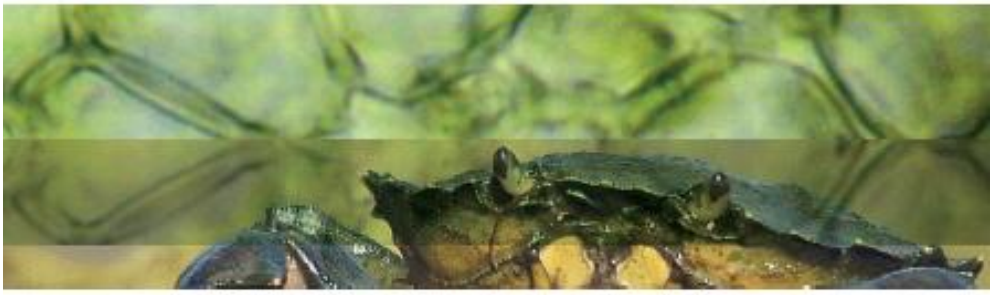
The Effects of Environmental Conditions and Mercury on Common Estuarine Species

An Integrated Ecological and Toxicological Approach

Sónia Isabel Almeida Costa



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The Effects of Environmental Conditions and Mercury on Common Estuarine Species

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Sónia Isabel Almeida Costa

Doctoral dissertation in Biology (Scientific area of Ecology) presented to the University of Coimbra

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CONTENTS

This thesis is based on the following manuscripts:

- **The influence of climate variability on *Carcinus maenas* population structure from a southern temperate estuary.**
  Costa, S., Marques, S.C., Primo, A.L. Baeta, A., Bessa, F., Pardal., M. A.
  Submitted to Scientia Marina.

- **The parasite *Sacculina carcini* in the crab *Carcinus maenas*: influence of environmental conditions, colour morphotype and gender.**
  Costa, S., Bessa, F., Pardal, M. A.
  Submitted to Crustaceana.

- **Differential sex, morphotype and tissue accumulation of mercury in the crab *Carcinus maenas*.**
  Costa, S., Viegas, I., Pereira, E., Duarte, A., Palmeira, C., Pardal, M. A.
  Water, Air, & Soil Pollution. *In press* (2011). DOI: 10.1007/s11270-011-0809-6

- **Kinetics of mercury accumulation and its effects on *Ulva lactuca* growth rate at two salinities and exposure conditions.**
  Costa, S., Crespo, D., Henriques, B., Pereira, E., Duarte, A., Pardal, M. A.
  Water, Air, and Soil Pollution 217, 689-699. (2011). DOI: 10.1007/s11270-010-0620-9

ABSTRACT

The main goal of the present thesis was to assess the effects of the environmental conditions and mercury on estuarine species. In order to achieve this purpose, different approaches were performed using different categories of organisms: a primary producer (the macroalgae *Ulva lactuca)* and two consumers (the crab *Carcinus maenas* and its parasite *Sacculina carcini*).

The thesis is divided in four main chapters, focusing on: 1) the consequences of two distinct hydrological periods (non-drought and drought) on the decapoda *C. maenas* from the Mondego estuary-Portugal; 2) the ecological role of the parasite *Sacculina carcini* on its host *C. maenas;* 3) the patterns of mercury accumulation in *C. maenas*; 4) the accumulation of mercury by *U. lactuca* and the effects on its relative growth rate.

The first chapter addresses the C. *maenas* dynamics under two contrasting environmental conditions in the Mondego estuary: non-drought and drought periods. In the drought years, salinity increased inside the estuary, displacing the estuarine brackish habitats to upstream areas. The drought event was characterized by higher crab abundance, mainly the recruits group. Although, the percentage of ovigerous females was not different, meaning that several other factors were also important for the recruitment level observed. Spatially, both periods presented the highest abundance of benthic crabs in the upstream estuarine area. The drought or non-drought conditions seemed to not interfere with the percentage of each gender and morphotype (green or red). Nonetheless, some spatio-temporal variations were observed within the estuary. While the males presented the highest percentage in the upstream area the females presented the lowest value. Moreover, the red morphotype was preferentially found in the estuary mouth. The increased abundance of *C. maenas* during drought periods certainly will have consequences for the ecosystem since it is an important species in the estuarine food webs.

In the second chapter, it was analysed the parasitism of *C. maenas* by the rizhocephalan parasite *S. carcini.* The infection, detected by the visible externae (parasite gonadas emerging externally) under the crab abdomen, presented the highest prevalence in the winter. It is known that the parasite changes the crabs'

behaviour and prevents moult. Probably, due to that, the red morphotype (resulting from a prolonged intermoult period) presented higher incidence of *S. carcini* externae. Additionally, the mouth of the estuary was the main area with sacculinized crabs. Apparently, the presence of the parasite tends to promote crabs migration to that area.

In the third chapter, different scenarios of mercury industrial discharges were studied. In the laboratory, experiments for assessing the accumulation of inorganic mercury into the tissues of *C. maenas* were assessed. Differences among genders, morphotypes and tissues were detected, depending on the mercury concentration. Additionally, differences were found among crab tissues that seem to be dependent on the major pathway of exposure, diet or water. Mercury accumulation by the crab was a rapid process and could represent a risk for the trophic chains.

In the fourth chapter the algae *U. lactuca* was studied under different conditions of exposure to mercury. Three concentrations: 5, 50 and 500 $\mu g.L^{-1}$ were tested, under static and renewal conditions and at two salinities (15 and 35). Three patterns were observed based on the models of substrate inhibition, linear regression and Michaelis-Menten equation. The different kinetics observed were related with the exposure conditions and metal concentration. The salinity influenced the mercury accumulation being higher at salinity 15 coinciding, although, to the lower relative growth rates. The lowest mercury concentration did not have a significant effect on relative growth rate, while the others concentrations caused an accentuated inhibition after 24 h. After 48 h the highest concentration was toxic to algae causing its death. The results show that the high affinity of *U. lactuca* for mercury could be useful for phytoremediation and for industrial wastewaters treatment.

Overall, based ont two long-term studies and two toxicologial tests, it was possible to contribute to a better understanding of some processes operating both at the physiological and at the population dynamic level.

RESUMO

O principal objectivo da presente tese foi avaliar o impacto das condições ambientais e do mercúrio em espécies estuarinas. Para atingir este fim, diferentes abordagens foram realizadas utilizando diferentes categorias de organismos: um produtor primário (a macroalga *Ulva lactuca*) e dois consumidores (o caranguejo *Carcinus maenas* e o seu parasita *Sacculina carcini*).

A tese escontra-se dividida em quatro capítulos principais, focando-se: 1) nas consequências de dois períodos hidrológicos distintos (seca e não seca) no crustáceo decapode *C. maenas* do estuário do Mondego – Portugal; 2) no papel ecológico do parasita *Sacculina carcini* no seu hospedeiro *C. maenas*; 3) nos padrões de acumulação de mercúrio em *C. maenas*; 4) na acumulação de mercúrio pela macroalga *Ulva lactuca* e os efeitos na taxa de crescimento relativa.

O primeiro capítulo aborda a dinâmica de *C. maenas* em duas condições ambientais contrastantes no estuário do Mondego: períodos de não seca e períodos de seca. Nos anos de seca a salinidade aumentou no estuário, deslocando os habitats estuarinos salobros para as áreas a montante. O evento de seca foi caracterizado por elevadas abundâncias de caranguejo, principalmente no grupo de recrutas. Todavia, a percentagem de fêmeas ovígeras não registou diferenças entre os períodos hidrológicos, o que significa que vários outros factores foram importantes para o nível de recrutamento observado. Espacialmente, ambos os períodos apresentaram maior abundância de caranguejos bentónicos na área estuarina mais a montante. As diferentes condições hidrológicas parecem não interferir na percentagem de cada género e morfotipo (verde ou vermelho). No entanto, algumas variações espacio-temporais foram observadas no estuário. Enquanto a percentagem de machos foi mais elevada na área estuarina mais a montante as fêmeas apresentaram nesse local a média o valor baixa. Ainda, o morfotipo vermelho foi preferencialmente encontrado na embocadura do estuário. O aumento do número de *C. maenas* durante períodos de seca certamente terá consequências para todo o ecossistema, uma vez que é uma espécie importante na cadeia alimentar estuarina.

No segundo capítulo analisou-se o parasitismo de *C. maenas* pelo parasita rizocefalo *S. carcini.* A infecção, detectada pela externa (gónadas dos parasitas

emergindo externamente) visível sob o abdómen do caranguejo, apresentou a maior prevalência no inverno. Sabe-se que o parasita provoca mudanças de comportamento dos caranguejos e impede as mudas. Provavelmente, devido a isso, o morfotipo vermelho (resultante de um período de intermuda prolongado) apresentou maior incidência de *S. carcini* externa. Ainda, verificou-se que a embocadura do estuário é a área que apresenta maior percentagem de caranguejos saculinizados. É plausível que a presença do parasita promova uma migração dos caranguejos para essa zona estuarina.

No terceiro capítulo foram estudados diferentes cenários de descargas industriais com mercúrio. No laboratório foram realizados testes para avaliar a acumulação de mercúrio inorgânico nos tecidos de *C. maenas* a partir de água contaminada. Foram detectadas diferenças de acumulação entre os sexos, morfotipos e tecidos, dependendo da concentração de mercúrio. Foram ainda observadas diferenças na concentração em vários tecidos do caranguejo que parecem ser dependentes da principal via de exposição: alimento ou água. A acumulação do mercúrio pelo caranguejo foi um processo rápido e pode representar um risco para as cadeias tróficas.

No quarto capítulo estudou-se a alga *U. lactuca* em diferentes condições de exposição ao mercúrio. Foram testadas três concentrações: 5, 50 e 500 $\mu g.L^{-1}$, em condições estáticas e de renovação do meio e em duas salinidades (15 e 35). Foram observados três padrões de acumulação com base nos modelos de inibição do substrato, regressão linear e equação de Michaelis-Menten. Estes modelos estão relacionados com as condições de exposição e concentração do metal. A salinidade influenciou a acumulação de mercúrio, que foi maior na salinidade 15 correspondendo, no entanto, à taxa de crescimento relativa mais reduzida. A menor concentração de mercúrio não teve efeito significativo sobre a taxa de crescimento, mas as outras concentrações causaram uma inibição acentuada após 24 h. Ao fim de 48 h a maior concentração testada foi tóxica para a alga causando a sua morte. Os resultados mostram que a grande afinidade de *U. lactuca* para o mercúrio pode ser útil para fitorremediação ou para o tratamento de efluentes industriais.

Em suma, através dos dois estudos de longo-prazo e dos dois testes toxicológicos realizados, foi possível contribuir para uma melhor compreensão de alguns processos que operam tanto ao nível fisiológico como populacional das espécies estuarinas analisadas.

Estuarine ecosystems: characteristics and main pressures

Estuaries are highly productive ecosystems (Kennish 2002; McLusky and Elliott 2004, Dolbeth et al. 2003) that provide different habitats with optimal conditions (good shelter, protection and food) for several groups of animals like invertebrates, fishes, and birds that use them as residency, nursery or as migration routes (Beck et al. 2001; Phil et al. 2002; Baeta et al. 2005; Martinho et al. 2007a). As areas of transition between distinct environments (land and sea) the estuaries are extremely ecologically demanding to its inhabitants. Within estuaries there are complex dynamic interactions characterized by a variety of inter-related biotic and abiotic factors and intensive chemical, physical and biological processes (Flindt et al. 1999; Molles 1999). In the estuaries the environmental conditions oscillate in time and space. Highly variations of temperature, salinity, oxygen, and water level occur subjugating the organism to tidal, diurnal and seasonal environmental changes. The dynamics and distribution of the few species that are able to live in the estuaries vary substantially between time periods and are affected both directly and indirectly by the hydrological conditions either at short and long-term (Silva et al. 2006; Marques et al. 2007; Baptista et al. 2010). The physical and chemical dynamics and the ecology of estuarine areas are strongly influenced by the runoff of freshwater from the land and the exchange of water with the adjacent open sea. The freshwater input influences estuarine hydrology by creating salinity gradients and stratification (Flindt et al. 1999) from the brackish waters of the upper reaches to the euryhaline downstream areas. That salinity regime is responsible for the distribution of organisms within estuarine waters (Thiel et al. 1995; Marques et al. 2006; Leitão et al. 2007). Moreover, water circulation due to tidal and freshwater currents is responsible for the transport of organisms, nutrient, and oxygen cycling (Molles 1999; Kimmerer et al. 2002; Gibson et al. 2003; Lillebø et al. 2004). The changes in hydrological regimes due to droughts, floods or water retention in dams, can significantly impact the estuarine species, mainly by the displacement of suitable habitats (Kimmerer 2002; Baptista et al. 2010).

The estuaries have been historically providing goods and services with high economic value. They are strategic locations that attracted populations to settle on the

proximity of them being main sites of industry and agriculture laboring. Moreover, they are places of navigation, recreation activities and the location of the biggest cities in the world (Kennish 1996; Mclusky and Elliot 2004; Martínez et al. 2007). Concomitantly with the demographic growth in the coastal areas there are an increasing demand for the ecosystems resources and a consequent rising of waste disposal. The intense human activities in the surroundings of estuaries have been leading to an over-exploitation of the natural resources supported by those ecosystems and to dramatic changes in land-use, contributing to the deterioration of the natural environment. The strong anthropogenic pressure effects include habitat reclamation, water quality impoverishment by agricultural, domestic and industrial effluent discharges, and nutrient enrichment with consequent eutrophication (Cloern 2001). Nutrient enrichment is seen as an important threat for the functioning of the estuarine ecosystem. Likewise, several effects of eutrophication have been described, including the stimulation of the growth of phytoplanckton and opportunistic macroalgae, due to the particular characteristics of these systems (shallow depth and reduced water exchange). A consequent reduction in seagrass beds, development of hynoxia and anoxia events result in some cases in fish and invertebrate fauna mass mortality (Raffaelli et al. 1998; Cloern 2001; Pardal et al. 2004; Dolbeth et al. 2007). These events may impact several key species and the entire trophic structure, resulting in an overall ecological impoverishment of the ecosystem (Raffaelli et al. 1998; Cardoso et al. 2004, 2008a).

Whithin estuaries the increasing levels of stressors, either of anthropogenic and natural origin, may originate strong implications for the supply of goods and services provided. Stress can be regarded as a perturbation with a negative effect on an area and thus a pressure which will reduce the ability to resist. The functions of the biological organization: cell, individual, population, community or ecosystem will be compromised (Elliot and Quintino 2007). In the last years there have been an increasing international conscientious towards assessing the current status of the estuaries and efforts to protect them. The aim is to assess and protect the ecological condition of these ecosystems in order to reduce, mitigate and/or compensate any adverse effects (McLusky and Elliott 2004). This concern resulted in recently developed worldwide legislation for the protection of water resources, such as the European Union Water Framework Directive (WFD, 2000/60/EC). This EU

Directive will mean a more rational use of water resources, but will also provide a framework for improve the water physicochemical quality, its ecological status, and an essential management framework of the highly valuable estuarine systems. Knowledge on land use, urban and industrial construction, waste disposal, pollution inputs (e.g. nutrient enrichment, pathogens, and chemical contaminants), resources exploitation and natural extreme events is thus essential in the decision-making process. Only with a global effort, extensive scientific knowledge and sound based decisions we will be able to achieve economic efficiency and ultimately, ecological sustainability (Martínez et al. 2007). Because anthropogenic impact and global climate changes has been increasing, studies focusing on natural and anthropogenic induced changes on coastal ecosystems are indispensable in order to mitigate its consequences.

Biological responses to the environmental variations and climate changes

The Earth is experiencing climate changes which the global warming is considered the main cause. Several human activities, such as combustion of fossil fuels, industrial expansion or widespread deforestation are contributing to accentuate the natural warming tendency (Houghton 2005). Consequently, it constitutes an unprecedented danger to biosphere. The identification and prediction of its consequences are nowadays a challenge for both scientists and environmental policy makers in order to acquire knowledge that enables effective management of ecosystems. Due to climate changes the frequency and magnitude of extreme climatic events are expected to increase (Mirza 2003; IPCC 2007). We could suppose, therefore, that rapid climatic change or extreme climatic events will alter biotic communities. The main impacts of extreme events in the estuarine ecosystems are thought to be associated with river flow, either by flood or drought events. The rainfall and freshwater flow can influence populations and communities` composition and structure (Livingston 1997; Kimmerer 2002). In the case of droughts, several authors have pointed out the effects of reductions in river flow for diverse estuarine species (Attrill and Power 2000; Christopher et al. 2006; Costa et al. 2007; Marques et al. 2007; Martinho et al. 2007b; Baptista et al. 2010).

Among the processes that could be affected by extreme whether episodes there is the recruitment (the input of new organism by reproduction). Most species abundance oscillations are caused by recruitment variability (van der Veer et al. 2000; Zheng et al. 2006). It could be a consequence of density-dependent factors acting mainly inside estuarine waters, such as predation, competition for food and refuge or density-independent factors acting essentially during the estuarine colonization phase such as hydrological features, coastal wind speed and direction, currents, salinity, turbidity, water temperature, and precipitation (Garvine et al. 1997; van der Veer et al. 2000; Zheng et al. 2006; Attrill and Power 2002; Martinho et al. 2009). Slight variations in daily mortality rates operating over the egg and larval stages are capable of generating high variations in recruitment (Heath 1992). Changes in precipitation and river runoff regimes, among other parameters, will significantly impact on coastal ecosystems, particularly on species whose larval stages depend on estuarine waters.

Environmental variations are stressors that affect biotic relationships between organisms, like the parasitism. Investigation of diseases of estuarine organisms caused by parasites is important to consolidate the knowledge about life in coastal waters. Parasitism is a fundamental and significant feature of nearly all natural systems, but less attention has been paid to this group than to others. The role of parasites in estuarine communities becomes more important as their distribution is widespread through coastal systems. Aside from studies on deleterious parasites that cause disease in humans, little effort has been dedicated to the understanding of potential changes in the parasite fauna of animal populations, especially those in aquatic systems (Marcogliese 2001). Evaluation of the potential response of parasites of aquatic organisms to environmental variations is influenced by the complexity of host–parasite systems that difficult the accurate predictions for biological systems. Climate-mediated stress may compromise host resistance and increase the occurrence of opportunistic diseases in the marine environment through shifts in the distribution of either hosts or pathogens (Harvell et al. 1999; Marcogliese 2001). Global climate change produces ecological perturbations, which cause geographical and phenological shifts, and alteration in the dynamics of parasite transmission, increasing the potential for host switching (Brooks and Hoberg, 2007). It is well known that parasites of aquatic organisms are sensitive to temperature change, not only in terms of direct effects on their life cycles and transmission but also on host biology (Marcogliese 2001).

