥6.4 nM (GR, –80% maximum inhibition recorded) and ≥12.8 nM (GPx and CAT) (planned contrast analysis) (Fig. IV.4.). Enzyme inductions ranged between 30% (GR) and 100% (GPx) and 200% (CAT) of control activity. In contrast, in Lima crabs significant inductions of GR and GPx activity were found for all FEN treatments, compared to controls. However, no changes in CAT activity were found for crabs collected at the Lima estuary (Fig. IV.4.). With respect to LPO levels, no differences among treatments were observed in Minho crabs (Fig. IV.4.). In Lima crabs, LPO levels were significantly increased at 1.6, 6.4 and 12.8 nM FEN, compared to controls, but returned to control levels at 25.6 nM (planned contrasts analysis, $p<0.05$).

Table IV.3. Results of the full–factorial two–way ANOVA carried out to assess the effects of fenitrothion exposure and the sampling site on the biomarkers assessed.

Parameter	Source of variation	*df*	*F*	p
Neurotransmission and energy metabolism				
AChE	Fenitrothion	5, 105	8.80	0.004
	Sampling site	1, 105	5.79	<0.001
	Fenitrothion × Sampling site	5, 105	1.14	0.344
IDH	Fenitrothion	5, 105	7.80	<0.001
	Sampling site	1, 105	2.36	0.128
	Fenitrothion × Sampling site	5, 105	2.44	0.039
LDH	Fenitrothion	5, 105	7.80	<0.001
	Sampling site	1, 105	2.36	0.128
	Fenitrothion × Sampling site	5, 105	2.44	0.039
Biotransformation and anti-oxidant defences				
GST	Fenitrothion	5, 105	62.14	<0.001
	Sampling site	1, 105	1.35	0.248
	Fenitrothion × Sampling site	5, 105	14.24	<0.001
GR	Fenitrothion	5, 105	7.83	<0.001
	Sampling site	1, 105	10.21	0.002
	Fenitrothion × Sampling site	5, 105	8.32	<0.001
GPx	Fenitrothion	5, 105	26.83	<0.001
	Sampling site	1, 105	31.33	<0.001
	Fenitrothion × Sampling site	5, 105	7.169	<0.001
TG	Fenitrothion	5, 105	1.12	0.356
	Sampling site	1, 105	1.44	0.233
	Fenitrothion × Sampling site	5, 105	5. 63	<0.001
CAT	Fenitrothion	5, 105	3.12	0.012
	Sampling site	1, 105	70.22	<0.001
	Fenitrothion × Sampling site	5, 105	3.28	0.009
Oxidative damage				
LPO	Fenitrothion	5, 105	3.86	0.003
	Sampling site	1, 105	12.70	0.001
	Fenitrothion × Sampling site	5, 105	3.22	0.010

AChE, acetylcholinesterase; *IDH*, $NADP^{+}$–dependent isocitrate dehydrogenase; *LDH*, lactate dehydrogenase; *GST*, glutathione *S*–transferases; *GR*, glutathione reductase; *GPx*, glutathione peroxidase; *CAT*, catalase; *TG*, total glutathione; *LPO*, lipid peroxidation.

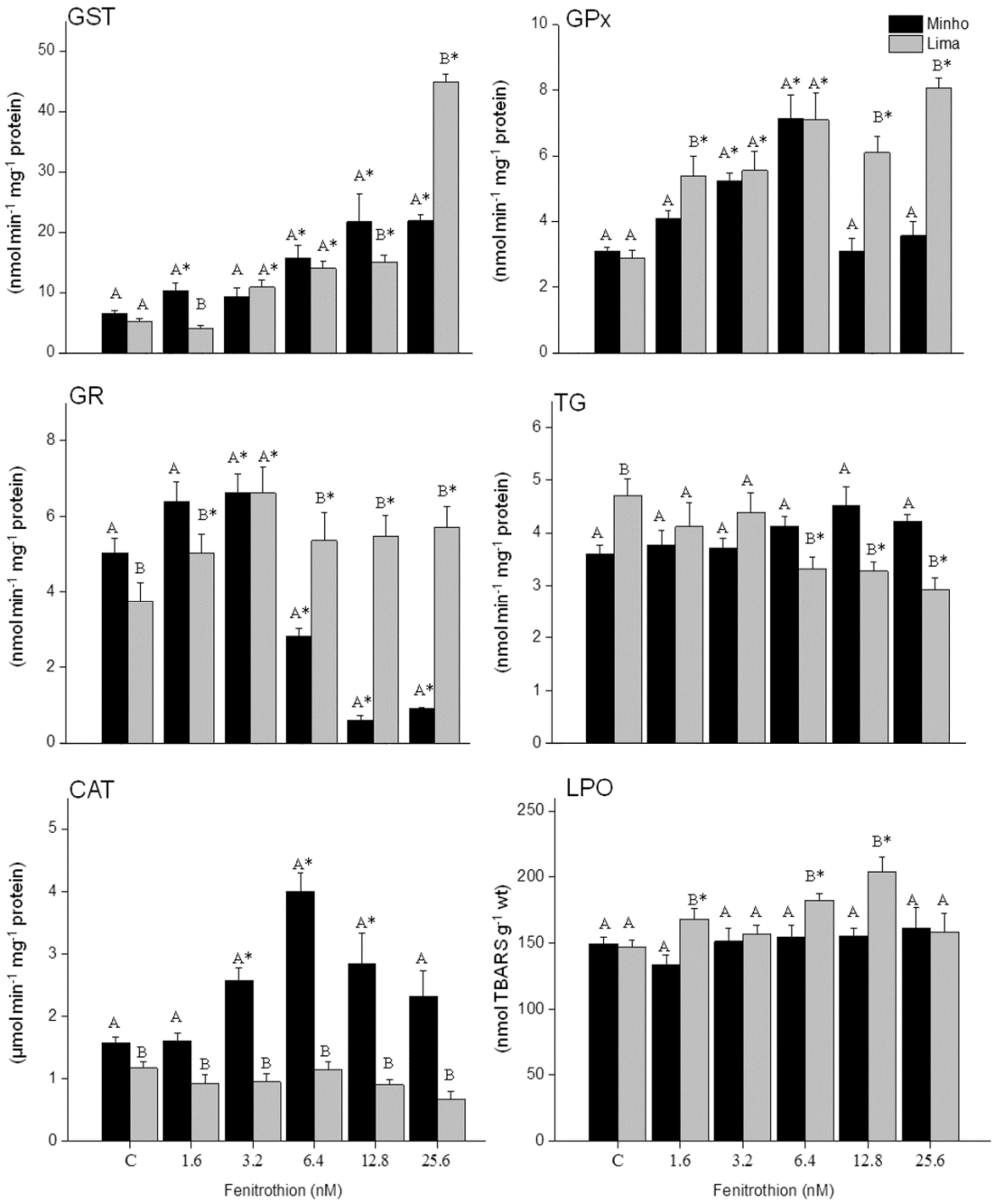

Fig. IV.4. Mean and corresponding standard error of glutathione *S*–transferases (GST), glutathione peroxidase (GPx) and reductase (GR), and catalase (CAT) activity, and total glutathione (TG) and lipid peroxidation (LPO) levels determined in the digestive gland of crabs from the Minho and the Lima estuaries exposed for 7 days to fenitrothion. Significant differences between sampling sites within each condition are identified by different capital letters (two–way ANOVA with planned pairwise comparisons, $p<0.05$). The asterisks indicate significant differences within

each estuary compared to the respective control group (two–way ANOVA followed by contrast analysis, $p<0.05$).

Concerning GST activity, significant increases relative to the respective controls were found in Minho crabs exposed to ≥6.4 nM FEN. A 3–fold maximum GST induction was recorded in Minho crabs exposed to ≥12.8 nM FEN (Fig. IV.4.). In Lima crabs, GST was significantly induced at ≥3.2 nM FEN, compared to controls (planned contrasts analysis, $p<0.05$). A 5–fold maximum GST induction was recorded in Lima crabs at 25.5 nM FEN (Fig. IV.4.).

4. Discussion

The results reported herein pointed out AChE as the predominant form present in the *C. maenas* leg muscle tissue. Because AChE and PChE exhibit different functions and sensitivity to pesticides (Sturm et al., 2000), and their ratio varies among tissues and among species (Nunes, 2011), characterising ChE forms is a requirement for their use in monitoring and environmental risk assessment. In the present study, the preferred substrate was found to be ATCh. BTCh and PTCh were also hydrolysed, but at a much slower rate.

Furthermore, this muscle form was highly sensitive to eserine sulphate (indicating it was a ChE rather than another esterase form) and BW284C51. In addition to this, it showed no significant response to iso–OMPA, further supporting the above conclusion. To our knowledge, previous studies addressing ChE characterisation in *C. maenas* date back to 1950 (Walop and Boot, 1950). The authors assessed ChE activity in muscle tissue using different substrates and eserine incubation. Similarly to the present study, they concluded that the muscle forms appeared to behave as ChE and that the preferential substrate was ATCh. However, Walop and Boot (1950) provided no information useful to elucidate about the potential presence of PChE in leg muscle. The present study thus confirms and further refines their results by indicating a main role in cholinergic neurotransmission of

muscle ChE, whose inhibition is expected to induce severe effects, leading to the death of the organisms in extreme scenarios. Studies with other crustacean species have also found AChE as the predominant form either in muscle tissue or whole body determinations, including in the estuarine copepod *Eurytemora affinis* (Forget et al., 2002), the marine copepod *Tigriopus brevicornis* (Forget et al., 1999), the grass shrimp *Palaemonetes pugio* (Key and Fulton, 2002), and the white shrimp *Litopenaeus vannamei* (García-de la Parra et al., 2006). The results presented in this study also indicate that it is adequate to measure only AChE in monitoring programs using *C. maenas* as sentinel species.

In vitro exposure to FEN significantly inhibited muscle AChE activity of crabs from both study sites. However, AChE of organisms collected from the polluted (Lima) estuary was more sensitive to FEN showing an IC_{50} value over 5.5–fold lower than that of crabs collected from the low impacted (Minho) estuary. Despite this, crabs collected from the Lima estuary appeared to be less sensitive to *in vivo* FEN exposure showing a significantly lower AChE inhibition and mortality than those from the low impacted estuary.

Values published in the literature for the effects of FEN on muscle ChE inhibition of other crustacean species are scarce. *In vitro*, an IC_{50} of 100 mM FEN was reported for *Palaemon serratus* muscle, a value close to the one reported here for Lima crabs (Bocquené et al., 1995). *In vivo*, Bhagyalakshmi and Ramamurthi (1980) reported a 24h IC_{50} of 5 mg L^{-1} for the freshwater field crab *Oziotelphusa senex*, following sublethal injections with FEN, a value near the 7–day IC_{50} for Minho crabs. These values are in agreement with the strong difference between *in vitro* (in the mM range) and *in vivo* (in the nM range) IC_{50} values presented herein. The higher *in vivo* toxicity is most probably due to the presence of the FEN oxon metabolite, which is also able to irreversibly inhibit AChE. The toxicity of OPs is related to the activity of cytochrome P450 (CYP 450) enzymes (Taylor and Feyereisen, 1996). These enzymes are responsible for the bioactivation of OPs, producing more potent oxygen analogues with higher

anticholinesterasic activity than their parent compounds (reviewed by Hyne and Maher, 2003).

Although the rate of chemical metabolisation is substantially lower in crustacean than in fish (Hyne and Maher, 2003), induction of CYP450 enzymes by FEN exposure was previously shown in the digestive gland of *Procambarus clarkii* (Escartín and Porte, 1996).

Taking into account that AChE has a crucial role in cholinergic neurotransmission, which in turn was shown to be involved in *C. maenas* locomotion (Sorenson, 1973), these results also suggest that Minho crabs exposed to FEN may show a stronger depression of locomotory behaviour than those from the Lima estuary. Other studies have found a reduction in swimming velocity of crustaceans, *i.e*. *Neomysis integer* and *P. serratus*, exposed to chlorpyrifos (Roast et al., 1999) and to FEN (Oliveira et al., 2013), respectively. In *P. serratus* the reduction of a locomotory function was also associated with significant inhibition of muscle ChE activity, which seemed to precede the impairment of swimming velocity at higher FEN concentrations (Oliveira et al., 2013).

Mechanisms involved in differential sensitivity to OPs in arthropods encompass decreased sensitivity of the target site given by mutations in AChE, reduced uptake, and increased metabolism and/or sequestration by detoxification enzymes such as carboxylesterases (CbE) or GST (Taylor and Feyereisen, 1996). Mutations in AChE appear to confer much higher sensitivity differences than those observed in the present study (Roush and McKenzie, 1987; Dunley et al., 1991; Taylor and Feyereisen, 1996).

The low fold difference in AChE sensitivity observed between Lima and Minho crabs, as well as the lack of direct correlation between *in vitro* and *in vivo* sensitivity, thus suggest that mutations affecting the physiological AChE target may not be the underlying mechanism explaining the moderate tolerance of Lima crabs. A role for GST, and possibly CbE, in the observed enhanced tolerance is suggested by the results obtained and would also be consistent with exposure in a moderately polluted estuary (Lima), where selection pressures are not expected to be maximal. CbE are involved in the metabolism of OPs (Jokanović et al., 1996; Wheelock et al.,

2008) by reducing the amount of pesticides available to cause impact on nervous and muscular AChE (reviewed by Wheelock et al., 2008). Increased levels of CbE activity were also linked to resistance of insects to pesticides (Wheelock et al., 2008). Furthermore, higher CbE activity has been found on the digestive gland of crustaceans, the main detoxification organ of this group of animals (Solé et al., 2006; Vioque-Fernández et al., 2007). Its scavenging and protective role may thus have contributed to the different tolerance of *C. maenas* to FEN found in this study. On the other hand, GST is known to be involved in the detoxification of FEN through demethylation in an *O*–alkyl conjugation reaction (Jokanović et al., 1996). In good agreement with this, crabs from both sites exhibited a strong GST induction following exposure to FEN. However, a limitation of the conjugation reaction appeared to take place in Minho crabs exposed to high FEN concentrations (25.6 nM), but not in Lima organisms. The strong inhibition in GR activity observed in Minho crabs exposed to ≥6.4 nM suggests that this limitation was probably caused by glutathione depletion. Most cells contain high concentrations of the glutathione tripeptide in its reduced form (GSH); the oxidised form (GSSG) representing a minor fraction of total glutathione levels (Jokanović et al., 1996). GSH, together with GR, GPx, and NADPH make up the glutathione redox system. Moreover, it acts both in demethylation reactions and as a substrate in the GPx–mediated conversion of hydrogen peroxide into water (Lushchak, 2011). In particular, GSH was shown to serve as an acceptor of methyl groups in the demethylation of FEN, greatly increasing the rate of this reaction (Eto, 1974). More recent studies also demonstrated the importance of GSH as a FEN detoxification pathway, together with the existence of an oxidation metabolism, in the crayfish *P. clarkii* (Escartín and Porte, 1996). Hence, in the present case, GSH may have been depleted directly through the GST– and GPx–mediated reactions and indirectly by inhibition of GSH regeneration. This hypothesis is also in agreement with the induction at low concentrations and return to control levels of GPx activity in Minho crabs exposed to ≥12.8 nM FEN. In keeping with this, the increased TG levels found in Minho crabs exposed to ≥12.8 nM most

probably reflect an excess of GSSG rather than high concentrations of GSH. Conversely, in Lima crabs GST, GR, and GPx activities were maintained at significantly high levels even in the highest exposure concentrations, whereas TG levels decreased with the FEN concentration. The TG trend appears thus to reflect a decrease in GSH, possibly caused by the notably high inductions of GST and GPx found in these animals, which could not be compensated through GR–mediated recycling. FEN exposure also elicited changes in CAT activity in crabs collected from the Minho, but not in those from the Lima estuary. Further, a biphasic bell–shaped response was observed in Minho test organisms. CAT is an anti-oxidant enzyme that acts on the conversion of hydrogen peroxide into water and oxygen (Bhagyalakshmi et al., 1984). Together with the observed GPx induction at lower concentrations, this suggests that a pesticide-induced generation of free oxygen radicals took place in these crabs (El-Sharkawy et al., 1994; Banerjee et al., 1999; Viarengo et al., 2007). The bell–shape trend is a typical response of detoxification and anti-oxidant biomarkers to contaminants. The initial increase in measured activity is caused by activation of enzyme synthesis. The subsequent decrease in enzymatic activity is considered to occur due to the enhanced catabolic rate and/or a direct inhibitory action of contaminants on the enzyme molecules (Cohen et al., 2001). Interestingly, despite the alterations observed in GPx activity, LPO levels remained constant and at control levels in Minho crabs. The increased levels of CAT activity, also found in these animals, may have contributed to minimise oxidative damage to cell membranes that could possibly result from the generation of free radicals during detoxification reactions. Other components of the anti-oxidant system, such as enzymes providing necessary cofactors (*e.g.*, glucose–6–phosphate dehydrogenase) and/or low molecular weight scavenging compounds (*e.g.*, vitamin C, vitamin E, carotenoids) (El-Sharkawy et al., 1994), may have also been involved in keeping low LPO levels. In Lima organisms, slight LPO levels were detected in crabs exposed to concentrations up to 12.8 nM, but not at 25.6 nM, suggesting that the strong GST induction observed at the higher FEN concentration may have accounted for the return of LPO to

control levels. Overall, these results suggest that FEN is able to induce GST activity and cause inhibition or decrease of other important components of the glutathione redox system in *C. maenas*. Furthermore, it suggests a role for this system in conferring differential sensitivity to FEN exposure. The differences in neurotoxicity, detoxification, and anti-oxidant defences found between the two crab cohorts were also accompanied by distinct patterns of energy metabolism (IDH, LDH) biomarkers. In the Minho cohort IDH and LDH exhibited complementary response patterns. In the Lima cohort, only IDH was significantly induced by FEN concentration. LDH is a crucial enzyme acting in the conversion of pyruvate into lactate; a process accompanied by the oxidation of NADH which maintains the NAD^+ levels. According to previous studies, induction of LDH activity is an indication of increased energy demand to cope with exposure to toxicant challenge (De Coen et al., 2001; Jo et al., 2001; Key and Fulton, 2002; Guimarães et al., 2012). It is considered particularly important in situations where high energy levels may be required in a short period of time (Jo et al., 2001). Conversely, IDH is considered as more efficient in ATP production (Moreira et al. 2006) and has also an active role in the anti-oxidant defence system, providing the NADPH used by GR to regenerate oxidised glutathione (GSSG) into reduced glutathione (GSH) (Jo et al., 2001). The current results suggest that in organisms from low impacted sites, LDH induction constitutes a first line response to FEN exposure and is essential to cope with the chemical challenge. Under strong chemical stress, caused by high exposure concentrations, additional energy input is generated by IDH induction, possibly also related to the increased need for replenishment of GSH (Jokanović, 2001). In organisms' acclimated to historical pollution, the energetic requirements imposed by the exposure appear to be met through the aerobic production pathway, with no relevant contribution from the anaerobic route. Bhagyalakshmi et al. (1984) also reported alteration of these enzyme activities upon crustacean exposure to this OP. LDH induction and IDH inhibition were found in the freshwater crab *Oziotelphusa senex senex* exposed to 100 mg L^{-1} FEN (Bhagyalakshmi et al., 1984). According to them, structural gill alterations resulted in reduced

oxygenation, and hence in IDH inhibition, which would be compensated by the enhancement of the anaerobic pathway. Nevertheless, the concentrations tested in that study were much higher than those tested in the current work.

In summary, the results suggest that crabs from the polluted Lima estuary are more tolerant to FEN exposure than those from the Minho, exhibiting lower AChE inhibition, enhanced phase II biotransformation and anti-oxidant defences, as well as altered aerobic energy metabolism. The differences in sensitivity were probably related to differences in detoxification and anti-oxidant defences, rather than to differential sensitivity in the AChE target site. The results pointed out a role for the glutathione redox system towards tolerance to FEN exposure. Future studies should focus on further investigating FEN metabolism in this species by addressing the contribution of CbE and GSH. The measurement of CbE activity and the levels of GSH and GSSG in FEN–treated crabs, in combination with the use of specific inhibitors of CbE activity and/or glutathione synthesis and recycling, will clarify this issue. Whereas the biomarkers assessed herein are important tools in evaluating the health status of these organisms (Maria et al., 2009; Martín-Díaz et al., 2009; Pereira et al., 2011), their responses may vary in relation to animals' sensitivity. Moreover, the present results show that historical exposure in even moderately contaminated sites may elicit enhanced tolerance in an ecologically relevant decapod possibly accounting for confounding effects. This should, thus, be taken into consideration to improve site-specific monitoring of environmental health and risk assessment procedures envisaging preservation of the structure and function of the affected ecosystems.

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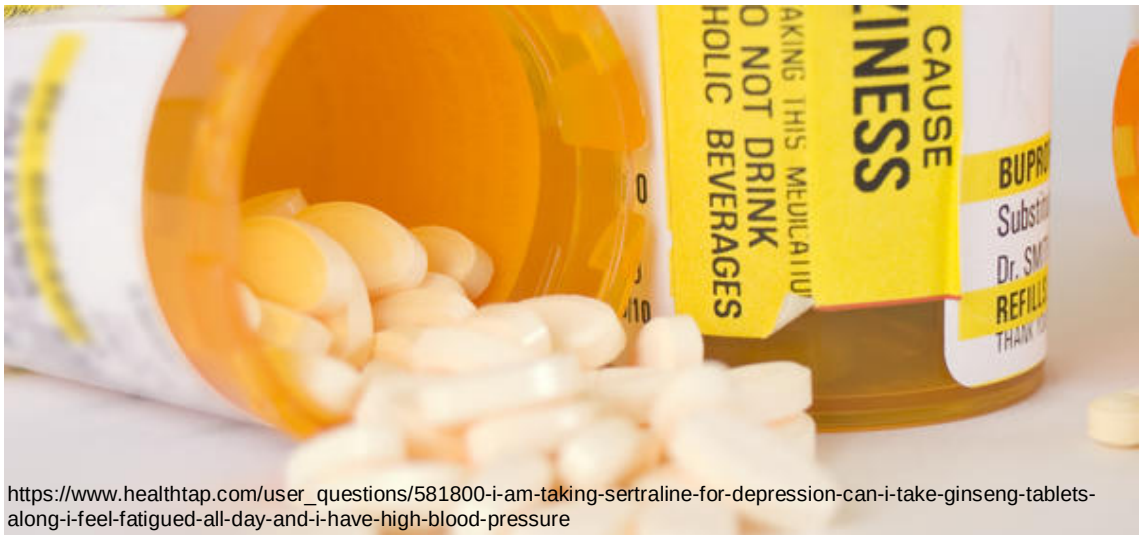
Chapter V

Accumulation and effects of sertraline in an estuarine decapod: importance of the history of exposure to chemical stress



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Accumulation and effects of sertraline in an estuarine decapod: importance of the history of exposure to chemical stress

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Abstract

Sertraline (SERT) is widely prescribed worldwide and frequently detected in aquatic systems. There is, however, a remarkable gap of information on its potential impact on estuarine and coastal invertebrates. This study investigated SERT accumulation and effects in *Carcinus maenas*. Crabs from a moderately contaminated and a low–impacted estuary were exposed to environmental and high levels of SERT. A battery of biomarkers related to SERT mode of action was employed to assess neurotransmission, energy metabolism, biotransformation, and oxidative stress pathways. After a seven–day exposure, SERT accumulation in crabs' soft tissues was found at ≥5 μgL^{-1}, which was higher in animals from the moderately polluted site. These crabs were also more sensitive to SERT, exhibiting decreased acetylcholinesterase activity, indicative of ventilatory and locomotory dysfunction, inhibition of anti-oxidant enzymes and increased oxidative damage at 0.05 μgL^{-1}. The Integrated Biomarker Response (IBR) index indicated their low health status. Crabs from the low impacted site showed non-monotonic responses of acetylcholinesterase suggestive of hormesis. The results pointed an influence of the exposure history on differential sensitivity to SERT and the need to perform evaluations with local ecological receptors in site–specific assessments, to increase relevance of risk estimations when extrapolating from laboratory to field conditions.

Keywords: Bioaccumulation, cholinergic neurotransmission, oxidative stress, exposure history, risk assessment, invertebrates

1. Introduction

Implementation of specific legislation worldwide is acting to reduce and mitigate the presence and effects of priority contaminants in aquatic systems. However, emerging contaminants (ECs) are placing a major challenge to ecologic risk assessment and mitigation programmes because they are excluded from standard chemical evaluations and their biological effects are still poorly known, despite their recognised importance. Among ECs, antidepressants and their metabolites have been raising a great deal of concern. They are widely prescribed and frequently detected in wastewaters (Calisto, 2009; González Alonso et al., 2010). In the European Union, their consumption increased by >80% in the last decade (OECD, 2012) due to escalating trends in mental health problems and psychiatric disorders. Their occurrence in aquatic systems is considered to be due to low removal rates of conventional wastewater treatments, resulting in the discharge of contaminated effluents into receiving waters, with major public health and environmental risks (Calisto, 2009; Santos et al., 2013). Conceived to trigger a specific biological response, several studies evidenced that antidepressants may induce effects on non–target species with unexpected consequences in behavioural and reproductive traits at concentrations within the ng L^{-1} range (Santos et al., 2010). This is the case for selective serotonin reuptake inhibitors (SSRIs) used to treat depression, anxiety, and personality disorders. In humans and other vertebrates, serotonin (5–HT) is involved in the neuromodulation of several hormone–dependent physiological processes. In invertebrates and lower taxa, 5–HT is involved in functions as diverse as bivalve reproduction (Croll et al., 1995) or cilia regeneration in protozoa (Rodríguez and Renaud, 1980). Based on high homology between receptors, relevant risk for undesirable outcomes may be expected for SSRIs in non–target invertebrates (Christen et al., 2010). In good agreement, sertraline (SERT), a potent SSRI with few side effects in humans, is considered the most acutely toxic SSRI towards freshwater species (*e.g.*, algae, daphnids, fish) (Henry et al., 2004; Sanderson et al., 2004; Christensen et al., 2007; Johnson et al., 2007).

Nevertheless, knowledge on its accumulation in and effects on coastal and estuarine key-invertebrates is scarce. Ecotoxicological data available is restricted, and mainly focused on its apparently less toxic counter partner fluoxetine, despite high human pressure and risk of contamination in these areas. Hence, research addressing the relationship between exposure, tissue accumulation and adverse effects of sertraline in such species is urgently needed, to provide empirical data for risk assessment and develop predictive approaches (Daughton and Brooks,2011).

It has been noted earlier that effects of pharmaceuticals should be better addressed by focusing on sensitive biomarkers related to their mode of action (MOA) (Christen et al., 2010). Nonetheless, most studies evaluating the effects of SERT employed conventional testing based on standard laboratory species and general endpoints such as survival, growth and reproduction. Also species and/or populations of different backgrounds (*e.g.*, geographic regions, genetic make–up, previous exposure history) may exhibit differential sensitivity to contaminants (Boets et al., 2012; Jin et al., 2012; Messiaen et al., 2013; Rodrigues et al., 2013a). However, this is seldom taken into consideration often limiting ecological relevance to field scenarios and accurate site–specific risk assessment. This work investigated the accumulation and sub–lethal effects of SERT exposure in *Carcinus maenas* originating from sites with differing contamination histories. *C. maenas* was chosen because it is a key invertebrate of European estuarine and coastal systems regularly used in laboratory and field studies addressing effects of contaminants, including pharmaceuticals (Mesquita et al., 2011; Pereira et al., 2011; Rodrigues et al., 2012). It is a good biological indicator reflecting the levels of environmental contamination (Bamber and Depledge, 1997). It is a voracious feeder, whose foraging behaviour is considered to be a structuring feature of marine and estuarine benthic communities (Raffaelli et al., 1989), and also a common prey of several species of crustacean, fish, aquatic birds, minks, otters, and seals (Klassen and Locke, 2007). Recently, it was observed that the history of chronic exposure to even moderate pollution may influence *C. maenas* sensitivity to chemical and

natural stress. Crabs under such conditions showed higher sensitivity to salinity stress but increased tolerance to organophosphate exposure possibly resulting from acclimation processes (Rodrigues et al., 2012; Rodrigues et al., 2013a). Hence, the working hypotheses were that: *i)* exposure to low environmental levels of SERT would cause tissue accumulation and alterations in sub-individual biomarkers, impairing the health status of *C. maenas*; ii) these effects would be dependent on the exposure history to moderate contamination of the crabs, providing relevant information to the ecotoxicological assessment involved in risk calculations. To test this sub–acute exposures to SERT were performed with crabs from two NW Iberian estuaries, a low–impacted and a moderately polluted by metals and polycyclic aromatic hydrocarbons (PAHs) (Rodrigues et al., 2013a). Ten biomarkers providing health status information were assessed for their potential involvement in SERT MOA (Govorunova et al., 2010; Mesquita et al., 2011; Abdel-Salam et al., 2013) and widespread use in integrated chemical-biological effects monitoring. They were related to neurotransmission, energy metabolism, biotransformation, and oxidative stress pathways: activity of acetylcholinesterase in the thoracic ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$–dependent isocitrate dehydrogenase (IDH) in the muscle; activity of glutathione *S*–transferases (GST), catalase (CAT), glutathione peroxidase (GPx) and reductase (GR), and levels of total glutathione (TG) and lipid peroxidation (LPO) in the digestive gland. AChE is a serine hydrolase acting in the cleavage of acetylcholine in synapses and neuromuscular junctions terminating the transmission of nervous impulse to postsynaptic cells. Blockage of AChE is life–threatening leading to paralysis and respiratory arrest. SERT was previously shown to inhibit human AChE activity and 5–HT and serotonergic neurons have been implicated in the modulation of cholinergic transmission in *Caenorhabditis elegans* (Müller et al., 2002; Govorunova et al., 2010), pinpointing the interest of AChE to assess SERT effects. To clarify the results, a characterisation of cholinesterase (ChE) forms in the crabs' thoracic ganglion was performed. This ganglion is

composed of interneurons and motoneurons that are important components of the ventilatory central pattern generator (Simmers and Bush, 1983). Catalytic properties of muscle AChE, involved in crabs' locomotion (Sorenson , 1973), were reported elsewhere (Rodrigues et al., 2013a). LDH and IDH are involved in anaerobic and aerobic pathways of energy production, respectively; their induction under chemical challenge provides additional energy to deal with toxicant exposure (De Coen et al., 1997; Jo et al., 2001; Rodrigues et al., 2013b). GST act in detoxification of xenobiotics, and defence against oxidative damage, by catalysing conjugation of phase I metabolites with reduced glutathione (GSH), facilitating excretion from the organism. GSH is also a direct scavenger of oxyradicals formed during detoxification and a cofactor of GPx in the conversion of hydrogen peroxide into oxygen and water (van der Oost et al., 2003). Oxidised glutathione (GSSG) is subsequently recycled in reactions involving GR activity. CAT is another anti-oxidant enzyme facilitating the conversion of hydrogen peroxide into less reactive components. When the balance between the generation of oxyradicals and its elimination by anti-oxidants is disrupted oxidative damage to macromolecules will occur (van der Oost et al., 2003). An influence of SERT on LPO and hepatic tissue damage was reported previously (Abdel-Salam et al., 2013) suggesting its putative usefulness here. All these parameters may be altered by xenobiotic exposure, hence their wide use as environmental biomarkers (Filho et al., 2001; van der Oost et al., 2003; Lushchak , 2011; Mesquita et al., 2011; Pereira et al., 2011; Rodrigues et al., 2013b). Finally, the Integrated Biomarker Response (IBR) index was used for interpretation of the multibiomarker responses and evaluation of health status.

2. Material and Methods

2.1. Study sites, crab sampling, and acclimation

Detailed description of the Minho and Lima estuaries and location of the sampling sites is provided elsewhere (Rodrigues et al., 2012). These

are transitional systems with different urban and industrial patterns of activity. The first one is part of the Natura 2000 network; it has low susceptibility to human influence and levels of environmental contamination (Ferreira et al., 2003; Reis et al., 2009; Guimarães et al., 2012). The second one receives wastewaters of industrial, soil leaching, livestock and urban origin. It shows high susceptibility to human influence, and moderate sediment concentrations of metals and PAHs, when compared with the Minho and other European estuaries (Ferreira et al., 2003; Guimarães et al., 2012).

A recent study on the population genetic structure of *C. maenas*, which encompassed sampling of 14 different locations (including the Minho estuary), found no significant genetic differentiation among sites separated by several 100 s of km along a 1,200 km stretch of the Iberian Peninsula coast (Domingues et al., 2010). This suggests that the patterns of physiological variation of *C. maenas* from the Minho and the Lima estuaries are likely to reflect environmental differences between these two systems. This is further supported by studies of Rodrigues et al. (2012, 2013a) reporting differential sensitivity of *C. maenas* from these sites to chemical and natural stresses. Crabs from the moderately polluted side showed enhanced tolerance to organophosphate exposure but increased sensitivity to salinity challenge, compared to those from the low impacted site; in both cases accordant with a history of exposure to low pollution levels in which selective forces are not expected to be maximal.

Intermoult male crabs were used in the two experiments (Minho: 4.2 ± 0.03 cm carapace width, mean ± standard deviation, SD; Lima: 4.5 ± 0.04 cm). The aim of the first experiment was to assess the kinetic properties of ChE of the thoracic ganglion of *C. maenas*. The thoracic ganglion was chosen to assess cholinergic transmission in the central nervous systems because of its role in the ventilatory function (Simmers and Bush, 1983) and its 5–fold higher enzymatic content compared to the cerebral ganglion (Walop and Boot, 1950). This ganglion contains interneurons and motoneurons both of which have been shown to be important components of the ventilatory central pattern generator (Simmers and Bush, 1983). In

addition, its motoneurons innervate the levator and depressor muscles controlling the ventilatory appendages. The second experiment was intended to assess the bioaccumulation and effects of SERT and provides a measure of the health status of the exposed crabs. Water temperature, pH, dissolved oxygen and salinity were measured in triplicate during the crab sampling. The mean values, and corresponding SD, obtained for these parameters, were in the range previously obtained for this same locations (Rodrigues et al., 2012).

In the laboratory, the crabs were placed in tanks (300 L) for 45 days, separated by the estuary of origin. The tanks contained filtered seawater (14 psu, near the level measured at the time of capture) and were continuously aerated. The temperature was kept at 15 ± 0.6ºC and the photoperiod at 14:10h day/night. During the acclimation period crabs were fed with frozen mussels every 2 days, before medium renewal.

2.2. Chemicals

All the reagents used, including SERT, were of analytical grade and were purchased from Sigma–Aldrich Chemical (Steinheim, Germany), except the Bio–Rad protein assay dye reagent that was purchased from Bio–Rad Laboratories, Inc.

2.3. Biochemical properties of ganglion ChEs

The measurement of ChE substrate preferences and responses to selective inhibitors allows for their classification either as AChE (widely used as biomarker of exposure and/or effect) or pseudocholinesterase (also known as butyrylcholinesterase, BChE) enzymes. Affinity of AChE is expected to be higher for acetylthiocholine (ATCh) than for butyrylthiocholine (BTCh) or propionylthiocholine (PTCh) (Rodrigues et al., 2013a). Following *in vitro* exposure, eserine sulphate is known to inhibit ChE activity providing an indication of the presence of non-specific esterases. Furthermore, AChE is strongly inhibited by 1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide (BW284C51) at

concentrations in the mM range, while tetramonoisopropyl pyrophosphortetramide (iso–OMPA) inhibits BChE. The characterisation was performed as described in Rodrigues et al. (2013a). Briefly, ChE activity was assayed in the presence of ATCh, BTCh or PTCh in concentrations ranging from 0.024 to 25.00 mM. Inhibition by eserine sulphate and BW284C51 was assayed in the range of 1.56 to 400 mM; iso–OMPA was tested at concentrations of 0.016 to 4 mM. ATCh was used as substrate in the assays with the three selective inhibitors. ChE activity was determined by the Ellman's method (1961). Protein concentrations in the samples were determined by the Bradford method (1976) using bovine γ–globulin as the standard.

2.4. SERT accumulation and effects in *C. maenas*

Crabs collected at each sampling site were exposed to three concentrations of SERT (0.05, 5.0, and 500.0 µg L^{-1}) for a seven–day period. A full–factorial experimental design was employed, with SERT concentrations and the site of crabs sampling as main factors. SERT concentrations were chosen to include a broad range, from levels reported in environmental samples to high exposures, to elucidate about possible differential effects caused by low and high treatments. Stock solutions of SERT were made in ultra-pure water for each tested concentration. Test solutions were prepared by dilution with filtered seawater. A control group with filtered seawater only was included in the exposure experiments. Crabs were exposed in groups of 4 per glass aquarium containing 4 L of test medium. Three glass aquaria were prepared per treatment. The exposure conditions were the same described for the acclimation period. Medium renewal was performed every 48h. Salinity, temperature, pH and dissolved oxygen were measured at 0h and 48h (before medium renewal) (Table V.I.). Mortality was observed daily. Organisms were carefully handled during the experiments to minimise possible discomfort to the animals. During the experiments, samples of the freshly prepared (0h) and old (48h) test media from the highest SERT treatment were collected for confirmation of real exposure concentrations. At the end of the exposure

period, sub–samples of ganglion, leg muscle and digestive gland from each surviving crab were collected, snap frozen, and stored at –80ºC until determination of selected biomarkers. The remaining whole–body soft tissues, hereafter referred to as soft tissues for simplicity, were pooled and frozen at –20ºC until chemical quantification of SERT and its major metabolite norsertraline (NORS).

Table V.1. Variation of salinity (S), temperature (T), pH, and dissolved oxygen (DO) values during the assays duration in freshly prepared medium (0h) and old (48h) test medium (mean ± SD).

Estuary	Minho		Lima	
	0h	48h	0h	48h
S (psu)	14.3 ± 0.2	14.2 ± 0.2	14.4 ± 0.3	14.3 ± 0.2
T (ºC)	15.0 ± 0.2	15.2 ± 0.3	15.2 ± 0.2	15.1 ± 0.2
pH	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1
DO (mg L^{-1})	8.5 ± 0.3	8.6 ± 0.2	8.6 ± 0.3	8.9 ± 0.3

2.4.1. Chemical analyses of SERT and NORS

Water samples from the crab tanks were extracted by solid phase extraction (SPE) with Oasis MCX cartridges (6 mL, 150 mg) (Waters, Milford, MA, USA). Briefly, prior to extraction SPE cartridges were conditioned with 6 mL of methanol followed by 6 mL of ultra-pure water pH 2. Then, 50 mL of water sample with pH adjusted to 2 was loaded onto the cartridge. After sample pre–concentration, the cartridges were rinsed with 5 mL of 2% acid formic in ultra–pure water, and were dried with vacuum for 20 minutes. Finally, SERT and NORS were eluted with 5 mL of 5% ammonium hydroxide in methanol. Extracts were evaporated until dryness under a gentle stream of nitrogen and reconstituted with 1 mL of acetonitrile. Finally, 10 µL of fluoxetine-d5 (1 mg L^{-1} in acetonitrile) was added to the extract as internal standard. Extracts were analyzed by ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS).

SERT and NORS concentrations in *C. maenas* soft tissues were

determined using a QuEChERS–based extraction method adapted from Ramalhosa et al. (2009) and Gratz et al. (2011). Prior to analysis, crab soft tissues were homogenised. For the extraction, 5 g of homogenised crab soft tissue was weighted into a 50 mL polypropylene centrifuge tube and 5 mL of water was added, the tube was capped and vortexed for 30 s. Then, 10 mL of acetonitrile was added and the tube was again vortexed for 1 min. Afterwards, the QuEChERS salts (6 g $MgSO_4$ and 1.5 g NaOAc) were added, vortexed for 3 min and centrifuged (3400 rpm, 3 min). Then, 8 mL of the supernatant layer was transferred to a dispersive SPE tube containing 900 mg $MgSO_4$, 300 mg PSA and 150 mg C_{18}, vortexed for 1 min and centrifuged (3400 rpm, 3 min). After this step, a volume of 6 mL of supernatant was transferred to a glass vial and was evaporated to dryness under a gentle stream of nitrogen. Finally, the extract residue was reconstituted with 1 mL of acetonitrile, filtered through a 0.2 µm PTFE syringe filter and 10 µL of fluoxetine-d5 (1 mg L^{-1} in acetonitrile) was added previously to UHPLC–MS/MS analysis.

Water and crab samples were analysed using a Nexera Ultra–High Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan) coupled with a triple–quadupole mass spectrometer detector LCMS–8030 with an electrospray ionization source (ESI), operating in positive ion mode. Chromatographic separation was performed on a Kinetex C18 column (150 x 2.10 mm i.d.; 1.7 µm particle size) (Phenomenex, USA) using 0.1% formic acid in ultra–pure water (A) and acetonitrile (B) as mobile phase, at a flow rate of 0.3 mL min^{-1}. The injection volume was 5 µL and the column was kept at 30 ºC. Mass spectrometer parameters were defined as follow: desolvation temperature 200 ºC, source temperature 200 ºC, nitrogen drying and nebulising gas flows 720 L h^{-1} and 120 L h^{-1}, respectively, and capillary voltage 1 kV. Argon was used as collision induced dissociation gas at a pressure of 230 kPa. SERT and NORS were analysed using multiple reaction monitoring (MRM) and quantification was made using fluoxetine-d5 as internal standard. Method detection limits (MDL) for SERT and NORS were 1.76 and 1.80 ng g^{-1} wet weight, respectively, in crab soft tissues, and 10 and 3 ng

L^{-1}, respectively, for water.

2.4.2. Biomarkers determination

Determination of AChE activity in ganglion and muscle tissues were performed as described above, using ATCh as substrate. All other biomarkers were determined as described for *C. maenas* in Rodrigues et al. (2012, 2013b). Briefly, the methods of Vassault (1983) and Ellis and Goldberg (1971) were employed to assess muscle LDH and IDH activity, respectively. LPO was determined in digestive gland homogenates by measuring spectrophotometrically the thiobarbituric acid reactive substances (TBARS), according to Filho et al. (2001). GST, CAT, GPx and GR activity, and TG levels were assayed in the post–mitochondrial supernatant. GST activity was determined according to the method of Habig et al. (1974). CAT activity was determined according to Clairborne (1985). GPx activity was assayed by following the method of Mohandas et al. (1984). GR activity was measured according to the method of Cribb et al. (1989) and TG levels were determined as described in Tietze (1969).

2.5. Data analysis

To investigate the biochemical properties of ganglion ChE, experimental curves based on the Michaelis–Menten equation were fitted to substrate affinity data and used to estimate maximal velocity (V_{max}), Michaelis–Menten constant (K_m) and their ratio (V_{max}/K_m). Significant differences in the kinetic parameters obtained for the two sampling sites were assessed by comparison of the regression slopes and intercepts of the kinetic curves. Data pertaining to specific inhibitors were analysed using the non-parametric Friedman test.

Two–way analysis of variance (ANOVA) with interaction, followed by contrast analysis, was used to investigate potential differences in the biochemical biomarkers in relation to SERT exposure concentrations and the estuary of origin of the crabs. ANOVA assumptions were tested using the Shapiro–Wilk and the Levene's test. Log–transformation of the data was

used when required to fulfill ANOVA assumptions.

A stress index, the Integrated Biomarker Response (IBR), was calculated for global interpretation of the multibiomarker responses assessed according to the description of Beliaeff and Burgeot (2002) following the subsequent modification by Guerlet et al. (2010). The IBR was calculated for each SERT concentration tested in the Minho and the Lima crabs as follows: individual areas A_i connecting the i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot were obtained through the formula:

$$A_i = 1/2 \sin(2\pi/n)\, S_i S_{i+1}$$

where S_i and S_{i+1} represent the individual biomarker scores (calculated from standardised data) and their successive star plot radius coordinates, and n represents the number of radii corresponding to the biomarkers used in the survey.

3. Results

3.1. Biochemical properties of ganglion ChEs

The preferential substrate to ganglion ChE was ATCh, followed by PTCh and BTCh (Table V.2.). Activity inhibition by excess substrate was never detected. The catalytic efficiency for ATCh was higher in Lima than in Minho crabs ($p<0.001$). Nevertheless, eserine sulphate and BW284C51 strongly inhibited ChE activity in a similar manner in crabs from both estuaries indicating these were true ChE and that the main form was AChE, respectively (Fig. V.1.). Incubation with iso–OMPA caused no relevant change in ChE activity (Fig. V.1.) further confirming the above.

3.2. SERT accumulation and effects in *C. maenas*

Salinity, temperature, pH, and dissolved oxygen were stable along the assays, within the expected values (Table V.1.). SERT concentration in freshly prepared medium of the highest test concentration was 566 ± 49 µg L^{-1} (mean ± SD). SERT exposure levels will, thus, be indicated as the nominal concentrations hereafter. In old test medium (48h), the concentration was 49% lower than in the freshly prepared. A residual level

of NORS (0.035% of the SERT concentration) was detected. After the seven–day exposure, accumulation of SERT in soft tissues was found in crabs from the Minho estuary exposed to 500 µg L^{-1} and in those from the Lima exposed to ≥5µg L^{-1} (Table V.3.). Tissue levels found in Lima crabs were considerably higher than those measured in Minho crabs. Accumulation of NORS in soft tissues was only observed in crabs exposed to 500 µg L^{-1} SERT. The accumulated levels were low, representing 19% and 11% of SERT accumulation in Minho and Lima crabs, respectively.

Table V.2. Kinetic parameters (Michaelis-Menten constant, K_m; maximal velocity, V_{max}; catalytic efficiency, ratio V_{max} /K_m) of cholinesterase activity from *C. maenas* collected at the Minho and the Lima estuaries. Values represent the mean ± standard error. Different letters indicate statistical significance at $p<0.05$.

Substrate	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1} prot)	V_{max}/K_m (µl min^{-1} mg^{-1} prot)
Minho			
Acetylthiocholine	0.125 ± 0.012[a]	416.46 ± 30.49	3409.24 ± 356.65[a]
Propionylthiocholine	0.234 ± 0.054	289.02 ± 58.47	1302.38 ± 125.57
Butyrylthiocholine	0.100 ± 0.018	13.06 ± 1.045	143.63 ± 24.59
Lima			
Acetylthiocholine	0.080 ± 0.010[b]	455.03 ± 80.02	5627.45 ± 556.65[b]
Propionylthiocholine	0.122 ± 0.007	247.67 ± 26.23	1588.68 ± 541.16
Butyrylthiocholine	0.118 ± 0.045	16.71 ± 1.34	201.12 ± 56.84

Significant differences between control animals from the Minho and the Lima sites were found for CAT, GPx and GR activities (Fig. V.2.). For CAT, the activity levels were higher in the Minho than in the Lima crabs, whereas for GPx and GR the inverse pattern was found with lower activities measured in Minho crabs. The levels of all the other biomarkers evaluated were similar between the two control groups ($p>0.05$). Two–way ANOVA indicated a significant main effect of SERT on the levels of TG and LDH activity (Table V.4.). Significant SERT × Sampling site interactions were found for all the remaining biomarkers assessed (Table V.4.), indicating that SERT elicited different responses in Minho and Lima crabs.

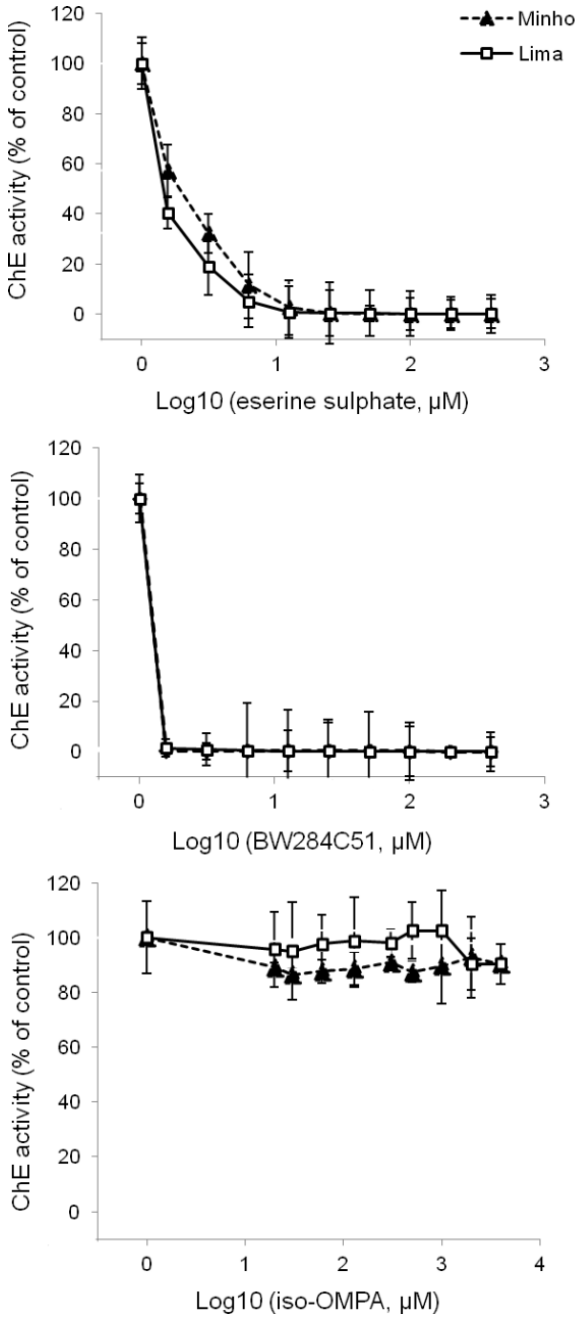

Fig. V.1. Effects of the specific inhibitors (eserine sulphate, BW284C51], and iso-OMPA), on cholinesterase (ChE) activity (mean ± standard error in % of control) in the thoracic ganglion of *C. maenas* from the Minho (black triangles) and the Lima (white squares) estuaries. Acetylthiocholine was used as substrate.

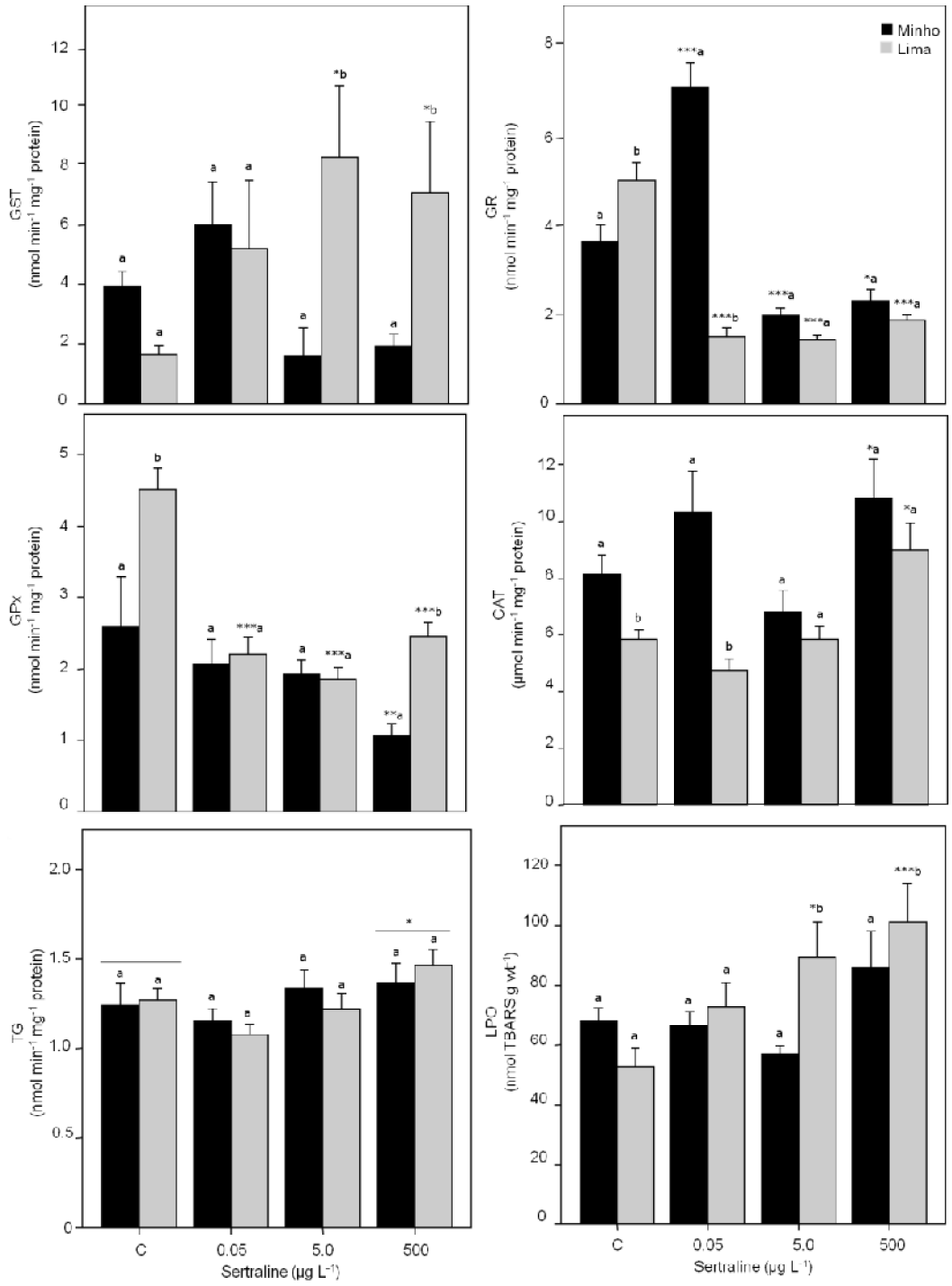

Fig. V.2. Mean and corresponding standard error of the activity of enzymes glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and reductase (GR), and catalase (CAT) activity, and the levels of total glutathione (TG) and lipid peroxidation (LPO) in the digestive gland of *C. maenas* from the Minho and the Lima estuaries exposed for 7 days to sertraline. Significant differences between sampling sites within each treatment are identified by different letters; the asterisks indicate

significant differences within each estuary compared to the respective control group (two-way ANOVA followed by contrast analysis; * $p<0.05$, *** $p<0.001$).

In Minho crabs, differences among treatments were related to cholinergic neurotransmission (AChEg activity) ($p<0.05$, Fig. V.3.) and anti-oxidant defences (GR, GPx, and CAT activity) ($p<0.05$, Fig. V.2.). For the remaining biomarkers, no differences were recorded between controls and SERT-treated crabs ($p>0.05$, Fig. V.4.).

AChEg activity of crabs exposed to the lowest and to the highest SERT treatments were within control levels. A significant increase (+24%, $p<0.05$) in AChEg activity was observed in those exposed to 5 µg L^{-1}, compared to controls, suggesting a hormetic effect of SERT in Minho crabs.

Differences among treatments were not found for GST activity, though its pattern of variation was similar to that of GR. But exposure to SERT caused marked increases (1.5 to 4–fold) in its coefficient of variation, compared to the control group, a recognised consequence of exposure to detrimental compounds. GR activity was significantly increased (+95%) in crabs exposed to 0.05 µg L^{-1}, relative to controls ($p<0.001$). Yet, compared to controls, significantly decreased activity was found in organisms exposed to ≥5.0 (–45%, –37%) ($p<0.001$). GPx showed a tendency for decreased activity with increasing SERT concentration, which became significant at 500 µg L^{-1}(–60%, $p<0.001$). An inverse pattern was observed for CAT with increased activity in animals treated with 0.05 µg L^{-1}(+26%) and 500 µg L^{-1}(+33%). A significant but slight increase in TG levels (~15%) was found for the highest test treatment, relative to controls. Interestingly, no changes among treatments were found for LPO levels.