Alternatively, environmental stress may have a deleterious effect on parasites causing infection rate and intensity within host populations to decrease (Lafferty 1997). The understanding of the dynamic change of parasite-host assemblages under a regime of global climate change is a subject poorly understood and more attention is need by the scientific community.

Species abundance and distributions are expected to be affected, simultaneously, by a number of anthropogenic and natural stressors (Thrush 2008). One possible approach allowing an overview of the potential stressor effects is to analyse the responses of various species across gradients of environmental stressors. The assemblages of species in ecological communities reflect interactions among organisms, as well, as between organisms and physical forcing. In this way, it is very important to understand the wide complexity of the climate pressure scenarios its causes and mechanisms. Moreover, it is crucial to know, and if possible to predict, its worldwide impacts. Studies at organism, population and community level processes are thus required for an integrative view of the response of an ecosystem to global climate change.

Aquatic ecosystems contamination: the mercury problems

Estuarine contamination

Due to their privileged location estuarine and coastal areas have long been subjected to multiple anthropogenic pressures, such as progressive industrialization and intensive agriculture leading to the deterioration of coastal and estuarine areas (Kennish 2002). Among the priority anthropogenic pressure affecting estuaries are those that compromise water quality like the chemical contamination. Due to those features, estuaries are one of the most endangered ecosystems on Earth. Among the most studied contaminants in estuarine and marine ecosystems, due to their toxicity and persistence in the environment, are metals. Besides natural sources such as weathering of rocks and volcanic eruptions, a wide range of human activities mobilize metals and are identified as more important point emissions like chlor-alkali production, mining activities, smelting refining and electroplating coal and oil combustion, cement production, waste incineration and lightning industry. In aquatic systems, metals tend to be associated with suspended particles because they have

high affinity for particulate matter. The vast majority of theses contaminants can settle and thus are stored into estuarine and marine sediments (Crist et al. 1994; Ramalhosa et al. 2001, Kim et al. 2004). In that group of contaminants is included the mercury. Growing awareness of the hazards of mercury exposure began in the late 1960s (Wiener et al. 2003). Despite recent settings of restrictions on anthropogenic emissions (e.g. the European Union Directive 82/176/EEC establishes a maximum value of 50 μg L^{-1} of Hg for the effluents of the chlor-alkali electrolysis sector), mercury buried in sediments for years may be released to the water column. This realease may be through disturbances induced by resuspension or changes in the physicochemical environment. Consequently, the mercury becomes available to aquatic organisms and to transport (Cardoso et al. 2008b; Kim 2004). In addition, the transport of mercury from the sediment to the water column may also occur via the activities of burrowing organisms or by higher trophic level organisms feeding on benthic invertebrates.

Remediation measures for contaminated areas

Currently, efforts are underway in many countries to control the release of contaminants (Schnoor et al. 1995) and to accelerate the breakdown of existing ones by appropriate remediation techniques. In the case of the heavy metals ions, the conventional methods include chemical precipitation, chemical oxidation and reduction, ion-exchange and activated-carbon adsorption. However, these processes have disadvantages including incomplete metal removal, mainly at low concentrations and relatively high capital expenditure and manpower as well as long term operating costs (Cochrane et al. 2006). Hence, efforts are in progress to develop more cost-effective approaches to treat large volumes of contaminated natural resources such as soil, ground water and wetlands. An emergent coast effective “green” technology in research with this aim is the phytoremediation that utilizes plants to remove, transform, or contain toxic chemicals located in soils, sediments, ground water, surface water, and even the atmosphere (Raskin et al. 1997; Susarla et al. 2002; Fereshteh et al. 2007). The plants concentrate elements and compounds from the environment and metabolize them in their tissues (Fereshteh et al. 2007). In situ phytoremediation involves placement of live plants in contaminated surface water, soil or sediment that is contaminated, or in soil or sediment that is in contact with contaminated ground water for the purpose of remediation. If the phytomechanism consists of only uptake

and accumulation as opposed to transformation of a contaminant, the plants may be harvested and removed from the site after remediation for disposal or recovery of the contaminants (Susarla et al. 2002). Soil and water contaminated with metals pose a major environmental and human health problem that is still in need of an effective and affordable technological resolution (Raskin et al. 1997). However, the phytoremediation is being developed as a potential remediation solution.

The mercury in the environment

Mercury occurs naturally and is distributed through the environment by both natural processes and human activities (EPA 2001). The Fig. 1 represent the mercury life cycle in the environment. Natural sources comprise volcanic activity, erosion of rocks and volatilization from the surface of oceans (Fig. 1). Therefore, anthropogenic

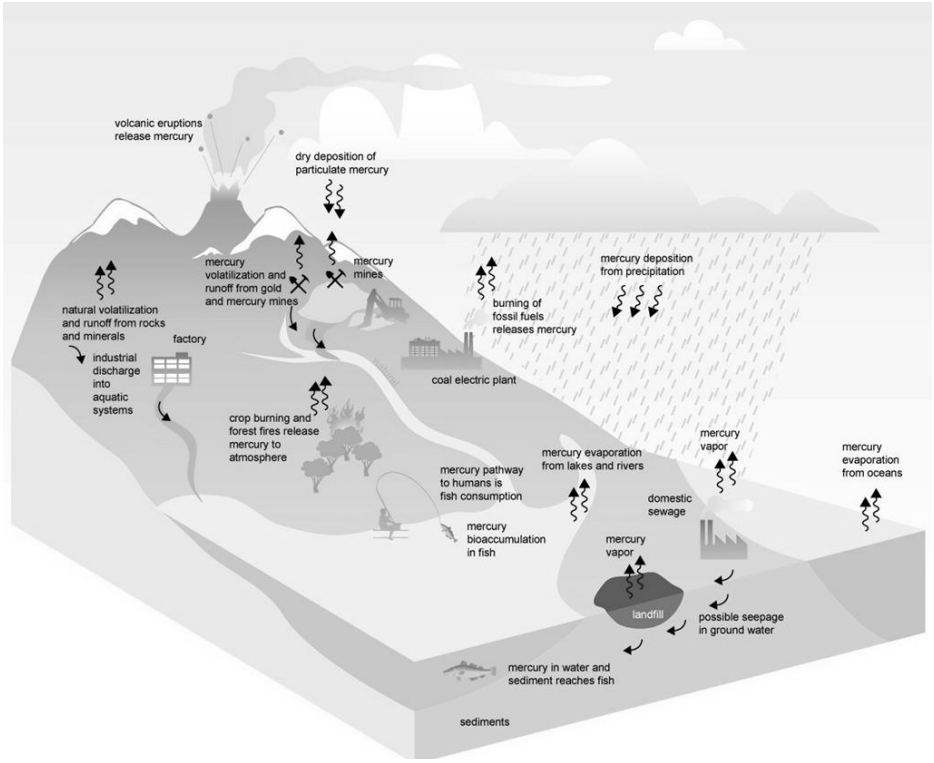

Fig. 1. Mercury life cycle in the environment, highlighting its natural and anthropogenic sources (EPA 2001).

activities are the main source of this metal into the environment. Several point emissions are identified as more important, mainly chlor-alkali production, mining activities, coal and oil combustion, pharmaceutical industry, antifouling paints in the

shipping industry, waste incineration (medical and municipal), and lightning industry (Kennish 1996; OSPAR 2000). Accordingly with the OSPAR Commission (2000) the annual amount of mercury emitted from natural sources is 2500 tons, while 3600 tons corresponded to anthropogenic inputs.

Mercury exists in the environment in three oxidation states: Hg0 (metallic), Hg_2^{2+} (mercurous), and Hg^{2+} (mercuric-Hg(II)). For each valence many chemical forms can occur in the solid, aqueous, and gaseous phases. The environmental chemistry of mercury is very complex, and subtle changes in chemical, physical, biological, and hydrologic conditions can cause substantial changes in its physical form and valence state (Wienner et al. 2006). Most of the mercury encountered in all environmental media, except in the atmosphere, is found mainly as inorganic salts and as organomercuric species, compounds defined by the presence of a covalent C-Hg bond. The presence of a covalent C-Hg bond differentiates organomercurics from inorganic mercury compounds that could be associated with organic material but without the C-Hg bond formation. The compounds most likely to be found under environmental conditions are the mercuric salts ($HgCl_2$, $Hg(OH)_2$ and HgS), methylmercury compounds like CH_3HgCl (methylmercury chloride) and CH3HgOH (methylmercury hydroxide) (USEPA 1997). Mercury is particularly reactive in the environment, shifting rapidly between the four interconnected compartments (atmospheric, terrestrial, aquatic and biotic) (Martins 2007). Some species stand out due to their importance in the global behaviour. The Fig. 2 shows a simplified view of the biogeochemical cycling of mercury in an aquatic ecosystem, highlighting the pathways and processes that influence exposure of biota to methylmercury. Most of the mercury in surface waters and sediments is typically inorganic Hg(II) that enters in the ecosystem by atmospheric deposition. The mercury cycle includes a complex set of biogeochemical processes, of which the methylation of inorganic Hg(II) is the most toxicologically significant transformation in the environmental mercury cycle because it greatly increases the bioavailability and toxicity of mercury. Methylmercury is readily bioaccumulated and transferred in food webs and can biomagnify to high concentrations in predatory fish and wildlife (Wiener et al, 2003). The methylation of mercury and the subsequent exposure of biota to methylmercury is greater in aquatic environments than in terrestrial ones (Wiener, 2006). In aquatic invertebrates, for instance, methylmercury

is much more readily assimilated and bioaccumulated than is inorganic mercury (Back and Watras, 1995; Lawson and Mason, 1998).

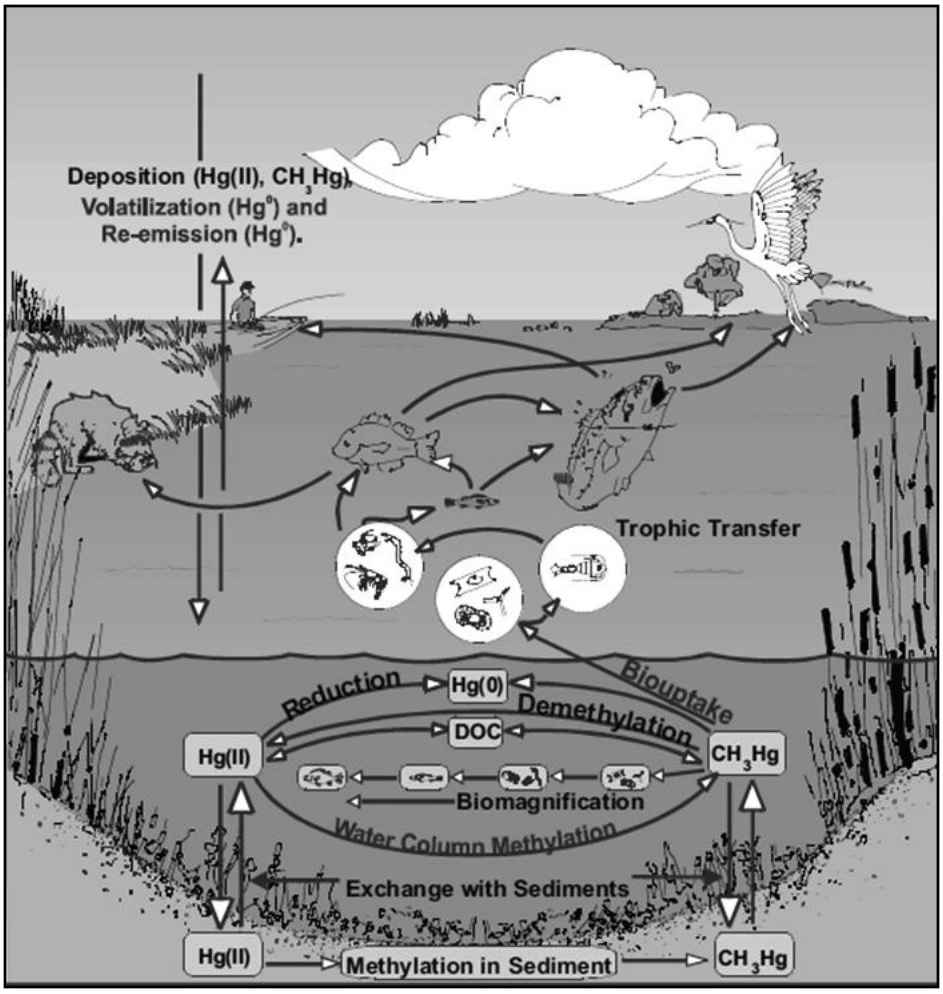

Fig. 2. Simplified cycling of mercury in aquatic systems (Wiener et al. 2003).

The presence of mercury in the landscape, whether from anthropogenic or natural sources, can devalue some of the services performed by aquatic ecosystems. The mercury is persistent and cannot be degraded into harmless products. It will therefore be permanently recycled through complex processes in the environment (OSPAR, 2000).

Interactions between mercury and biota

Mercury has no recognized role in the organisms' metabolism but can accumulate on their body being toxic to them (Boening 2000). Its toxicological effects include neurological damages, reduction of reproduction capacity, growth inhibition, development abnormalities and behaviour changes (Wiener et al. 2003; Caldéron et al. 2003; Tchounwou et al. 2003). The health effects are related to the period of exposure, mercury form and exposure route (Clarkson et al. 2003). The toxicity of mercury to aquatic organisms is affected by both biotic and abiotic factors, including the speciation, the environmental conditions (e.g. temperature, salinity and pH), the organisms' sensitivity and tolerance and the life history stage (Beckvar et al. 1996). The two main pathways for mercury incorporates in biota are the dissolved fraction, through direct adsorption to body surfaces, and particulate intake (Wang and Fisher, 1999). The relative proportion from each route will differ with species and the mercury bioavailability in water and diet (Rainbow 2002). The incorporation of mercury in organisms depends on the availability of the metal (its ability to bond and/or cross biological barriers that separate the organisms from the surrounding environment). Mercury has high affinity for lipids, allowing movement across cell membranes, which can interfere with cell metabolism (Pinho et al. 2002). The steps of the mercury uptake process include the transport of mercury to the absorbent epithelium (e.g. the gills), its movement across the diffusion barriers (e.g. mucous, membranes) to the blood and the internal distribution of mercury through the circulatory fluids. The extent of accumulation and the uptake rate are a function of the permeability of the absorbent membrane and of other tissues, which can establish a barrier to the intake and transfer of the contaminant (Barron 2003). Aquatic organisms can obtain mercury from food, water, and sediment. Once inside the organism, mercury becomes available to biotransformation into other chemical species. The biotransformation can promote the elimination, detoxification, isolation, redistribution or activation of the metal. Since the elimination rate from tissues is very slow in relation to uptake rate, the mercury easily accumulates in the organisms (Wiener et al. 2003) and it can be transferred and magnified along the trophic chains (Watras and Bloom 1992; Laporte et al. 1997; Lawson and Mason 1998; Blackmore and Wang, 2004). Eventually, cand find its way to economically important species, and ultimately to humans (Coelho et al. 2008).

The concentration of mercury in an organism during its lifetime can display a decrease defined as dilution growth (Meili 1997). This dilution is not considered as an elimination contribution, since the amount of contaminant is not changed due to growth. Some studies suggest the presence of detoxification strategies by some species to cope with high mercury concentrations, such as synthesis of metal binding proteins (phytochelatins in primary producers and metallothioneis in the heterotrophics) (Pedersen and Lundebye 1996; Gupta et al. 1998; Berthet et al. 2005; Maria et al. 2009). Generally, mercury accumulates in the trophic chain after the transformation of inorganic mercury in methylmercury, mostly in the surface of sediments, where the decay of organic matter also occurs (Lucotte et al. 1999). Because the primary producers are very important in aquatic systems the accumulation of mercury in primary producers has extensive implications for the throphic chains. On the other hand, invertebrates also play a key role in the mercury cycle because they constitute the diet of several fish species, being a major source of mercury to higher trophic levels (Tremblay and Lucotte 1997).

Despite, all the mentioned above, environmental mercury research remains an area of substantive scientific progress. Considering the mercury toxicity for the biota, it is essential to evaluate the processes behind its behavior and the consequences for the ecosystems.

General aims and thesis structure

The increasing anthropogenic contamination and environmental changes that affect the aquatic ecosystems have caused the need to assess its ecological influence on the natural communities. The concern with the consequences of stressors in the aquatic systems arises as they represent very important and productive habitats, being an important support for commercial fishery and recreation activities. Defining methods for describing and predicting effects of stressors is an important challenge. Therefore, the present thesis aimed to investigate whether different categories of stressors produce differential responses in the biota. As a complement to traditional laboratory-based ecotoxicological approaches or small scale field experiments, field surveys describe patterns that emerge from the effects of several factors and process

and thus include the effect of interactions between stressors (Thrush et al. 2008). Inspired by this approach, field data of the green crab *Carcinus maenas* and its parasite *Sacculina carcini* were analysed and ecotoxicological experiments based in the mercury accumulation, either with the same species *C. maenas, a* predator, and a primary producer, the macroalgae *Ulva lactuca,* were conducted. These studies were conceptually divided into four chapters that are the core of the thesis. Each chapter is a published paper or a manuscript in submission. In the end, a general discussion an overview of the results integrates and synthetized the work developed.

In the last years in Portugal have been notable the rapid shift between floods and droughts in consecutive years and it have been reflected in the Mondego river basin runoff (INAG - Portuguese Water Institute, http://snirh.inag.pt/ and IM - Portuguese Weather Institute, http://web.meteo.pt/pt/clima/clima.jsp). In addition, in the last years, severe droughts were recorded, with significant reductions in precipitation and river runoff, when compared to the long-term average values. As a consequence, a change in the structure and composition of estuarine planktonic, macrobenthic and fish communities of the Mondego estuary have been observed, as a response to the upward displacement of brackish habitats (e.g. Marques et al. 2007; Martinho et al. 2007a; Grilo et al. 2011). Due to the importance of the macrobenthonic crab *C. maenas* in several estuaries, a time field-based series data of *C. maenas* abundance distributions from the Mondego estuary, were investigated to understand the effects of environmental conditions in this species` dynamics. In the chapter: “The influence of climate variability on *Carcinus maenas* population structure from a southern temperate estuary” the *C. maenas* time series provided an opportunity to investigate the crab ecology over two contrasting environmental conditions, non-drought years and drought years. We try to understand the role of drought in the abundance of benthic crab and its plancktonic life stage zoeae I. It is essential to evaluate the magnitude in which environmental and climatic variables, both at local and global scales, influence the species dynamics processes in estuarine areas. Local patterns including the precipitation, the river runoff and salinity regimes play an important role in determining the community structure and function. The climate influences aquatic species directly through physiology, as well as indirectly through affecting interactions with predators, prey, parasites and competitors in addition by regulating suitable habitat (Ottersen et al. 2004). The infection of *C. maenas* by the parasite *S. Carcini,*

externally visible, is influenced by environmental factors and also influence the demography of the green crabs. That interaction was studied in the second chapter: "The parasite *Sacculina carcini* in the crab *Carcinus maenas*: influence of environmental conditions, colour morphotype and gender".