In Lima crabs, differences among treatments were found for all the biomarkers assessed, except TG levels (Figs. V.2., V.3. and V.4.). Compared to controls, decreased AChEg and AChEm activity was detected at concentrations ≥0.05 µgL^{-1} (–20%, –56%, –59%, $p<0.05$) and ≥5 µg L^{-1} (–34%, –25%, $p<0.05$), respectively. Energy metabolism enzymes showed a

tendency to increase with the exposure concentration which was significant at 500 µg L^{-1} for LDH (+32%, $p<0.05$) and ≥5 µg L^{-1} for IDH (+52%, +100%, $p<0.05$). Increased GST activity, relative to controls, was found at 5 µg L^{-1}(~5-fold increase, $p<0.05$) and 500 µg L^{-1} SERT (~4-fold). An opposite trend was observed for GR and GPx activities, which were markedly inhibited in all SERT treatments (GR: –70%, –71%, –63%; GPx: –51%, –59%, –46%; $p<0.001$). CAT activity was significantly increased but only in crabs exposed to 500 µg L^{-1} (+54%). TG levels were weakly increased only at 500 µg L^{-1}. LPO levels were markedly increased in all SERT treatments (38%, 69%, 92%, $p<0.05$) compared to controls, indicating damage to cellular macromolecules.

Table V.3. Concentration (mean ± SD, ng g^{-1}ww) of sertraline and norsertraline found in the soft tissues of *C. maenas* from the Minho and the Lima sampling sites, after a seven-day exposure experiment.

Exposure	Sertraline	Norsertraline
Minho		
Control	*n.d.*	*n.d.*
0.05 µg L^{-1}	< 1.76[§]	*n.d.*
5.0 µg L^{-1}	<1.76[§]	*n.d.*
500 µg L^{-1}	605 ± 18	115 ± 2
Lima		
Control	*n.d.*	*n.d.*
0.05 µg L^{-1}	<1.76[§]	*n.d.*
5.0 µg L^{-1}	15.3 ± 1.7	<1.80[§]
500 µg L^{-1}	1010 ± 10	111 ± 1

n.d., not detected; [§], method detection limits

Overall, after seven days, significant changes in biomarkers of cholinergic transmission in the central nervous tissue and muscle, and anti-oxidant defences and oxidative damage in the digestive gland were elicited by the lowest SERT exposure levels in crabs originating from the moderately polluted site but not in those from the low impacted site (Table V.5.). The IBR indicated distinct biological effects in crabs from these sites, providing a qualitative measure of the stress caused by SERT exposure

(Fig. V.5.). No important differences among SERT treatments were noted in Minho crabs. But in Lima crabs all SERT treatments triggered high stress levels, although much stronger at ≥5 µg L^{-1}. The distinction among test treatments was provided essentially by the activities of AChEg, AChEm, IDH, and GST.

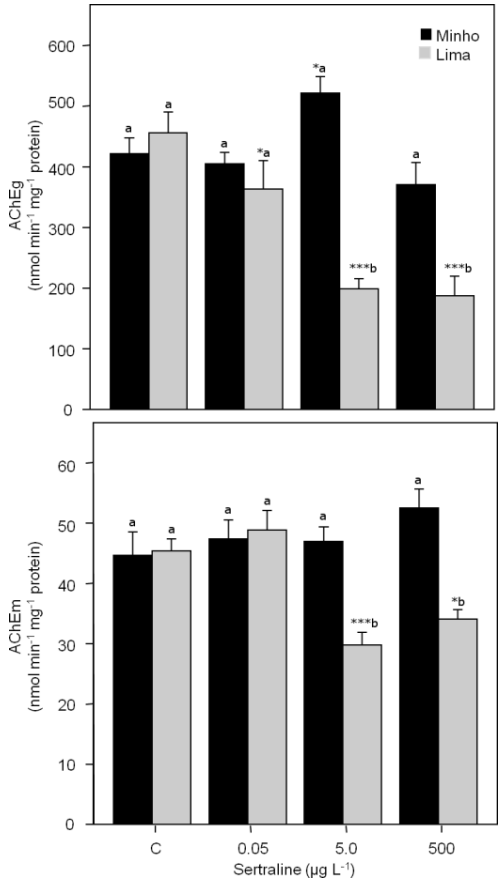

Fig. V.3. Mean and corresponding standard error of ganglion and muscle acetylcholinesterase (AChEg and AChEm, respectively) activity determined in *C. maenas* from the Minho and the Lima estuaries exposed for seven days to sertraline. Significant differences between sampling sites within each treatment are identified by different letters; the asterisks indicate significant differences within each estuary compared to the respective control group (two-way ANOVA followed by contrast analysis; * $p<0.05$, *** $p<0.001$).

Table V.4. Results of the full-factorial two-way ANOVA to assess the effects of sertraline exposure and the sampling site on the biomarkers assessed. *AChEg,* ganglion acetylcholinesterase, *AChEm*, muscle acetylcholinesterase, *LDH*, lactate dehydrogenase, *IDH,* $NADP^+$-dependent isocitrate dehydrogenase, *GST,* glutathione *S*-transferases, *GR*, glutathione reductase, *GPx,* glutathione peroxidase, *CAT,* catalase, *TG,* total glutathione, *LPO,* lipid peroxidation.

Parameter	Source of variation	*df*	*F*	p
Neurotransmission				
AChEg	Sertraline	3, 94	8.90	<0.001
	Sampling site	1, 94	33.62	<0.001
	Sertraline × Sampling site	3, 94	12.89	<0.001
AChEm	Sertraline	3, 94	4.32	0.007
	Sampling site	1, 94	18.25	<0.001
	Sertraline × Sampling site	3, 94	7.72	<0.001
Energy metabolism				
LDH	Sertraline	3, 94	3.62	0.016
	Sampling site	1, 94	0.72	0.789
	Sertraline × Sampling site	3, 94	0.24	0.867
IDH	Sertraline	3, 94	8.18	<0.001
	Sampling site	1, 94	35.70	<0.001
	Sertraline × Sampling site	3, 94	5.95	0.001
Biotransformation and anti-oxidant defences				
GST	Sertraline	3, 94	0.66	0.578
	Sampling site	1, 94	4.59	0.035
	Sertraline × Sampling site	3, 94	3.00	0.035
GR	Sertraline	3, 94	41.73	<0.001
	Sampling site	1, 94	35.49	<0.001
	Sertraline × Sampling site	3, 94	47.90	<0.001
GPx	Sertraline	3, 94	14.01	<0.001
	Sampling site	1, 94	14.65	<0.001
	Sertraline × Sampling site	3, 94	4.78	0.004
CAT	Sertraline	3, 94	6.84	<0.001
	Sampling site	1, 94	20.32	<0.001
	Sertraline × Sampling site	3, 94	2.86	0.042
TG	Sertraline	3, 94	4.14	0.009
	Sampling site	1, 94	0.23	0.635
	Sertraline × Sampling site	3, 94	0.76	0.518
Oxidative damage				
LPO	Sertraline	3, 94	5.40	0.002
	Sampling site	1, 94	2.60	0.111
	Sertraline × Sampling site	3, 94	2.81	0.044

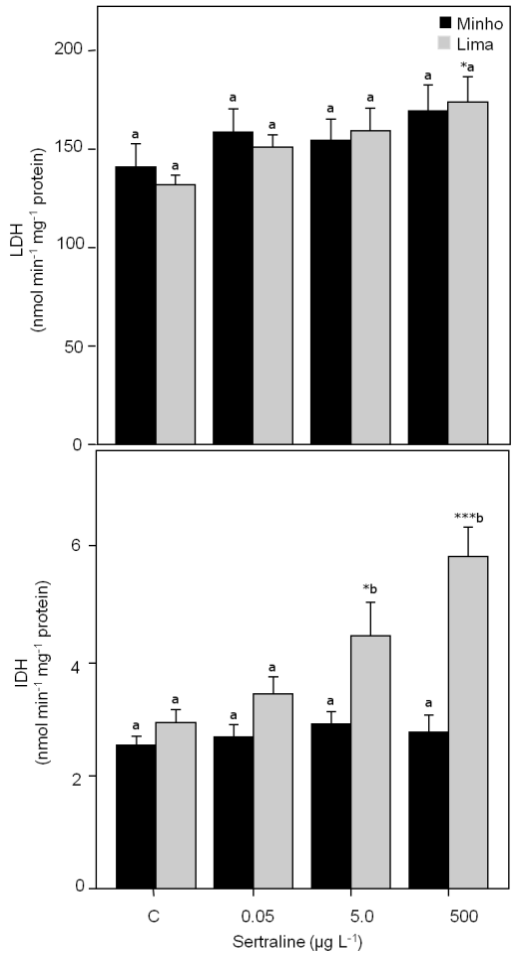

Fig. V.4. Mean and corresponding standard error of lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) activity determined in the muscle of *C. maenas* from the Minho and the Lima estuaries exposed for seven days to sertraline. Significant differences between sampling sites within each condition are identified by different letters; the asterisks indicate significant differences within each estuary compared to the respective control group (two-way ANOVA followed by contrast analysis; *p<0.05, ***p<0.001).

4. Discussion

SERT has been detected at concentrations as high as 0.037 µg L^{-1} in surface waters (USA) (Schultz et al., 2010) and 0.100 µg L^{-1} (Norway) (Weigel et al., 2004) and 0.106 µg L^{-1} (China) (Yuan et al., 2013) in hospital

effluents. Moreover, predicted environmental concentrations (PECs) range from 0.14 µg L^{-1} to 17.1 µg L^{-1} for untreated wastewaters (Styrishave et al., 2011). Thought SERT is highly prescribed worldwide and the most acutely toxic SSRI to standard crustacean (Henry et al., 2004; Christensen et al., 2007), there is a notorious lack of information on its effects in estuarine and coastal species. Most studies available investigated effects of fluoxetine, which exhibits different properties. It is a racemic mixture of two lipophilic enantiomers with a non-linear kinetics, whereas SERT has linear kinetics and only one enantiomer in its commercial formulation (Hiemke and Härtter, 2000).

Table V.5. Summary of the changes observed in the parameters evaluated in *C. maenas* from the Minho and Lima estuaries exposed to sertraline. Increase (↑), decrease (↓), and no alteration (~) are indicated for each endpoint in relation to the respective control group. Legend provided in Table V.4.

	Site of origin			
	Minho		*Lima*	
Parameters	0.05 µg L^{-1}	5.0 µg L^{-1}	0.05 µg L^{-1}	5.0 µg L^{-1}
SERT in tissues	~	~	~	↑
AChEg	~	↑	↓	↓
AChEm	~	~	~	↓
LDH	~	~	~	~
IDH	~	~	~	↑
GST	~	~	~	↑
GR	↑	↓	↓	↓
GPx	~	~	↓	↓
CAT	~	~	~	~
TG	~	~	~	~
LPO	~	~	↑	↑

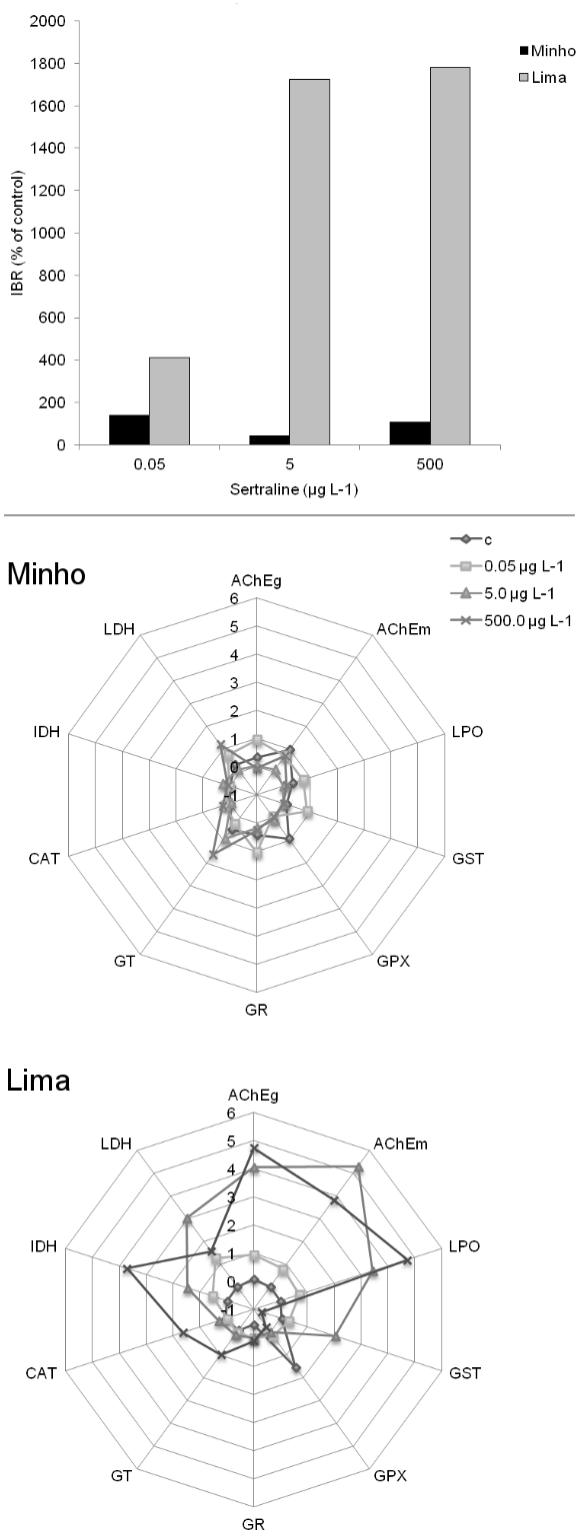

Fig. V.5. Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *C. maenas* from the Minho and Lima estuaries exposed to sertraline (top). Contribution of each biomarker to the IBR value and the discrimination among treatments in each sampling site (star plots). Legend provided in Table V.4.

Also, its main metabolite, norfluoxetine, is considered more potent than the parent compound to inhibit the reuptake of 5–HT (DeVane, 1999). In contrast, inhibition of 5–HT reuptake by NORS is lower than that exhibited by SERT (DeVane, 1999). In this study, the seven–day exposure to waterborne SERT elicited notable tissue accumulation in crabs from both study sites, but clearly higher (almost the double) in those from the moderately polluted Lima site. Very low NORS/SERT ratios were found in crabs from both sites, supporting the hypothesis of low metabolisation and/or elimination by the shore crab. Information related to the metabolisation pathway of SERT is limited. The available data resulted from studies with suspensions of mammalian liver microsomes, which demonstrated that SERT was N–demethylated by cytochrome P450 (CYP 450) enzymes (Obach et al., 2005). In aquatic invertebrates the levels of total CYP are substantially lower than those found in vertebrates (Livingstone, 1998), suggesting that low metabolisation of SERT is to be expected in the former. The low NORS/SERT ratio (0.00035) found in the medium of the highest test concentration after 48h exposure supports this. Also, although data are only available for freshwater species, other studies detected concentrations of NORS in fish tissues greater than those of the parent compound (Schultz et al., 2010; Lajeunesse et al., 2011). It is also of note that SERT is expected to undergo oxidative metabolism as its major elimination pathway (DeVane, 1999). Hence, dysfunction of the digestive gland could impair its elimination, which may also explain the higher tissue concentrations found in Lima crabs. Animals from this site exposed to levels causing bioaccumulation also showed significantly reduced anti-oxidant defences and high extent of oxidative damage to lipid macromolecules, both particularly cytotoxic, with multiple effects on enzyme activity and ATP production, as well as the initiation of apoptosis (Lesser, 2006). Because the potential of SERT to inhibit the reuptake of 5–HT is higher than that exhibited by NORS, these results suggest that its effects in decapods may be more severe than those induced in vertebrate species, which usually show much higher NORS/SERT ratios.

SERT levels detected in *C. maenas* tissues are considerably higher than those reported for mussel *Geukensia demissa* (0.1 to 1.4 ng g^{-1} ww) in San Francisco Bay (Klosterhaus et al., 2013). Such measurements were, however, obtained under conditions favouring extensive dilution, as the winter season and the relatively well–flushed system of San Francisco Bay (Klosterhaus et al., 2013). To our knowledge no other study investigated bioaccumulation of SERT in estuarine and/or coastal organisms either in field or laboratory studies. This data indicate potential for bioaccumulation in *C. maenas*, raising concern on possible biomagnification over the trophic chain and risk to humans through dietary consumption as this crab is common prey of fish species in human diet. In some regions it is also exploited by seafood industries producing shellfish pastes, empanadas and patties for human consumption.

Different biomarker response patterns were observed in Minho and Lima crabs with non–monotonic responses elicited in Minho crabs. Globally, the pattern of variation of control groups is in good agreement with previous observations in crabs from these sites (Rodrigues et al., 2012). The biphasic U–shaped AChEg response of Minho crabs, contrasting with the enzymatic decrease observed in Lima organisms raised the question of whether these differences could be attributed to different ChE forms in the thoracic ganglion of these cohorts. Previous investigations indicated the presence of true ChE and ATCh affinity in *C. maenas* ganglion, but possible relevant presence of BChE remained to elucidate (Walop and Boot,1950). Here, ganglion ChE characterisation confirmed AChE as the predominant form in both cohorts. Moreover, this form was showed no response to iso–OMPA, a BChE inhibitor, further supporting our conclusion. Similar findings were reported for the central nervous system of other marine crustacean (Monserrat and Bianchini, 1998). Given this, the consideration of a hormetic effect of SERT on the enzyme activity seems more plausible.

Hormetic dose–response curves, with typical occurrence of opposite effects at low and high doses, were described for several chemicals, including neurotransmitters; responses with a 10– to 100–fold stimulatory

range were reported (Calabrese and Blain, 2011). Moreover, hormetic responses were found also in high–risk or more sensitive individuals, as the Lima crabs appear to be. In such cases the biphasic concentration–response relationship becomes shifted to the left (Calabrese and Baldwin, 2002). Considering this, AChEg responses of Lima crabs appear to be already on the inhibitory side of the U–shape concentration–response relationship, whereas those of Minho crabs are still on the stimulatory phase. The pattern of AChEm is also consistent with this, further supporting that Lima crabs were more sensitive to SERT than Minho ones. Increases in AChE activity, associated with altered locomotor behaviour, and biphasic responses in behavioural traits, were observed in other marine crustacean exposed to fluoxetine (Guler and Ford, 2010; Mesquita et al., 2011). Interestingly, it was shown recently that serotonergic neurons, and 5-HT signalling, regulate cholinergic neurotransmission in *C. elegans* through both stimulatory and inhibitory inputs (Govorunova et al., 2010). While no explanation is available for the paradoxical observations in crustacean, it may be hypothesised that SERT would affect both stimulatory and inhibitory responses through differential affinity to receptor subtypes, or differential activation of the same receptor in different neurons, that may lead to dual stimulatory or inhibitory inputs to cholinergic neurotransmission (Govorunova et al., 2010), consequently affecting AChE activity. Noteworthy is also the possibility of AChE inhibition by SERT (Müller et al., 2002), which could contribute to the decreased activity found. SSRIs were shown to inhibit AChE activity in human serum and erythrocyte membrane. This interaction is labile (Müller et al., 2002) suggesting that upon continuous exposure strong inhibition of AChE activity may occur but recovery may be possible following remediation measures. Nevertheless, both up- or down–regulations of AChE activity may have serious detrimental consequences to the crabs. Increased AChE expression and activity is a hallmark of cells undergoing apoptosis (Zhang and Greenberg, 2012). On the other hand, both lower cholinergic transmission and/or AChE inhibition will depress crabs

ventilatory and locomotory functions (Sorenson, 1973; Simmers and Bush, 1983), setting difficulty to find food and increasing risk of predation.

The increase in LDH (at 500 µg L^{-1} SERT) observed in Lima crabs could provide additional energy to readily cope with the chemical stress induced by the exposure (De Coen et al., 2001). However, not only moderate changes were observed as the anaerobic pathway appears to be relevant only at very high concentrations, with concomitant SERT accumulation in tissues. The intense IDH increase (at ≥5 µg L^{-1}) found in Lima crabs appears to be a more important mechanism to meet the energy requirements imposed by SERT exposure. IDH is considered as the most efficient pathway in ATP production. It is also active in the anti-oxidant defence system by supplying the NADPH necessary to GR–mediated regeneration of GSH (Jo et al., 2001). Likewise, previous studies have found that Lima crabs seem to cope with toxicant challenge by increasing the aerobic pathway rather than the anaerobic route (Rodrigues et al., 2013a).

GST activity was another biomarker showing non–monotonic responses in the Minho crabs. Moreover, at ≥ 5.0 µg L^{-1} inverse effects were observed in Minho (decrease) and in Lima (induction) animals, again suggesting differential sensitivity to SERT. In Minho crabs, the increased levels of CAT and LPO indicate that SERT is able to cause the production of oxyradicals leading to oxidative stress. In animals with a history of exposure to moderate contamination this may not be compensated by the anti–oxidant system resulting in oxidative damage. Conversely, in animals originating from low impacted sites it is possible that alternative detoxification pathways may become active, preventing oxidative damage and possibly leading to reduced contribution of phase II biotransformation to SERT detoxification and elimination. Reports on the effects of SERT in detoxification and anti-oxidant defences of aquatic organisms are not available in the literature. Previous investigations on the effects of fluoxetine on *C. maenas* showed that, besides increasing locomotion, this SSRI could induce AChE, GST, and GR activity and TG levels in Minho crabs at high exposure levels (120–750 µg L^{-1}) (Mesquita et al., 2011),

contrasting with the lower exposure levels and effects found in the present study. Also in this case no oxidative damage was found in the exposed animals. This is, however, consistent with low metabolisation rate and higher toxicity of the active metabolite than fluoxetine, relative to SERT.

The results indicate that crabs from the moderately contaminated site are more susceptible to SERT, showing considerably lower health status than those from the low impacted site. Previous investigations supports that chronic exposure of *C. maenas* to moderate levels of contamination in this estuary may elicit differential sensitivity to further environmental contamination and natural stress (Rodrigues et al., 2012; Rodrigues et al., 2013a). The integration of biomarker responses (IBR) revealed that SERT caused low stress in Minho crabs with no clear differences among exposure levels. Lima crabs showed remarkable stress increases even at concentrations as low as 0.05 µg L^{-1} SERT (over 200%). Biomarkers linked to ventilatory, locomotory, and anti-oxidant functions, and oxidative damage, were the most affected by SERT. Also, reduced health status was observed at concentrations not causing relevant SERT accumulation in crab tissues. Biomarkers provide crucial early–warning measures of bioavailability and effects caused by environmental disturbance that may reflect at population levels. These results highlight the suitability of biomarkers involved in cholinergic neurotransmission, detoxification, anti–oxidant defences and oxidative damage to assess contamination by SSRIs in *C. maenas*.

An important point here is that data employed to derive species sensitivity distributions or predicted no effect concentrations (PNECs) for hazard risk calculations are usually based on bioassays assessing conventional endpoints (*e.g.*, survival, growth, and reproduction) performed with standard species (or clones), or organisms originating from pristine or low–impacted sites. The present results suggest that estimates derived from such procedures may not be sufficiently protective, as detrimental effects may go undetected using such an ecotoxicological approach. The study demonstrates the occurrence of changes with ecologically relevant repercussions in crabs from the moderately impacted

estuary at SERT concentrations four orders of magnitude lower than those observed for crabs from the low impacted site. Such life threatening changes would be missed if regular testing approaches would be used. Moreover, impaired health status was found for crabs from the moderately polluted site at concentrations about three orders of magnitude lower than persistent changes found in the most sensitive freshwater species tested up–to–date, using reproduction as endpoint (Henry et al., 2004). Concerns are further deepened by the fact that additive effects to algae and aquatic invertebrates may be caused by mixtures of SERT with other SSRIs (Christensen et al., 2007) frequently found in environmental samples (*e.g.*, fluoxetine, citalopram). This work supports that more sensitive MOA–related endpoints should be used to assess SSRIs toxicity (Christen et al., 2010), at broad concentration ranges to encompass the possibility of different responses at low and high exposure levels (Calabrese and Blain, 2011). The results also stress the importance of tailored site–specific criteria and risk assessment, involving testing with local ecological receptors, and accounting for dynamic natural and man–induced environmental change, to improve accuracy in extrapolation from laboratory testing to field conditions.

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Chapter VI

Joint effects of salinity and the antidepressant sertraline on the estuarine decapod *Carcinus maenas*

Joint effects of salinity and the antidepressant sertraline on the estuarine decapod *Carcinus maenas*

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Abstract

Concurrent exposure of estuarine organisms to man–made and natural stressors has become a common occurrence. Numerous interactions of multiple stressors causing synergistic or antagonistic effects have been described. However, limited information is available on combined effects of emerging pharmaceuticals and natural stressors. This study investigated the joint effects of the antidepressant sertraline and salinity on *Carcinus maenas*. To improve knowledge about interactive effects and potential vulnerability, experiments were performed with organisms from two estuaries with differing histories of exposure to environmental contamination. Biomarkers related to sertraline mode of action were employed to assess effects of environmentally realistic concentrations of sertraline at two salinity levels. Synergism and antagonism were identified for biomarkers of cholinergic neurotransmission, energy production, anti–oxidant defences and oxidative damage. Different interactions were found for the two study sites highlighting the need to account for differences in tolerance of local ecological receptors in risk evaluations.

Keywords: Sertraline, salinity, interactive effects, acetylcholinesterase, oxidative damage.

1. Introduction

Estuarine and coastal organisms are exposed to numerous environmental factors (*e.g.*, food availability, pathogens, toxins, dissolved oxygen, temperature, salinity) that may act as natural stressors influencing their physiology and behaviour. In many systems they are also influenced by pollution inputs resulting in exposure to multiple stressors (Heugens et al., 2001). This co–exposure often leads to interactions causing effects that can not be predicted from those elicited by single stressors (Turja et al., 2014). Exposure to a toxicant may constrain tolerance of organisms to a range of environmental factors. Conversely, such factors may in turn influence the toxicity of aquatic pollutants (Heugens et al., 2001). The ability of organisms to cope with combined stress will determine their health status and fitness. Hence, prediction of combined effects of multiple stressors is essential to increase the ecological relevance of results obtained in the laboratory relative to field scenarios, and refine risk calculations aimed at the protection and management of affected ecosystems (SCHER et al., 2013).

Among natural stressors, salinity has major influence on the distribution and physiology of estuarine species. Estuaries exhibit high salinity variations due to their transitional character. Given their privileged location they are main centres of urban and industrial settlement, receiving point and nonpoint discharges of chemicals derived from these activities. Various studies found interactions between salinity and toxicants from different classes in aquatic organisms (reviewed by Heugens et al., 2001). However, the available literature reports mostly on interactions involving classical or priority compounds. Studies addressing combined effects with emerging contaminants are urgently needed to improve prediction capacity and site–specific risk assessment (SCHER et al., 2013).

Current disease trends indicate depression as one of the three leading causes of burden of disease worldwide by 2030 (Mathers and Loncar, 2006), raising the need to focus on effects in non-target organisms of emerging antidepressants such as sertraline (SERT). This selective serotonin reuptake inhibitors (SSRIs), often detected in aquatic systems

(Santos et al., 2010), increases serotonin (5–hydroxytryptamine [5–HT]) levels in the synaptic cleft and, consequently, serotonergic neurotransmission (Stahl, 1998). Found in vertebrates and invertebrates, 5–HT plays several different roles in these organisms (reviewed by De–Miguel and Trueta, 2005). It regulates functions so diverse as sleep and appetite in humans (Murphy, 1990), ovarian growth in crustaceans (Fingerman, 1997), reproductive processes in bivalves (Croll et al., 1995), egg laying and penile erection in gastropods (Muschamp and Fong, 2001), ciliary reaction in nudibranches (Pavlova et al., 1999) and cilia regeneration in protozoans (Rodríguez and Renaud, 1980). Despite this, there is a paucity of information on interactive effects of salinity and sertraline in estuaries species.

The green crab *Carcinus maenas* is a key invertebrate widely distributed in European estuarine and coastal habitats. Earlier works demonstrated its interest as biological indicator (Bamber and Depledge, 1997) and its usefulness to evaluate the impact of environmental stressors (Pereira et al., 2011). This species is also sensitivity both to SSRIs and salinity stress (Towle and Weihrauch, 2001; Mesquita et al., 2011; Rodrigues et al., 2012).

The main objective here was to investigate the combined effects of SERT and salinity on bioaccumulation and biomarkers of neurotransmission, energy metabolism, anti–oxidant defences and oxidative damage of *C. maenas*. The sublethal parameters were chosen for their relationship to SERT mode of action (MOA), involvement in cellular processes influenced by salinity stress, and to provide a link between physiological changes and possible ecological consequences (Müller et al., 2002; Rodrigues et al., 2012; Abdel-Salam et al., 2013). Additionally, they are widely recognised as providing useful early–warning indications on the health status of affected organisms, which contributes to their broad use in monitoring studies. The effects of SERT were evaluated in sub–acute exposure experiments carried out at two different salinity levels (14 psu and 35 psu). The history of exposure to moderate contamination was previously found to influence sensitivity of *C. maenas* to toxicants and to

salinity stress (Rodrigues et al., 2012; Rodrigues et al., 2013). Hence, to improve understanding of the potential vulnerability, the experiments were conducted with organisms from two NW Iberian estuaries with different levels of environmental contamination. At the end of the experiments, concentrations of SERT and its major metabolite norsertraline (NORS) were determined in the soft tissues of the crabs to obtain information on uptake, detoxification, and elimination. A battery of ten endpoints was then evaluated. Altered cholinergic neurotransmission was assessed by determining acetylcholinesterase activity in the thoracic ganglion (AChEg) and muscle tissue (AChEm). Effects on anaerobic and aerobic energy production pathways were investigated by determining the activity of enzymes lactate dehydrogenase (LDH) and $NADP^+$–dependent isocitrate dehydrogenase (IDH) in muscle tissue. Biotransformation and anti–oxidant defences were evaluated by measuring in the digestive gland activity of glutathione *S*–transferases (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT), as well as the levels of total glutathione (TG). Oxidative damage was assessed by quantifying the extent of lipid peroxidation (LPO) in the digestive gland.

2. Material and Methods

2.1 Crab sampling and acclimation

Test organisms were collected at the mouth of Minho and Lima estuaries (Rodrigues et al., 2012). Minho estuary shows low susceptibility to human influence and levels of environmental contamination (Ferreira et al., 2003; Guimarães et al., 2012). Lima estuary receives pollution from industrial, soil leaching, livestock, and urban effluents. It is highly susceptible to human influence and exhibits moderate concentrations of metals and polycyclic aromatic hydrocarbons (PAHs) in sediments relative to Minho and other European estuaries (Rodrigues et al., 2012 and references therein). Analysis of genetic structure of crabs sampled in 14 locations (including Minho estuary) along the NW coast of the Iberian Peninsula showed no relevant genetic differentiation among the studied

populations (Domingues et al., 2010). Physiological variation of crabs from these estuaries is thus expected to reflect environmental differences between these two systems. This is in agreement with investigations on the responses of crabs from these sites to chemical and natural stress (Rodrigues et al., 2012; 2013).

Intermoult male crabs were collected in late autumn using trawl nets (Minho: 4.3 ± 0.03 cm carapace width, mean ± SD, n = 96; Lima: 4.5 ± 0.03 cm carapace width, n = 96). Mean values of water temperature, pH, dissolved oxygen and salinity were similar to previous measurements in these locations (Rodrigues et al., 2012). In the laboratory, crabs were accommodated in 300 L tanks with aerated filtered seawater (14 psu) for 30 days. Temperature was 15 ± 0.5ºC and photoperiod was 14:10h day/night. Half of the animals were then placed at 35 psu and the other half was kept at 14 psu for another ten days. The lowest salinity level (14 psu, BW) was chosen to be near values measured at the time of capture. The highest salinity level (35 psu, SW) was chosen to represent full strength seawater. During acclimation, crabs were fed frozen mussels every other day.

2.2. Exposure experiments

Exposure to low (0.05 µg L^{-1}, LowS) and high (5.0 µg L^{-1}, HighS) sublethal SERT concentrations lasted for 7d. Test concentrations represent a level found in natural environments and a predicted environmental concentration (PEC) from psychiatric hospital wastewaters (Styrishave et al., 2011). Test media were made by dilution of stock solutions (prepared in ultra-pure water). Controls contained only filtered seawater of the respective salinity level. Twelve crabs were exposed per treatment. Each glass aquarium contained 4 animals in 4L of medium. Exposure conditions were as described for acclimation. Medium renewal was performed every 48h. Salinity, temperature, pH, and dissolved oxygen were measured at 0h and 48h.

After exposure, the thoracic ganglion and sub–samples of leg muscle and digestive gland were isolated from each crab, flash frozen in liquid

nitrogen, and stored at –80ºC until biomarker determinations. The remaining whole–body soft tissues (*e.g.*, digestive gland, muscle, ganglia, skin, gills, gonads) were pooled and frozen at –20ºC for later quantification of SERT and NORS.

2.3. Chemical analysis

Quantification of SERT and NORS in tissues was performed in a Nexera Ultra-High Performance Liquid Chromatograph coupled with a tandem mass spectrometer (LCMS-8030 Shimadzu) and a Kinetex C18 column (150 x 2.10 mm *i.d.*; 1.7 µm) (Phenomenex) as described in supplementary information. Prior to analysis, crab soft tissues were homogenised and processed by a QuEChERS-based extraction method. Method detection limits for SERT and NORS were 1.76 and 1.80 ng g^{-1} wet weight, respectively.

2.4. Measurement of biochemical biomarkers

All biomarkers were determined as described by Rodrigues et al. (2013). AChE activity in muscle and ganglion samples was determined according to the Ellman's method (1961). Muscle LDH and IDH determinations were performed following the methods of Vassault (1983) and Ellis and Goldberg (1971), respectively. In the digestive gland, LPO was evaluated by measuring the thiobarbituric acid reactive substances (TBARS), as adapted by Filho et al. (2001). GST activity was determined by the method of Habig et al. (1974); GR activity was measured through the method developed by Cribb et al. (1989); GPx activity was assessed following the method of Mohandas et al. (1984); CAT activity was assayed according to Clairborne (1985); and TG levels were determined through the method of Tietze (1969). Protein concentration in samples was done through the Bradford's method (Bradford, 1976).

2.5. Data analysis

To investigate potential interactions between SERT, salinity and the exposure history, data was analysed as described by De Coninck et al. (2013). Three–way analysis of variance (ANOVA) was performed to assess significance of main effects and the four interaction terms for all biomarkers. Significant Sertraline × Estuary interactions indicated that the effect of SERT was dependent on the origin of crabs. Significant Sertraline × Salinity × Estuary interactions could possibly indicate that Sertraline × Salinity (SeSa) was different in crabs of the two estuaries. Because, interpretation of three–way interactions is complex, detailed analysis by two–way ANOVA was subsequently used to clarify the significance of the interaction term in each estuary and as formal statistical test of the independent action (IA) model of combined effects of the two stressors (Fournier et al., 2006; De Coninck et al., 2013). The IA model was chosen because it has been recognised as suited to predict combined effects of dissimilarly acting stressors (Jonker et al., 2004). A balanced design was always used, with the same number of replicates in each treatment and biomarker analysed. The logarithmic transformation was applied to the data to fulfil ANOVA assumptions and allow testing deviations from the IA model. Statistical significance was accepted for $p<0.05$. All analyses was done with SPSS v22.

Concordance with the predictions expected under IA model of the effects induced by the interaction SeSa was investigated as follows. The evaluation was done by applying equations (1) and (2) presented in De Coninck et al. (2013), as derived from the formulation of the IA model by Bliss (1939) and Faust et al. (2003). Combined effects of the two factors were predicted from observed effects in the individual treatments using the equations:

$$E_{SeSa}, \text{predicted} = E_{Se} + E_{Sa} - E_{Se} \times E_{Sa} \quad (1)$$

and

$$E_i = (Y_{control} - Y_i) / Y_{control} \quad (2)$$

where, E_i is the observed fractional effect of treatment *i* on biomarker *Y* relative to control treatment, and *i* is either *Se* (low or high SERT concentration), *Sa* (salinity levels tested) or *SeSa*. E_i can be positive, when the biomarker decreases compared to control, or negative, when it increases compared to control. The value of each biomarker in the combined treatment was predicted from the combination of equations (1) and (2) (De Coninck et al., 2013) using the arithmetic mean of the values observed in the control ($Y_{control}$), the SERT only treatment (Y_{Se}) and the salinity only treatment (Y_{Sa}):

$$Y_{SeSa,\ predicted} = Y_{Se} \times Y_{Sa} / Y_{control}.$$

The lower salinity level tested was taken as control because it corresponds to local salinity at the time of capture and represents the transitional character of estuaries. Significant deviation from the IA model was accepted when SeSa was statistically significant at $p<0.05$. Such interactions were classified as synergistic if the observed effect in the combined treatment was higher than the effect predicted by IA model: if $E_{SeSa,\ observed} > E_{SeSa,\ predicted}$ in cases where $E_{SeSa,\ observed} > 0$ (*i.e.* when the combination of the two factors causes a reduction of the biomarker compared to control) or if $E_{SeSa,\ observed} < E_{SeSa,\ predicted}$ and $E_{SeSa,\ observed} < 0$ (*i.e.* when the combination of factors causes an increase of the biomarker compared to control). They were classified as antagonistic when the observed effect was lower than the predicted effect: if $E_{SeSa,\ observed} < E_{SeSa,\ predicted}$ and $E_{SeSa,\ observed} > 0$ or if $E_{SeSa,\ observed} > E_{SeSa,\ predicted}$ and $E_{SeSa,\ observed<0}$.

3. Results

Throughout the experiments, water parameters were kept stable in all test treatments. Dissolved oxygen and pH ranged between 6.8-7.0 mg L^{-1} and 8.1-8.2, respectively. Salinity varied between 14.2-14.4 and 34.9-35.2 psu. Temperature ranged between 15.0-15.3°C. At the end of the exposures, relevant SERT accumulation in soft tissues was observed in the

HighS treatment in crabs from the Minho site exposed in SW (15.0 ± 0.4 ng g^{-1}wt, mean ± SD) and in those from the Lima exposed in BW (15.3 ± 1.7 ng g^{-1}wt) and SW (18.9 ± 0.3 ng g^{-1}wt). Levels in the remaining groups were not detected or were below the limits of detection. NORS was generally not detected in tissues, or below the limit of detection, irrespective of the salinity level and source of the crabs.

Overall, the three–way ANOVA revealed significant effects of SERT, salinity or the estuary for all biomarkers analysed (Tables VI.1., VI.2., VI.3. and VI.4.). Significant effects of the interaction salinity × estuary were observed for AChEm, GST, and GPx. In Minho crabs, GST activity decreased from BW to SW, whereas AChEm and GPx tended to increase with salinity. Responses of each of these biomarkers in Lima crabs were inverted relative to those from the Minho crabs (Figs. VI.1. and VI.2.).

Differential responses to SERT of Minho and Lima crabs were found for neurotoxicity, biotransformation, anti–oxidant defences and oxidative damage, as indicated by significant SERT x Estuary interactions (Tables VI.1., VI.2., VI.3. and VI.4.). Lima crabs generally showed decreased activity of AChE, GR and GPx, induction of GST and higher LPO levels, relative to control (Figs. VI.1. and VI.2.). Minho crabs exhibited increased AChEg activity in the HighS treatment, compared to control. Induction in LowS and inhibition in HighS treatments were also found for GST ad GR.

Significance of SeSa was found for AChEm, LDH, IDH, GST, GR, and CAT activities (Tables VI.1., VI.2., and VI.3.). The three–way interactions further suggested that for some biomarkers SeSa elicited different responses in Minho and Lima crabs. Detailed analysis by two–way ANOVA performed separately for each site revealed significant effects of SeSa on AChEm, GST, GR, CAT, and LPO for Minho crabs, and on AChEm, LDH, IDH and GR for Lima crabs (Tables VI.1., VI.2., VI.3. and VI.4). The two cohorts responded differently to SERT exposure in SW, suggesting that the previous exposure history may influence the interactive effects of SeSa on crabs' physiological responses. Occasionally, different interaction effects were found for LowS and HighS (Figs. VI.1. and VI.2.).

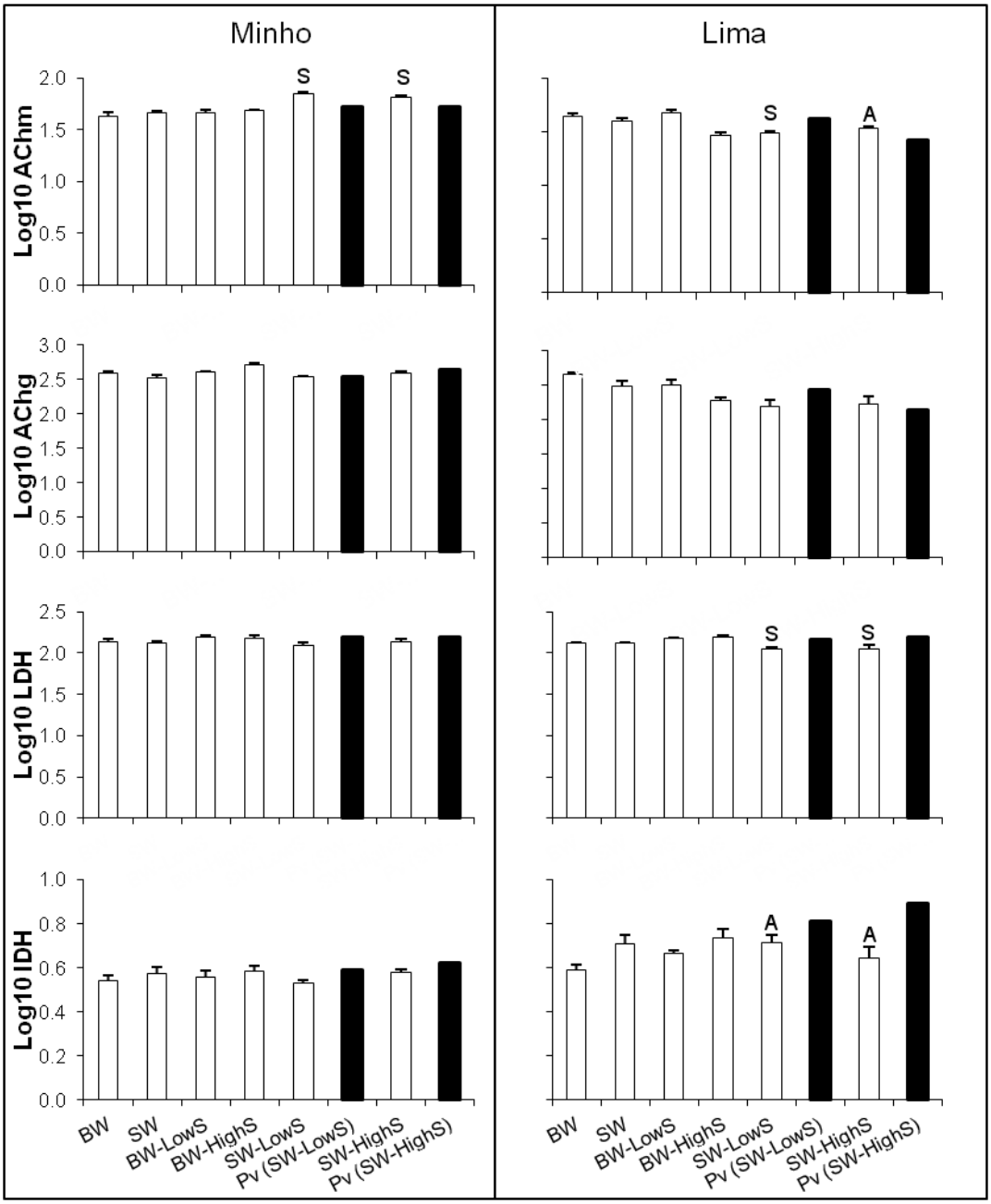

Fig. VI.1. Biomarkers determined in *C. maenas* from the Minho and Lima estuaries. Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle. Error bars represent the standard error of the mean. *BW* (control), brackish water (14psu); *SW*, seawater (35psu); *LowS,* low sertraline level (0.05 µg L^{-1}); *HighS,* high sertraline level (5.0 µg L^{-1}); *Pv,* predicted value; *S*, synergism; *A*, antagonism.

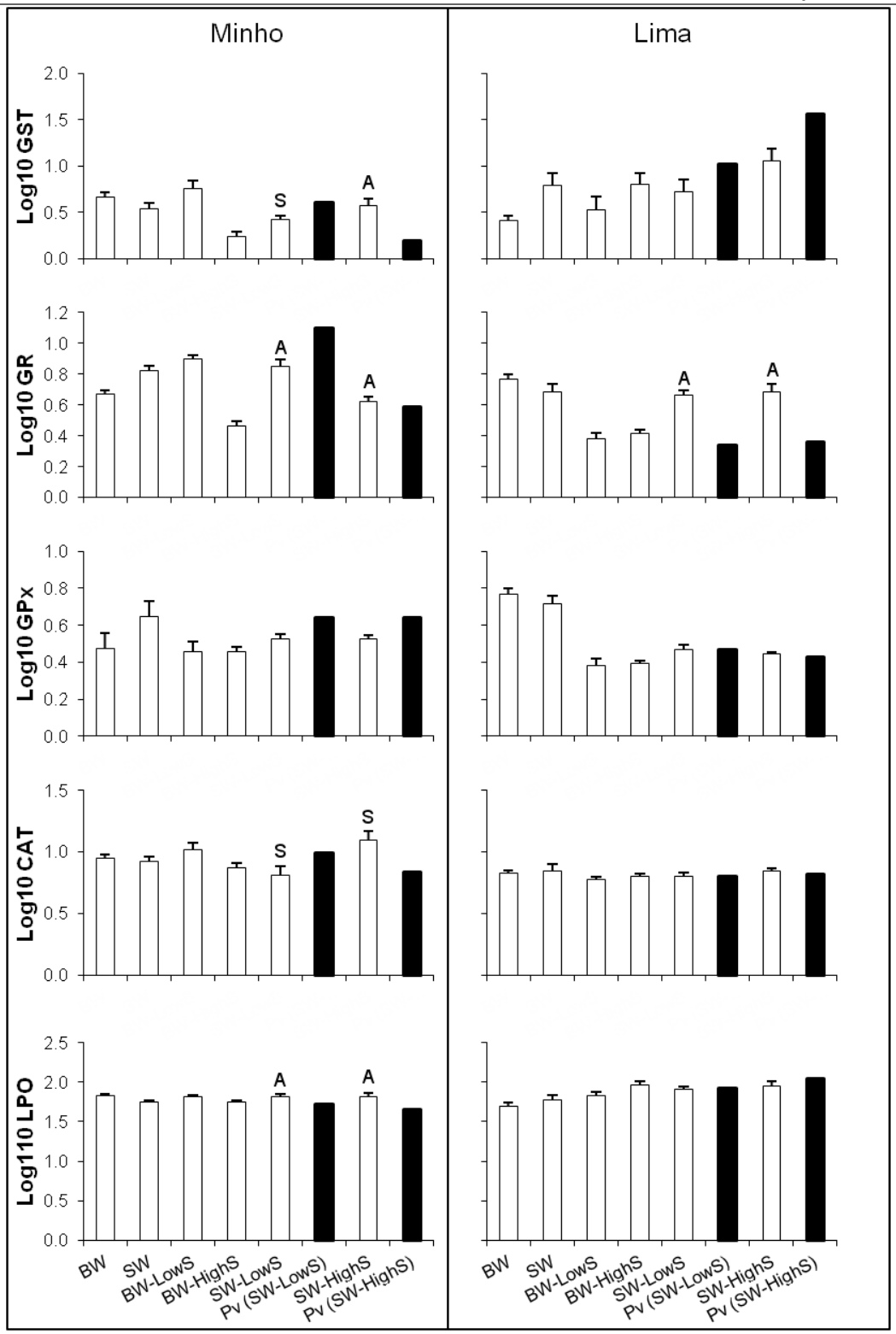

Fig. VI.2. Biomarkers determined in *C. maenas* from the Minho and Lima estuaries. Activity of glutathione *S*–transferases (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT), and the levels of lipid peroxidation (LPO). Error bars represent the standard error of the mean. Legend provided in Fig. VI.1.

Table VI.1. Results of the full-factorial two-way ANOVA to assess the effects of sertraline exposure, salinity, and the sampling site on neurotransmission biomarkers.

Parameter	Source of variation	*df*	*F*	p
AChEm				
Three-way ANOVA	Sertraline	2, 132	3.796	0.025
	Salinity	1, 132	4.883	0.029
	Estuary	1, 132	107.087	<0.001
	Sertraline × Salinity	2, 132	5.794	0.004
	Sertraline × Estuary	2, 132	22.057	<0.001
	Salinity × Estuary	1, 132	36.360	<0.001
	Sertraline × Salinity × Estuary	2, 132	11.173	<0.001
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	4.677	0.013
	Sertraline × Salinity (Lima estuary)	2, 66	11.587	<0.001
AChEg				
Three-way ANOVA	Sertraline	2, 132	4.836	0.009
	Salinity	1, 132	16.364	<0.001
	Estuary	1, 132	40.960	<0.001
	Sertraline × Salinity	2, 132	0.914	0.404
	Sertraline × Estuary	2, 132	12.527	<0.001
	Salinity × Estuary	1, 132	2.287	0.133
	Sertraline × Salinity × Estuary	2, 132	1.904	0.153
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	0.940	0.396
	Sertraline × Salinity (Lima estuary)	2, 66	1.442	0.244

AChEg, ganglion acetylcholinesterase; *AChEm*, muscle acetylcholinesterase.

In Minho crabs, synergistic effects of SeSa on AChEm were observed, with higher activity relative to predictions under IA model (Fig. VI.1.) indicative of upregulation of cholinergic transmission. Synergistic and antagonistic effects on GST were observed in LowS and HighS levels, respectively (Fig. VI.2.): crabs in the SW–LowS treatment showed lower than predicted biotransformation; those in the SW–HighS treatment showed biotransformation induction compared to IA. Globally, decreased biotransformation was found relative to BW controls. For GR, only antagonistic effects of SeSa were found (Fig. VI.2.): in the SW–LowS treatment, the activity was lower than predicted; in the SW–HighS treatment the activity was higher than expected. For CAT, synergistic effects were found leading to lower than predicted activity in the SW–LowS treatment and higher in the SW–HighS. For LPO, the combined exposure caused antagonistic effects leading to higher than expected oxidative damage for both SERT levels.

In Lima crabs antagonistic and synergistic effects of SeSa in AChEm were found. Compared to IA predictions, lower activity was observed in SW–LowS and higher activity was measured in SW–HighS (Fig. VI.1.). Concerning GR, similar to Minho crabs, only antagonism was observed, with higher than expected activity. Synergism was additionally found for LDH activity and antagonism was observed for IDH. In both pathways energy production was lower than predicted by IA model, and suggests exposure in SW may limit energy production to cope with toxicant-induced energetic requirements.

Table VI.2. Results of the full-factorial two-way ANOVA to assess the effects of sertraline exposure, salinity, and the sampling site on energy metabolism.

Parameter	Source of variation	*df*	*F*	p
LDH				
Three-way ANOVA	Sertraline	2, 132	0.228	0.797
	Salinity	1, 132	15.766	<0.001
	Estuary	1, 132	2.627	0.107
	Sertraline × Salinity	2, 132	3.747	0.026
	Sertraline × Estuary	2, 132	0.234	0.791
	Salinity × Estuary	1, 132	1.589	0.210
	Sertraline × Salinity × Estuary	2, 132	0.848	0.430
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	0.897	0.413
	Sertraline × Salinity (Lima estuary)	2, 66	3.951	0.024
IDH				
Three-way ANOVA	Sertraline	2, 132	1.204	0.303
	Salinity	1, 132	0.436	0.510
	Estuary	1, 132	41.566	<0.001
	Sertraline × Salinity	2, 132	4.088	0.019
	Sertraline × Estuary	2, 132	0.866	0.423
	Salinity × Estuary	1, 132	0.513	0.475
	Sertraline × Salinity × Estuary	2, 132	2.520	0.084
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	0.862	0.427
	Sertraline × Salinity (Lima estuary)	2, 66	4.182	0.020

LDH, lactate dehydrogenase; *IDH*, $NADP^+$-dependent isocitrate dehydrogenase.

Table VI.3. Results of the full-factorial two-way ANOVA to assess the effects of sertraline exposure, salinity, and the sampling site on biotransformation and anti-oxidant defences.

Parameter	Source of variation	*df*	*F*	p
GST				
Three-way ANOVA	Sertraline	2, 132	0.591	0.555
	Salinity	1, 132	4.661	0.033
	Estuary	1, 132	11.875	0.001
	Sertraline × Salinity	2, 132	3.924	0.022
	Sertraline × Estuary	2, 132	9.952	<0.001
	Salinity × Estuary	1, 132	8.420	0.004
	Sertraline × Salinity × Estuary	2, 132	3.555	0.031
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	18.161	<0.001
	Sertraline × Salinity (Lima estuary)	2, 66	0.346	0.709
GR				
Three-way ANOVA	Sertraline	2, 132	30.184	<0.001
	Salinity	1, 132	33.523	<0.001
	Estuary	1, 132	33.476	<0.001
	Sertraline × Salinity	2, 132	5.951	0.003
	Sertraline × Estuary	2, 132	29.543	<0.001
	Salinity × Estuary	1, 132	2.892	0.091
	Sertraline × Salinity × Estuary	2, 132	15.871	<0.001
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	6.267	0.003
	Sertraline × Salinity (Lima estuary)	2, 66	14.264	<0.001
GPx				
Three-way ANOVA	Sertraline	2, 132	19.852	<0.001
	Salinity	1, 132	3.948	0.049
	Estuary	1, 132	1.742	0.189
	Sertraline × Salinity	2, 132	0.337	0.714
	Sertraline × Estuary	2, 132	7.045	0.001
	Salinity × Estuary	1, 132	4.530	0.035
	Sertraline × Salinity × Estuary	2, 132	0.688	0.504
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	0.589	0.558
	Sertraline × Salinity (Lima estuary)	2, 66	0.145	0.865
CAT				
Three-way ANOVA	Sertraline	2, 132	1.179	0.311
	Salinity	1, 132	0.133	0.716
	Estuary	1, 132	22.356	<0.001
	Sertraline × Salinity	2, 132	5.759	0.004
	Sertraline × Estuary	2, 132	0.423	0.656
	Salinity × Estuary	1, 132	0.285	0.594
	Sertraline × Salinity × Estuary	2, 132	5.058	0.008
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	7.032	0.002
	Sertraline × Salinity (Lima estuary)	2, 66	0.044	0.957
TG				
Three-way ANOVA	Sertraline	2, 132	1.887	0.156
	Salinity	1, 132	0.271	0.604
	Estuary	1, 132	6.599	0.011
	Sertraline × Salinity	2, 132	1.178	0.311
	Sertraline × Estuary	2, 132	1.319	0.271
	Salinity × Estuary	1, 132	2.748	0.100
	Sertraline × Salinity × Estuary	2, 132	0.309	0.734
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	0.902	0.411
	Sertraline × Salinity (Lima estuary)	2, 66	0.522	0.596

GST, glutathione *S*-transferases; *GR*, glutathione reductase; *GPx*, glutathione peroxidase; *CAT*, catalase; *TG*, total glutathione.

Table VI.4. Results of the full-factorial two-way ANOVA to assess the effects of sertraline exposure, salinity, and the sampling site on oxidative damage.

Parameter	Source of variation	*df*	*F*	p
LPO				
Three-way ANOVA	Sertraline	2, 132	7.408	0.001
	Salinity	1, 132	0.900	0.344
	Estuary	1, 132	5.374	0.022
	Sertraline × Salinity	2, 132	0.262	0.770
	Sertraline × Estuary	2, 132	6.960	0.001
	Salinity × Estuary	1, 132	1.026	0.313
	Sertraline × Salinity × Estuary	2, 132	1.978	0.142
Two-way ANOVA	Sertraline × Salinity (Minho estuary)	2, 66	3.178	0.048
	Sertraline × Salinity (Lima estuary)	2, 66	0.433	0.650

LPO, lipid peroxidation.