Not only the climate changes influences the life history of organism, its population structure and ecosystems relations. Anthropogenic contamination is also an important stressor. Considering the effectiveness bioaccumulation of mercury, it is important to focus on the base and intermediate trophic levels in an effort to assess the routes by which mercury reaches the top. By this reason, was investigated how the mercury accumulates in the tissues of the *C. maenas,* when exposed to a short time contamination at different concentrations. That concerns is analysed in the third chapter: "Sex, morphotype and tissue accumulation of mercury in the crab *Carcinus maenas"*. Macroalgae, as primary producers, are either an important pathway for mercury incorporation in organisms. To understand the mercury accumulation by macroalgae when exposed to various exposure conditions a laboratory experiment was performed. The fourth chapter: "Kinetics of mercury accumulation and its effects on *Ulva lactuca* growth rate at two salinities and exposure conditions" deals with the kinetics of inorganic mercury accumulation by the macroalgae and its effects on growth rate when exposed also to short time contamination. The experiment was carried at different experimental conditions that simulated different scenarios of contamination. It is important to know if the mercury accumulation patterns will be similar considering the mercury gradient existent in aquatic systems. Furthermore, that chapter focuses in the potential use of *U. lactuca* as phytoremediator.

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CHAPTER I

The influence of climate variability on *Carcinus maenas* population structure from a southern temperate estuary

Abstract

From December 2003 to November 2008 two distinct contrasting environmental conditions were identified in the southern Mondego estuary-Portugal: a non-drought period corresponding to the years 2004, 2006 and 2007 and a drought period corresponding to the years 2005, 2008 and 2009 associated with variations in the runoff. During the drought period, salinity increased inside the estuary. Changes in the *Carcinus maenas* population were found between the two periods. During drought periods increased the planktonic zoea I life stage and the benthic crabs' abundances and biomass driven mainly by the increase of crabs under 5 mm. This represented a strong recruitment causing an enhancement in *C. maenas* stocks. The ovigerous female abundance did not explained the differences in the recruitment. This means that other factors either of physiological and ecological order in the subsequent larvae and early juveniles' stages were involved. Spatially, both periods presented the highest abundance of benthic crabs in the upstream area sampled. The seasonal peak of density of benthic *C. maenas* during the drought periods was recorded in spring due to the high new recruits. In the non-drought periods the highest densities were registered in the autumn but with much lower values. The occurrence of each gender and each morphotype (green or red) seems to be independent of the period but some spatio-temporal variations were registered. The increased abundance of *C. maenas* during drought periods may have an important effect on the estuarine communities, since this species is a both predator and prey of several other species.

Keywords

Carcinus maenas · abundance · recruitment · drought · environmental factors · temperate estuary

Introduction

Estuaries are among the most ecologically and economically important ecosystems on Earth because they are habitat of important species and provide several goods and services. The rational equilibrium between the man use of the estuarine ecosystem and their preservation is essential. This need and preoccupation is expressed through several national and international regulations that have been developed to protect and recover transitional and coastal areas worldwide (Airoldi and Beck 2007). Those ecosystems are highly sensitive habitats subjected to different impacts. They are characterized by wide fluctuations in the environmental conditions at different time and space scales, determining the distribution of species inhabiting them. Nowadays, climate change is a focus of concern because it represents a potential dramatic impact on the structure and functioning of estuarine ecosystems (Atrill and Power 2000; Harley et al. 2006). Consequently, climate is becoming an important component to consider when monitoring these ecosystems.

The severity and frequency of extreme weather events such as droughts, floods and heat waves tend to increase and its impact on ecosystems will be certainly worrying. In terms of global climate change, several environmental factors are expected to have direct impacts on estuarine and marine systems such as temperature change, sea-level rise, the availability of water and associated nutrients from runoff, precipitation and wind patterns (Kennedy et al. 2002; Harley et al. 2006). Those changes are likely to affect the physical and biological functioning of these ecosystems, including changes in their composition, biodiversity and productivity (Harley et al. 2006; Martinho et al. 2007b; Primo et al. 2009). The direct effects of climate change impact the performance of individuals at various stages in their life history cycle via changes in physiology, morphology and behaviour (Harley et al. 2006). Climate impacts also occur at the population level via changes in transport processes that influence dispersal and recruitment and at the community level through the interacting species (Harley et al. 2006). Consequently, temporal and spatial patterns of population and species abundance will suffer alterations. Besides species have dealt with climate variability throughout their evolutionary history, the rapid rate of change currently observed is a challenge for species and ecosystems functioning (IPCC 2007). For instance, geographic distribution and abundance of several species will alter according

to their thermal tolerance and ability to adapt (Harley et al. 2006). Species that are unable to migrate or compete with other species for resources may face extinction (Kennedy et al. 2002). Most recent climate-related research in marine environments focuses on one single environmental parameter, frequently the temperature (as reviewed by Harley et al. 2006). However, understanding the climate change impacts only based on relationships between temperature and the biota is simplistic and insufficient (Harley et al., 2006). It is important try to understand the complexity of processes causing the abrupt marine ecosystem shifts (Alheit 2009; Kraberg et al. 2011). In Portugal during the last two decades were recorded differences in the precipitation regimes. For example, drought conditions have been frequently documented in several relatories (Portuguese Weather Institute, http://www.meteo.pt /pt/clima/clima.jsp). This phenomenon is of greater concern due to the resulting water retention in dams that lower even more the freshwater input into the estuaries.

The structure of natural populations is the result of several distinct processes. In the marine environment recruitment (supply of new individuals by reproduction) has long been recognised to affect this structure (Gaines and Bertness 1992; van der Veer et al. 2000; Mosknes 2002; Martinho et al. 2009). Recruitment regulates marine benthic communities and is determined by physical and biological processes occuring during the pelagic larval phase, settlement and juvenile growing phases (Zeng et al. 1997; Mosknes 2002; Bramanti 2003; Queiroga et al 2006; Martinho et al. 2009). For instance, larval dispersion in early stages has several advantages, such as the potential for colonization of new habitats, gene flow and minimization of intra-specific competition (Martinho et al. 2009). Recruitment success in marine species seems to be regulated mostly by density-independent factors related with the surrounding environment and climate. Nevertheless, density-dependent mechanisms such as predation, cannibalism, and feeding (Gaines and Bertness 1992; van der Veer et al. 2000; Mosknes 2004) have also important influence.

One of the most abundant species that recruit and inhabits both hard and soft intertidal and shallow habitats of the coast line and estuaries is the European green crab *C. maenas* (Cohen et al. 1995). Actually, this crab can be looked upon as a nearly cosmopolitan species of the temperate regions of the world. Its native range encompasses a wide geographical area, from Mauritania through Atlantic Europe to northern Norway and Iceland. Moreover, recently has become established in South

Africa, eastern Australia, Tasmania, the Patagonian coast of South America, and the Atlantic and Pacific coast of North America (Gillespie et al. 2007). The intermoult crabs exhibit a range of carapace colours from green through orange to red coloration, reflecting increasing lengths of intermoult duration (McGaw et al. 1992). Different physiological and behavioural responses are known to take place in this species, in relation to gender, size and carapace coloration (Lee et al.; Reid et al. 1997). This intraspecific variability of *C. maenas* reflects the adaptive responses of each individual to deal with the highly variable environmental conditions (McGaw and Naylor 1992; Reid et al. 1997). Its life cycle is complex and alternates between benthic adult and planktonic larval stages. It includes four pelagic zoeae and a megalopal stage. Ovigerous females move to the lower parts of the estuaries where hatching occurs (Queiroga et al. 1994). Hatched larvae perform active vertical migrations, attaining their highest position in the water column during ebb that enhances their export from estuaries. The megalopa is the stage that reinvades the estuary in order to settle and metamorphose into juvenile crabs. The megalopa metamorphoses to first crab instars 4-9 weeks after hatching, depending on the temperature (Queiroga et al. 1996). The green crab is considered a key-species in estuaries and some studies have confirmed that crab predation is an important factor structuring marine benthic communities (Raffaelli et al. 1989; Grosholz et al. 2000; Floud and Williams 2004). It is postulated that one of the key factors for successful propagation of the *C. maenas* is a match between physiological reactions (with particular effect of temperature on reproductive cycle and larval release), and the ecological conditions for larval survival and recruitment (Sprung 2001). Accordingly, the study of environmental factors determining the interspecific and intraspecific interactions is crucial to understand the distribution and density of *C. maenas.* Therefore, the aims of the present work were to assess the impact of drought events on *C. maenas* population dynamics. Additionally, its population structure was examined at different spatio-temporal timescales in the scope of the climate variability.

Material and Methods

Study area

The Mondego River estuary (40^0 80` N, $8^0$ 50` W), is a small, warm-temperate, intertidal system located on the western coast of Portugal (Fig. 1). It consists of two arms with very different hydrological features, delimiting the Murraceira Island. Tides in this system are semi-diurnal, and at the inlet the tidal range is 0.35-3.3 m. The northern arm is deeper (5-10 m during high tide, tidal range 0.5-3.5 m), constituting the main navigation channel and the location of the Figueira da Foz harbour. The southern arm is shallower (2-4 m during high tide), and is characterized by large areas of intertidal flats exposed during low tide. Environmental conditions in the Mondego estuary provide a large variety of aquatic habitats for populations of planktonic, nektonic and benthic species (Marques et al. 2006; Martinho et al. 2007a; Grilo et al. 2011). A total of 4 different areas throughout the estuary were sampled (Fig. 1) comprising both arms representing the major environments found in the estuarine system.

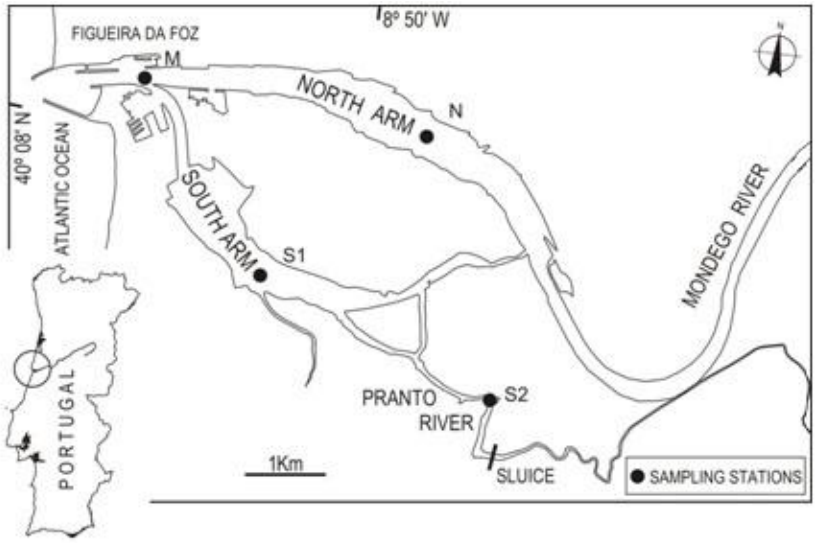

Fig. 1. Mondego estuary map showing the location of the four sampling stations (M, S1, S2 and N).

Sampling procedure

From December 2003 to November 2009 benthic *C. maenas* were obtained at each sampling area by using a 2 m beam trawl with one tickler chain. The campaigns were performed during the night, at high tide of spring tide. In the laboratory, all *C. maenas* crabs were counted and examined for sex, size (carapace width, CW - the maximum width between the tips of lateral spines, to the nearest mm), reproductive condition (occurrence of ovigerous females) and colour morphotype ("green" and "red", according to McGaw et al. 1992). Crabs were measured with a vernier caliper used to the nearest 0.1 mm, and when necessary with an ocular carrying a micrometric eye piece. Biomass was calculated by using the regression equations established for this species by Baeta et al. (2005): $AFDW=0.00005CW^{2.8586}$, N=90, r=0.99; $AFDW=0.00005CW^{2.885}$, N=98, r=0.99, for females and males respectively. Abundance was standardized as number of individuals per 1000 m^{-3}.

From December 2003 to November 2009, zooplankton samples were obtained at each sampling area by horizontal subsurface tows (using a 335 µm mesh plankton net, mouth diameter: 0.5 m), equipped with a Hydro-bios flowmeter, during high tide. The samples were preserved in a 4% buffered formaldehyde seawater solution. They were subsequently sorted and the individuals were identified and counted. Abundance was standardized as number of larvae per m^{-3}.

Environmental variables

At each sampling, water temperature (ºC) and salinity were recorded *in situ* with sensors (WTW). A database concerning precipitation and river runoff was acquired from the Portuguese Water Institute, station Soure 13/01G 12G/01A (INAG, http://snirh.pt/) and are presented in the Fig. 2. Compared to the mean precipitation regime for central Portugal during 1971–2000 (75 mm/month), the year of 2006 corresponded closely to an average precipitation (76 mm), except for some above-mean precipitation in October leading to flooding events. The years of 2004 (39 mm), 2005 (38 mm), 2007 (50 mm), 2008 (36 mm) and 2009 (48 mm) showed periods with below mean precipitation. In 2005 and 2008, two of the biggest droughts of the last 80 years occurred in Portugal (Portuguese Weather Institute). Besides the below-mean precipitation registered, the runoff in 2004 (133 138 dam^3) and 2007 (167 536 dam^3)

was higher than the average of the six years studied (126 941 dam^3), while in 2005 (32 928 dam^3), 2008 (69 451 dam^3) and 2009 (117 718 dam^3) was lower. Thought based on the joint analysis of precipitation and runoff values, the years were grouped in two contrasting periods: non-drought period (years of 2004, 2006 and 2007) and drought period (years of 2005, 2008 and 2009).

Data analysis

C. maenas stages were analysed by season, sampling station and according to the non-drought and drought periods. The seasons were defined as: summer (June-August), autumn (September-November), winter (December-February) and spring (March-May). Salinity anomalies were calculated by subtracting the mean seasonal value from the mean value of the all respective seasons of the six years analysed. The results of *C. maenas* structure are presented by estuarine area (N, S1, S2 and M), season and period (non-drought or drought). The benthic crabs were divided in groups to improve the analysis, according with the size (individuals with carapace width under 5mm - also often referred in the text as recruits, between 5 and 30mm and higher than 30mm), with the sex (male or female) and with the morphotype (green or red).

PERMANOVA + for PRIMERsoftware (PRIMER v6 & PERMANOVA + v1, PRIMER-E Ltd.) was used to perform a non-parametric permutational multivariate analysis of variance (PERMANOVA) to test for differences in the *C. maenas* population structure between periods and to test differences in salinity and temperature. Either, within each period PERMANOVA was carried out to test differences among seasons and sampling stations. The analysis was based on Euclidian distances between samples, considering the factors as fixed and unrestricted permutation of raw data. When necessary, *a posteriori* multiple comparisons were used to test for differences between/within groups for pairs of levels of factors. Additionally, a cluster analysis was performed based on the resemblance among abundances of crab size groups distributions by estuarine area applied to non-drought and drought periods. Finally, Spearman rank-order correlation was carried out to investigate the relationships among the biological parameters and the temperature, salinity and runoff. When necessary data were fourth root transformed.

Results

Environmental variables

Throughout the study period, both precipitation and river runoff presented seasonal and annual variations (Fig. 2).

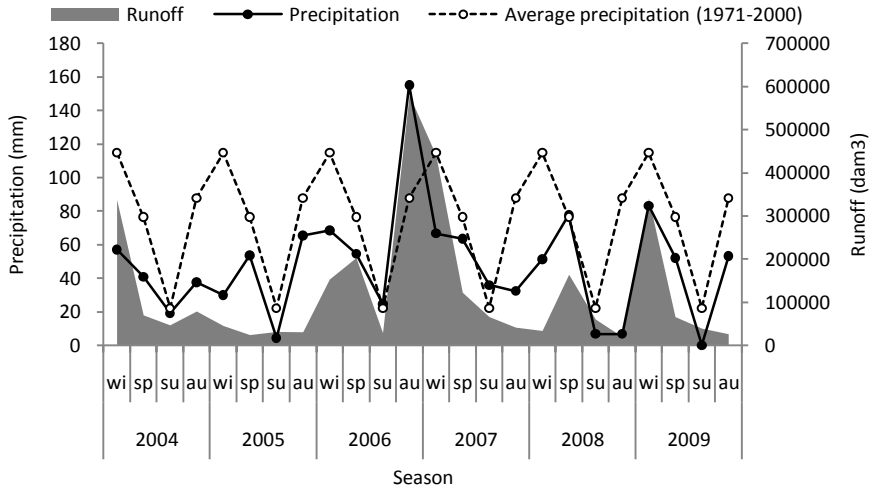

Fig. 2. Seasonal variation of precipitation, runoff and long-term average precipitation (1971-2000) for the centre of Portugal (wi-winter; sp-spring; su-summer; au-autumn).

Estuarine water temperature and salinity (Fig. 3) had a similar variation pattern during the sampling period, with lower salinities and lower temperatures in winter months. The year 2008 showed the highest annual mean water salinity (32.6) and temperature (17.6 ºC). During the drought period, low precipitation and runoff values led to an increase in salinity in the estuary (Table 1). In the non-drought period, 2004 and 2006 registered negative anomalies in the values of salinity, for all seasons, while in 2007 were found in warmer seasons. The years 2005, 2008 and 2009, as drought years, resulted in positive anomalies (Fig. 3). Mean salinity values were significantly higher during the drought period (Pseudo F¼=12.70, *P*(perm)<0.05), for all sampling stations (Table 1). The mean water temperature presented no significant differences between non-drought and drought period (Pseudo F¼=0.036, *P*(perm)>0.05). Nevertheless, the higher mean was recorded in the station S2 during drought period

(18.9 ^{0}C) while the lowest was in the station M of winter during the non-drought period (15.0 ^{0}C) (Table 1).