4. Discussion

The last decade witnessed a surge in the detection of antidepressants in aquatic environments. Concurrently, climate changes are causing hydrological alterations worldwide. In Atlantic and Southern European Regions, extreme sea level events are previewed to increase the risk of coastal floods (IPCC, 2014). Frequency and intensity of heat waves are also likely to increase in these areas. Among others, these events cause salinity shifts influencing distribution, abundance and physiology of estuarine organisms. Interactions between contaminants and salinity were reported for several classes of pollutants (reviewed by Heugens et al., 2001). Low salinity increases metal toxicity by decreasing speciation and increasing bioavailability (Bjerregaard and Depledge, 1994; Hall and Anderson, 1995). Toxicity of organophosphates increases with salinity, possibly due to their higher bioaccumulation and degradation half–lives in seawater (Johnston and Corbett, 1985; Brecken-Folsen et al., 1994). Toxic interactions between PAHs and salinity may also occur, related to increased solubility of PAHs at lower salinity (which augments their bioavailability and toxic risk) and/or to PAH–induced structural alterations in gills causing osmoregulatory disruption (Whitehead, 2013 and references therein). This

work focused on the overlooked interaction between an emergent pharmaceutical and salinity.

Here SERT accumulation in crabs' tissues was higher in SW than in BW. Moreover, Minho crabs coped better with the exposure in BW than Lima crabs. SERT and NORS residues were detected previously in tissues of feral fish at concentrations up to 4.27 and 28.9 ng g^{-1}, respectively (Daughton and Brooks, 2011 and references herein). However, SERT metabolism is expected to occur through the action of cytochrome P450 and flavin–containing monooxygenases (Obach et al., 2005). These enzymes are known to show lower capacity in marine invertebrates relative to vertebrates (Livingstone, 1991; Solé and Livingstone, 2005), consistent with the present study, in which no relevant bioaccumulation of NORS was observed. Altogether, data indicated that SERT accumulates more readily in marine crustaceans with a history of exposure to chemical stress and that salinity may be expected to increase SERT bioavailability to and/or accumulation in decapods.

For most biomarkers tested, similar responses to salinity were observed in the two cohorts. But opposite trends were found for AChEm, GST and GPx in Minho and Lima crabs. The alterations in AChE activity suggest an upregulation in cholinergic transmission due to greater locomotory activity in Minho crabs under SW and in Lima crabs in BW. Previous experiments by Bolt and Naylor (1985) have shown that walking activity of *C. maenas* augments under low and high salinities to avoid both hyposaline and hypersaline environments. Muscle cholinergic transmission was also shown to be involved in crabs' locomotion (Sorenson, 1973). An influence of salinity in oxidative stress enzymes triggering differences in activity levels was described for this and other estuarine crustaceans (Freire et al., 2011). The results indicate the two cohorts show different sensitivity to salinity, in agreement with previous studies on the effects of the stressor on *C. maenas* from these estuaries (Rodrigues et al., 2012).

Significant SERT x Estuary interactions indicated differential sensitivity of the cohorts of various biomarker responses The most important alterations were observed in Lima crabs and encompassed decreased AChE

activity, increased biotransformation GST, downregulation of anti-oxidant defences and increased oxidative damage, as indicated in previous observations on the toxicity of SERT towards *C. maenas* (Rodrigues et al., *submitted*). However, both synergistic and antagonistic interactions of SeSa were identified for AChEm, LDH, IDH, GST, GR, CAT and LPO. For AChEm, GST, GR and CAT these were influenced by the source of the crabs. The synergism in AChEm of Minho crabs resulted in clear upregulation of activity, but downregulation was observed in Lima crabs due to synergism in the SW–LowS treatment and antagonism in the SW–HighS level. Induction of this enzyme was also found after exposure of Minho crabs to fluoxetine (Mesquita et al., 2011). This induction was positively correlated with locomotory activity and suggests stimulation in cholinergic neurotransmission upon exposure to SSRIs. In favour of this, there are indications that these compounds may not only interact with the serotonin transporter but also with acetylcholine receptors (Garcia–Colunga et al., 1997). Investigations in *Caenorhabditis elegans* showed that *eat-6* is a component of the pathway that couples 5–HT signalling and acetylcholine neurotransmission (Govorunova et al. 2010). This gene is expressed in ventral cord cholinergic neurons. It mediates 5–HT inputs to cholinergic neurons, regulating acetylcholine pre–synaptic neurotransmission, and has a post–synaptic role at the body wall neuromuscular junctions (Govorunova et al., 2010). According to that study, both stimulatory and inhibitory inputs of serotonergic neurons to cholinergic neurotransmission may be expected through the regulation of different receptor subtypes at different cellular targets, resulting both in synergistic or antagonistic regulation. This cross–regulation could explain the divergent response patterns to SeSa of Minho and Lima crabs. Surprisingly, *eat-6* encodes a Na^+/K^+–ATPase α subunit which is expected to share about 76% identity with a Na^+/K^+–ATPase α subunit of *C. maenas* at the protein level. Activity of Na^+/K^+–ATPase was first described in leg nerves of *C. maenas* (Skou, 1957) and later was shown to be involved in the regulation of extracellular osmolytes by driving the increased uptake of Na^+ and Cl^- across the gills of hyperosmoregulating crabs (Towle and Weihrauch, 2001). It is also of note

that earlier studies have shown a rapid decrease of 5–HT in anterior and posterior gills of *C. maenas* under hypoosmotic stress (Zatta, 1987). Complete sequencing of the Na^+/K^+–ATPase α subunit of *C. maenas* and investigation of its gene and protein expression in both *in vitro* and *in vivo* studies will help to elucidate the mechanisms behind the interactive effects found and provide additional early warning biomarkers of exposure to SSRIs in marine invertebrates.

Synergistic (LDH) and antagonistic (IDH) interactions found in Lima crabs limited the increase in energy metabolism usually found in animals challenged with toxicant stress (Jo et al. 2001; Rodrigues et al. 2012; 2013). This common response allows to deal with additional energic requirements triggered by the exposure. Further, muscle gluconeogenesis, where LDH is involved, might be one of the pathways implicated in the metabolic adjustment of aminoacids during hypo– and hyperosmotic stress (Schein et al., 2005).

As an osmoconformer at >25.5–27 psu, the shore crab has less oxygen demands with osmoregulation when in SW than in BW. 5–HT is also able to decrease the frequency of forward beating of *C. maenas* scaphognathites, the modified exopodites that pump rhythmically to draw fluid through the gill chambers (Berlind, 1977), possibly contributing to the lower energy production.

The synergistic and antagonistic interactions found for biotransformation and anti-oxidant enzymes reflected differing effects of SW–LowS and SW–HighS concentrations in both cohorts. GST are enzymes catalysing the conjugation of contaminants with GSH, and may also exert an anti-oxidant role. GPx is responsible for the reduction of lipid and hydroperoxides with concomitant oxidation of GSH. GR recycles the oxidised glutathione restoring GSH levels. CAT also converts hydrogen peroxide into less reactive components. In humans SERT has been shown to decrease the generation of ROS, increase the activity of superoxide dismutase and CAT, and decrease the levels of LPO (reviewed in Lee et al., 2013). Data for Minho crabs appear to be consistent with this, as indicated by the low GST activity detected and the synergistic effects for CAT,

particularly in SW–HighS. Also, though the antagonistic interaction found for LPO indicated that exposure to SERT in SW caused higher than expected levels of oxidative damage, these were within control levels. Salinity was previously found to influence *C. maenas* anti–oxidant defences (Rodrigues et al., 2012). Following observations showing that high NaCl levels lead to oxidative stress, a link between high salinity and ROS generation has also been suggested (Freire et al., 2011 and references herein). Contrary to the low impacted site, and despite upregulation of GST and GR activity caused by SeSa, Lima crabs could not overcome the stress imposed by the toxicant, exhibiting very important extent of oxidative damage, as observed also in BW.

Altogether the results pointed different SeSa interactions for the two cohorts. SERT accumulation was higher in SW in both estuaries, but more intense life threatening effects appear to occur following exposure of Lima crabs in SW, as indicated by AChE, GST and LPO responses. Downregulation of neuromuscular transmission may have a reflex at the population level by impairing food search, and reproductive behaviour, and increasing the risk of predation as well. Enhanced AChE activity is known to have a pivotal role in apoptosis (reviewed by Zhang and Greenberg, 2012). It has been established that increased AChE expression or activity is detected in apoptotic cells, after apoptotic stimuli *in vitro* and *in vivo*, so that cells in which AChE is over–expressed undergo apoptosis more easily than controls (Zhang and Greenberg, 2012). The reduced anti-oxidant defences and high extent of oxidative damage to lipid macromolecules causes additional concern since they are particularly toxic to cells, interfering with the activity of multiple enzymes and ATP production, and triggering apoptosis initiation (Lesser, 2006). According to the results the MOA–related biomarkers tested here appear to be more sensitive than common regulatory endpoints indicating likely hazardous responses. In a recent study concerning exposure to oil in the marine environment, comparison of species sensitivity distributions (SSDs) based on oxidative stress biomarkers to a SSD for whole–organism responses

indicated that the selected biomarkers were, on average, 35–50 folds more sensitive than the whole–organism effect (Smit et al., 2009). Future work should focus on investigating such a pattern for SSRIs. SSDs are very often used to estimate ecological risk for chemical substances. Previous investigations have shown, however, that for most toxicants SSDs include too few species (Kefford et al., 2005; Hickey et al., 2008), not representative of local ecological receptors in the habitat of interest. Often they are based on data obtained with organisms reared under controlled conditions or from more pristine sites. The interactions between SERT and salinity found here, and their dependence on the source of the crabs, support the need for more site–specific assessments with local species/populations to improve predictions to field scenarios and establish appropriate protective thresholds.

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Chapter VII

Spatial and temporal variation in integrated biomarker responses of a decapod in estuaries with varying levels of abiotic stress and heavy metal contamination

Spatial and temporal variation in integrated biomarker responses of a decapod in estuaries with varying levels of abiotic stress and heavy metal contamination

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In preparation.

Abstract

An integrated chemical biological effects monitoring was performed in 2010 and 2012 in two NW Iberian estuaries under different anthropogenic pressure. The aim was to verify the usefulness of a multibiomarker approach, using *Carcinus maenas* as bioindicator, to reflect the health status of the species in relation to abiotic variation and diminishing contamination by heavy metals. Sampling sites were assessed for metal levels in sediments and *C. maenas*, water abiotic factors, and biomarkers (neurotoxicity, energy metabolism, biotransformation, anti–oxidant defences, and oxidative damage). Seasonal and inter–annual variability was observed in the parameters measured. Biomarkers indicated differences between the study sites and seasons in 2010 and an improvement in health status in 2012. Integrated biomarker response (IBR) index depicted sites with higher stress levels; principal component analysis (PCA) associated biomarker responses to environmental variables. In 2012 a relevant decrease in sediments contamination and high variation in temperature, salinity and nutrient levels were found. Data suggests the integrated multibiomarker approach is useful to the early diagnosis of remediation measures in impacted sites.

Keywords: Heavy metals, early–warning biomarkers, extreme climate events, integrated chemical biological effects monitoring, estuaries, contamination history.

1. Introduction

Concern around water quality and its sustainable management has increased throughout the years. In 2000 the European Commission adopted policies to protect and manage superficial and groundwater. The Water Framework Directive (WFD, Directive 2000/60/EC) established an innovative approach based on natural boundaries, the River basins, and was set at achieving a good ecological and chemical status of all water bodies by 2015 (Laane et al., 2012). Following issuing of the directive, initial assessments were performed and restoration measures were implemented for several aquatic systems. Despite this, data on follow–up and routine monitoring, providing information on spatial and temporal scales, is still required. Particularly, there is a need for data that may improve prediction on biota responses to restoration measures in multiple stressor contexts (Hering et al., 2010). Recovery of biotic communities is the ultimate goal. Though, it shows limited interest as parameter to assess potential indications of amelioration. Recent evidence indicates that functional recovery of aquatic ecosystems disturbed by human action may be achieved in a feasible temporal horizon but is usually a long process showing average times of 10 to 20 years (Jones and Schmitz, 2009). Moreover, although different levels of biological organisation would be expected to respond on different time scales, both community– and ecosystem–level variables appear to operate on contemporary time scales (Jones and Schmitz, 2009). Hence, other biological effect parameters that may respond in shorter time scales and could anticipate the suitability of management actions and the potential success of recovery should be sought and incorporated in follow–up programmes. In this sense, biomarker responses can be of significant contribution to the weight–of–evidence approaches required in such cases. Biomarkers reflect the integrated impact of natural and man–made chemical stressors to which the organisms may be exposed, giving crucial information on health status of species. They provide early warning signals of exposure and potential adverse outcomes that may reflect to population–level effects (Chapman et

al., 2013). As biological effects measured at the sub–individual or individual levels (*e.g.*, molecular, cellular, metabolic, physiological, behavioural) they should respond on a much shorter time scale than community–level variables. Furthermore, batteries of cost–effective specific biomarkers may be combined in integrated multibiomarker response indices useful to identify sites where organisms are under higher or lower stress levels (Marigómez et al., 2013; Rodrigo et al., 2013). Also, given their valuable contribution to assess exposure to and effects of the complex mixture of anthropogenic contaminants found in ecosystems, providing added information on their bioavailability, biomarkers have been considered as a strategic tool in assessment of environmental quality of coastal waters (Allan et al., 2006; Picado et al., 2007). Under these considerations they have been recommended for inclusion in Portuguese monitoring programmes (Picado et al., 2007).

Contamination by heavy metals and its impact in estuarine organisms is a continuous priority due to their persistence in environment and abiding life–threatening effects. The estuary of the Lima River, in NW Iberia Peninsula, shows high susceptibility to human influence (Ferreira et al., 2003). It is under important industrial pressure (*e.g.*, harbour, shipyard, paper mill), and also receives wastewaters of husbandry and livestock origin. Its sediments were previously shown to be contaminated by heavy metals, and to a lesser extent with polycyclic aromatic hydrocarbons (PAHs), which were associated to reduced health status of local relevant fish species, such as *Anguilla anguilla* and *Pomatoschistus microps* (Guimarães et al., 2009; Guimarães et al., 2012). Hence, further investigation of sediments contamination by heavy metals and their effects in key structuring species was needed. In recent years, in addition to some protection measures and in consequence of generally hard economic conditions, several industries have diminished (including the shipyard) or ceased their activity. Moreover, intense temperature variation and drought events have been reported in the area. This provided thus an interesting case study to investigate temporal and spatial trends of metal contamination in sediments and biota how multibiomarker responses

would reflect such changes in anthropic activity and abiotic variation. This study was, therefore, intended to carry out an integrated chemical biological effects evaluation in two sites located in the terminal part of Lima estuary and two sites located in the terminal part of the estuary of the Minho River during the years of 2010 and 2012. The latter is under low industrial and urban pressure (Ferreira et al., 2003). It is part of the NATURA 2000 network, exhibiting generally low levels of environmental contamination by heavy metals and PAHs, despite having some localised sources of contamination (Reis et al., 2009; Guimarães et al., 2012).

Carcinus maenas was selected for the present study as recommended for biomarker measurements in Portuguese monitoring programmes (Picado et al., 2007), and because there is a need for information on the effects of Lima contamination in benthic invertebrates with key role in its trophic chains. Hence, the study sites were chosen according to the life history of *C. maenas* (Queiroga, 1996) and to be comparable as much as possible. Assessments of metal levels in sediments and *C. maenas*, water abiotic factors and biomarkers were performed in two key seasons of the year. The set of biomarkers selected was previously employed in resident fish species: acetylcholinesterase (AChE, neurotoxicity), lactate (LDH) and $NADP^+$–dependent isocitrate (IDH) dehydrogenases (anaerobic and aerobic energy production, respectively), glutathione *S*–transferases (GST, phase II biotransformation), glutathione peroxidase (GPx), glutathione reductase (GR), and total glutathione (TG) (as anti–oxidant defences), and lipid peroxidation (LPO, as measure of oxidative damage to macromolecules). The integrated biomarker response (IBR) index was used to depict sites with different stress levels and spatial and temporal evolution of the integrated responses to environmental change. Principal component analysis (PCA) allowed investigating patterns of biomarker responses and their association to heavy metal contamination and abiotic stress.

2. Material and methods

Minho and Lima estuaries (NW Iberian coast) were selected as different contamination scenarios. In each estuary sampling sites, one upstream and another downstream, were selected to be at approximately the same distance from the mouth of the estuary (Fig. VII.1.). Minho estuary sites were located in Seixas (upstream, MU) and Moledo (downstream, MD) villages. Lima estuary sites were located in Porto Velho (upstream, LU) and Cabedelo (downstream, LD) villages. The selection of these sites in each estuary was related to life history of the species. Younger crabs are expected to occupy upstream regions with lower salinity, whereas older crabs inhabit the mouth of the estuary where reproduction takes place (Queiroga, 1996). Sampling, and all animal experiments were conducted in compliance with ethical guidelines of the European Union Council (Directive 2010/63/EU of 22th September) for the protection of animals used for experimentation and other scientific purposes.

2.1. Chemicals

The reagents used were of analytical grade or suprapur® grade (65% nitric acid) and were purchased from Sigma–Aldrich Chemical (Steinheim, Germany), except the Bio–Rad protein assay dye reagent that was purchased from Bio–Rad Laboratories, Inc. Metal standard solutions (zinc, copper, lead, nickel, chromium, cadmium) were prepared with the 1000 mg L^{-1} stock solutions (Panreac) and ultrapure water.

2.2. Water, sediments, and crab sampling

In each sampling campaign, water physico–chemical parameters (*i.e.* temperature, salinity, conductivity, pH, dissolved oxygen) were measured during low tide with a multiparametric sea gauge WTW multi 340i with appropriate probes (pH Sen Tix 41 and Tetracon 325) (Table VII.1.).

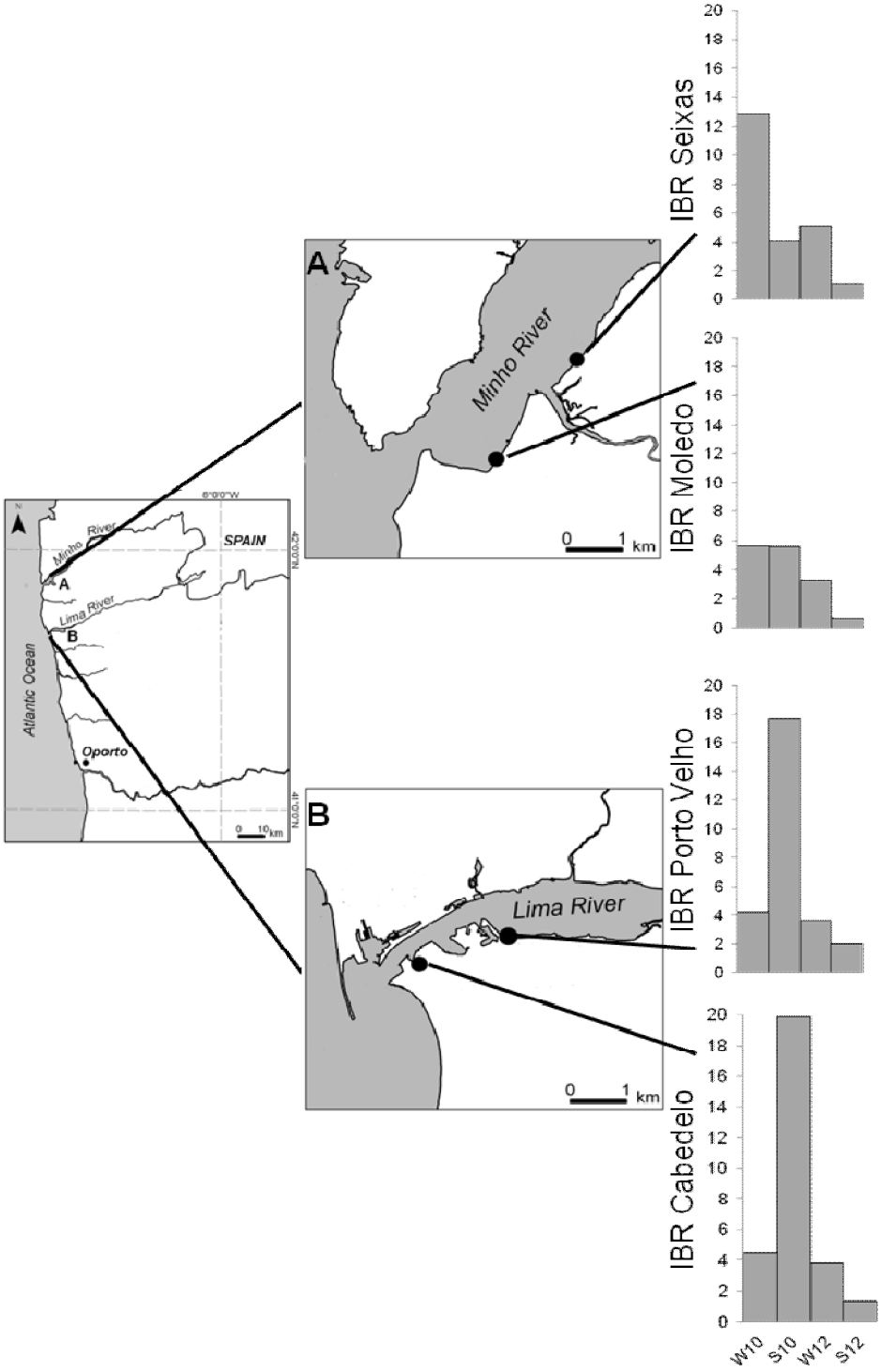


Fig. VII.1. Location of the sampling sites at the estuary of (A) Minho River (Seixas and Moledo), and at the estuary of (B) Lima River (Porto Velho and Cabedelo). Graphics represent the integrated biomarker response (IBR) indices of each sampling site in winter and summer 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively).

Water samples were collected, in triplicate, and stored at −20ºC until further analysis. These samples were used for later determination of nitrites, nitrates, ammonium, phosphates, iron, silica, and hardness by colorimetric methods using a Hanna 200 photometer. Sediment samples were collected, in triplicate, from the top layer to determine heavy metals concentrations.

Intermoult male crabs (n=16–22 per site and season), with complete appendices, were captured using hand baited or trawl nets in low tide, during winter and summer of 2010 and 2012. Once in the laboratory, crabs were allowed to deplete gut contents for 24h. After this period, crabs were ice–anaesthetised, measured (width and weight) (Table VII.2.) and sacrificed. Sub–samples of muscle (AChE, LDH, IDH) and digestive gland (GST, GPx, GR, TG, LPO) were collected for biomarkers determination and stored at –80ºC until further analysis. Remaining whole–body soft tissues were used to prepare three pools per site/season in each year, to metals quantification. Samples were stored at –20ºC until analysis.

2.3. Chemical analysis of sediments and tissue samples

In sediments and tissue samples zinc, copper, lead, nickel, chromium, and cadmium were analysed by High Resolution–Continuum Source Atomic Absorption Spectrophotometer (HR–CS–AAS, ContrAA 700, Analytik Jena), according to American Public Health Association (APHA) recommendations (APHA, 1992). Lead, nickel, chromium, and cadmium were quantified by electrothermal atomization and zinc and copper by flame atomization.

The samples were pre–treated by acid mineralization using microwave–assisted digestion (1200 W MARS–X, CEM) in HP–500 Plus Teflon vessels (CEM). The optimised microwave digestion temperature was 180ºC (20 min), at a maximum pressure of 200 psi.

Table VII.1. Seasonal and inter–annual variation in the water abiotic parameters measured.

Sampling site	Season	T	Sal	Cond	pH	DO	NO_2^-	NO_3^-	NH_4^+	PO_4^{3-}	Fe	SiO_2	$CaCO_3$
Minho													
Upstream	W10	10.89 (0.55)	0.02 (0.00)	113.50 (0.87)	7.35 (0.04)	10.66 (0.11)	0.01 (0.00)	1.11 (0.21)	0.04 (0.01)	0.23 (0.04)	0.06 (0.02)	3.73 (0.10)	33.33 (5.44)
	W12	15.07 (0.15)	7.28 (0.58)	10.24 (0.81)	8.04 (0.03)	8.62 (0.14)	0.02 (0.00)	0.55 (0.16)	0.12 (0.01)	0.07 (0.01)	0.07 (0.00)	2.17 (0.04)	102.90 (3.63)
	S10	20.37 (2.12)	0.88 (0.40)	158.05 (89.87)	7.82 (0.13)	10.01 (0.11)	0.01 (0.00)	0.62 (0.10)	0.04 (0.01)	0.12 (0.01)	0.07 (0.03)	2.23 (0.24)	20.00 (0.00)
	S12	24.04 (0.10)	35.00 (0.01)	39.43 (1.7)	7.70 (0.10)	7.73 (0.07)	0.02 (0.00)	5.10 (2.51)	0.19 (0.04)	0.03 (0.01)	0.06 (0.00)	0.19 (0.01)	106.70 (2.84)
Downstream	W10	11.46 (0.58)	1.75 (0.55)	211.91 (38.71)	8.11 (0.24)	10.99 (0.24)	0.03 (0.01)	0.69 (0.19)	0.03 (0.00)	0.13 (0.03)	0.04 (0.01)	3.50 (0.12)	30.00 (4.71)
	W12	12.33 (0.17)	16.84 (0.62)	20.09 (0.68)	8.24 (0.05)	8.62 (0.10)	0.05 (0.00)	1.44 (0.47)	0.18 (0.03)	0.06 (0.03)	0.06 (0.00)	1.51 (0.13)	84.60 (5.97)
	S10	19.06 (2.07)	14.47 (5.29)	212.70 (98.73)	8.36 (0.20)	9.97 (0.69)	0.01 (0.00)	0.79 (0.08)	0.18 (0.14)	0.06 (0.01)	0.02 (0.00)	0.99 (0.43)	60.00 (0.00)
	S12	16.10 (0.14)	36.87 (0.41)	45.43 (0.05)	8.20 (0.00)	7.92 (0.48)	0.02 (0.00)	1.22 (0.47)	0.19 (0.03)	0.02 (0.01)	0.05 (0.00)	0.23 (0.05)	93.10 (4.74)
Lima													
Upstream	W10	12.68 (0.85)	5.12 (1.23)	208.80 (79.68)	7.97 (0.13)	11.16 (0.22)	0.04 (0.01)	0.52 (0.15)	0.12 (0.07)	0.32 (0.11)	0.12 (0.07)	3.12 (0.02)	600.00 (37.71)
	W12	13.73 (0.07)	25.1 (0.02)	30.90 (0.05)	8.18 (0.01)	8.97 (0.05)	0.06 (0.00)	1.62 (0.34)	0.34 (0.06)	0.04 (0.01)	0.04 (0.00)	1.36 (0.17)	1063.30 (9.02)
	S10	18.65 (1.44)	12.16 (3.84)	497.30 (0.00)	8.04 (0.05)	8.98 (0.13)	0.63 (0.01)	1.47 (0.15)	0.41 (0.05)	0.38 (0.06)	0.23 (0.08)	2.21 (0.05)	440.00 (0.00)
	S12	19.40 (0.40)	33.07 (0.90)	44.93 (1.40)	8.10 (0.00)	7.99 (0.40)	0.21 (0.01)	2.88 (0.44)	0.33 (0.06)	0.03 (0.01)	0.04 (0.00)	0.66 (0.06)	638.00 (7.03)
Downstream	W10	11.58 (0.65)	9.93 (1.79)	179.40 (47.58)	8.28 (0.23)	10.97 (0.08)	0.00 (0.00)	0.45 (0.08)	0.04 (0.02)	0.18 (0.04)	0.11 (0.08)	3.04 (0.13)	1100.00 (48.99)
	W12	15.77 (0.32)	29.00 (0.36)	36.83 (0.42)	8.10 (0.04)	10.44 (0.39)	0.02 (0.00)	0.33 (0.16)	0.21 (0.01)	0.11 (0.06)	0.06 (0.00)	1.01 (0.10)	1048.70 (17.47)
	S10	18.17 (1.35)	19.17 (1.18)	249.32 (127.82)	7.74 (0.36)	9.54 (0.06)	0.02 (0.00)	0.93 (0.04)	0.07 (0.01)	0.10 (0.02)	0.05 (0.01)	1.59 (0.21)	960.00 (0.00)
	S12	25.00 (0.10)	35.01 (0.30)	40.00 (1.70)	7.8 (0.04)	7.80 (0.40)	0.02 (0.00)	1.88 (0.47)	0.21 (0.03)	0.02 (0.01)	0.06 (0.00)	0.43 (0.04)	636.80 (26.57)

Values represent the mean and the corresponding standard deviation (within brackets) of temperature (T, ºC), salinity (Sal, psu), conductivity (Cond, mS cm^{-1}), pH, dissolved oxygen (DO, mg L^{-1}), nitrites (NO_2^-, mg L^{-1}), nitrates (NO_3^-, mg L^{-1}), ammonium (NH_4^+, mg L^{-1}), phosphates (PO_4^{3-}, mg L^{-1}), iron (Fe, mg L^{-1}), silica (SiO_2, mg L^{-1}) and water hardness ($CaCO_3$, mg L^{-1}). The measurements were performed in samples collected during the winter and summer of 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively).

Table VII.2. Morphometric measures of the crabs collected from the Minho and Lima estuaries.

Sampling site		Season	Weight (g)	Width (mm)
Minho	*Upstream*	W10	11.02	37.50
			(0.63)	(0.85)
		W12	17.10	42.23
			(5.10)	(4.07)
		S10	21.93	46.25
			(1.09)	(0.90)
		S12	43.66	57.43
			(16.54)	(6.57)
	Downstream	W10	29.42	51.63
			(2.07)	(1.09)
		W12	20.37	44.82
			(6.45)	(4.92)
		S10	42.45	58.60
			(1.79)	(0.96)
		S12	38.16	41.58
			(10.79)	(3.36)
Lima	*Upstream*	W10	51.49	61.00
			(5.33)	(2.16)
		W12	22.02	45.05
			(7.44)	(4.88)
		S10	27.25	47.80
			(1.66)	(1.18)
		S12	29.98	51.18
			(9.45)	(5.18)
	Downstream	W10	15.11	40.89
			(1.05)	(1.03)
		W12	40.56	55.41
			(21.87)	(10.53)
		S10	46.76	62.20
			(2.10)	(1.23)
		S12	27.24	48.22
			(9.66)	(5.34)

Values represent the mean and the correspondent standard error (within brackets) of weight (g) and width (mm). The measurements were performed in crabs captured during the winter and summer of 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively).

Before analysis, the remaining whole–body soft tissues were lyophilized, ground and homogenised. Then, 0.2–mg portions were digested with 10 mL of a 27% (v/v) nitric acid solution (Reis and Almeida, 2008). Sediments were air dried (to constant weight), homogenised and

sieved through 2–mm screens; 0.3–g samples were digested with water (1 mL) and 65% nitric acid (7 mL) (Reis et al., 2009). The atomic absorption parameters were optimised following guidelines provided by the equipment's manufacturer. External calibration method was used for metal quantifications and analytical procedure was checked using samples spiked with known amounts of metal. Satisfactory mean recoveries (84.5–122.7%) were obtained for all the metals studied. All samples were analysed in triplicate.

2.4. Biomarkers determination

All biomarker determinations were performed as described by Rodrigues et al. (2013) for *C. maenas.* Protein concentrations were determined, in triplicate, by the Bradford method (1976) adapted to microplate. All cuvette assays were quantified in a Jasco 6405 UV/VIS spectrophotometer. Microplate determinations were carried out with a Bio Tek Power Wave 340 microplate reader. All biochemical analyses were performed at 25ºC.

2.4.1. Neurotoxic effects and energy metabolism

Briefly, muscle AChE was assayed according to Ellman's method (1961). Tissue was homogenised (phosphate buffer pH 7.2, 0.1 M), centrifuged (3,300 ×*g*, 5 min at 4ºC), and the supernatant collected to determine AChE activity. Muscle LDH activity was determined by the method of Vassault (1983). Muscle was homogenised (Tris/NaCl buffer pH 7.2, 50 mM), centrifuged (3,300 ×*g*, 3 min at 4°C), and the supernatant was recovered to determine the enzyme activity. Determination of muscle IDH activity was done through the method developed by Ellis and Goldberg (1971). Tissue was homogenised (Tris/NaCl buffer pH 7.8, 50 mM) and centrifuged (3,300 ×*g*, 15 min at 4°C) and the supernatant used to assess IDH activity.

2.4.2. Biotransformation, anti–oxidant defences, and oxidative damages

Biomarkers of biotransformation, anti–oxidant defences, and oxidative damage were determined in the digestive gland of crabs. The digestive gland was homogenised (1:10 wt v^{-1}) in phosphate buffer (pH 7.4, 0.1 M). Part of the homogenate was used to determine LPO levels using the method adapted by Filho et al. (2001). Remaining homogenate was centrifuged (14,000 ×*g*, 20 min at 4ºC) and the post–mitochondrial supernatant used to measure GST, GPx, and GR activities, as well as TG levels. GST activity was determined according to the method of Habig *et al*. (1974). GPx activity was measured according to Mohandas et al. (1984). GR activity was determined through the method described by Cribb et al. (1989). TG levels were assayed as described in Tietze (1969).

2.5. Data analysis

Given abiotic characteristics of the sites and the life history of the shore crab, all analyses were performed separately for upstream and downstream sites. One–way analysis of variance (ANOVA) followed by planned contrasts for pairwise comparisons were used to investigate differences in biomarkers measured. Assumptions of normality and homogeneity of variances were checked *a priori* using the Kolmogorov–Smirnov and the Levene's tests, respectively. The logarithmic transformation was applied to data whenever needed to fulfil the ANOVA assumptions. All univariate tests were performed with SPSS 19.0.

The Integrated Biomarker Response (IBR) index was used as stress indicator allowing for the overall interpretation of the multibiomarker responses. IBR was calculated according to Beliaeff and Burgeot (2002) and the subsequent modification described by Guerlet et al. (2010), for each sampling campaign as follows: individual areas A_i connecting the i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot were obtained through the formula:

$$A_i = 1/2 \sin(2\pi/n)\, S_i S_{i+1}$$

where S_i and S_{i+1} represent the individual biomarker scores (calculated from

standardised data) and their successive star plot radius coordinates, and *n* represents the number of radii corresponding to biomarkers used in the survey.

Biomarker patterns of response assessed and their relation to environmental variables were investigated through principal component analysis (PCA). Chemical measurements and biomarkers determined were entered as quantitative variables. A composite qualitative variable grouping the measurements according to estuary, sampling year and season was included as supplementary factor. Only principal components (PC) with Eigen values >1 were accepted. PCA interpretation was based on the examination of correlations between the variables and the PCs obtained. All multivariate analyses were carried out in FactoMineR. Significant differences were accepted for $p<0.05$.

3. Results

3.1. Water abiotic parameters

Water temperature increased in all sampling sites from 2010 to 2012, except in MD during the summer (Table VII.1.). The highest temperatures were registered during the summer 2012 in MU (24ºC) and LD (25ºC), corresponding to increases of almost 4ºC and 7ºC compared to the values reported for these same sites in the summer 2010 (Table VII.1.). In general, sampling sites located in Lima estuary also exhibited higher water temperature than those located in Minho estuary. Dissolved oxygen globally accompanied this trend, with lower values found in 2012. Salinity was generally higher in the Lima sites than in the Minho, and in the summer 2012 upstream, relative to values obtained in 2010 and normally expected for this part of the estuaries (Table VII.1.). Conductivity decreased from 2010 to 2012 in all sampling sites and seasons. The highest levels of nitrites, ammonium, phosphates, and Fe were found in LU in the summer of 2010 (0.63, 0.41, 0.38, and 0.23 mg L^{-1}, respectively). MU exhibited higher levels of nitrates during the summer of 2012 (5.10 mg L^{-1}). Water samples collected from the Lima estuary also showed high

hardness when compared with the samples from Minho; the highest $CaCO_3$ concentration (1100.00 mg L^{-1}) was observed during the winter 2010 in LD.

3.2. Chemical analysis

MD sediments showed the lowest metal levels in all the sampling campaigns (Table VII.3.). Sediments from LD exhibited the highest concentrations of heavy metals, in particular of Zn, Cu, Cr, and Ni. These were in general remarkably higher than those found in MD. In some cases by over an order of magnitude, as for Zn and Cr during the winter 2010, and by about two orders of magnitude, as for Pb and Cu during the winter 2010 and the summer 2012, respectively (Table VII.3.). All sampling sites, except MU, showed lower levels of metals in 2012 compared with those determined in samples collected in 2010. In MU, Zn, Ni, and Cd showed the highest concentrations. MU also exhibited higher levels of metals than LU in all the campaigns, except in the summer 2010. Metal levels found in crabs whole–body soft tissues (Table VII.4.) were fairly concordant with the trend observed for sediments. In general the highest tissue accumulation was recorded during 2010 in LD. In those crabs Zn, Cu, Ni, and Cd were the metals found in higher levels. In 2012, metal levels in tissues dropped off in the Lima sampling sites to levels comparable to those found in Minho crabs (Table VII.4.).

3.3. Biomarkers

On average, crabs were larger at the mouth of the Minho estuary (MD) compared with the upstream site (MU) (Table VII.2.). In Lima estuary they were fairly comparable in size between upstream (LU) and downstream (LD) sites (Table VII.2.). Analysis of covariance revealed an influence of the animals' size on LDH ($p<0.05$), TG ($p<0.01$), and LPO ($p<0.01$) of crabs collected upstream. Despite this, significant differences between sites, seasons or years were still found for these biomarkers after correction for the size of the animal. For the remaining biomarkers and sampling sites no influence of the crabs' size was observed.

Table VII.3. Concentration of metals (µg g^{-1} dry weight) determined in sediment samples collected from the Minho and Lima estuaries.

Sampling site	Season	Zn	Cu	Pb	Ni	Cr	Cd
Minho							
Upstream	W10	85.77 (9.11)	11.53 (2.37)	1.36 (0.03)	34.12 (3.34)	19.86 (2.22)	41.77 (11.31)
	W12	62.20 (6.61)	9.28 (0.97)	5.25 (0.66)	14.33 (0.21)	16.02 (2.96)	0.05 (0.01)
	S10	57.33 (15.01)	5.70 (2.57)	1.33 (0.67)	22.97 (8.98)	10.54 (6.26)	18.90 (9.90)
	S12	85.75 (2.99)	23.16 (1.39)	14.09 (0.45)	26.87 (1.45)	35.95 (4.05)	0.25 (0.01)
Downstream	W10	25.37 (4.08)	14.70 (2.45)	0.33 (0.07)	3.51 (1.29)	3.45 (0.90)	6.40 (1.97)
	W12	17.39 (3.06)	2.13 (0.17)	1.25 (0.09)	1.61 (0.13)	2.13 (0.08)	0.01 (0.00)
	S10	49.90 (12.80)	3.23 (1.05)	0.60 (0.17)	10.30 (3.47)	7.88 (1.27)	17.87 (11.09)
	S12	15.58 (2.11)	2.23 (0.07)	1.09 (0.05)	1.83 (0.41)	2.10 (0.06)	0.01 (0.00)
Lima							
Upstream	W10	65.73 (5.01)	12.43 (10.86)	1.11 (0.19)	6.43 (0.33)	11.09 (1.76)	33.30 (5.10)
	W12	45.47 (3.49)	5.84 (0.49)	4.82 (0.83)	4.19 (0.34)	11.49 (0.36)	0.06 (0.02)
	S10	75.90 (3.64)	4.67 (3.04)	0.94 (0.42)	9.33 (6.11)	6.96 (4.35)	29.53 (20.40)
	S12	41.76 (2.04)	5.71 (0.78)	3.79 (0.19)	2.82 (0.14)	9.51 (0.06)	0.05 (0.00)
Downstream	W10	309.37 (15.52)	295.50 (28.53)	33.30 (39.92)	63.06 (46.11)	120.40 (111.47)	62.43 (52.62)
	W12	200.99 (49.50)	140.21 (48.18)	18.11 (1.52)	33.92 (6.82)	59.47 (9.19)	0.06 (0.01)
	S10	283.83 (6.93)	271.90 (19.15)	8.76 (1.49)	74.23 (1.68)	164.80 (91.85)	1.87 (0.46)
	S12	47.97 (5.24)	8.10 (1.42)	4.57 (1.17)	7.81 (1.49)	7.58 (1.38)	0.04 (0.01)
ERL		150	34	467	20.9	81	1.2
ERM		410	270	218	51.6	370	9.6

Values represent the mean and the corresponding standard deviation (within brackets) of zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), chromium (Cr) and cadmium (Cd). The measurements were performed in samples collected during the winter and summer of 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively). The "effects range-low" (ERL) and "effects range-medium" (ERM) derived by Long et al. (1995) are shown in mg L^{-1}.

Table VII.4. Concentration of heavy metals (µg g^{-1} dry weight) determined in tissues of *C. maenas* collected at the Minho and Lima estuaries. Legend provided in Table VII.3.

Sampling site	Season	Zn	Cu	Pb	Ni	Cr	Cd
Minho							
Upstream	W10	136.03 (18.12)	44.01 (23.57)	0.36 (0.04)	10.45 (4.34)	0.31 (0.08)	13.22 (5.31)
	W12	106.90 (4.90)	101.00 (5.00)	0.30 (0.01)	4.52 (0.47)	0.46 (0.08)	1.27 (0.14)
	S10	148.33 (25.33)	82.15 (18.47)	0.47 (0.06)	11.77 (7.77)	0.89 (0.12)	9.17 (3.78)
	S12	93.60 (2.40)	134.30 (3.50)	0.33 (0.04)	9.04 (0.32)	0.30 (0.02)	0.51 (0.08)
Downstream	W10	139.32 (24.07)	37.62 (15.33)	0.31 (0.08)	2.53 (1.19)	0.27 (0.10)	2.45 (0.97)
	W12	102.30 (10.2)	83.30 (9.60)	0.34 (0.03)	2.15 (0.51)	0.32 (0.04)	0.28 (0.05)
	S10	154.02 (22.70)	35.08 (8.06)	0.38 (0.18)	3.54 (0.98)	0.37 (0.03)	7.42 (4.19)
	S12	113.30 (1.10)	68.20 (1.80)	0.34 (0.03)	5.24 (1.38)	0.64 (0.06)	1.30 (0.23)
Lima							
Upstream	W10	128.79 (25.01)	68.07 (10.86)	1.78 (0.39)	2.84 (0.45)	0.44 (0.21)	11.78 (3.10)
	W12	125.10 (6.60)	70.20 (1.60)	0.24 (0.02)	2.17 (0.04)	0.48 (0.01)	1.50 (0.10)
	S10	146.67 (38.69)	97.73 (13.94)	0.81 (0.47)	3.98 (2.86)	0.61 (0.02)	15.33 (5.40)
	S12	119.10 (5.20)	56.20 (1.40)	1.29 (0.27)	2.91 (0.63)	0.60 (0.10)	0.16 (0.04)
Downstream	W10	134.56 (18.53)	123.38 (38.72)	1.44 (0.87)	29.74 (16.19)	1.89 (0.98)	27.44 (12.62)
	W12	112.70 (2.20)	111.70 (10.00)	0.57 (0.06)	5.75 (0.28)	0.38 (0.04)	1.01 (0.04)
	S10	178.40 (26.43)	186.17 (29.19)	1.56 (0.79)	54.33 (16.48)	2.33 (0.67)	12.64 (3.66)
	S12	103.10 (8.10)	40.00 (2.40)	0.88 (0.04)	2.35 (0.52)	1.33 (0.55)	0.24 (0.10)

3.3.1. Upstream sites

Differences in AChE activity were found only in relation to sampling season (Fig. VII.2.). Crabs collected in Minho estuary during the winter of 2010 showed higher AChE activity (+50–55%) than those collected in the summer of the same year ($p<0.05$). In 2012, no significant differences

between sites or seasons were found. Also, AChE activity was remarkably higher in the summer 2012 (~3 folds) than in the summer 2010 ($p<0.05$). Significant differences were recorded between the winter 2010 and 2012 in crabs collected from the Lima estuary, with higher AChE activity in 2012 ($p<0.05$). Conversely, LDH activities registered in the summer 2010 were higher than those determined in the winter, reaching +40% in LU crabs ($p<0.05$) (Fig. VII.3.). Significant differences between sites (+35% in LU crabs compared to MU) were found during the summer 2012 ($p<0.05$). Considering IDH activity, MU crabs exhibited significantly lower values in the summer compared to the winter (60–70%) in both 2010 and 2012 (Fig. VII.3.). In the winter of the two years, IDH activity was higher in MU than in LU (>50%) ($p<0.05$).

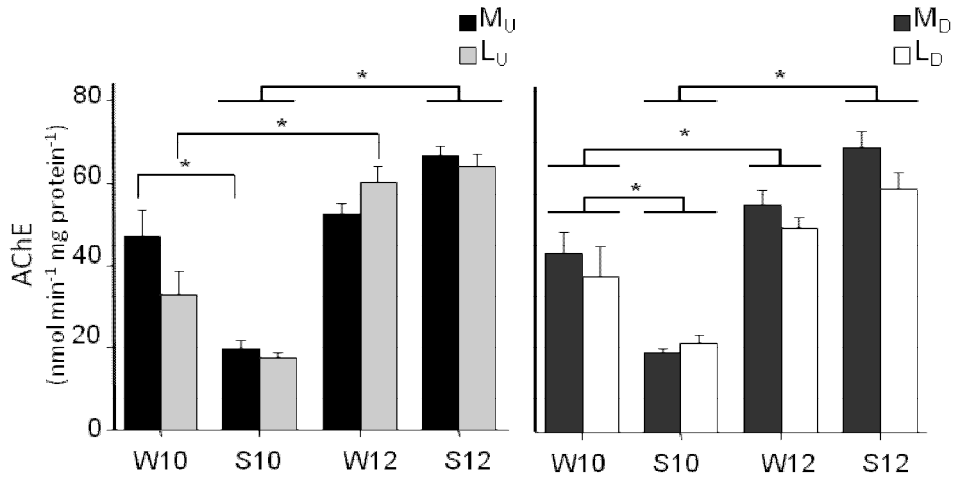


Fig. VII.2. Mean and corresponding standard error of acetylcholinesterase (AChE) activity determined in the muscle of crabs from the Minho (Seixas, *MU* and Moledo, *MD*) and the Lima estuaries (Porto Velho, *LU* and Cabedelo, *LD*) in the winter and summer 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively). Significant differences are identified by asterisks (one–way ANOVA followed by planned contrasts; * $p < 0.05$).

Clear differences between sampling years, sites and seasons were found for GST activity. GST activity was significantly higher in 2010 in all sampling stations, except in MU during the summer ($p<0.05$) (Fig. VII.4.). Significantly higher GST activity was recorded during the summer ($p<0.05$) of 2010 in LU. In the Minho estuary, significantly higher activity was recorded in the winter 2010 compared to the summer 2010 and to the winter 2012 ($p<0.05$). In 2012, however, the inverse trend was found, with higher activity measured in the summer. Differences between Minho and Lima sites were only observed in winter (>45%) and summer (>90%) of 2010 ($p<0.05$).

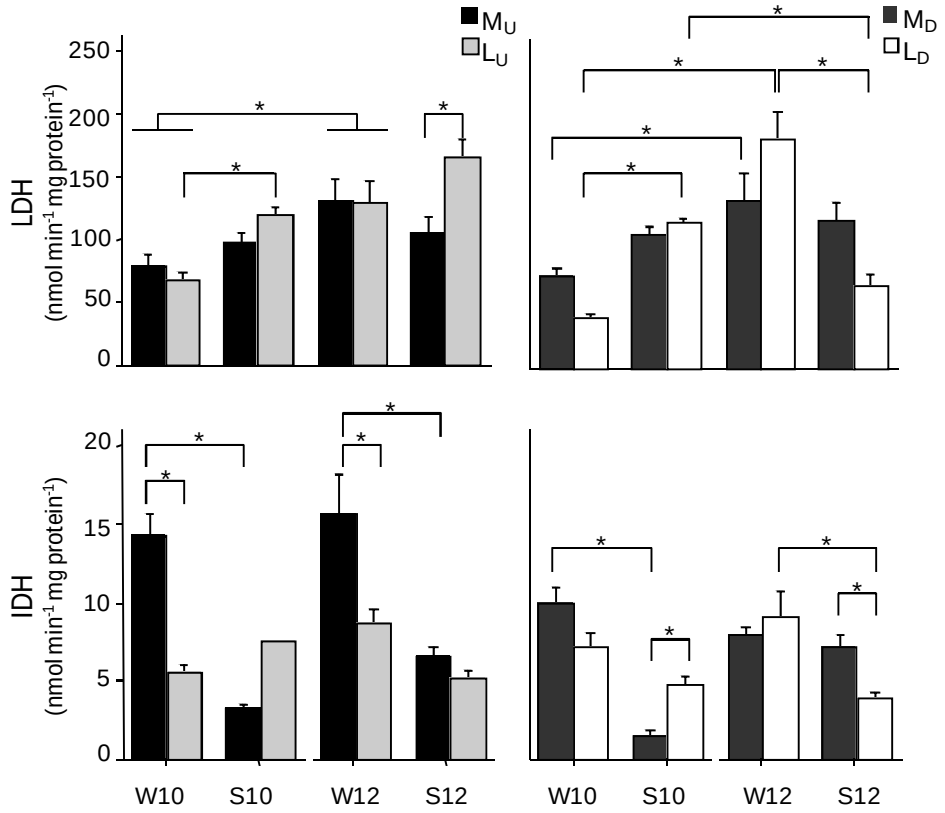


Fig. VII.3. Mean and corresponding standard error of lactate (LDH) and $NADP^+$–dependent isocitrate (IDH) dehydrogenases activity determined in the muscle of crabs from the Minho (Seixas, *MU* and Moledo, *MD*) and the Lima estuaries (Porto Velho, *LU* and Cabedelo, *LD*) in the winter and summer 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively). Significant differences are identified by asterisks (one–way ANOVA followed by planned contrasts; *$p<0.05$).

No significant differences in GPx activity were found between upstream sites (Fig. VII.4.). Activity levels were, however, much lower in 2012 (~80%) than in 2010. GR activity was found to differ between sites in 2010; differences of about 75% and 45% were found during the winter and the summer, respectively ($p<0.05$) (Fig. VII.4.). TG levels varied according to the season and the sampling site (Fig. VII.4.). Levels determined in the winter were higher than those determined in the summer in both years (>30%). Also, in 2010 crabs captured in LU exhibited higher TG levels than those captured in MU, in both the winter and the summer ($p<0.05$). LPO levels were significantly higher (by about 2.5 folds) in LU than in MU crabs during the winter 2010 ($p<0.05$) (Fig. VII.5.). In addition, on average they were higher in the summer compared to the winter, except in LU during the winter 2010 ($p<0.05$).

3.3.2. Downstream sites

Differences in AChE and LDH activity were mainly related to the sampling year and season. AChE activity was lower in 2010 (–22% in the winter and –68% in the summer, $p<0.05$) relative to 2012 (Fig. VII.2.). In addition, in 2010 lower activity (–50%) was found in the summer, relative to the winter season ($p<0.05$). As to LDH, in 2010 higher activity was found in the summer than in the winter particular in LD (by ~2.8 folds, $p<0.05$) (Fig. VII.3.). The inverse pattern was found in 2012 with higher activity in LD crabs during the winter (~2.7 folds) compared to the summer ($p<0.05$) (Fig. VII.3.). LDH activity in LD crabs was also much higher (about 4.5 folds) in the winter 2012 than in the winter 2010. Analysis of IDH activity indicated differences between sites of 3 and 1.8 folds during the summer 2010 and the summer 2012, respectively ($p<0.05$), although in 2010 higher activity was observed in LD crabs and in 2012 higher activity was found in MD crabs (Fig. VII.3.).

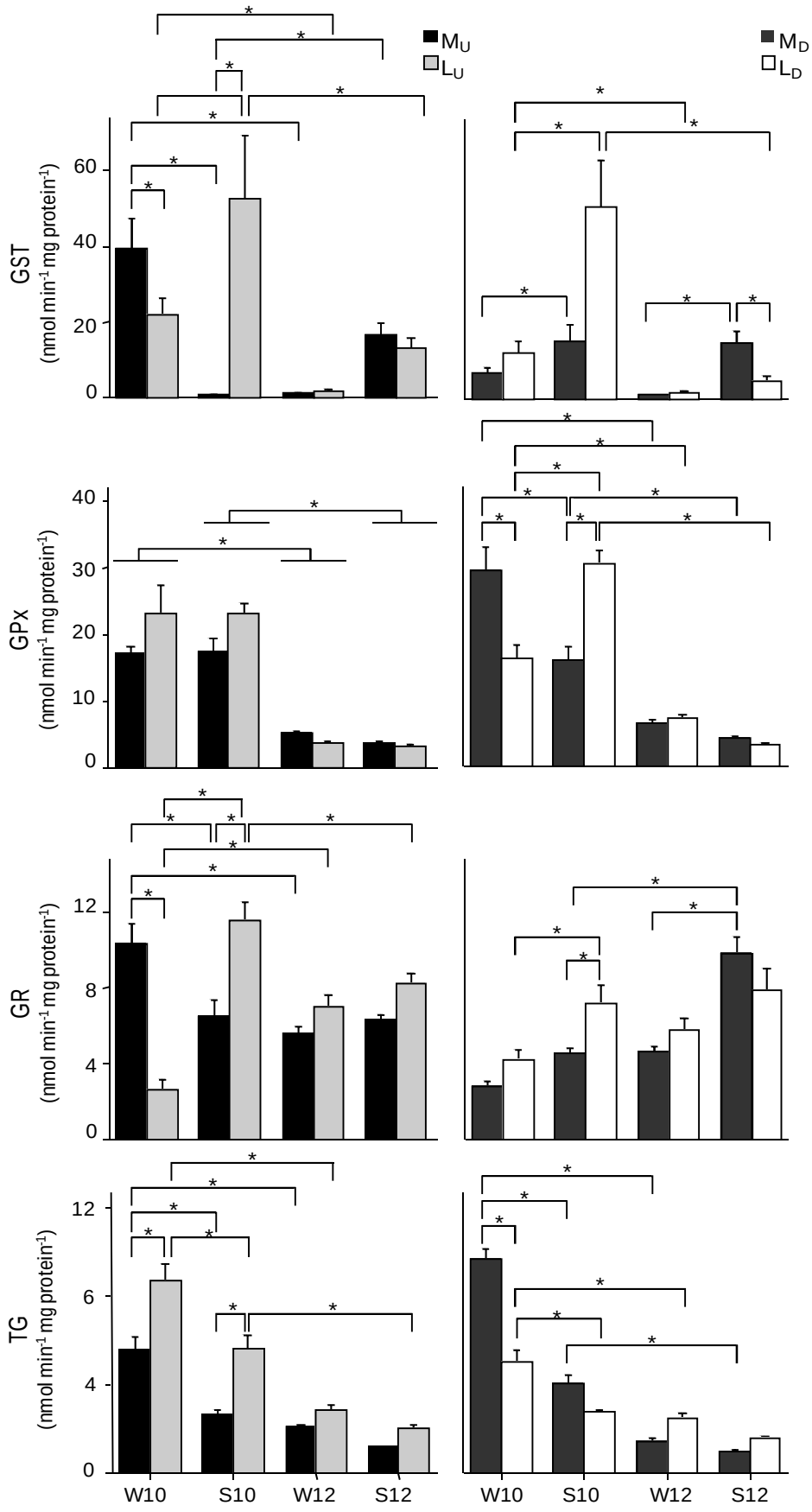


Fig. VII.4. Mean and corresponding standard error of glutathione *S*–transferases (GST), glutathione reductase (GR) and glutathione peroxidase

(GPx) activity, and the total glutathione (TG) level determined in the digestive gland of crabs from the Minho (Seixas, *MU* and Moledo, *MD*) and the Lima estuaries (Porto Velho, *LU* and Cabedelo, *LD*) in the winter and summer 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively). Significant differences are identified by asterisks (one–way ANOVA followed by planned contrasts; * $p < 0.05$).

GST activity was strikingly higher during the summer 2010 in LD (over an order of magnitude in some cases) than in the remaining sampling campaigns ($p<0.05$) (Fig. VII.4.). Apart from that, GST activity was on average higher in the summer than in the winter and in 2010 compared to 2012 ($p<0.05$). Concerning GPx, higher activities were recorded in 2010 compared to 2012 ($p<0.05$) (Fig. VII.4.). Differences between sites were found in 2010. In this year MD crabs exhibited higher (+45%) activity in the winter than LD crabs, whereas the inverse pattern was observed in the summer ($p<0.05$).