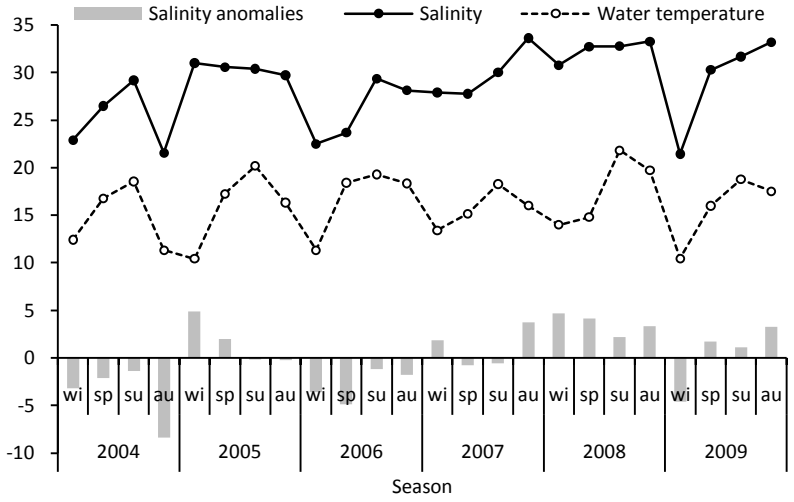

Fig. 3. Seasonal variation of bottom salinity, temperature (ºC) and salinity anomalies, obtained from the average of the values measured in the four sampling stations, for the study period (2004 to 2009), in the Mondego Estuary.

Table 1. Mean salinity and temperature (± standard error) per sampling station (M; S1; S2 and N) of the Mondego estuary water, for non-drought period (means of the years 2004, 2006 and 2007) and drought period (means of the years 2005, 2008 and 2009). The values with an *asterisk* are statistically different (PERMANOVA, p (perm)<0.05).

Estuarine area	Salinity		Temperature (°C)	
	Non- drought	Drought	Non-drought	Drought
M	30.7 (±0.9)*	32.8 (±0.9)*	15.0 (±0.5)	15.2 (±0.6)
S1	30.6 (±0.7)*	33.3 (±0.5)*	15.6 (±0.5)	16.1 (±0.8)
S2	23.7 (±1.0)*	29.5 (±0.7)*	17.3 (±0.9)	18.9 (±1.1)
N	22.8 (±1.8)*	27.1 (±1.4)*	15.7 (±0.6)	16.0 (±0.8)

C. maenas population abundance and structure

The abundance and biomass of *C. maenas* are represented in the Fig. 4. In the plankton samples exclusively was found the first larval phase (zoea I). This life stage constituted 57% of the total zooplankton decapoda larvae identified during the six years. Indeed, during winter, autumn and spring *C. maenas* zoeae I formed the dominant fraction of the decapods larvae in the Mondego estuary (91%, 76% and 67%, respectively), while in summer constituted only 27%.

The comparisons performed with PERMANOVA demonstrated that the mean of drought period was significantly higher from the mean of non-drought for total abundance of benthic *C. maenas* (Pseudo-F=14.08, *P*(perm)<0.05), for its biomass (Pseudo-F=14.24, *P*(perm)<0.05), for each size groups (recruit: Pseudo-F=8.87, *P*(perm)<0.01; 5-30 mm: (Pseudo-F=10.47, *P*(perm)<0.01: >30mm: (Pseudo-F=7.97, *P*(perm)<0.01), and for the planktonic zoeae I (Pseudo-F=5.29, *P*(perm)<0.05) (Fig. 4A, 4B and 4C). Relatively to that larval stage the peak of abundance was registered in the S2 station of spring drought (3.93 ind.m^{-3}) and the lowest mean was recorded in the S2 of non-drought summer (0.04 ind.m^{-3}). Although, in average, the highest values were found in S1 station (non-drought, 1.4 ind.m^{-3}; drought, 2.1 ind.m^{-3}). When statistically analysed each period individually considering the factors season and sampling station, the PERMANOVA performed revealed no significant differences for zoea I abundances between stations. Although, for drought period the autumn presented lower mean than warmer seasons (Table 2). Instead, for the benthic *C. maenas* abundance and population structure was highlighted its variability at spatial and temporal scale (Table 2). A seasonal pattern was apparent along the estuary, being the lowest densities recorded in winter. The highest total abundance of benthic *C. maenas* peak occurred in spring of the drought period at the station S2 (1763 ind.$1000m^{-2}$). This peak had a predominance of juveniles smaller than 5 mm width (987 ind.$1000m^{-2}$) representing 56% to the total density observed. On the other hand, in the spring of the non-drought period the recruits corresponded only to 26%. Those small crabs had settled recently, corresponding to an apparent high recruitment during drought conditions. Despite the high spring total abundance observed during drought (Fig. 4A), no statistical differences were found (Table 2). This was probably due to the high standard deviations. Moreover, the peak of total abundance during the non-drought period was registered in the same sampling station that the drought (S2) but in the autumn (321 ind.$1000m^{-2}$). This peak was proved statistically by PERMANOVA (Table 2).

While the non-drought period presented a seasonal statistical different abundance in the three crab size groups (*P*(perm)<0.05, Table 2), the drought period only presented for the recruits. For that group it was found that summer, spring and autumn samples of non-drought period were similar and significantly higher than winter ones. Instead, in the drought periods two groups of similarity were recorded

Table 2. Summary of PERMANOVA results for *C. maenas* comparisons within periods (non-drought and drought). Significant differences are marked as *p < 0.05 and **p < 0.01. Pairwise comparisons results are summarised for each significant factor. wi-winter; sp-spring; su-summer and au- autumn; St-sampling station; Se-season; M, S1, S2 and N are the estuarine sampling stations.

NON-DROUGHT												
		Total abundance (ind.1000m^2)		**Biomass (g.1000m^2)**		**<5mm (ind.1000m^2)**		**5-30mm (ind.1000m^2)**		**>30mm (ind.1000m^2)**		**Zoea I (ind. 1m^3)**
	Df	***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***
Se	3	8.44**	au>wi,sp,su	3.87*	au>wi,sp,su	5.89**	au,sp,au>wi	4.03**	sp,au>wi	8.32**	au>wi,sp,su	1.59
St	3	30.20**	S2>M,S1,N S1>N	8.65**	S2>M,S1,N	13.73**	S2>M,S1,N N<M,S1	33.04**	S2>M,S1,N	11.04**	S2>M,S1,N	2.08
SexSt	9	1.52		1.48		2.46*	sp: S2>M, S1,N	0.71		1.71		0.80
		Females (%)		**Males (%)**		**Ovigerous females (%)**		**Green morphotype (%)**		**Reds morphotype (%)**		
	Df	***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		
Se	3	2.77**	su>wi	1.84**		1.93		3.518*	au,su>sp	4.361**	sp>wi,su,au	
St	3	7.43**	M,S1,N>S2	7.74	S2>M,S1,N	1.73		15.12**	S2>M,S1,N S1>M	17.94**	M,N>S1>S2	
SexSt	9	1.39		1.57		0.574		0.99		1.538		
DROUGHT												
		Total abundance (ind.1000m^2)		**Biomass (g. 1000m^2)**		**<5mm (ind.1000m^2)**		**5-30mm (ind.1000m^2)**		**>30mm (ind.1000m^2)**		**Zoea I (ind. 1m^3)**
	Df	***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***
Se	3	1.23		1.90		5.52**	sp,su>wi,au	2.29		0.31		3.362* sp,su>au
St	3	22.07**	S2>S1>M,N	11.34**	S1,S2>M,N	15.93**	S2>S1>M,N	35.05**	S2>S1>M,N	12.03**	S2>S1>M,N	2.301
SexSt	9	1.03		1.214		1.09		0.70		1.02		1.006
		Females (%)		**Males (%)**		**Ovigerous females**		**Green morphotype (%)**		**Red morphotype (%)**		
	Df	***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		***Pseudo-F***		
Se	3	6.03**	su,au>wi	4.05**	wi>sp,su,au	2.28		4.51**	au,su>wi su>sp	2.66		
St	3	3.57**	M,S1,N>S2	5.67**	S2>M,S1,N	1.73		7.98**	S2>M,S1,N	7.73**	M, S1,N >S2	
SexSt	9	2.94		4.87**	wi: S2,N>M sp: S2>M	0.79		2.48*	wi: S2>M, S1 sp: S2>M, S1, N au: S2>S1	1.24		

(Table 2) with the warmer seasons (summer and spring) presenting significant higher abundances than the coldest ones (autumn and winter). In the non-drought period significant interaction was found between station and season in the recruits group (Table 2). This occurred due to the different results at the four seasons (the higher S2 mean only occurred in the warmer seasons). Finally, the abundance of benthic crabs was variable among estuarine areas (Fig. 4B, Table 2) but the means were always higher in the S2 sampling station.

In respect to biomass, the highest mean of drought period (422 ind.1000m^{-2}, station S2), did not corresponded to the highest abundance peak. This is a consequence of the presence in those samples of high densities of crabs larger than 30 mm (150 ind.1000m^{-2}). Contrariwise, in the non-drought period the total density and biomass peaks coincided (autumn, station S2) since larger crabs contributed with 43% of the density and the recruits corresponded only to 6%. Considering the mean abundance calculated for the non-drought period the crabs larger than 30mm represented 43% of the samples while the recruits constituted only 10%. Instead, in the drought period, the crabs smaller than 5mm represented 35% of the total *C. maenas* abundance, while the crabs larger than 30mm provided only 25%.

The structure of the *C. maenas* population in respect to the genders is presented in the Fig. 5. It was possible observe that the percentage of females and males caught throughout the two periods was variable. Relatively to the females carrying eggs, they were found through all the seasons (Fig. 4C). In average, the ovigerous females accounted only 0.9% of all females caught. While the peak of females` abundance in the non-drought period was registered in the station S1 during spring (11.5 ind.1000m^{-2}), in the drought period was in the station N during summer (8.0 ind.1000m^{-2}). However, the percentage of ovigerous females did not presented significant seasonal neither spatial differences in any period (*P*(perm)>0.05, Table 2). On the contrary, the percentage of all females was significantly lower in the station S2, while the pattern for the males was the opposite, on both periods.

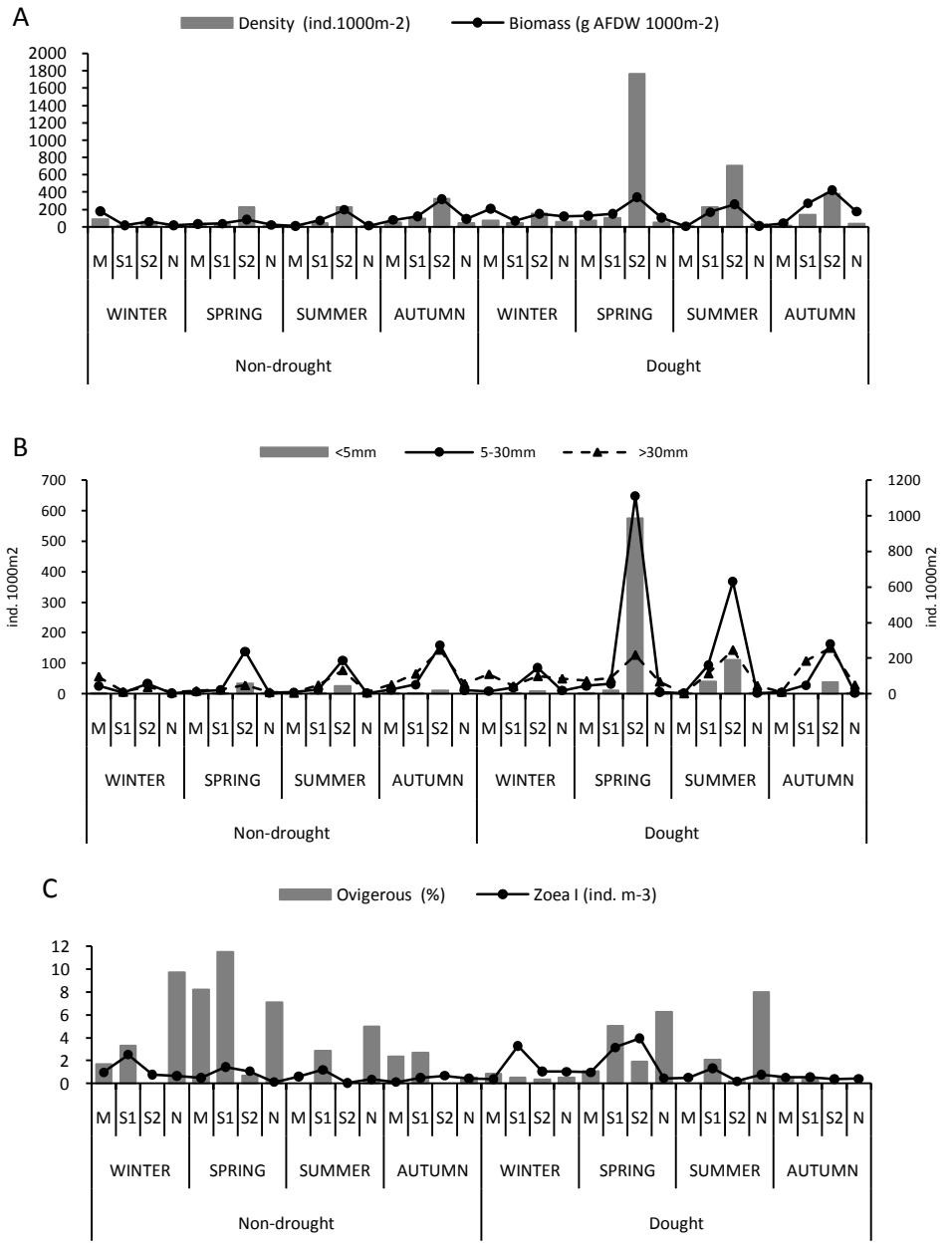

Fig. 4. Seasonal variation of the *C. maenas* structure by estuarine sampling station (M, S1, S2 and N), in the non-drought period (2004, 2006 and 2007 years mean) and in the drought period (2005, 2008 and 2009 years mean). A- Abundance of total benthic population and biomass: B- Abundance of the three size carapace width groups; C- Percentage of ovigerous females and abundance of zoea I.

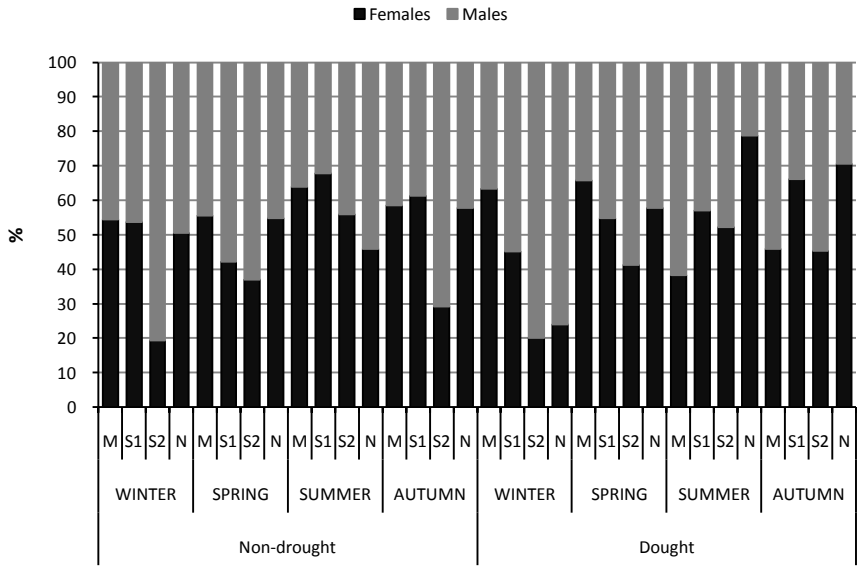

Fig. 5. Seasonal percentage of *C. maenas* males and females by estuarine sampling station (M, S1, S2 and N), in the non-drought period (2004, 2006 and 2007 years mean) and in the drought period (2005, 2008 and 2009 years mean).

Green and red *C. maenas* of both genders were recorded during sampling but the proportion of the green morph was always higher, on both periods (Fig. 6). The lowest value for green crabs was registered in the station M of the drought period (52.9%). By looking to all estuarine areas it is also possible to see the area M as the one where the highest percentage of red crabs was recorded (Fig. 6). This area corresponds to the mouth of the estuary that has typically high salinities (Table 1). Moreover, within each period significant differences were found among seasons, with the autumn and summer presenting the highest percentage of green crabs. The red morph presented the higher mean in the spring of the non-drought period, but no differences were found during the drought (Table 2). Statistically, the mean percentage of each morphotype seemed be independent of the period, due to the lack of significance in the comparisons (Pseudo-F=0.02, *P*(perm)>0.05). However, during the drought period the percentage of red crabs in the station S1 approached to the M and N stations means (Fig. 6, Table 2).

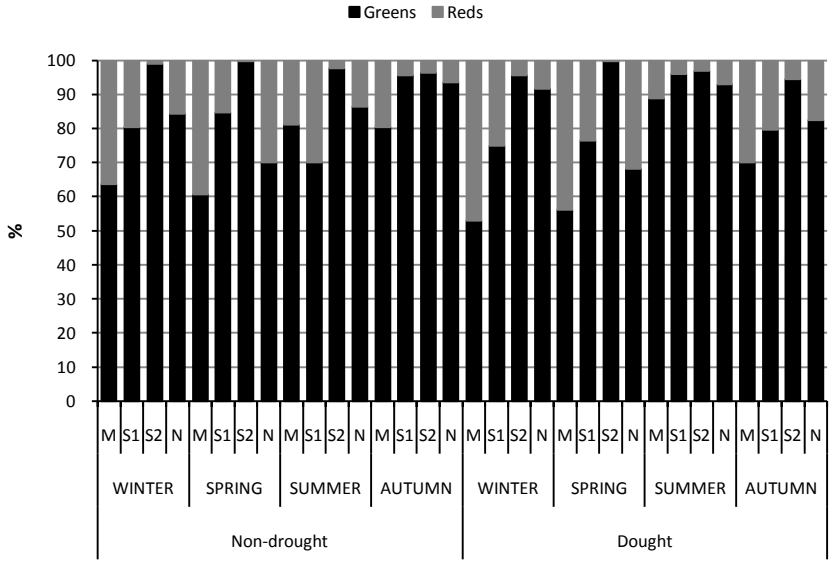

Fig. 6. Seasonal percentage of *C. maenas* morphotype (green and red morphs) by estuarine sampling station (M; S1; S2; N), in the non-drought period (2004, 2006 and 2007 years mean) and in the drought period (2005, 2008 and 2009 years mean).

The cluster analyses including the densities of the three *C. maenas* size groups are presented in the Fig. 7. The first dichotomy of the two dendrograms clearly separated the upstream station S2 samples from the rest of the estuarine areas. Interesting is that in non-drought conditions the stations M and S1 presented higher similarity while the N appeared in a separated group. On contrary, in the drought periods the estuarine area N was grouped with the station M and the S1 approached to the S2 crab population structure. Overall, population structure in the four estuarine areas presented a higher similarity in the non-drought period than in the drought (Fig. 7).

Some correlations between the structure of *C. maenas* population and the parameters salinity, temperature and runoff were detected using the Spearman rank-order correlation. The total abundance was positively correlated with temperature (r_s=0.456, p<0.05) and salinity (r_s=0.585, p<0.01), while the total biomass was positively related only with salinity (r_s=0.544, p<0.01). Moreover, strong correlations were found between the temperature and either the recruits (r_s=0.722, p<0.001) and the group of crabs between 5 and 30 mm (r_s=0.451, p<0.05), but not with the larger

crabs (>30mm - r_s=0.186, p>0.05). These results are in agreement with the higher abundances of recruits registered in the warmer seasons (spring or summer), constituting the main recruitment timing, while the larger crabs than 30 mm were significantly more abundant during the autumn (Table 2). Instead, the salinity presented positive correlations with the three size groups (r_s=0.498, p<0.05; r_s=0.530, p<0.01, r_s=0.530, p<0.01, respectively for recruits, 5<crabs<30 and >30 mm). The runoff was negatively correlated only with recruits (r_s=-0.509, p<0.05). Concerning the life stage zoea I no correlation was found (p>0.05).

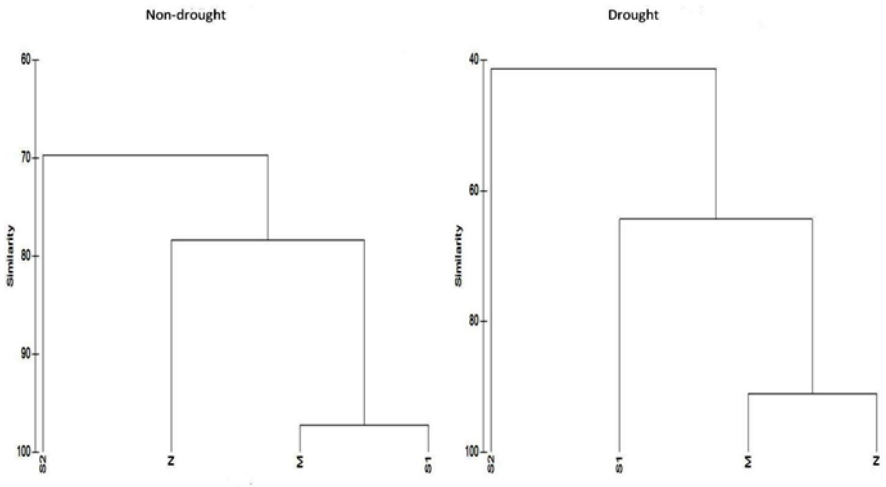

Fig. 7. Results from hierarchical clustering showing the variation in *C. maenas* structure within each periods: non-drought (2004, 2006 and 2007 years mean) and drought (2005, 2008 and 2009 years means), for the estuarine areas (M, S1, S2 and N). The clusters were performed based on the abundance of each size carapace width groups (<5mm; 5-30mm and >30mm).

Discussion

The Mondego estuary is used by *C. maenas* as habitat, spawning and recruitment area, (Baeta et al. 2005; Bessa et al. 2010). Crab egg production in this estuarine system took place throughout the whole year but the presence of ovigerous females was variable at both periods. Paula (1993) classified *Carcinus* as a permanent irregular spawner. It seems to be the behaviour of the species in the Mondego estuary, independently of the hydrological period and besides no statistical differences among seasons. Instead, for the Southern North Sea population, D`Udekem D`acoz (1994)

suggested that large females can breed twice or three times each year in the same intermoult (in winter, in spring and sometimes at begin of summer). Small females would breed usually only once, in spring. In the Ria Formosa lagoon (Southern Portugal), the occurrence of *C. maenas* larvae was limited to the period between October and May, when the water temperature stayed below 23°C (Sprung 2001). In Maine, eastern USA coast (northern end of the species range) the occurrence of ovigerous females is restricted to the warmer months (Berrill 1982).

Part of larval development of *C. maenas* takes place in the nearby coastal water (Queiroga 1996; Sprung 2001). Consequently, zoea I was the only zoeal stage found in the estuarine plankton samples of the present study. However, this larval stage is an important part of the decapoda plankton in the Mondego estuary due to its high percentage. According to Queiroga et al. (1996), older zoeae are dispersed progressively offshore, using selective tidal stream transport. Avoidance of stressful water conditions of estuarine areas in respect to temperature, salinity and oxygen content and either reduction of predation have been claimed to explain larval exportation (Forward and Richard 1987; Queiroga et al. 2006). Meagalopa is the stage reinvading the estuaries, moving into specific areas where benthic recruitment occurs (Queiroga et al. 2006). Besides the higher recruitment in the drought periods, the distribution of the recruits in the estuarine areas was similar. This means that the main area for settlement was the same corresponding to the S2 station. The present finding corroborate previous studies in the Mondego estuary, (Baeta et al. 2005; Bessa et al. 2010) indicating this area as the preferred site for benthic recruitment. The characteristics of this area probably are advantageous to the settlement of *Carcinus* megalopae. According to Baeta et al. (2005) the S2 station correspond to the shallowest subtidal area in the estuary, being characterized by muddy bottoms, high percentage of algae biomass and turbid waters, providing more food resources and shelters. In the present study, M and N estuarine areas together represented a small part of the recruits meaning that are not appropriated areas for settlement. The north arm sediments are mostly composed of medium to coarse sand (Baeta et al., 2005; Bessa et al. 2010) and it is known that shore crab megalopae avoid open sand (Moksnes 2002). In general, the abundance of small crabs in the S2 area is high and constitutes a reservoir of small individuals in the Mondego estuary. The area S1,

corresponding to the south arm of the estuary, also presented high abundance of recruits but with much lower values. This area is characterized by muddy sediments and a *Zostera noltii* bed (Cardoso et al. 2010; Grilo et al. 2011) that could also represent a structured substrate providing some refuge from predation.

The *C. maenas* is a resident species in the estuary. As result its physiology and its population structure is not independent of the hydrological and meteorological conditions reflected in the estuarine system. In drought years, as a consequence of the decrease in precipitation and storage of freshwater in dams, a decrease in runoff occurred inside the Mondego river. Thereafter, the seawater intrusion into the estuary led to changes in salinity. During drought events, above-mean salinity occurred throughout the Mondego estuary at all studied estuarine areas. For the *C. maenas* population we found induced drought effects, mainly in its abundance and structure. The drought year's conditions were reflected in significant increase in the crab` abundance driven by the high percentage of recruits. Similar results to the present work have been found in Thames estuary (Attrill and Power 2000) where the catches of *C. maenas* were significantly higher during the dry period. Other studies in the Mondego estuary also described the induced droughts changes at several levels of the estuarine communities. The studies of Primo et al. (2009) reported induced drought effects in zooplanktonic assemblages of Mondego estuary while Martinho et al. (2007b) stated the influence on fish communities. Primo et al. (2009) observed that the dry period was associated with an increase in zooplankton density, a reduction in the seasonality and a higher abundance and prevalence of marine species throughout the year. Whereas Martinho et al. (2007b) found a decline in the abundance of the fishes *Pomatoschistus microps* and *Pomatoschistus minutes*. Different groups of organisms may respond differently to drought conditions, depending on its physiology and ecology. The drought conditions also induced changes in the structure of the crab population in each estuarine area. During non-drought period the estuarine areas presented a more even crab structure than during the drought period.

In a natural population, if excluded the migrations, abundance increases through recruitment and decreases due to catch and natural mortality (Zheng and Cruse 2006). In the drought period the larger recruitment exceed a lot the catches and natural mortality resulting in a large increase in the total population abundance. The species

recruitment variation may be explained not only by changes in the environmental factors, but also by changes in spawning biomass, competition, and predation (Zheng and Cruse 2006). In the present study it was not possible to measure the spawning biomass. Nevertheless, the abundance of ovigerous females was not different among periods while the zoeae I and recruits' abundance were higher in the drought period. This indicates that other causes actuated resulting in the larger presence of zoea I and recruits.

The temperature is one the main limiting factor for *C. maenas* distribution because it affects directly the life cycle by influencing the survival of the larvae, juveniles and adults, or indirectly by influencing the development of reproductive organs and embryos (Vinuesa et al. 2007). Larvae of species living in estuarine zones could be expected to be adapted physiologically to variations in water parameters. But often show considerable changes in their survival rates and development duration depending on the environmental conditions (Nagaraj 1993; Paula 1993). Larvae of *C. maenas* do not complete development to the megalopal stage at salinities below 20 and the survival of zoeae I decrease with increasing temperature and decreasing salinity (Nagaraj 1993). Nonetheless, in the present study, the analysis did not revealed correlations between neither temperature or salinity and zoeae I abundance. Instead, positive correlation was found between temperature and the recruits. The recruitment in the Mondego estuary occurs all year, but preferentially in spring or summer months when the temperatures are higher. On the Swedish west coast, for example, recruitment starts only between August and September (Eriksson and Edlund 1977), while in the Dutch Wadden Sea occurs in mid-June, after mild winters, and in the beginning of August, after cold winters (Beukema 1991). Other factors determining larval survival and success of recruitment are of ecological nature and comprise the presence of predators (predation on eggs, larvae, or juveniles), the food availability, appropriate currents (Zheng and Cruse 2006; Mosknes et al. 1998) or other additional factors that also could not be evaluated in the present analysis. It is known that settlement or benthic recruitment (transition from a pelagic larval to a benthic phase) and early juvenile life stages are known to be critical periods in the life cycle of many benthic organisms because of high predation pressure (Mosknes et al. 1998; Bramanti et al. 2003; Mosknes 2004).

Overall, some explanations could be proposed to explain the apparent larger recruitment during the drought period in the Mondego estuary. Probably, in the drought period the viability of the eggs and larvae development was higher due to the favourable combination of environmental conditions and ecological factors. During the non-drought period, the larvae could be swept out of the estuary due to the higher freshwater flow having more difficulty to get inside the estuary to settle. Yet, we did not find a significant correlation with runoff. As well, we could either hypothesize that during the drought period due to the decrease of resident estuarine fishe species in the Mondego estuary (Martinho et al 2007b), or other species that usually include the yearly life stages of *C. maenas* in their diet, origin a consequent decrease of predation pressure. Finally, both physiological condition and the ecological framework may match and lead to good recruitment or may not match and lead to poor or no recruitment (Sprung 2001).

Relatively to the crabs` morphotype, the lack of statistical significance between the periods could be an indicative that drought conditions did not have a significant effect in the physiological mechanism responsible for the colour change (from green to red). Its causes are not well known but evidences suggest that carapace colour is an indicator of intermoult duration being the red crabs in longer intermoult (Reid et al. 1997). As expected, the red colour morph was more associated with the areas M and N representing subtidal zones that are preferred by the red crabs (Reid et al. 1997). Red crabs are generally less tolerant of environmental stress than green crabs, including prolonged exposure to low salinity (McGaw and Naylor 1992; Lee et al. 2003). Additionally, the structure of the *C. maenas* population in relation to males and females abundance was independent of the periods, but the lower percentage of females in the upstream estuarine area S2 could be explained due to the lower salinity. Females are known to prefer water of higher salinity than males (Dries and Adelung 1982).

The data set obtained from the Mondego estuary allowed an assessment of the effects of drought in the crab population abundance and structure. The study showed that drought conditions, which are predicted to increase in frequency and duration at global scale over the next years, enhance the abundance of *C. maenas,* mainly the recruits. That increasing obviously has consequences to the crab dynamics but has

subsequently a great impact on the estuarine ecosystem, mainly in the trophic webs, because that shore crab is both predator and prey for a wide range of estuarine species (Mosknes et al. 1998; Floud and Williams 2004; Baeta et al. 2006). Additionally, because this species tend to support efficiently extreme environmental events they could easily colonize several kind of systems around the world. Currently, it is clear that changes in the amount of freshwater flowing into an estuarine ecosystem may have significant effects on the communities established.

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CHAPTER II

The parasite *Sacculina carcini* in the crab *Carcinus maenas*: influence of environmental conditions, colour morphotype and gender

Abstract

Parasitism is increasingly recognized as an important factor that can influence the structure and function of natural communities. The presence of externae of the parasite *Sacculina carcini* was investigated in a population of the crab *Carcinus maenas,* from the Mondego estuary, Portugal. The prevalence of crabs with externae ranged between 0.5% and 10%. A seasonal pattern of presence of the *Sacculina carcini* externae was observed with the highest values recorded in winter and the lowest in summer. The parasite affected males and females crabs indiscriminately, but relatively to the morphotypes the reds crabs presented higher prevalence of externae comparatively with the green ones. The width class [25, 35 mm[was the one with the highest average of infection. The mouth of the estuary was the area where the most sacculinized crabs were caught. Apparently, the presence of the parasite tends to promote a crabs migration to the mouth, which coincides with the preferential spawning area of unparasitized crabs. The *Sacculina-Carcinus* infection dynamics influence the crabs's demography and its life history.

Keywords
Sacculina carcini · *Carcinus maenas* · parasite · Mondego estuary

Introduction

The ecosystems enclose a wide set of interactions between the organisms. Parasitism is one that is increasingly recognized as an important factor influencing the structure and function of natural communities. It derives from the parasite`s ability to inflict significant host mortality and to decrease the reproductive output of the host population (Mouritsen and Poulin 2002). Parasites are controlled by two habitats: the internal environment and the ecological conditions. The parasite-host system is fairly complex, being under the influence of a set of biotic and abiotic factors. Parasites usually lack a variety of morphological and accessory features as an evolutionary result of living inside a rather stable internal environment of their hosts (Combes 2001). Often, they are dependent on the host to attain vital functions. If the host experiences a certain perturbation in its external environment, this will be reflected in the parasite as well.

In Europe, a number of parasites are known to infect *Carcinus maenas*, including the common rhizocephalan barnacle, *Sacculina carcini* (Goddard et al. 2005). Its life cycle involves a mature female, which is located in the abdominal brood chamber of the host, being fertilized by male cryptic dwarf that remain near the female for the duration of the latter's lifetime and fertilize all its broods. Subsequently, female releases a series of nauplii broods that develop lecithotrophically, metamorphose into cyprids larvae after 5–6 days, and become competent to settle after 3-4 days. Female cyprids settle on the exoskeleton of a host crab and metamorphose into the stage kentrogon (Glenner et al. 2000). The kentrogon penetrates the crab exoskeleton with a hollow stylet and injects the primordial parasite, a vermigon larva, into the hemolymph (Glenner and Høeg 1995; Glenner et al. 2000). The vermigon is the first stage of the internal phase (or interna), after which the parasite produces an external virginal reproductive body (externa) (Glenner and Høeg 1995; Høeg 1995). That structure emerges in the brood chamber of the host, a position that provides support, protection, and adequate ventilation (Høeg 1995). The interna grows by developing a profuse branched root system. Within its interior forms the nucleus, which ultimately comes to lie beneath that part of the host skin where the externa will emerge (Høeg and Lützen 1995; Glenner et al. 2000). *S. carcini* has severe and durable effects on the growth, morphology, physiology, and behaviour of the host crab (Thresher et al. 2000).

The parasite castrates both male and female crabs and feminizes the males. The behaviour of both genders is changed since both respond to the externa and parasite's eggs as their own eggs (Høeg and Lützen 1995). The sacculinized host becomes a parasite genotype with a crab phenotype (O'Brien and van Wyk 1985).

The large worldwide distribution of the host *C. maenas* (originally from the Atlantic coast of Europe, has established populations in North America, southern Australia, and South Africa (Cohen et al. 1995)) demonstrates the high ability of this species to tolerate a large range of conditions in different habitats. The intermoult crabs exhibit a range of carapace colours from green through orange to red coloration, reflecting increasing lengths of intermoult duration (McGaw and Naylor 1992). Different physiological and behavioural responses are known to take place in this species, in relation to gender, size and carapace coloration (Lee et al. 2003; Reid et al. 1997). This intraspecific variability reflects the phenotypic adaptive responses of each individual in relation to their ability to withstand environmental variability (McGaw and Naylor 1992; Warman et al. 1993; Reid et al. 1997).

The interactions parasite-host are, indubitably, an important factor that influences the structure of communities and food webs (Mouritsen and Poulin 2002; Fredensborg et al. 2006). For that reason, they should not be disregarded in ecological studies. The role of parasites in the ecosystems is similar to that of predators and herbivores by reducing the abundance of certain species (Mouristen and Jensen 2006). The studies of Goddard et al. (2005) showed that *S. carcini* has a large effect on survivorship of *C. maenas* because infected crabs died more than uninfected. In this context, and in order to understand the ecological role of the parasite *S. carcini* in the *C. maenas* population from Mondego estuary, Portugal, the objectives of the present study were: a) to examine in detail the parasite abundance in the host *C. maenas* from the Mondego estuary, comparing the incidence of parasitism between host genders, colour morphtypes and size classes b) to assess the spatiotemporal parasite abundance along the estuarine salinity gradient.

Material and Methods

Study area

The Mondego estuary is a small, warm-temperate, intertidal system located on the western coast of Portugal (Fig. 1). It consists of two arms with very different hydrological features, separated by the Murraceira Island. The northern arm is deeper (5-10 m during high tide, tidal range 0.5-3.5 m), constituting the main navigation channel and the location of the Figueira da Foz harbour. The southern arm is shallower (2-4 m during high tide), and is characterized by large areas of intertidal flats exposed during low tide.

Field and laboratory procedures

Samples of *C. maenas* were collected monthly or bimonthly from June 2005 to May 2010 at four sampling stations (M, S1, S2 and N1) (Fig. 1). The sampling programme took place during the night at high water of spring tides, using a 2 m beam trawl with one tickler chain and 5 mm mesh size in the cod end. Temperature and salinity were registered for all sampling stations. Each survey consisted of three hauls towed for an average of 5 minutes each at all stations, covering at least 500 m^2, each haul. In the laboratory, the catch was divided into two groups according to whether or not they were infected by *S. carcini* (being only registered the presence or absence of the externa). Using this criterion of infection means that the group of uninfected crabs may include individuals that were only internally infected, ensuring a conservative analysis. Crabs were counted and carapace width (CW, the maximum width between the tips of lateral spines) was recorded. Crabs were measured with a vernier caliper to the nearest 0.1 mm, and when necessary with an ocular carrying a micrometric eye piece. Colour morphotypes were determined visually following the method of McKnight et al. (2000). Individuals displaying green, white or yellow colouration of the ventral carapace were categorized as green. Crabs with a red or orange ventral surface were considered red.