In 2012 no significant differences were found among sites or seasons (Fig. VII.4.). As in crabs collected in upstream sites, GR activity was always higher in the summer (Fig. VII.4.). Significant differences between sites (~60%) were observed in the summer 2010 ($p<0.05$). As to seasonal variation, significant differences were observed for LD in 2010 ranging 72%, and for MD in 2012 ranging more than 2 folds. TG levels were higher in 2010 compared to 2012. Significant differences among sites (of about 50%) were observed during the winter 2010 ($p<0.05$) (Fig. VII.4.). Winter levels of MD crabs were also significantly higher (by about 50 to 70%) than levels measured in the remaining sampling campaigns ($p<0.05$) (Fig. VII.4.). LPO levels were significantly higher (by about 2–4 folds) in LD crabs sampled during the summer 2010, relative to the remaining sampling campaigns ($p<0.05$) (Fig. VII.5.).

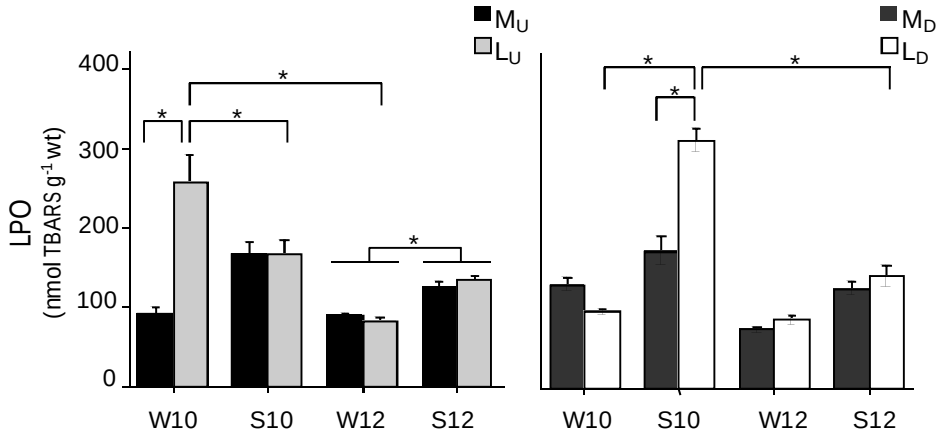
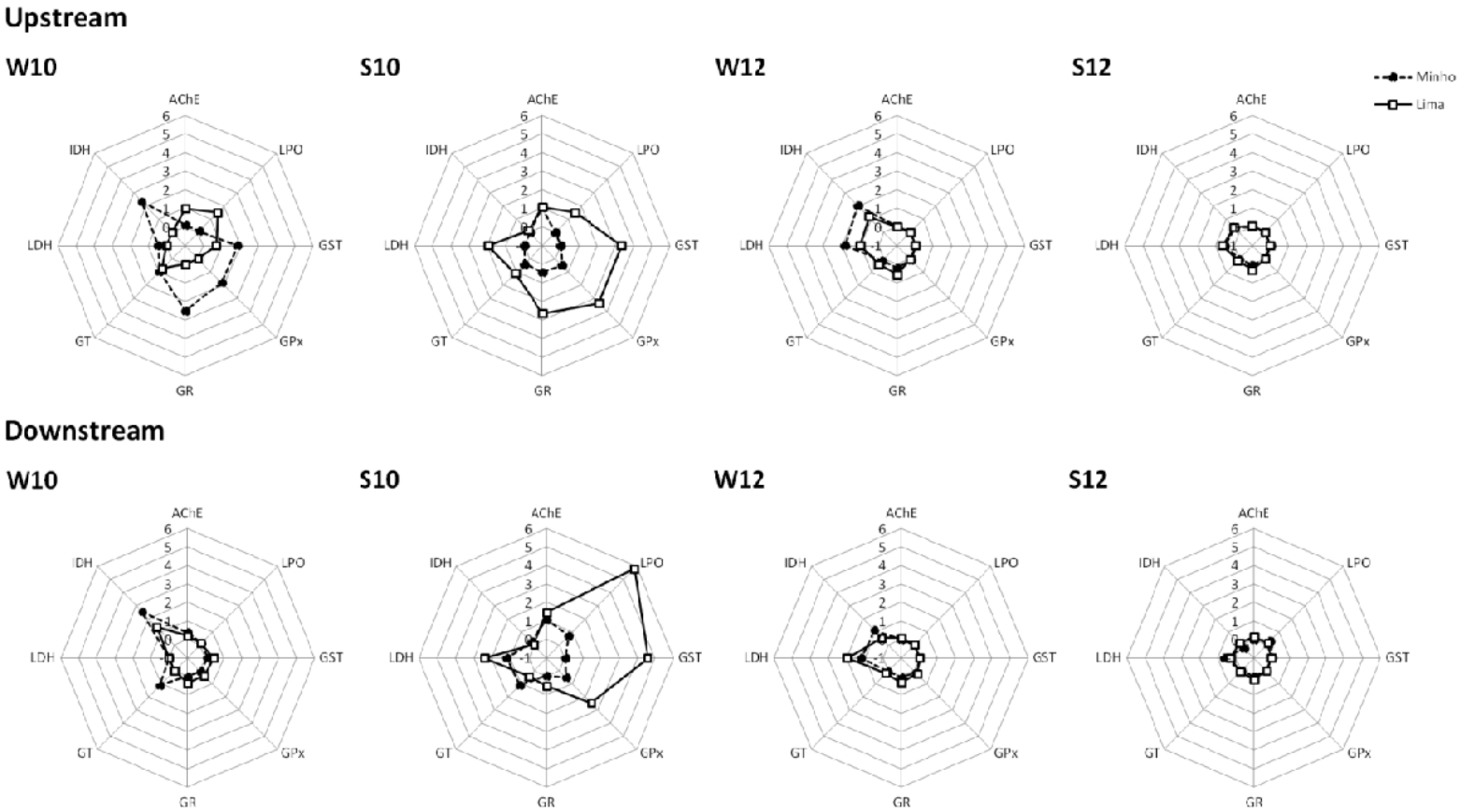


Fig. VII.5. Mean and corresponding standard error of lipid peroxidation (LPO) levels determined in the digestive gland of crabs from the Minho (Seixas, MU and Moledo, MD) and the Lima estuaries (Porto Velho, LU and Cabedelo, LD) in the winter and summer 2010 (W10 and S10, respectively) and 2012 (W12 and S12, respectively). Significant differences are identified by asterisks (one–way ANOVA followed by planned contrasts; * $p < 0.05$).

3.3.3. Integration of multibiomarker responses

IBR indicated distinct stress levels between the Lima and Minho sites in 2010, giving a qualitative measure of the stress to which animals were exposed (Fig. VII.1.). The highest IBR values were found in MU during the winter 2010 and in LU and LD in the summer 2010. IBR values obtained for LD were about 3.5 folds higher than those obtained for MD. The distinction between upstream sites was provided mainly by IDH, GR, GPx and GST in the winter 2010 and by GST, GPx, GR and LDH in the summer 2010 (Fig. VII.6.). The discrimination between downstream sites in the summer 2010 was given by LPO, GST, GPx and LDH. In 2012, all sampling sites showed low IBR values, which were below 4 in the winter and 2 in the summer in MD, LD and LU. Moreover, star plots indicated no clear distinction between sites either upstream or at the mouth of the estuaries. In both years and across sites and seasons, MD was the site exhibiting the lowest stress levels (Figs. VII.1. and VII.6.).

Fig. VII.6. Star plots representing the biomarkers assessed in the present study (acetylcholinesterase, *AChE*, lipid peroxidation, *LPO*, glutathione *S*–transferases, *GST*, glutathione peroxidase, GPx, glutathione reductase, GR, total glutathione, TG, lactate dehydrogenase, LDH, and $NADP^+$–dependent isocitrate dehydrogenase, IDH) used to compute the IBR/n index that were measured in Minho (○) and Lima (▲) estuaries during the winter and summer 2010 (W10 and S10, respectively) and winter and summer 2012 (W12 and S12, respectively).



3.3.4. Linking biomarker responses to environmental variables

In the PCA performed for upstream sites four components expressing 76% of the total variability observed in the data were retained. The first two principal components (PCs) summarised 55% of the total inertia, expressing about 2.6 times more variability than the remaining components (Fig. VII.7.). PCA interpretation was, therefore, based on these PCs. PC1 was mainly linked to heavy metal contamination by Cd, Zn, Pb, and Cr and abiotic factors (Fig. VII.7.). Cd in sediments and tissues, Zn in tissues, the levels of phosphates, silica, dissolved oxygen, conductivity and Fe all showed high positive correlations with the axis, ranging from 0.92 to 0.62. Pb and Cr in sediments, nitrates and salinity showed high negative correlations with the axis, ranging from –0.89 to –0.63 (Fig. VII.7.). Biomarkers associated with this axis were related to anti–oxidant defences (GPx, r = 0.73; TG, r = 0.70), neurotoxicity (AChE, r = –0.65), and to a lesser extent with oxidative stress (LPO, r = 0.38). The axis established a gradient opposing the sampling campaigns showing lower levels of heavy metal accumulation on average (those performed in 2012) to campaigns showing higher bioaccumulation (2010). In particular, 2012 campaigns tended to show lower bioaccumulation of Cd and Zn, and higher AChE activity, as well as higher levels of Pb and Cr in sediments and increased salinity and nitrate levels. Sampling campaigns of 2010 tended to exhibit higher tissue accumulation associated with higher levels of GPx, TG, and LPO, as well as increased levels of phosphates, silica, dissolved oxygen, conductivity, and Fe. PC2 was mainly associated to contamination by Ni, Cr, and Cu in sediments, which showed positive correlations with the axis (ranging between 0.68 to 0.95) and ammonia, water hardness, and pH showing negative correlations with the axis (ranging between –0.77 to –0.92). LDH was also significantly associated with this axis (r = 0.35). The axis produced a gradient opposing sampling sites tending to show higher levels of Ni, Cr and Cu in sediments but lower levels of ammonia, pH and hardness (Minho in the winter 2010) to sites under stronger influence of these abiotic factors and increase LDH activity (Lima in the summer 2012).

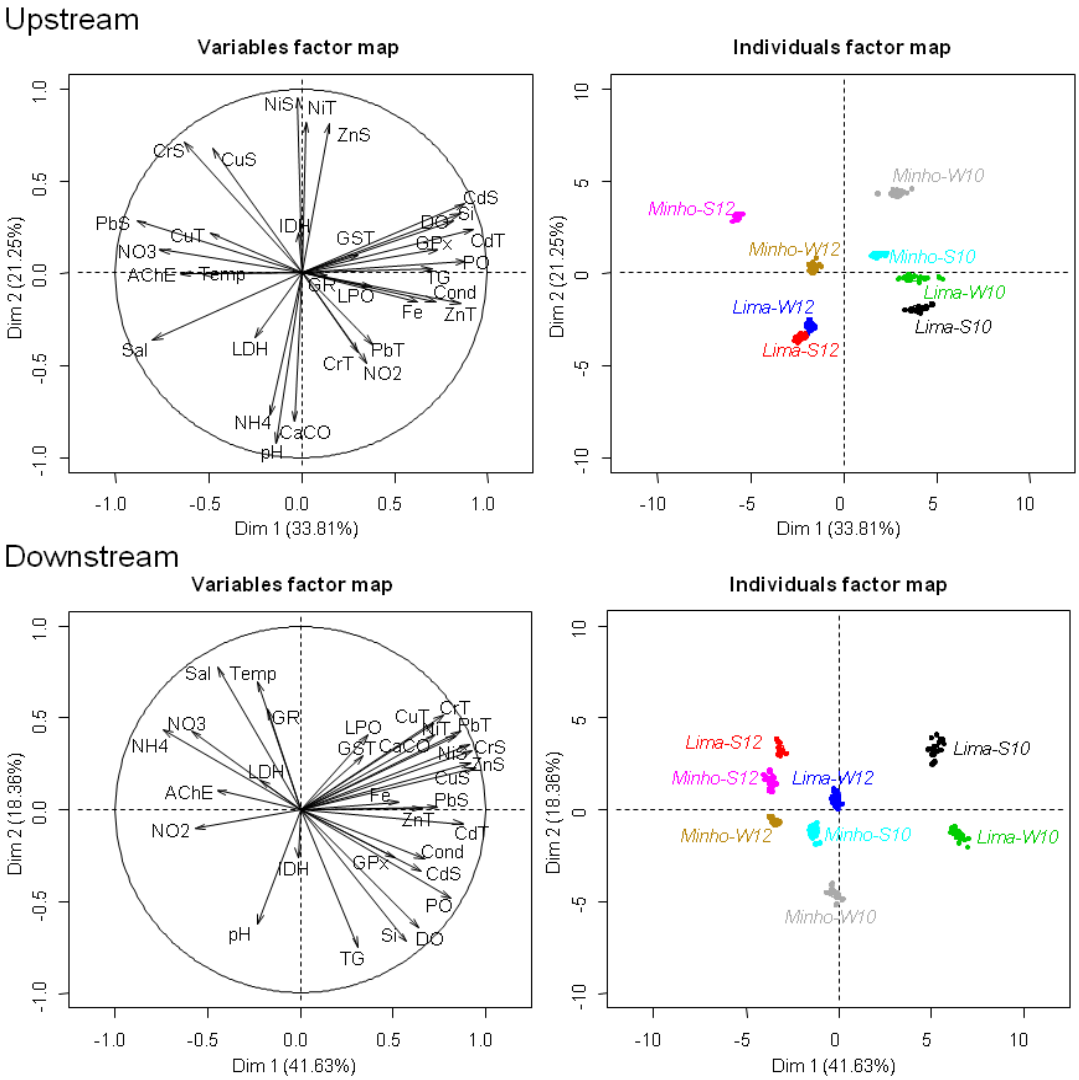


Fig. VII.7. Results of the PCA defined by the biomarkers in *C. maenas,* the abiotic variables, and the concentrations of metals in sediments and tissues. Legend provided in Tables VII.1. and VII.3.

In the PCA performed for downstream sites, four components expressing 79% of the total variability observed in the data were retained. The first two PCs summarised 60% of the total inertia, expressing three times more variability than the remaining components (Fig. VII.7.). Hence, PCA interpretation was based on these PCs. In this case high correlations were generally observed between the different heavy metals and their levels in sediments and crab tissues. PC1 was highly associated with contamination by Cu, Ni, Zn, Cr, Cd, Pb, and phosphates, all showing

correlations with the axis above 0.80. The biomarkers showing significant positive correlations with the axis were GPx (r = 0.51), LPO (r = 0.37), GST (r = 0.34) and TG (r = 0.31). Ammonia (r = –0.74), nitrates (r = –0.59), nitrites (r = –0.57) and AChE (r = –0.45) showed negative correlations with the axis. The axis opposed Minho (winter and summer) and Lima (summer) sites sampled in 2012, showing lower contamination levels, higher AChE activity and nutrient levels, to Lima (winter and summer) in 2010. The latter tended to show high levels of metals in sediments and tissues as well as higher levels of biotransformation (GST), anti–oxidant defences (GPx, TG), and oxidative damage (LPO). PC2 was positively correlated with salinity (r = 0.78), temperature (r = 0.70), GR (r = 0.54), and LPO (r = 0.41) and negatively correlated with pH (r = –0.62), dissolved oxygen (r = –0.64), silica (r = –0.72), and TG (r = –0.75). Sampling campaigns performed in the Lima during the summer tended to be under increased influence of these abiotic factors on average.

4. Discussion

Biomonitoring studies in estuarine habitats are particularly complex due to the numerous variables that might influence biological responses of the organisms. Generally, contaminants are present in complex mixtures and their behaviours are influenced by abiotic factors and their seasonal fluctuations (Farcy et al., 2013). Confounding factors such as salinity, temperature, and nutrient levels must be taken into account because they may influence several biological responses as well as the behaviour of many contaminants (Handy et al., 2003; Tankoua et al., 2011; Rodrigues et al., 2012; Farcy et al., 2013). Multibiomarker responses have been recommended as of great importance for inclusion in programmes with such monitoring requirements (Picado et al., 2007). The present study investigated temporal and spatial trends in heavy metal contamination and biomarkers responses in two NW Iberian estuaries. In particular, in the latest years there was a decrease in contamination inputs to the Lima estuary due to some protection measures and alterations in industrial

activities. The estuary was previously shown to be polluted by heavy metals and other contaminants (Villaverde et al., 2008; Guimarães et al., 2012), thus providing an interesting context to evaluate the sensitivity and usefulness of multibiomarker responses to assess potential amelioration of polluted sites.

Measurements of water temperature and salinity in the study sites indicated high increases in 2012, particularly in the summer, compared to 2010 and to previous water quality assessments performed in 2006 in MU, MD and LD (Guimarães et al., 2012). Nutrient levels (nitrites, nitrates, phosphates) were also increased in the summer 2012, compared to previous years, denoting some level of eutrophication and loss in water quality. This variation is consistent with the climatological context of 2012 in Portugal and the expected trends of occurrence of extreme climate events (Lima et al., 2014). This was a year of significant drought and warm temperatures, triggering high number of forest fires.

The consequently diminished River flow and increased tidal influence should have contributed to the marked alterations in temperature and salinity. These conditions may have also promoted the concentration of nutrients originating from nearby agricultural and other human activities, which under regular precipitation levels would be diluted to safe quality levels. The concentrations of heavy metals found in sediments of the study sites were in general low, in many instances below the “effects range–low” (ERL) derived by Long and colleagues (1995). For some metals and sampling campaigns, however, they exceed either the ERL or the “effects range–medium” (ERM), the range within which detrimental effects would frequently occur (Long et al., 1995).

Worrisome levels of heavy metals were found especially in 2010, which decreased to levels below ERLs in 2012. In 2010, LD exhibited concentrations of Cd, Cu and Ni above the ERM and concentrations of Zn and Cr above the ERL. This site showed the highest total sum of heavy metals. In 2012, only Zn, Cu and Ni persisted in levels above the ERL. As to the Minho estuary, in 2010 MU showed levels of Ni (>ERL) and Cd (>ERM) raising concern; in MD Cd levels were also above the ERM. The presence of

several industries nearby the Lima estuary (*e.g.*, paper–mill, shipyard, agriculture) and its high demographic density should have contributed to the elevated concentrations of metals observed in 2010. However, in recent years there was a closure (a shipyard included) or decrease in activity of many industries in this region, which together with some protection measures were main drivers of reduction of pollutant levels observed in 2012. In general, the contamination levels found in the Lima and Minho sites were considerably higher than those observed in 2006 (Guimarães et al., 2012). Exception to this trend was MD in 2012, which contamination levels were back to the levels reported in 2006. The levels of Cd found in this study were above those found for other Portuguese sites showing moderate levels of contamination (Caçador et al., 2012; Pereira et al., 2012). For the remaining metals, the concentrations found were below those reported by Pereira et al. (2012) for all sites but LD. Concerning the concentration of metals in tissues, Zn and Cu were those accumulating in higher levels. A previous study carried out in Seixal Bay has shown that *C. maenas* is prone to accumulation of essential metals Cu, Cr, and Zn (Caçador et al., 2012). Moreover, this green crab has been pointed out as good bioindicator of heavy metal contamination in monitoring programmes (Caçador et al., 2012). The tissue levels found by the authors are close to those reported in the present study. As noted by Guimarães et al. (2012) in *P. microps* from the Minho and Lima estuaries, the accumulation of Zn and Cu was lower in the sampling sites with higher concentrations of these metals, namely in the Lima estuary. Abiotic parameters may be responsible for the different patterns of accumulation observed in crabs from these estuaries. Low salinity, for instance, may decrease metal speciation and increase bioavailability (Heugens et al., 2001). On this regard it is of note that, apart from the summer 2012, salinity in the Lima estuary is usually higher than in the Minho, possibly contributing to such dissimilarities.

Although the concentration of contaminants (in sediment and tissues) gives important information on the contamination levels and bioavailability in a given ecosystem, it provides limited evaluation of the possible

detrimental effects that exposure to contamination might cause. The inclusion of biomarkers in integrated chemical and biological monitoring has thus been advised as suitable tool to assess the impact of such exposure and assess good ecological status of water bodies (Allan et al., 2006).

AChE has been successfully used over the last decades as biomarker of neurotoxicity in monitoring programmes. Inhibition of the enzyme activity of 20% or over is taken has indication of exposure to anti–cholinesterasic agents (Ludke et al., 1975). These agents are primarily carbamates and organophosphate pesticides (Galgani and Bocquene, 1990; Rodrigues et al., 2013a). But other contaminants such as metals, PAHs, and pharmaceutical products were also shown to inhibit AChE activity (Frasco et al., 2005; Solé et al., 2010; Rodrigues et al., 2013b). Some of them were found in sediments from the Lima River at moderate concentrations compared with the Minho estuary and other European estuaries (Villaverde et al., 2008; Guimarães et al., 2012; Pereira et al., 2012). Additionally, abiotic factors like temperature and salinity are known to influence AChE activity, including in *C. maenas* (Cailleaud et al., 2007; Tankoua et al., 2011; Rodrigues et al., 2012). In *C. maenas*, neurotoxic effects leading to inactivity of AChE may compromise the ability to capture preys and to escape from predators, and the reproductive behaviour as well, threatening their survival and success (Sorenson, 1973; Mesquita et al., 2011; Rodrigues et al., 2013a). Here a marked increase in AChE activity was noted from 2010 to 2012. Higher AChE was also associated to lower levels of Cd in sediments and tissues and Zn in tissues in upstream sites, and to Cd, Zn and Cu in sediments and tissues downstream, as indicated by the PCA. This is consistent with the work of Frasco et al. (2005), who demonstrated that AChE could be inhibited by these metals. This also suggests that in 2010, animals would generally be at lower health condition. This trend is additionally consistent with the combination of lower contamination and higher salinity observed in 2012. Both could be expected to result in increased AChE activity with low differences between the study estuaries (Rodrigues et al., 2012). Other authors also found

higher levels of AChE measured in digestive gland of *Carcinus aestuarii* associated with lower levels of contaminants in tissues (Ricciardi et al., 2010).

Environmental contamination may increase energy requirements leading to depletion of energy reserves and jeopardizing the success of the organisms and the rapid response for energy demand to cope with life–threatening situations (*e.g.*, predator escape, feeding capacity). LDH is an important enzyme of anaerobic glycolysis. It is responsible for the provision of energy under limiting oxygen conditions and is often associated with burst swimming ability and locomotory–related behaviours, such as feeding and escape to predators, by providing additional muscle power (Wells et al., 2001). Environmental contaminants such as metals, PAHs, pesticides may often trigger increases in LDH activity (Vijayavel and Balasubramanian, 2006; Guimarães et al., 2012; Rodrigues et al., 2013a; Rodrigues et al., 2013b) to cope with the additional energetic requirements imposed by the exposure. Under such situations the energy produced may be redirected from growth, reproduction and regular activities such as feeding. The present results suggest that upstream and downstream organisms react differently to environmental stress. In the summer 2012, Lima crabs exhibited higher LDH activity in upstream sites and Minho crabs showed increased activity in downstream sites. A balance between exposure to abiotic and chemical stress, together with slight differences in size, may be at the origin of these differential responses. Indeed, PCA indicated that LDH activity appeared to be inhibited by Ni and Zn and tended to be increased under conditions of higher salinity, pH, ammonia, and water hardness. It is also of note that besides contaminants, effects of size and salinity on LDH were previously observed in estuarine invertebrates (Tankoua et al., 2011; Rodrigues et al., 2012; Rodrigues et al., *Submitted*).

Under a scenario of contamination, the induction of enzymes related with biotransformation and anti–oxidant defences is considered evidence of enhanced detoxification capacity of toxicants and the reactive oxygen species (ROS) generated during such processes. The glutathione (GSH)

enzymes are important intervenients in the protection against oxidative damages. In the present study, GST, GPx, and GR activity were assessed to evaluate seasonal and inter–annual responses. GST conjugate electrophilic contaminants to ease their excretion from the organism, while GPx converts hydrogen peroxides into water, both with the consumption of GSH and formation of oxidised glutathione (GSSG). GR is responsible for the regeneration of GSSG into GSH to be used by GST and GPx. When one of these mechanisms fails, the entire cycle is compromised. Factors such as contaminant exposure and nutrient levels in the water, food availability, temperature and salinity, which are highly variable over the year, may influence their enzymatic responses (Paital and Chainy, 2010; Guimarães et al., 2012; Ben-Khedher et al., 2013). GST, GPx, TG, and LPO tended to be higher in 2010 and in the Lima estuary and correlated with higher levels of metals, dissolved oxygen and conductivity. GST was highly enhanced in crabs from the Lima estuary particularly in the summer 2010. Increased variability in activity was also observed in this estuary. Other studies reported higher GST levels in organisms from estuaries contaminated with organic compounds and metals (Ait Alla et al., 2006; Martín-Díaz et al., 2008; Guimarães et al., 2009; Pereira et al., 2009; Tankoua et al., 2011; Ben-Khedher et al., 2013). The induction of GST activity, promoting the biotransformation of toxicants, acts to reduce their biological effects. The presence of contaminants other than metals and the possible interaction with seasonal variation in abiotic factors may have also contributed to the variability observed. Altogether these constrains may additionally explain the GST induction found in 2010 in the upstream site of Minho estuary during the winter. In 2012 the activity levels exhibited a marked decrease consistent with the lower contamination in sediments and tissues and the decrease in pollutant inputs. Although GR activity was also increased in the Lima sites during the summer 2010, it appears that this was insufficient to replenish GSH levels, especially in the downstream site, and prevent oxidative damage. GSH is of key importance to the maintenance of intracellular redox homeostasis and defence against oxidative damage. Of note is also the higher GR activity found in 2012 in downstream sites in

association to higher salinity and temperature. Previous studies on the response of *C. maenas* from these sites to salinity challenge showed that the enzyme activity could increase with changes within this range (Rodrigues et al., 2012). In addition, anti–oxidant enzymes are highly influenced by water temperature often showing higher activity levels in warmer seasons (Lushchak, 2011; Guimarães et al., 2012). Despite the changes in GR and GPx activities, the glutathione–mediated anti–oxidant defence mechanisms were overwhelmed and damage to cellular macromolecules occurred as indicated by significant LPO levels detected in Lima crabs compared to those from the Minho estuary. Positive correlations between metal contamination (Cd, Ni, Pb) and LPO levels were also found in *C. maenas* captured from a contaminated lagoon in Tunisia (Ben-Khedher et al., 2013). Altered anti–oxidant defences and increased LPO were also found previously in yellow eel and common goby from the Lima estuary (Guimarães et al., 2009; Guimarães et al., 2012).

The IBR index provided an overall measure of the health status of the organisms under study. The index gives useful qualitative information about environmental stress, even in situations of diffuse pollution and mixture of compounds (Beliaeff and Burgeot, 2002) and has been successfully applied in several species, including under the WFD (reviewed by Marígomez et al., 2013; Beliaeff and Burgeot, 2002; Bocquené et al., 2004; Hagger et al., 2008; Pereira et al., 2011). Here, crabs from the Lima estuary (upstream and downstream) collected in the summer 2010 were at lower health status than those from the remaining sites and seasons. The upstream site in the Minho estuary also showed high IBR during the winter 2010 although clearly lower than the values found in the Lima estuary during the summer. These results were not unexpected as contamination of this site by metals and PAHs was observed in sediments and *P. microps* tissues collected in 2006 (Guimarães et al., 2012), raising the need to further evaluate bioavailability and effects in other resident species. Crabs collected in the downstream site of Minho estuary consistently showed low IBR values and good health status over the two sampling years. In upstream sites, biotransformation and anti–oxidant biomarkers were the

more prominent in terms of diagnosis of environmental stress. Downstream, biomarkers of biotransformation, anti–oxidant defences, anaerobic metabolism, and oxidative damage indicated sites with stronger detrimental effects and lower health status of the organisms. Despite the strong variation in abiotic factors that occurred in 2012, the IBR decreased in the affected sites so that no distinction in health status of the crabs was found between sampling sites either upstream or downstream. Two relevant indications arise from this: the animals were able to cope with the strong variation in water abiotic factors without major health impacts, and, despite the potential interaction between contaminants and abiotic variables and their influence on the biological effects measured, this multibiomarker approach reflected mainly the contamination levels at which the crabs were exposed. Although metal levels in tissues showed only a slight decrease in crabs collected in 2012, animals from the most impacted sites recovered their good health status, which suggests that the accumulated metals are possibly bound to GSH and metallothioneins and, hence not able to cause oxidative stress. Earlier studies have shown that GSH is an important player in the detoxification of free radicals generated from Zn, Cd and Cu through the formation of chelation complexes (Perrin and Watt, 1971; Mason and Jenkins, 1995). In addition to this, in *C. maenas* digestive gland some metals (*e.g.*, Cd, Zn, Cu) may also be bound to metallothioneins (Pedersen et al., 1998), preventing oxidative damage.