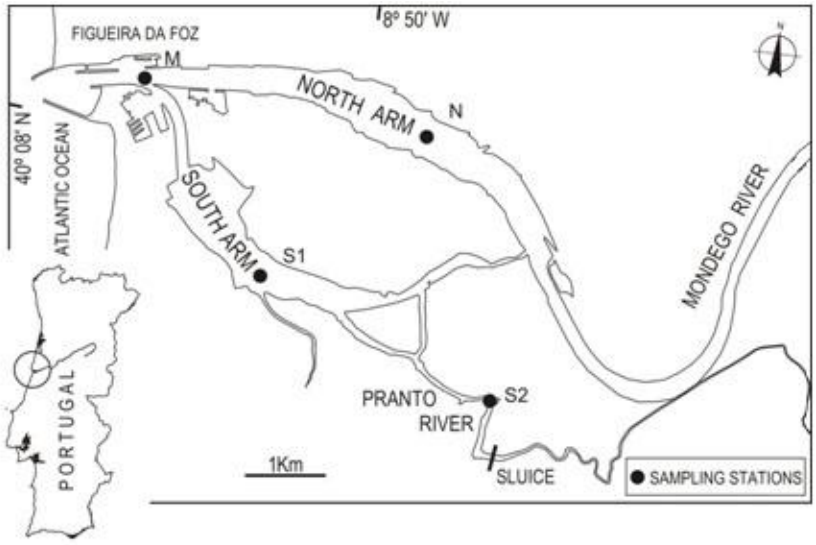

Fig. 1. The Mondego estuary: location of the 4 sampling stations (M; S1; S2; N).

Data analyses

Pearson Product Moment Correlation was performed between the percentage of crabs infected and the estuarine water temperature and salinity. The significance in data involving multiple comparisons: seasons (summer, autumn, winter and spring), sampling stations (M, N1, S1 and S2) and years (2005/06, 2006/07, 2007/08, 2008/09, 2009/10) were tested using one way analysis of variance (ANOVA) or Kruskal-Wallis One Way Analysis of Variance on Ranks. The years were defined from June to May. Pair-wise comparisons (gender and morphotypes) were done using *t*-test. The data not meeting the test assumptions were square root or logarithm transformed.

Results

The average temperature and salinity (Fig. 2A), show a clear seasonal pattern during the five-year period, characteristic of temperate regions. Generally, water temperature and salinity presented lower values in winter months. There were not found statistical differences among years with these two water parameters (temperature: F=0.0768, p=0.988, salinity: F=0.928, p=0.474). Although, between

sampling stations (Fig. 2B) there were found statistical differences for salinity values (F=35.357, p<0.001), following the sequence M=S1>N>S2, but for temperature the differences were not significant (F=2.580, p=0.060).

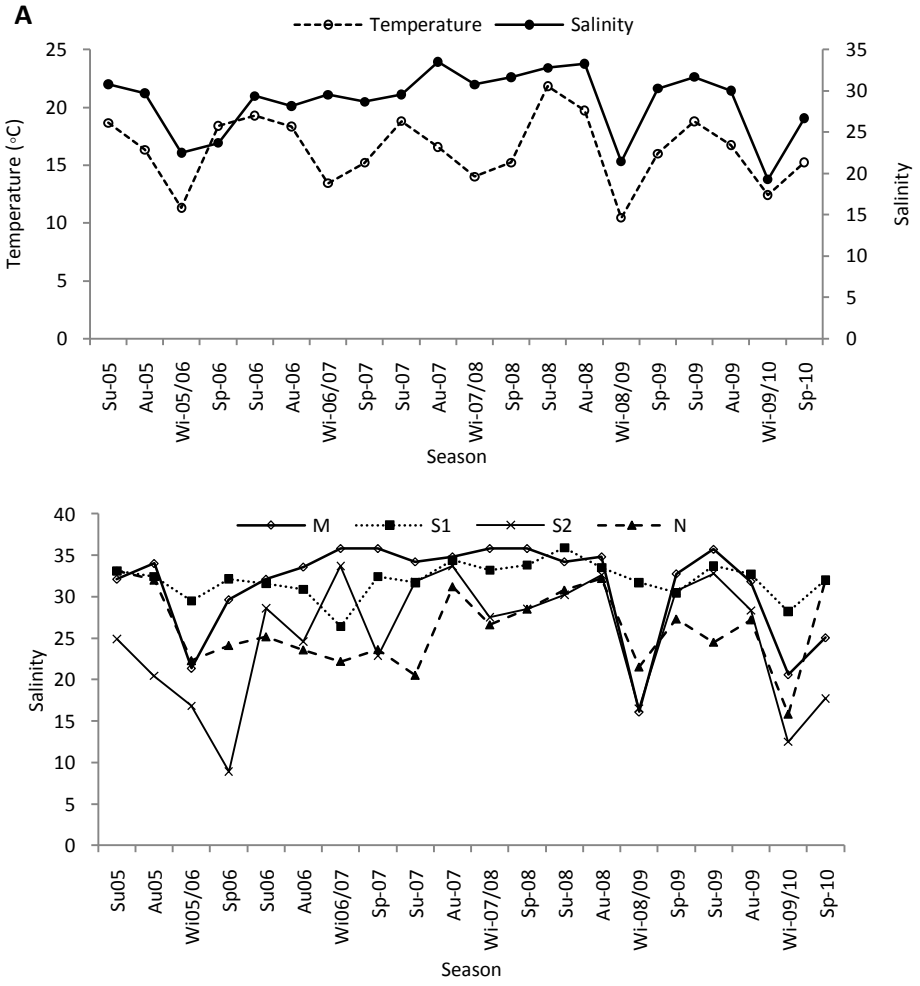

Fig. 2. Average seasonal variation of the temperature and salinity of the Mondego estuary water (A) and seasonal salinity variation in each estuarine sampling area (M, S1, S2 and N) (B).

A total of 40232 crabs were analysed (16482 females, 15038 males, and 8712 immature), from which 866 were infected with the parasite *S. carcini* (externa).The average prevalence of infection was 3.6%±0.6 (standard error). All crabs with externa of *S. carcini* were in intermoult phase and all females were non-ovigerous. No externae

were found on immature crabs. The smallest sacculinazed crab caught was a green female with 5.1 mm of carapace width and the biggest was a red male with 62.4 mm.

A seasonal pattern in the crab infection by *S. carcini* was observed with the highest values been recorded in winter and the lowest in summer (Fig. 3A). There were a negative correlation between parasitized crabs and temperature (Pearson correlation: r=-0.692, p=0.000731), and with salinity (r=-0.509, p=0.0218). Generally, the number of crabs infected increased in colder seasons and decreased in the warmer ones. One-way ANOVA confirmed the differences between seasons (F=4.593, p=0.017), and Tukey`s test allow to verify that the winter percentage of infected crabs was statistically different from the summer one (q=4.992, p=0.013). Nevertheless, the differences between the other seasons were not confirmed statistically (p>0.05). The percentage of infection was not statistically different among years (F=0.639, p=0.643).

From the 866 infected crabs, 535 were females and 331 males. The parasite affected males and females indiscriminately, as shown by the results of the *t*-test performed to assess the differences between genders, analysed as the percentage of infected male or female relatively to the total respective gender caught (t=1.033, p=0.308). Instead, the percentage of infected green and red crabs, relatively to the total respective morphotype caught, oscillated during the sampling period (Fig. 3B). There were registered significant differences (t= -5.084, p<0.001) between them. The parasite infected 10.9%±1.5 (average±standard error) of the red crabs and 3.0%±0.6 of the green ones.

For a detailed analysis, the infected crabs were distributed in six classes of 1 cm class carapace width (Fig. 4) from 5 to 65 cm. The one way ANOVA revealed significant differences between classes (F= 56.161, p<0.001). The intermediate class [25, 35mm[registered the highest percentage of infected crabs (29%±2, average ± standard error) and the smallest class [5, 15mm[the lowest percentage (0.3%±0.2).

Analysing the distribution of the infected crabs in the four estuarine sampling areas (Table 1) could be observed that local M (estuary mouth) presents the highest average percentage of parasitism. The correlation between the salinity and the percentage of crabs infected was significant (Pearson Correlation: r=0.263, p=0.0186) and positive, indicating that parasitized crabs are more associated with areas of higher salinity.

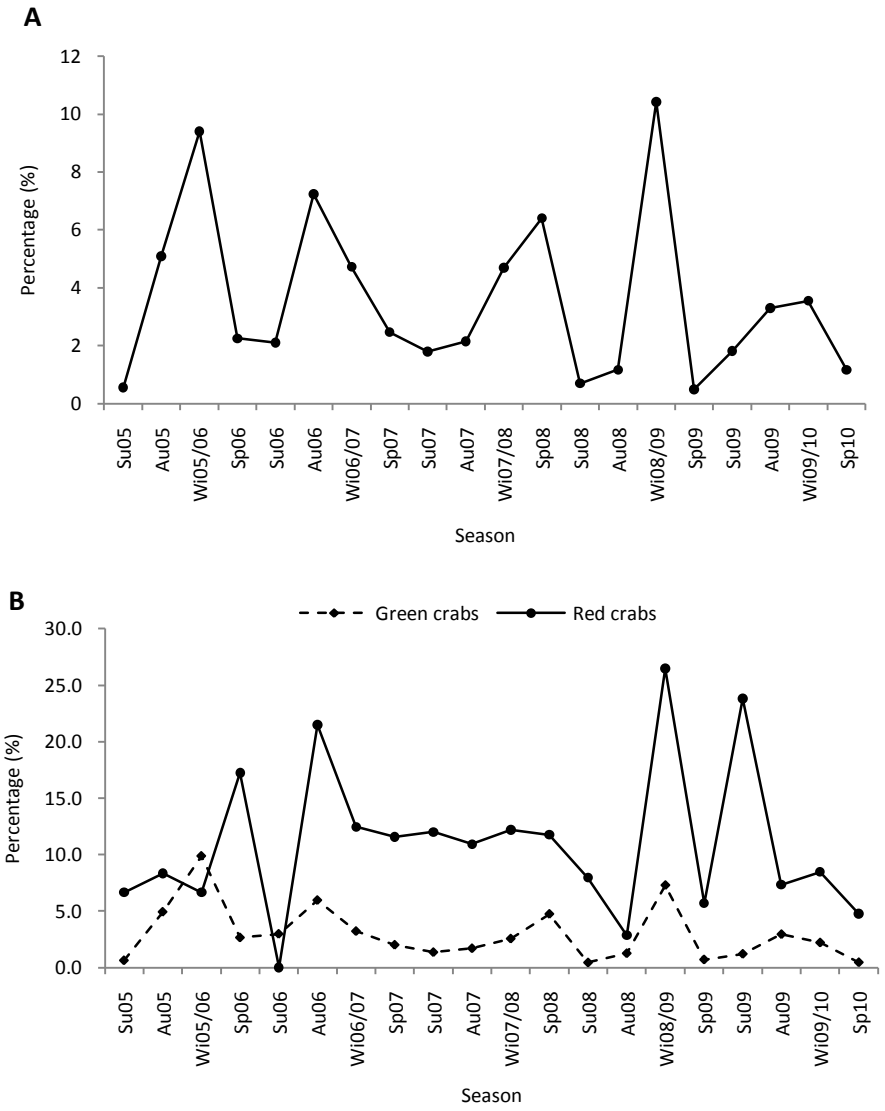

Fig. 3. Percentage of infected crabs relatively to the total individuals caught (infected plus uninfected, n=40232) (A) and percentage of infected crabs by morphotype relative to the total crabs caught with the same characteristics of the infected group (males and females, green or red, n=47208) (B).

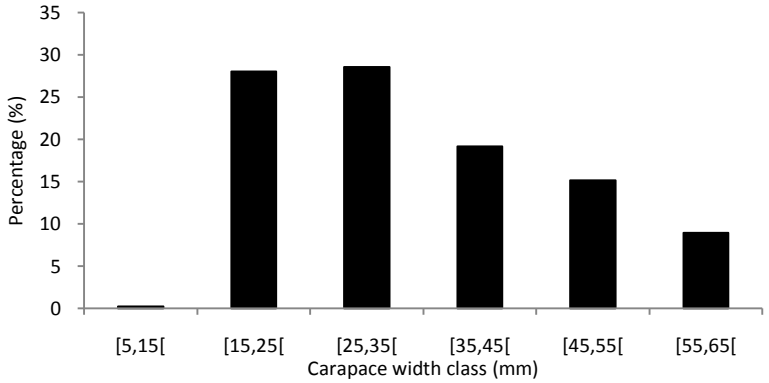

Fig. 4. Percentage of infected crabs in the different carapace width classes (both genders, n=866).

Table 1-Percentage of [5,65mm[class *C. maenas* caught, infected and total, distributed for each sampling estuarine station (M; S1; S2-and N). The values are average ± standard deviation (n=866).

Average percentage (%)	Estuarine station			
	M	S1	S2	N
Infected crabs	65 (±7)	26 (±6)	3 (±1)	5 (±2)
Total crabs caught	17 (±3)	22 (±3)	51 (±4)	10 (±2)

Discussion

The parasitism could have an important influence in the structure of animal communities. Our study shows that the abundance of crabs with externae of *S. carcini* differs among estuarine areas (Table 1) morphotypes (Fig.3B) and class sizes (Fig. 4). Those results indicate that the presence of the parasite influence the *C. maenas* population dynamics. In the Mondego estuary, the incidence of *S. carcini* is similar to the results of Rasmussen (1973) for Denmark, but lowers than the results for other European countries (Table 2). Furthermore, both Mondego crab genders are equally infected. Also, Mathieson et al. (1998) found the same pattern in the Forth Estuary, Scotland.

Table 2. Percentage of the parasite *S. carcini* in the crab *C. maenas* (adapted from Thorchin et al. (2001)).

Locality	Prevalence	Reference
Denmark	0-10%	Rasmussen (1973)
Ireland	0.3-64%	Minchin (1997)
Scotland	6.4-47%	Mathieson et al. (1998)
France	50%	Bourdon (1960)
France	20%	Bourdon (1963)
France, Spain, Sweden	0-78%	Thorchin et al. (2001)
Portugal	0.5-10%	Present study

S. carcini is known to induce "parasitic sterilization", reducing testes weight (Zetlmeisl et al. 2010) and exerting a very extensive host control (Høeg 1995). It profoundly influences the crab`s behaviour. Most Rhizocephala infestations result in complete sterilization since female hosts never carry eggs and the gonopores of the both genders degenerate or disappear completely (Høeg 1995). Also, Ritchie and Høeg (1981) observed how the externa, in occupying the very small place as the embryos in an ovigerous female, effectively mimics a brood of eggs. This explains why all infected females caught were non-ovigerous. Furthermore, Rasmussen (1959) observed how sacculinized crabs assume the migrational pattern of ovigerous females. The estuary mouth presented the highest salinity and was the sampling area where the infected crabs were more abundant (Table 1). Moreover, the mouth was not the area were the total crabs (infected plus non-infected) with the characteristics of infected crabs (class [5, 65 mm[, males and females) were caught in higher percentage (Table 1). It excludes the possibility of consider that were caught more infected crabs in this area because there are more crabs there. Although, mouth coincides with the area to where the ovigerous females migrate in, constituting the preferential spawning area (Baeta et al. 2005). From the above, we could suppose that those facts could indicate that infected crabs in the Mondego estuary tend to migrate to the mouth. In fact, host male assume the female behaviour (Høeg and Lützen 1995) providing maternal care for the externa rather than attempting to remove the parasite from their body. Like the ovigerous females choose the estuary mouth to spawning, the parasitized males also go to that estuarine area increasing there the percentage of crabs with externa. Other authors also reported that sacculinized crabs migrate offshore when externa attain sexual maturity, just healthy brooding female crabs do (Rasmussen 1954; Lützen 1984). No

matter ecological and behavioural differences between both genders (Lee et al. 2003), the parasite infected them indiscriminately, probably due to its capacity to change the male behaviour feminizing it, as previous explained. By another hand, the positive correlation between salinity and percentage of infected crabs indicates that infected crabs are associated with areas of higher salinities.

All crabs parasitized with externae of *S. carcini* were in intermoult phase and the parasite did not infected immature hosts. This fact is in agreement with other studies that establish that parasitized crabs cease moulting (O'Brien and Skinner 1990). The literature on the development of *S. carcini* in *C. maenas* thoroughly documents a marked reduction in the growth rate of infected crabs. It leads to cessation of further ecdysis upon emergence of the externa of the parasite (parasite maturation) (Høeg 1995). The parasite arrest the moult cycle of the host (Thresher et al. 2000), known as parasitic anecdysis (O'Brien and Van Wyk 1985). Because moult is essential for crab's growth, the cessation of moulting induced by maturation of *S. carcini* would still cause infected crabs to be smaller than non-infected crabs.

In the present study we find higher percentage of parasite prevalence in red crabs comparatively with the green ones. Nevertheless, the red crabs are frequently the morphotype less abundant in the ecosystems. The carapace colour depends on the duration of intermoult with newly moulted crabs being bright green gradually turning red during prolonged intermoult as the crab becomes older (McGaw et al. 1992). As long as it carries an externa the crabs remain at intermoult stage (Høeg 1995). Based in this prepositions, we could assume that infected crabs due to the cessation of moult will turn red justifying in this way the higher prevalence observed in that morphotype. Red crabs are stronger and more successful in mating competitions than green crab (Reid et al. 1997). Although, this mating success appears to be achieved at the expense of a lower physiological tolerance to natural variations - higher susceptibility to hypoxia and salinity stress (Reid and Aldrich 1989; Lee et al. 2003) and to anthropogenic pollution (Styrishave et al. 2000; Dam et al. 2006). Because the parasite trade off crab growth we can hypothesize that they will turn red and, consequently, will become physiologically more vulnerable. Still, because feminized males have an female ovigerous behaviour, they will increase in areas to where ovigerous females migrate to release the eggs. The reproduction of the species is either affected because

sacculinized females do not reproduce. Overall, the *C. maenas* dynamic will be influenced by the parasite presence.

The seasonal pattern in the crab infection presented the highest values in winter seasons and the lowest in summer. A negative correlation between temperature and incidence of externae was recorded. This could be explained by the heavy mortality of externae in warmer seasons, suggested by the studies of Heath (1971). Additionally, the duration of the internal phase from invasion to emergence of the externa could be 1-3 years (Høeg 1995). We could believe that infection of Mondego estuary crabs occurs in spring or summer with development of interna, and that the externa emerge some months later in the autumn or winter, when the temperature of water is lower. This is suggested by the increase of crabs with externa parasite in winter, and the subsequent decrease in spring and summer corresponding probably to the internal infection. In *S. carcini* it seems to be the temperature, rather than host species or size, that determining the length of the internal period (Høeg and Lützen 1995). According to some authors the duration of the internal phase of the parasite development requires 6-12 months in the English Chanel, 18 months on the Atlantic coast of Spain, and 3 years in Danish waters (Høeg and Lützen 1995). An additional explanation proposed to the parasite life cycle is that the reproduction of *Sacculina* is synchronized with reproduction of its host (Day 1935). In this way, the food reserves normally used by the developing ovary are used by the parasite. It is suggested that an equally valid explanation might be that it is the developing interna, rather than the externa, that requires optimal conditions for growth, being such conditions particularly found in the spring and summer season of growth, moulting, and ovarian development (Heath 1971).

The knowledge of the parasite dynamics in the ecosystems is very important and can originate huge variations in populations' abundance and structure. This study indicates that *Sacculina-Carcinus* infection dynamics influence the demography of the green crabs and its life history, including implications for reproductive potential. In addition to its functional role, *S. carcini* also change significantly the phenotype of their host. The behaviourally modified host crab could occupy an ecologically important role in the Mondego estuary.

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CHAPTER III

Differential Sex, Morphotype and Tissue Accumulation of Mercury in the Crab *Carcinus maenas*

Abstract

Carcinus maenas is an invasive species of recognised economical and ecological importance in which mercury accumulation could be a pathway for bioamplification through food webs. Little information is available about differential accumulation between crab sexes and morphotypes. Taking this in mind, a set of different industrial discharge scenarios were investigated in 96-h laboratory experiments for assessing the accumulation of inorganic mercury from contaminated seawater into the tissues of *C. maenas*. Three groups of crabs (green males, green and red females) where exposed to 5, 50 and 250 µg $Hg.L^{-1}$. Differences among sexes, morphotypes and tissues were detected, depending on the mercury concentration. The muscle did not show differential accumulation between sexes or morphotypes. For mercury-exposed crabs, the contami-nant was accumulated preferably in the gills (more than 75%) while, in control experiments, it was in the internal organs, muscle and hepatopancreas, and gills corresponded to less than 31% of the total mercury quantified. The different tissue contamination seems dependent on the major pathway of exposure, diet or water. Mercury accumulation by the crab was a rapid process and could represent a risk for the environment only after 96h. In a scenario of a discharge point of 250 µg L^{-1} all tissues of crabs exposed would attain a very close, or even exceed the threshold concentration value for human consumption (0.5 $mg.kg^{-1}$).

Keywords

Carcinus maenas · mercury accumulation · morphotypes · sexes

Introduction

Mercury (Hg) occurs naturally in nature. Nevertheless, the pollution episodes associated with this metal result from human activities, such as metal smelting, coal production, chemical synthesis and use, and waste disposal (Tchounwou et al. 2003). The presence and behaviour of mercury in aquatic systems is of great interest since it is a highly deleterious environmental pollutant with recognised mutagenic and teratogenic effects (Calderón et al. 2003; Tchounwou et al. 2003). It is not known to perform any essential biochemical function (Bowen 1966), and the contamination problems are enhanced when it enters the food web via biomethylation and bioaccumulation processes (Andres et al. 2002, Cardoso et al. 2008; Laporte et al. 1997; Lawson and Mason 1998; Watras and Bloom 1992). Even though the inorganic mercury (Hg (II)) accounts for 95% of the metal present in seawaters, marine organisms, especially those occupying higher trophic levels, generally accumulate organic mercury to higher levels than they do inorganic mercury (Bjerregaard and Christensen 1993). This occurs perhaps because organic mercurials pass easily across biological membranes, since they are more lipid-soluble than inorganic mercury (Horvat 2005).

Industrial discharges of mercury to waterways under the form of inorganic salts have been common practice (Tchounwou et al. 2003). Because of this and due to its toxicity, much action to reduce uses and emissions are being highlighted in international regulations (e.g. European Union, Directive 82/176/EEC that regulates the mercury discharges by chlor-alkali electrolysis industry effluents which should not exceed 50 $\mu g\ L^{-1}$). Presently, in the European Union Countries, this metal is considered a priority hazardous substance and the cessation or phasing out of mercury discharges, emissions and losses will be required by 2020 under Water Framework Directive-UE (2000/60/CE).

A study of contamination at the organism level and at the main organ level gives a better understanding of the overall accumulation mechanisms involved during exposure (Inza et al. 1998). Some estuarine species have been used in laboratory experiments with this intent: e.g. the blue crab *Callinectes sapidus* (Andres et al. 2002), the Normay lobster *Nephrops norvegicus* (Canli and Furness 1995), the bivalve *Scrobicularia plana* and the polychaete *Hediste diversicolor* (Cardoso et al. 2009). The

European green crab *Carcinus maenas* (Linnaeus 1758) has also been used frequently in toxicity assessments (e.g. Bamber 1997; Bjerregaard al. 1990, 1991; Laport et al. 1997). It is an aggressive and voracious predator that has an important feature in structuring marine and estuarine benthic communities (Raffaelli et al. 1989). *C. maenas* is a widely distributed epibenthic portunid crab that inhabits hard and soft intertidal shallow habitats of European coasts and estuaries. It is also found in the north-western Atlantic and due to its eco-physiological plasticity towards natural or anthropogenic variations, has recently invaded some areas in southern Africa, Australia and Pacific coast of North America (Cohen et al. 1995). It is now accepted that *C. maenas* is an invasive species that can have significant impacts, both economically and ecologically (Grosholz and Ruiz 1995). This species is one of the most important and exploited natural resources in temperate estuarine systems and therefore can represent a major pathway for human mercury consumption (Coelho et al. 2008).

Despite its common name, the intermoult European green crabs exhibit a range of carapace colours from green through orange to red colouration, reflecting increasing lengths of intermoult duration. The crabs that have recently moulted are green in appearance while crabs in late intermolt are most likely to be dark red or brown and often referred in the literature as red (McGaw and Naylor 1992; Reid et al. 1997; Lee et al. 2003; Styrishave et al. 2004). Green and red crabs will be the nomenclature used in this paper. Variation between green and red *C. maenas* is proposed based on the hypothesis that colour and its correlates result from prolonged inter-moult in adult male crabs as a strategy for maximising mating success. Small adult males, with little chance of mating successfully, must rapidly grow by moulting to increase in size and strength, in order to get access to females, thereby maintaining a green colour. In contrast, large males with physical strength to ensure paternity suspend growing, and remain in late intermoult, allocating energy into reproduction. This reproductive success in red crabs seems be achieved at the expense of physiological tolerance (Reid et al. 1997; Styrishave and Andersen 2000; Dam et al. 2006). Additionally, red individuals are characterised by a greater load of epibionts and a thicker carapace (Reid et al. 1997; McKnight et al. 2000). The shift from a “growth strategy” to a “reproduction strategy” appears to exert a profound effect on male shore crab biology (Styrishave et al. 2004). These assumptions are valid for males, but the relationship

between colour and moult state in female *C. maenas* is less clear. All the investigations into colour and its correlates have concentrated on the male crabs. As in the males, there are females of all colours from green to red. Observations by some of the authors in laboratory situations have shown that, unlike the males, some female crabs retain the red colour through the moult, possibly as a result of retaining material for vitellogenesis (the eggs are usually a pinkish red colour) (Reid et al. 1997).

Different physiological and behavioural responses in that species are known to take place not only in relation to carapace colour but also to sex and size. *C. maenas* females are different from males morphologically, ecologically and behaviorally (Lee et al. 2003). However, the studies on trace metal handling in *C. maenas* have been carried out predominantly on male crabs. Additionally, a number of studies have demonstrated that there is a series of response variations correlated to the carapace colour, which were as well made predominantly with male morphs. These differences include respiratory and osmoregulatory physiology, response to desiccation, moult cycle pattern, chelal morphometry, prey selection, incidence of chelal autonomy and hence handicap and mating pattern, as reviewed by Reid et al. (1997) and Styrishave et al. (2004).

The great *C. maenas* intraspecific variability reflects the phenotypic adaptive responses of each individual in relation to their ability to withstand environmental variability (Abelló et al. 1997; McGaw and Naylor 1992; Reid et al. 1997; Warman et al. 1993). Overall, no laboratorial study mentions the differential metal accumulation between male and female and between the female crab morphotypes, as in such study. Crabs have been lumped into just two colour groups, green and red (Reid et al. 1997).

Considering the above-mentioned reasons, the main objectives of this work were: (a) to document short time inorganic mercury accumulation by crabs from water column in three different contamination scenarios, at laboratory conditions; (b) to compare male and female accumulation patterns; (c) to compare female morphotype accumulation patterns and (d) to evaluate the existence of differential tissues accumulation.

Material and Methods

Materials and reagents

All materials used in the experiments and for the storage, processing and analysis of samples was washed in Derquim 3%, rinsed in Milli-Q water (MQW), soaked in 25% HNO_3 for at least 24 hours and subsequently rinsed with MQW. Mercury standard solution (analytical grade), containing 1001±2 $mg.L^{-1}$ of mercury (II) as $Hg(NO_3)_2.H_2O$, in HNO_3 5 $mol.L^{-1}$, was purchased from Merck. Working solutions were prepared by diluting the mercury standard solution with MQW.

Experimental set-up

Crabs were caught at Mondego estuary (Figueira da Foz, Portugal, 40°08`N, 8°50`W) using a 2-m beam trawl. The crabs were transported to the laboratory in isothermal bags and divided into three groups: green males, green females and red females of similar size classes to avoid major inter-individual variations (3.5-4.5 cm carapace width, corresponding to 2+ years-old individuals (Baeta et al. 2005)). The distinction between colour morphotypes was based on the abdomen colouration. Red males were not included in the present study due to their scarcity. To evaluate the total mercury accumulation ability of the studied organisms in the field, four crabs of each group were excised, and hepatopancreas, muscle, exoskeleton and gills were freeze-dried. The dried tissues were macerated prior to analysis of total mercury. The other crabs were acclimated to laboratory conditions (20°C and 14L:10D) for 1 week in glass aquariums with seawater (salinity 30), continuously bubbled with air and mechanically filtered. Only non-damaged crabs with hard carapace were used. The animals were fed ad libitum twice a week, with a blend of mussel bits. Seawater was changed after each meal. After the acclimatation, the crabs were individually contaminated in glass containers with 300 ml of continuously aerated seawater. The experimental design included a control (seawater only) and three treatment groups, each replicated four times. Nominal mercury concentrations of 5, 50 and 250 $\mu g.L^{-1}$ were prepared by dilution of appropriate amounts taken from the mercury standard solutions ($Hg(NO_3)_2.H_2O$)) and filtered seawater collected at the coast (1 km north of the estuary). Crabs were not fed during the 96 hours of the experiment.

Although, the concentrations tested in this study are higher than those found in natural waters contaminated by mercury (e.g. Coelho et al. 2008), the rationale for using high mercury concentrations should be seen in the context of the short exposure period and the simulation of a possible contamination by wastewaters rich in mercury. The different scenarios simulated at laboratory were: the contamination caused by industries, in which effluents cannot contain more than 50 $\mu g.L^{-1}$; contamination by effluents after dilution (5 $\mu g.L^{-1}$) and an accidental spillage of non-treated effluent (250 $\mu g.L^{-1}$).

Analytical procedures

Crabs were excised as mentioned above, and the dried homogenised tissues were analysed for total mercury by thermal decomposition atomic absorption spectrometry with gold amalgamation, using a LECO AMA-254. Accuracy of the results was assessed by using the certified reference material (CRM) Tort-2 (lobster hepatopancreas) and Dorm-2 (dogfish muscle) for the highest contaminated samples. The average of daily recovery percentage of the CRM analyses was 107.7±1.5% (average±standard error, n=30).

Data analysis

Prior to statistical analyses, normality and homogeneity of variance were checked and, when necessary, logarithm transformations were performed. Differences between field and control crabs, females versus males and green versus red females were compared using the *t*-test. Differences among treatments were tested performing a one-way analysis of variance (ANOVA), using the data of the three crab groups pooled. The Dunnett test was performed when a significant difference occurred to compare the treatment groups to the control group. Between tissues the differences were tested using a one way ANOVA, followed by a Tukey`s test adjusted pairwise comparisons amongst groups. The null hypothesis was rejected when $p<0.05$.

Results

During the experiment, no mortality was recorded. Comparisons of mercury content between field and control crabs showed no statistical differences in the mercury content for the tissues analysed (p<0.05). In the exposed crabs, the accumulation was observed to follow the trend of water mercury concentrations (Figs. 1 and 2).

Results of one way ANOVA showed significant differences between concentrations (p<0.001) in all tissues (Table 1). The mercury accumulated in the exoskeleton and gills of crabs at control conditions was significantly lower than the accumulated at 5, 50 and 250 $\mu g.L^{-1}$. For hepatopancreas and muscle, the mercury contents of control crabs were only significantly lower than that in the exposed crabs at 50 and 250 $\mu g.L^{-1}$.

The t-test showed significant differences between green males and green females for exoskeleton and gills at control, 50 and 250 $\mu g.L^{-1}$ (Fig. 1), being lower in females. The differences for hepatopancreas were only found at 5 and 250 $\mu g.L^{-1}$. The muscle did not present a differential accumulation between sexes.

The comparison between female morphotypes revealed statistical differences but only at the highest water mercury concentration. Those differences were found for all tissues except for muscle, with the red females presenting the highest values (Fig. 2).

One-way ANOVA revealed significant differences among tissues (P<0.001; Table 2). For control and all water mercury concentrations, the lowest tissue mercury level was found in the exoskeleton but levels were only statistically significant at control and 5 $\mu g.L^{-1}$, for all crabs groups studied and at 250 $\mu g.L^{-1}$ for the green female group. The highest amount of mercury was always recorded in the gills for all exposed crabs. For green male and red female, it was similar to muscle values.

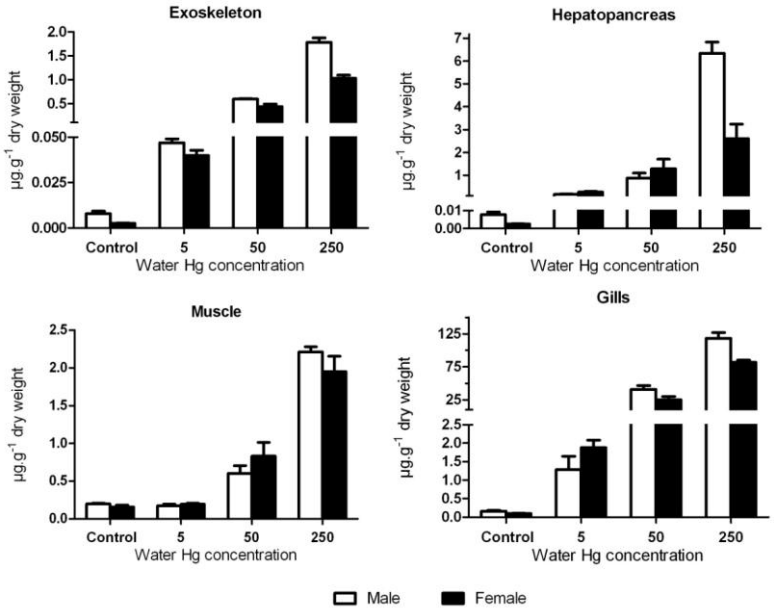

Fig. 1. Tissue mercury concentration (µg.g^{-1} dry weight) in green males and females. Values with an asterisk are statistically different (p<0.05).

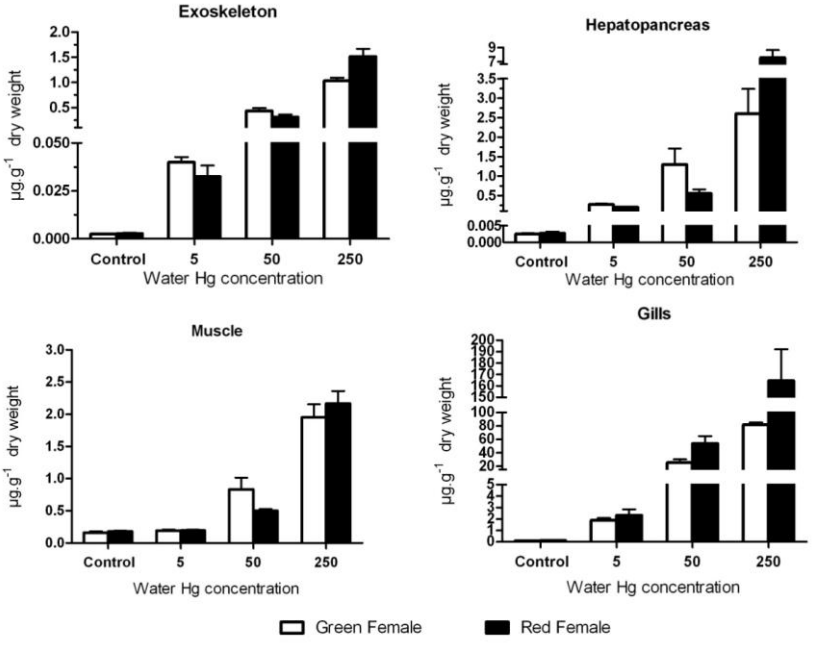

Fig. 2. Tissue mercury concentration (µg.g^{-1} dry weight) in green and red females. Values with an *asterisk* are statistically different (p<0.05).

Table 1 - Results of one-way ANOVA to test the differences among treatments.

Tissue	F	*p*	Dunnett test
Exoskeleton	519.60	<0.001	ctl≠5 ctl≠50 ctl≠250
Hepatopancreas	34.93	<0.001	ctl=5 ctl≠50 ctl≠250
Muscle	306.30	<0.001	ctl=5 ctl≠50 ctl≠250
Gills	730.13	<0.001	ctl≠5 ctl≠50 ctl≠250

Ctl- control; 5 - 5 µg L^{-1}; 50- 50 µg L^{-1}; 500- 500 µg L^{-1}

Table 2. Results of One Way ANOVA for the differences between tissues for all crabs groups and concentrations.