PCA clearly discriminated the most contaminated sites and seasons from the less impacted ones. It also provided important associations between environmental variables and the biological effects assessed, supporting the results of the IBR and a deeper comprehension of the spatial/temporal trends in variability. The fact that the biomarkers assessed were moderately associated with the environmental variables suggest that other contaminants, besides those measured here, were also contributing to the lower health status found in the 2010 and particularly in the Lima estuary. Overall seasonal and inter–annual variability in environmental contamination, water abiotic variables and biomarker responses was observed. On top of this, however, the battery of

biomarkers employed was able to reflect differences between the study sites in 2010 and the improvement in health status of the most affected sites in 2012, indicating its usefulness to early diagnosis of remediation measures aiming at the recovery of biotic communities.

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Chapter VIII

General discussion, conclusions, and final remarks

General discussion, conclusions, and final remarks

1. Discussion and conclusions

Over the past years investigation on the effects and impact of environmental pollutants in invertebrates has increasingly evolved to the use of bioassays and biomarkers as biological effects tools providing crucial information for environmental risk assessment (Neuparth et al., 2005; Moreira et al., 2006; Pereira et al., 2009; Aguirre–Martínez et al., 2013a; Aguirre–Martínez et al., 2013b). Biomarkers give useful warning signs of exposure to pollution before the onset of irremediable damage at higher levels of organisation, and thus with higher impact on populations, communities, and ecosystems. In this thesis a combination of laboratory and field studies, and specific and general stress sub-cellular biomarkers was employed. The work addressed effects of important pollutants, and their relation to abiotic stress in populations of *C. maenas* from estuaries under differing levels of environmental contamination, contributing with relevant data and knowledge to environmental risk assessment.

The species chosen was abundant along the north-western Iberian coast and was sensitive to priority and emerging contaminants (EC), allowing to detect tissue accumulation and effects induced by exposure levels with environmental relevance. It also allowed to assess spatial and temporal dynamics of heavy metal bioaccumulation and health status of crabs in the field confirming its interest and recommendation for regional monitoring purposes (Picado et al., 2007).

Apart from the monitoring, it is essential to study sublethal effects of pollutants in estuarine invertebrates, and crustaceans in particular. This allows for better understanding and knowledge on their modes of action and putative impact and recognition of management actions required for protection of species with key structuring roles. Additionally, it provides empirical data to improve predictive risk models, an ever more important issue. Species may, however, show different sensitivity to toxicants due to

inherent characteristics of a particular local area, as their habitat or ecology, or their previous exposure to pollution, which may enhance tolerance or increase sensitivity to additional chemical stress. Also, in face of the complexity of the estuarine environment, particularly in cases of moderate contamination levels where responses may be more prone to confounding from abiotic factors (Pereira et al., 2011), a single biomarker would hardly provide a reliable measurement of the effects of contaminants and the health status of the test species. In this sense, a suite of biomarker responses was used in this thesis. These were intended to elucidate about exposure, response, and susceptibility to contaminants (Timbrell, 1998), allowing an evaluation of the stress induced and potential differences in sensitivity between crabs from the Minho and Lima estuaries. These estuaries are located 20 km apart from each other, and are both mesostratified, but are under different human pressure resulting in differential levels of pollution.

As biomarker tools are likely to be influenced by other biotic and abiotic parameters, the experimental design and sampling procedures gain particular importance in both field and laboratory investigations. These were thus carefully taken into consideration in this Thesis. Sampling procedures were as similar as possible and homogeneous in what concerns the size of the organisms, age, gender, moult stage, which are factors that could influence some biomarkers (Pereira et al., 2009; Mesquita et al., 2011). Measurement of several abiotic factors and nutrient levels in the field, as well as comparison with sites of similar characteristics in the low impacted estuary were essential for the interpretation of chemical data and biological responses in the monitoring study. Measurements of salinity, temperature, pH or ammonium levels during laboratory acclimation and exposure experiments were crucial to minimise confounding and ensure the effects observed were caused by the contaminants under investigation.

The chemical stressors investigated in the exposure experiments elicited alterations in relevant subcellular biomarkers in some cases related to tissue accumulation of the tested substances. Sublethal exposure to waterborne fluoranthene (FLU) (Chapter II) induced concentration-

dependent tissue accumulation in *C. maenas* detectable through both gas chromatography – mass spectrometry (GC-MS) and fixed fluorescence wavelength (FF). The application of the FF method to determine polycyclic aromatic hydrocarbon (PAH) compounds and metabolites in fish bile (Balk et al., 2011; Almeida et al., 2012) and urine and haemolymph of crab species (Dissanayake and Galloway, 2004; Watson et al., 2004) provides important measures of exposure and susceptibility to these toxicants. This is an expeditious method, clearly less expensive than the use of GC-MS. Its application to the determination of FLU and its metabolites in crabs' digestive gland and muscle showed high correlation with determinations by GC-MS in whole body-soft tissues. Because it requires only a very small amount of sample FF also allows obtaining data for each individual analysed, avoiding the need for pooling of whole tissues from several animals as was required to obtain enough analytical sample for GC-MS determinations in the present work. This is an important issue as it provides a measure of the variability observed in response to the exposure.

Interestingly, muscle acetylcholinesterase (AChE) activity, a biomarker of neurotoxicity, was found to be inhibited by FLU. Although literature reports lack some consistency on this, it is thought that lipophilic compounds such as PAHs can pass through phospholipid monolayers increasing membrane unsaturation and hydrophobicity and, hence, influence membrane-bound enzymes (Donato et al., 2000; Roche et al., 2002), as is the case of AChE. Nevertheless, the results of this study support the hypothesis of Kang and Fang (1997) that PAHs with three or more aromatic rings could cause higher inhibition of AChE activity than compounds with less rings. Also relevant was the induction of the glutathione redox system (glutathione *S*–transferases, *GST*, glutathione reductase, *GR*, total glutathione, *GT*, $NADP^+$-dependent isocitrate dehydrogenase, *IDH*) in the exposed crabs that was able to protect against oxidative damage to lipid macromolecules. This is a common response mechanism to chemical stress that allow invertebrates to overcome toxicant challenge and concomitant effects of reactive oxygen species

(ROS) generated during detoxification processes (Lushchak, 2011). Because FLU is a priority substance commonly found in the aquatic environment, in sediments, particulate matter, and water (Baumard et al., 1998; van Hattum et al., 1998), the results raise concern about the impact of continued exposure to this PAH.

Of the biomarkers employed in the above study those involved in cholinergic transmission, energy metabolism, biotransformation, and oxidative stress were the most sensitive, with effects at environmentally relevant concentrations, and thus most promising for use in subsequent studies. However, though previous works showed that depending on the crustacean species, and its natural habitat, both hypo- or hypersalinity conditions could influence ROS and the activity of some anti-oxidant enzymes (Freire et al., 2011), data on the effects of this natural stressor on biotransformation and oxidative stress biomarkers of *C. maenas* were not available. This issue also gained importance due to the increase in frequency of extreme events, resulting from the actual climate changes scenario, which drives important salinity shifts. The experiments of Chapter III revealed that salinity was able to influence several biomarkers in *C. maenas.* Salinity challenge altered not only AChE, as previously observed for other invertebrates (Scaps and Borot, 2000; Pfeifer et al., 2005; Menezes et al., 2006; Cailleaud et al., 2007), but also the activity of anaerobic and aerobic energy production enzymes (lactate dehydrogenase, *LDH*, IDH). It also caused alterations in anti-oxidant defences (GR, TG) and the levels of oxidative damage (lipid peroxidation, *LPO*), confirming the hypothesised influence on oxidative stress biomarkers. Interestingly, for several of these parameters this influence was dependent on the sampling site. Indeed, organisms collected at the Minho estuary (low pollution) exhibited alterations in AChE and GR activity upon on salinity exposure in the range of 5 to 45 psu. The effects of salinity were more dramatic in Lima crabs, triggering alterations in AChE, LDH, IDH, GR, TG, and LPO. The responses of AChE suggest an increase in cholinergic transmission due to greater locomotory activity at low salinity in Minho and high salinity in Lima crabs. This is consistent with previous experiments by Bolt and

Naylor (1985), who have shown that walking activity of estuarine *Carcinus* augments under low and high salinities to avoid both hypo- and hypersaline environments. Also, generation of ROS and the development of oxidative stress may occur in response to different stressors, one of them being osmotic challenge (Lushchak, 2011). Upon exposure to the stressor the equilibrium between pro-oxidants, such as ROS, and anti-oxidants molecules and enzymes, as for instance those of the glutahione redox system, may be disrupted leading to oxidative damage (Livingstone, 2001; Lushchak, 2011). According to the results, *C. maenas* from the Lima estuary were more susceptible to salinity stress than crabs from the Minho estuary. Chronic exposure of the former to environmental contamination therefore appeared to affect sensitivity of these organisms. Salinity may represent an additional stress possibly increasing ROS generation more easily in Lima crabs. Noteworthy is the observation that depending on the origin of the crabs, both hypo- and hypersalinity were found to influence biomarkers of neurotransmission, aerobic energy production, and anti-oxidant defences, as previously noted by Freire et al. (2011). These results deepened knowledge on how physiological processes directly involved in the responses to pollution are modulated by salinity in a key estuarine crustacean. The work also clarified the need to address potential confounding effects of salinity in biomonitoring studies using these biomarkers.

The differential responses to salinity stress displayed by Minho and Lima crabs, though not unexpected, supported follow-up investigations to address their sensitivity to environmental contamination and its possible consequences to environmental risk assessment. Exposure to the model organophosphate (OP) fenitrothion (FEN) confirmed differential sensitivity of crabs from the low impacted and the polluted estuary (Chapter IV). *In vitro* studies revealed higher sensitivity of Lima crabs to FEN than those from the Minho estuary. Surprisingly, in *in vivo* experiments, crabs from the moderately polluted Lima estuary were more tolerant to the compound than those from the low impacted site. Mainly, Lima crabs exhibited lower mortality, lower inhibition of AChE, enhanced phase II biotransformation

and anti-oxidant defences. In contrast, Minho crabs showed higher mortality and AChE inhibition, lower biotransformation induction and inhibition of anti-oxidant enzymes. AChE inhibition is the primary mode of action of OP. In arthropods several mechanisms have been described that may enhance tolerance to these compounds. Namely, decreased sensitivity of the target site conferred by mutations in this enzyme, reduced uptake of the contaminant and increased metabolism and/or sequestration by detoxification enzymes such as carboxylesterases (CbE) or GST. Mutations in AChE appear to confer much stronger differences than those observed in the present study (Roush and McKenzie, 1987; Dunley et al., 1991; Taylor and Feyereisen, 1996). The fact that an inverse response pattern was observed between *in vitro* and *in vivo* sensitivity also suggests that mutations affecting the physiological AChE target should not be responsible for the moderate tolerance of Lima crabs. This is further supported by a previous investigation on the genetic structure of *C. maenas* collected in several sites spreading along a 1,200 km stretch of the Iberian coast, encompassing the studied estuaries (Domingues et al., 2010a; Domingues et al., 2010b). Based on studies with microsatellite markers the authors found no significant genetic differentiation of these populations. A role for GST, and possibly CbE, in the enhanced tolerance seems therefore plausible and consistent with exposure in a moderately polluted estuary (Lima). CbE are known to participate in the metabolism of OP (Jokanović et al., 1996; Wheelock et al., 2008), including in crustaceans, by sequestering the compounds, which become less available to inhibit AChE activity (Wheelock et al., 2008 and references therein). GST acts on the detoxification of FEN by demethylation in an O-alkyl conjugation reaction. This reaction occurred in a limited extent in Minho crabs, relative to Lima ones. This limitation could be related to depletion in the glutathione molecule, as indicated by the reduced glutathione levels measured and the inhibition of GR and GPx activities in Minho crabs. The glutathione molecule serves as an acceptor of methyl groups in the demethylation of this compound (Eto, 1974) and is important in the detoxification pathway of FEN in other crustaceans (Bhagyalakshmi et al.,

1984). The study confirmed the differential sensitivity to contamination of crabs from the study sites and pointed out the involvement of the glutathione redox system in enhanced tolerance to this OP.

Exposure to sertraline (SERT) (Chapter V) also brought interesting, unexpected results. Increasing knowledge on the possible effects of antidepressants in non-target species is of utmost importance. Indeed, from current disease trends it has been estimated that by 2030 depression will be one of the three leading causes of burden of disease worldwide (Mathers and Loncar, 2006). In this context, consumption of antidepressants and the risk of contamination of the aquatic environment will certainly increase in the next decades. In this Thesis, following exposure to sertraline also different accumulation levels and biomarker responses were observed in Minho and Lima crabs. Tissue accumulation of this antidepressant was lower in Minho crabs, which also showed non-monotonic responses of ganglion AChE and GST activity, with induction at low environmental concentrations and return to control values at higher exposure levels. For muscle AChE only increasing activity was observed, relative to controls. These results were in good agreement with previous findings for other similar selective serotonin reuptake inhibitors (SSRIs) in marine crustacean (Guler and Ford, 2010; Mesquita et al., 2011). These reported increases in AChE activity, in some cases linked to altered locomotory behaviour, as well as biphasic responses in behavioural traits. However, in Lima crabs, in addition to higher tissue accumulation, SERT mainly caused important decreases in ganglion AChE (AChEg) and muscle AChE (AChEm) activity, indicative of ventilatory and locomotory dysfunction, inhibition of anti-oxidant enzymes and increased oxidative damage at a concentration as low as 0.05 µg L^{-1}. The different responses of AChE activity may be caused by a crosstalk between serotonergic and cholinergic neurotransmission. Recent evidence was provided by studies with *Ceriodaphnia elegans*, which showed that serotonergic neurons, and serotonin (5-HT) signalling, regulate cholinergic neurotransmission through both stimulatory and inhibitory inputs (Govorunova et al., 2010). SERT may possibly induce both stimulatory and inhibitory responses

through differential affinity to receptor subtypes, or differential activation of the same receptor in different neurons. There is also the possibility that SERT may directly inhibit AChE (Müller et al., 2002) since it was previously observed SSRIs could inhibit its activity in human serum and erythrocyte membrane through a labile interaction (Müller et al., 2002). Though the mode by which these differential responses occur deserve further investigation, up-regulation of AChE expression and activity is typical of apoptotic cells (Zhang and Greenberg, 2012), suggesting the crabs are under serious distress. On the other hand, decreased cholinergic transmission or AChE inhibition may depress crabs ventilatory and locomotory functions (Sorenson, 1973; Simmers and Bush, 1983), creating feeding difficulties and increasing the risk of predation.

The increased levels of catalase (CAT) observed in the exposed crabs also indicate that SERT elicits the production of ROS leading to oxidative stress. In animals with a history of exposure to moderate pollution, these effects could not be compensated, causing oxidative damage to lipid macromolecules.

SERT bioaccumulation and toxicity towards *C. maenas* was additionally influenced by salinity (Chapter VI). In crabs from both sites, increased bioaccumulation was found in seawater (35 psu, *SW*) for SERT concentrations within the range of predicted environmental levels for hospital effluents (5 µg L^{-1}), though this increase was comparatively stronger in Minho than in Lima crabs. Responses of muscle AChE, LDH, IDH, GST, GR, and CAT were dependent on the salinity level as previously observed. Both synergistic and antagonistic effects were identified depending on the SERT and the salinity levels. Moreover, interaction differences were found for Minho and Lima crabs, again suggesting an influence of the previous history of exposure to moderate pollution. For example, synergism in AChEm of Minho crabs resulted in higher than expected enzyme activity. Conversely, higher than expected AChEm inhibition in Lima crabs was caused by synergism in the low SERT concentration and antagonism in the high SERT level. Effects of the interaction between SERT and salinity on AChEm may be mediated by *eat-*

6, a gene that encodes a *C. elegans* Na^+/K^+-ATPase α subunit (Govorunova et al., 2010). Homology searches revealed that *eat-6* is expected to share about 76% identity with a Na^+/K^+-ATPase α subunit of *C. maenas* at the protein level. This gene belongs to the pathway that couples 5-HT signalling and cholinergic neurotransmission. Furthermore, it regulates pre- and postsynaptic cholinergic neurotransmission at neuromuscular junctions located in body wall (Govorunova et al., 2010). In the present case, Na^+/K^+-ATPase is active in *C. maenas* leg nerves (Skou, 1957) and is also involved in the regulation of extracellular osmolytes by driving the increased uptake of Na^+ and Cl^- across the gills of this species (Towle and Weihrauch, 2001).

Synergistic and antagonistic interactions were also found for biotransformation and anti-oxidant enzymes, which reflected dose-level dependent effects. In Minho crabs combined effects were identified for GST, GR, CAT, and LPO. Although these interactions resulted in higher than expected LPO in SW, the levels measured were near control values, hence, apparently not life threatening. In Lima crabs combined effects were found for LDH, IDH, and GR. In these crabs the combined exposure appeared to be energetically more costly. As previously observed, oxidative damage was of significant importance in these crabs. However, it was dependent only on SERT exposure.

Globally, crabs from the low impacted site were more sensitive to FEN but comparatively less affected by salinity stress and SERT exposure. Those with a past history of exposure to chemical stress were more tolerant to FEN but showed stronger detrimental effects of salinity and SERT.

The field investigations (Chapter VII), carried out in 2010 and 2012, showed that heavy metal contamination in sediments globally increased from 2006 to 2010 (Guimarães et al., 2012). Additionally, crabs from the mouth of Lima estuary exhibited lower health status than those from the Minho site. However, in 2012, contamination by metals of Lima sediments decreased to values only slightly higher than those found in 2006 for this location, while the crabs' health status improved. The industries and

maritime traffic in the area of the Lima estuary and its high demographic density were the probable cause for the contamination found in 2010. In recent years several of them closed or decreased their activity (shipyard included), contributing to the decrease in contamination. The high River flow resulting from intense raining conditions may also have caused a resuspension of metals from sediments into the water column that may have ended up in open sea.

As expected, important correlations between abiotic parameters, heavy metals in sediments and tissues, and the biomarkers measured were found. The integrated biological responses (IBR) index provided an overall measure of the health status of the organisms under study and their positive health recovery. Crabs collected at the mouth of Minho estuary consistently showed low IBR values and good health status over the two sampling years. In upstream sites biotransformation and anti-oxidant biomarkers were the more prominent in terms of diagnosis of environmental stress. Downstream, biomarkers of biotransformation, anti-oxidant defences, anaerobic metabolism, and oxidative damage indicated sites with stronger detrimental effects and lower health status of the organisms. In spite of the enhanced tolerance to metals and pesticides previously detected in Lima crabs, the combination of chemical and biological parameters used allowed to detect variations in the health status of animals from the more heavily contaminated estuary. Another relevant aspect is that the biomarkers assessed were moderately associated with the environmental variables, heavy metals, and abiotic parameters. This suggests that other contaminants, besides those measured in this study, were also contributing to the lower health status found in the 2010 and particularly in the Lima estuary. Altogether the results evidenced seasonal and inter-annual variability in contamination, water abiotic variables, and biomarker responses. On top of this, however, the battery of biomarkers employed was able to reflect differences between the study sites in 2010 and the improvement in health status of the most affected sites in 2012, indicating its usefulness to early diagnosis of remediation measures aiming at the recovery of biotic communities.

Overall, the results of this Thesis raise concern about the differential levels of contamination found in these estuaries and their impact not only in the health status of the species but also in relation to the assessment of the effects of contaminants. Historical exposure in even moderately contaminated sites was able to induce enhanced tolerance to cadmium and FEN (Mesquita et al., s*ubmitted*; Chapter IV) in an ecologically relevant decapod, but increased sensitivity to salinity and SERT. This is particular relevant when considering that data employed to derive species sensitivity distributions or predicted no effect concentrations for hazard risk calculations are usually based on bioassays assessing conventional endpoints (*e.g*., survival, growth, and reproduction) performed with standard species (or clones), or organisms originating from pristine or low impacted sites. According to the results of this Thesis estimates derived from such procedures may lead to either over or underestimation of the risk. In the first case (FEN) these estimations may be too protective; in the second case they may not be sufficiently protective, as detrimental effects may go undetected using such an ecotoxicological approach. For SERT for example (Chapter V), the study demonstrates the occurrence of changes potentially affecting the population, in crabs from the moderately impacted estuary at concentrations four orders of magnitude lower than those observed for crabs from the low impacted site. Such life threatening alterations would not be perceived if regular testing approaches would be used. In the same line of reasoning, impaired health status was found for crabs from the moderately polluted site at concentrations about three orders of magnitude lower than persistent changes found in the most sensitive freshwater species tested up-to-date, using reproduction as endpoint (Henry et al., 2004).

Relative to species sensitivity distributions, the results support the need for tolerance data on more species, from more taxonomic groups from widespread localities, in order to better meet the required assumptions previously adressed by Kefford et al. (2005) and Hickey et al. (2008). As suggested by these authors, the use of rapid toxicity tests, which are less rigorous than conventional tests, to approximate experimentally the

sensitivity of many species quickly and in approximate proportion to naturally occurring communities may be a more efficient approach. These tests are less rigorous but require less effort to conduct, relative to conventional testing. They thus allow the quick generation of data for many species and the construction of less biased species sensitivity distributions (Kefford et al., 2005). Globally, the results of this Thesis indicate the importance of tailored site-specific criteria and risk assessment procedures, involving testing with local ecological receptors, and accounting for dynamic natural and man-induced environmental change, to improve accuracy in extrapolation from laboratory testing to field conditions.

2. Final Remarks

The studies presented in this Thesis have provided new knowledge on how priority and ECs affect an important estuarine decapod typical of Portuguese systems, and their implications to environmental risk assessment. The specific conclusions of the work developed may be summarised as follows:

- Significant accumulation of waterborne FLU was found in tissues of *C. maenas* following subacute exposure to environmentally relevant concentrations. The bioaccumulation observed was accompanied by increases in detoxification and enzymatic anti-oxidant defences in the digestive gland, as well as neurotoxic effects in the muscle translated in inhibition of acetylcholinesterase activity;
- Fixed wavelength fluorescence measurements provided the most sensitive endpoint of exposure and a cost-effective, expeditious method to assess the uptake and availability of FLU and its metabolites for potential dietary transfer to higher trophic levels;
- Exposure to salinities ranging from 4 to 45 psu significantly influenced *C. maenas* biomarkers of neurotransmission (AChE), energy metabolism (LDH, IDH), anti-oxidant defences (GR, TG) and oxidative damage (LPO), providing new data on modulation of physiological processes and setting

the need to account for its influence in pollution monitoring programmes;

- Depending on the site of origin of the experimental crabs (the low impacted or the moderately contaminated estuary), both hypo- and hypersalinity were found to influence those biomarkers; salinity stress had more negative effects in crabs originating from the moderately polluted estuary significantly influencing AChE, LDH, IDH, GR, TG, and LPO;
- AChE was confirmed to be the most predominant cholinesterase form in *C. maenas* muscle and ganglion, confirming its interest for inclusion in monitoring and environmental risk assessment programmes;
- Similarly to salinity, differential sensitivity to FEN (lethality and AChE inhibition) was observed for crabs originating from the low impacted and the moderately polluted estuaries; the enhanced tolerance probably occurs by way of induction of the glutathione redox system;
- Subacute exposure to SERT elicited relevant alterations in biomarkers of neurotransmission (related to ventilatory and locomotory functions), anti-oxidant defences and oxidative damage, indicating their usefulness to assess environmental contamination by this psychopharmaceutical;
- Significant effects of SERT were found in the absence of relevant bioaccumulation of the compound or its main metabolite;
- Crabs from the polluted estuary were much more sensitive to SERT than those from the low impacted one (by four orders of magnitude) and than the most sensitive freshwater species tested up–to–date using reproduction as endpoint (by about three orders of magnitude);
- Both antagonistic and synergistic effects of SERT and salinity were observed, which for some biomarkers were different at low and high concentrations, and were influenced by the estuary of origin of the crabs;
- Spatial and temporal variation in heavy metal contamination and bioaccumulation suggest an improvement of the quality of the polluted estuarine from 2010 to 2012;
- Differences in heath status of crabs from the contaminated estuary were found, consistent with their higher sensitivity to salinity stress and the emerging compound tested;
- Overall, chronic exposure in even moderately contaminated sites may

elicit differential sensitivity to contamination in an ecologically relevant decapod, despite its broad adaptation ability, which favours its well known invasive behaviour;

- Adaptation processes leading to both enhanced tolerance or sensitivity appeared to occur by way of altered responses of biotransformation and anti-oxidant defences, including the glutathione redox system, which should be taken into consideration to improve site-specific monitoring and environmental risk assessment;
- To improve the ecological relevance of risk estimations, ecotoxicological testing for environmental risk assessment of toxicants, including selective serotonin reuptake inhibitors should be broadened to include assessments of populations with different backgrounds and more sensitive biomarkers related to their mode of action. For SSRIs broad concentration ranges should be tested to encompass the possibility of different responses elicited by low and high exposure levels.

3. Future perspectives

From the work carried out several questions emerged that opened perspectives for future research. The results of Chapter II suggest the determination of PAH-type compounds (parent compounds and metabolites of naphthalene, pyrene, benzo(a)pyrene) in digestive gland and muscle by fixed wavelength fluorescence as useful in pollution monitoring programmes. Application of this method to field samples may provide a rapid and inexpensive alternative to determine exposure to PAHs, the origin of PAH contamination (*i.e.* petrogenic, pyrogenic) and the extent of accumulation in tissues. Investigation of this hypothesis in future studies should focus on the measurement of PAH-type compounds and metabolites in urine and haemolymph samples through this method, for validity purposes, in ecosystems with different levels of PAH contamination. These measurements should be correlated with chemical determinations by GC-MS and biomarkers of biotransformation and oxidative stress to better evaluate this possibility.

Results of the exposure to FEN revealed important contributions of the glutathione redox system to differential sensitivity that should be addressed in future studies. The experiments should include exposure of *C. maenas* to FEN in combination with specific inhibitors of CbE activity and/or of glutathione synthesis. Measurement of CbE activity and reduced and oxidised glutathione levels under such exposure conditions will provide clear confirmation of the involvement of this system in enhanced tolerance to the organophosphate.

Exposure experiments carried out with crabs from both estuaries suggested that the Na^+/K^+-ATPase α subunit, involved in the *C. maenas* osmoregulation may play a role in the modulation of cholinergic responses elicited by SERT. Complete sequencing of the Na^+/K^+-ATPase α subunit of *C. maenas* and investigation of its gene and protein expression in both *in vitro* and *in vivo* studies will bring new pertinent knowledge to this issue. Such data should be compared with the determination of cholinergic, serotonergic, and dopaminergic neurotransmitters to elucidate the mechanisms behind AChE alterations and the interactive effects found between SERT and salinity. They may also provide additional early warning biomarkers of exposure to SSRIs in marine invertebrates. *In vitro* assays with SERT may also clarify a possible inhibitory effect of the compound in the enzyme activity, and other possible mechanisms by which differential responses may occur in Minho and Lima crabs.

The field study indicated that routine monitoring of the estuaries investigated in this thesis should be carried out in the future to confirm whether there is a sound improvement of environmental quality in the Lima estuary or just a transient amelioration. Additionally, the use of rapid toxicity tests performed with a panoply of different compounds and ecological receptors from distinct representative taxonomic groups of the study estuaries could provide tolerance data to calculate species sensitivity distributions for risk assessment procedures aiming at supporting management decisions of local authorities.

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