Treatment	Green males		Green females		Red females	
	F	Tukey test	*F*	Tukey test	*F*	Tukey test
Control	29.15*	E<H=G=M	18.31*	E<G=H=M	52.00*	E<G<H<M
5 µg L^{-1}	81.82*	E<M=H<G	98.15*	E<M=H<G	117.94*	E<M=H<G
50 µg L^{-1}	117.95*	M=E=H<G	62.99*	E=M=H<G	185.96*	E=M=H<G
250 µg L^{-1}	948.29*	E=M<H<G	249.97*	E<M=H<G	242.53*	E=M<H<G

E- exoskeleton; H-hepatopancreas, M-muscle and G-gills
*p<0.001

At 5 µg L^{-1} the concentration of mercury found in the gills was 28, 47 and 71 times higher than exoskeleton levels, respectively for green males, green females and red females. For 50 µg.L^{-1} it was 69, 59 and 174 times and for 250 µg.L^{-1} was 66, 80 and 109 times higher. At control conditions, the mercury concentration in the gills was only 21, 40 and 41 times higher than in the exoskeleton. Additionally, for red female group, at control conditions, the highest value was found at hepatopancreas while, for green males and females, the muscle presented no significant differences from hepatopancreas or gills. At 5 µg.L^{-1}, the mercury concentration found in the gills was 7, 10 and 12 times higher than that in muscle while at 50 µg.L^{-1} in gills were 68, 30 and 129 times and at 250 µg.L^{-1} 53, 42 and 76 times, respectively for green males, green

females and red females. Instead, in the control conditions, the muscle was the tissue with the highest mercury concentration (1.2, 1.6 and 1.4 times higher than in gills, respectively for green males, green females and red females). In control conditions, gills corresponded to less than 31% of total mercury in the analysed tissues, while in all the other experiments represented more than 75%. For exposed crabs at 5 $\mu g.L^{-1}$ the mercury accumulated followed the same trend for the three crab groups (muscle and hepatopancreas with similar values but higher than exoskeleton and lower than gills). At 50 $\mu g.L^{-1}$ the pattern was different (exoskeleton, muscle and hepatopancreas values were similar and much lower than in gills). For control conditions, hepatopancreas and muscle mercury concentration sum accounted for 67%, 76% and 73% of total mercury quantified, respectively for green males, green females and red females while, for the concentrations tested, represented less than 23%.

Table 3. Mercury accumulation rates ($\mu g.g^{-1}$ dry weight day^{-1}).

Tissue	Green males		
	5 µg L^{-1}	***50 µg L^{-1}***	***250 µg L^{-1}***
Exoskeleton	0.010 (±0.001)	0.146 (±0.003)	0.443 (±0.023)
Hepatopancreas	0.014 (±0.005)	0.194 (±0.055)	1.557 (±0.121)
Muscle	0.004 (±0.004)	0.109 (±0.026)	0.511 (±0.017)
Gills	0.299 (±0.090)	10.203 (±1.417)	29.535 (±2.154)
Tissue	**Green females**		
	5 µg L^{-1}	***50 µg L^{-1}***	***250 µg L^{-1}***
Exoskeleton	0.009 (±0.001)	0.009 (±0.001)	0.009 (±0.001)
Hepatopancreas	0.028 (±0.007)	0.028 (±0.007)	0.028 (±0.007)
Muscle	0.008 (±0.003)	0.008 (±0.003)	0.008 (±0.003)
Gills	0.448 (±0.051)	0.448 (±0.051)	0.448 (±0.051)
Tissue	**Red females**		
	5 µg L^{-1}	***50 µg L^{-1}***	***250 µg L^{-1}***
Exoskeleton	0.007 (±0.001)	0.007 (±0.001)	0.007 (±0.001)
Hepatopancreas	0.012 (±0.005)	0.012 (±0.005)	0.012 (±0.005)
Muscle	0.014 (±0.003)	0.014 (±0.003)	0.014 (±0.003)
Gills	0.556 (±0.132)	0.556 (±0.132)	0.556 (±0.132)

The results are the means more or less standard error (n=4).

The mercury accumulation rates increased with the increase in concentration of mercury in the water as shown in Table 3. The highest mercury accumulation rates were observed for gills at all concentrations tested and crab groups, and the lowest was observed in the exoskeleton or in the muscle depending on crab group and concentration (Table 3).

The duration of experiment was enough for all tissues of crabs exposed to 250 $\mu g.L^{-1}$ to be very close or even exceed the threshold concentration of 0.5 $mg.kg^{-1}$ for human consumption. At 50 $\mu g.L^{-1}$, only the gills exceeded the limit.

Discussion

At low concentrations, mercury compounds are toxic to living organisms. It is important to know by how much their concentration may be in above the normal range before effects on marine or estuarine organisms can be detected or commercial species become unsuitable as food (Bryan 1971). In the present study, the total concentration of mercury accumulated by the crabs followed the increase in surrounding medium. The accumulation at concentrations of 5 $\mu g.L^{-1}$ was enough to cause differences from control crabs but only at exoskeleton and gill tissues. Instead, 50 $\mu g.L^{-1}$ (maximum limit admissible for effluents from chlor-alkali electrolysis industry) and 250 $\mu g.L^{-1}$ caused a significant mercury accumulation only after 96 hours of exposure, at all tissues. These concentrations caused an accumulation in the crab tissues higher than the legislation allowed for human consumption. For field crabs, the results were in agreement with the ones found by Coelho et al. (2008) for Mondego estuary crabs, and they were not statistically different (p>0.05) from control, which attests no experimental contamination.

C. maenas presents many sex differences including maximum size, moult frequency and distribution on the shoreline (Reid et al. 1997; Lee et al. 2003) that may influence the contaminant accumulation patterns. For instance, Coelho et al. (2008), for field contaminated crabs, stated that females tend to accumulate more mercury than males in less contaminated areas whereas, in high environmental contamination sites, this situation was reversed. In the present study, some differences between males and females were detected as well. The organs with direct water contact

(exoskeleton and gills), at control conditions and at the highest mercury concentration, were significantly lower in females (p<0.05), while at 50 µg.L^{-1}, only the exoskeleton was significantly lower. Instead, the muscle (internal organ) did not show a differential mercury accumulation between sexes. For the hepatopancreas, the mercury levels found were significantly different between sexes but only for crabs exposed at 5 and 250 µg.L^{-1}. At the lowest water mercury concentration tested, the females presented the highest value, while at the highest concentration, presented the lowest, comparatively to the respective males.

The variability in uptake and accumulation of metals is often explained in terms of changing its bioavailability due to changing physicochemical conditions in the environment. The physiological condition of the individual *C. maenas* may also influence metal accumulation (Bjerregard 1990, 1991; Bondgaard et al. 2000). In this way, certain stages in the life of the animal could make it more susceptible to contaminants from the ecosystem. The higher mercury accumulation in the exoskeleton, heatopancreas and gills by red females could be caused by the different physiological tolerance and behaviour between *C. maenas* morphotypes. It is known that crab morphotype presents ecological and physiological differences. For instance, green crabs are better osmoregulators (Reid et al. 1989) and better oxyregulators (Reid and Aldrich 1989) while red crabs demonstrate narrower physiological tolerance (Lee et al. 2003) playing a part in control of its distribution in the shoreline (McKnight et al. 2000). Red shore crabs are more vulnerable to environmental stress of both natural and anthropogenic origin, and these stressors may operate in concert to exert a negative effect on shore crab physiology (Styrishave et al. 2004).

Besides the high accumulation of mercury in the tissues, no mortality was registered either for green or red crabs during the exposure tests in the present study but, for instance, Styrishave et al. (2000) over a cadmium 75-day exposure period, found a significantly higher mortality occurring in red crabs. The same pattern was observed by Dam et al. (2006) for crabs exposed to pyrene within 56 days. These authors stated that red individuals have a reduced tolerance to the contaminants. Our study was an acute test (96 h), but probably, if it lasted more days, some deaths could be registered. Styrishave et al. (2000) investigated the accumulation of cadmium in hepatopancreas, gills, muscle and haemolymph of starved and fed green and red crabs

during a 40-day exposure to 2 and 6 µM Cd^{2+} and did not find significant differences between colour morphs in gills and muscle. Instead, green crabs accumulated significantly more cadmium in the hepatopancreas and haemolymph than red crabs, at 6 µM Cd^{2+}. On the contrary, in the present study, the differences between green and red females were statistically significant not only for hepatopancreas but also for exoskeleton and gills. This difference was just for the highest concentration tested being the mercury content higher at red morph. In addition, at higher concentration of cadmium (6 mM Cd^{2+}), Styrishave et al. (2000) observed a tendency towards higher cadmium levels in muscle of red crabs than in the green crabs besides, without statistic significance, like in our study with mercury. This indicates differences in the contaminant kinetics between the morphotypes mainly at high water mercury concentrations and suggests that metal toxicity is conditioned by the crab's ability to accumulate it in the organs.

Styrishave and Andersen (2000) observed differences in hepatopancreas fatty acid between morphotypes caught at different seasons. Green crabs were found to have significantly higher levels of fatty acids. This could be an indicative of the "poor" conditions of red crabs because fatty acids are the principal energy source constituting the highest part of hepatopancreas lipid content. In other study, Styrishave et al. (2000) found that fatty acid content of green crabs was unaffected after cadmium exposure while red crabs showed a significant decrease in hepatopancreas fatty acid content, which was dose-dependent. The ability to accumulate cadmium in the hepatopancreas has been related to the physiological condition of the crabs. Bjerregaard (1991) stated that crabs in "good" condition accumulate lower amounts of cadmium in carapace, muscle and hypodermis and more cadmium in the hepatopancreas than crabs in "poor" condition. This is presumable for detoxification and/or excretion (Dam et al. 1006) by this organ. The physiological parameters also affect the efficiency with which individual crabs transported cadmium from the haemolymph to the hepatopancreas (Bjerregaard 1990). This shows that the crab's physiological status has a strong influence in the contaminant accumulation which was also revealed at the present study. In this context, the termination of growth, presented by red crabs, is likely to exert a profound effect on shore crab biology (Styrishave et al. 2004). The same authors speculated that cytochrome P-450 enzyme

family (CYP enzymes), involved in moulting, are also involved in the detoxification of xenobiotics and that such CYP enzymes are increased in certain stages of the moult cycle and are low in others. Such variations in CYP enzyme levels may in turn affect the tolerance of shore crabs to environmental stress and explain why crabs that are moulting frequently (green) are more tolerant to environmental stress than crabs that have terminated moulting (red). CYP enzymes hydroxylate a number of xenobiotics such as PAHs, thereby making them more water-soluble, which facilitates excretion (Styrishave et al. 2004). If green crabs, as consequence of higher growth rates, have higher CYP enzymes levels or have a higher capacity for inducing CYP enzymes than red crabs during PAH exposure (Rewitz et al. 2003), they may be better capable of metabolising some contaminants and/or excreting them. The difference in tolerance to heavy metals cannot be explained in a simple manner by differences in CYP enzyme expression, although indirect effects on, for example, membrane lipids may be involved (Styrishave et al. 2004).

The red colouration apparently develops during a particular prolonged intermoult, and consequently, such individuals are characterised by a greater load of epibionts, including cuticular biofilm and a thicker carapace (Reid et al. 1997; Laporte et al. (2002a). Laporte et al. (2002a) observed large amounts of mercury trapped on the crab cuticular biofilm after contamination with inorganic mercury. This could be an explanation to the highest mercury found in the gills and the exoskeleton of red females comparatively with the green females at 250 µg L^{-1} mercury. This agrees with some experiments examining the uptake and sequestration in phytoplankton tissue (e.g. Mason et al. 1996; Lawson and Mason 1998) showing that inorganic mercury is readily retained by unicellular algae. There are also evidences that inorganic mercury bind within the outer membrane of epithelial tissues (Domouhtsidou and Dimitriadis 2000). Nevertheless, the respective part of mercury taken up in the exoskeleton and gills tissues and that adsorbed on the biofilm could not be quantified in the present study. Mercury adsorbed on the surface or stored in the gill cuticle and carapace could be lost at moult, enabling its extrusion.

The differences in the crab tissues contamination recorded seem to be dependent on the major pathway of exposure, diet or water. In low contamination areas, represented by field crabs and control group, the preferential accumulation in the

internal organs, muscle and hepatopancreas, suggests that the major source of contamination is food (Coelho et al. 2008). It is known that mercury biomagnifies along food webs (Watras and Bloom 1992; Lawson and Mason 1998) and the highest trophic-level organisms accumulate mercury mostly from food rather than direct uptake from water (Evans et al. 2000; Laport et al. 2002). For the shrimp *Penaeus monodon,* Soundarapandian et al. (2010) also found a higher accumulation in the hepatopancreas and lower in gills. In field and control crabs, the lower mercury concentrations were found in the exoskeleton, which was foreseeable due to periodic moulting (Coelho et al. 2008). Contribution from dissolved fraction seems to be low, and it is reflected on the reduced mercury burden in gill tissue. Since *C. maenas* is a predator species, and the Mondego estuary has been used as a reference condition because no local source of mercury contamination is known (Coelho et al. 2006 and references therein), the results for field and control results are in accordance with the above statements. Instead, in highly contaminated areas, water exposure (mainly to inorganic mercury) seems to be the major pathway of incorporation (Laporte et al. 2002a). The present investigation has shown that the concentration of mercury in seawater had a considerable effect on its accumulation by *C. maenas.* Accumulation in the gills has been associated with a higher intake of water inorganic mercury, as the gills are in contact mostly with the dissolved and particulate species (Laporte et al. 1997). That direct contact of gills with water mercury resulted in the high level of mercury uptake and the highest mercury accumulation rates observed. Nevertheless, accumulation rate is affected not only by crab physiology but also by external factors such as the form of the metal, its speciation, and the potential complexing ligands in the medium (Andres et al. 2002). One of the primary controls exerted over accumulation is the transport of mercury across the membranes - the gut membrane for uptake from food, and the gill for uptake directly from water (for aquatic organisms), which are the interfaces between the organism and its close environment. The respective permeability and retention ability of these two structures determine the potential uptake of mercury from water or food. For uptake from water, the gill dominates over uptake through to the organism's carapace because of its larger surface area, the rapid movement of water across the gills surface and the physiology of the gills, which is well designed for accumulation of chemicals from solution (Laporte et al. 2002b). There is

an intense convection of water on their external side as well as of haemolymph on their internal side. For the same reasons, and besides being directly exposed to the surrounding environment as the gills, the exoskeleton accumulated relatively small amounts of mercury. Laporte et al. (1997), too, found particularly important mercury accumulation in gill tissue, and smaller levels in the carapace and internal organs.

The uptake pathways of mercury in the blue crab (*Callinectes sapidus*) were studied by Andres et al. (2002) and Laporte et al. (2002b) who reported that inorganic mercury uptake across the gill and intestinal tissues of the crab occur at a fast rate. This is due to the relatively unspecific nature of the uptake, with the potential for accumulation of mercury by a variety of pathways, both active and passive. It is tempting to think that the gills role is that of a protective barrier limiting access of the metal toward other compartments of the body, especially to internal tissues in the case of direct contamination from water. When mercury contacts with gills, one proportion of the metal taken up is retained in the gill tissue and other is left free to distribute inside the organism. Laport et al. (2002a) observed that only about 1% of the total mercury input from *C. maenas* was recovered in the effluent fluid from *in vitro* perfused gills and could thus be considered available to distribute inside the animal, via the circulatory system. This shows that the portion of mercury entering the gills, successfully crossing the different cellular layers to reach the circulating haemolymph, is very low compared with the portion that stays trapped in the gill tissue. They observed that the inorganic mercury was histochemically accumulated at two distinctly different locations in the gills: at the cuticular surface in direct contact with the contaminated medium and at high local levels in the central vacuole of gill nephrocytes. Besides, the gills account for no more than 1% of the shore crab body weight; they could accommodate as much as 33% of the total body burden of inorganic mercury (Laporte et al. 1997). The existence of cuticular layers covering the surface of the gills lowers the permeability of the gill but also constitutes a pool of binding sites for mercury (Andres et al. 2010). This explains the highest mercury content found in the gills at all concentrations tested. The mechanisms behind the internalisation of gills mercury route are unclear, but Laporte et al. (2002a) suggest endocytosis as the process.

In contrast with the high accumulation rate in the gills, the internal organs muscle and hepatopancreas, present lower accumulation rates. They are affected by water

mercury probably more slowly due to the gills barrier that incorporate the biggest part of inorganic mercury as referred to before. After, the first barrier transposed the mercury and, upon reaching the haemolymph, it will be absorbed by the hepatopancreas, since the role of this organ appears to be that of a "sponge" to mop up excess of heavy metals from the circulatory system and consequently keep the haemolymph heavy metals at a fairly normal level (Bryan, 1964). This seems to be a way to prevent other organs from being affected, thus limiting the mercury distribution to other internal target organs and, probably, its toxic effects to the organism.

In summary, this present work suggests that the mercury legislation limit of 50 µg L^{-1} and a hypothetical scenario of a point discharge of 250 µg L^{-1} cause significant accumulation on *C. maenas* tissues. It could represent a risk for population that uses that species as food resource because the highest concentration induces accumulation of mercury in crabs leading to concentrations above the limit set for human consumption. Additionally, since *C. maenas* is a prey for some fishes and birds, it could become a pathway to mercury accumulation through the food web. We can also highlight that sexes, morphotypes and tissues show different accumulation patterns and that large variability in the way individual European shore crab handle mercury is closely related to its physiological condition. This study provides, too, important information regarding the life-stage-dependent adaptation of *C. maenas* to anthropogenic stress, contributing to the elucidation of the mechanisms underlying the different accumulation and to evaluate the ecotoxicological significance of the presence of mercury in marine decapod crustaceans.

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CHAPTER IV

Kinetics of mercury accumulation and its effects on *Ulva lactuca* growth rate at two salinities and exposure conditions

Abstract

The research on metals effect on macroalgae has been focused on Cd, Cu, Zn and Pb and no study dealt with the effects of mercury on macroalgae growth rate. Also, the kinetic of accumulation is not well known. The main aims of this work were to assess the mercury accumulation kinetics of *Ulva lactuca* and its effects on growth rate. Three concentrations were tested: 5, 50 and 500µg.L^{-1}, under static and renewal conditions and at two salinities (15 and 35), during 72 h. The mercury accumulation kinetic patterns were different according to the exposure conditions and metal concentration, but were always a very fast process (hours). Three patterns were established based on the models substrate inhibition, linear regression and Michaelis-Menten equation. Statistical differences in the mercury accumulated were recorded depending on the salinity values and exposure conditions, being higher at salinity 15 and at renewal tests, corresponding to the lower relative growth rates. The lowest mercury concentration did not have an effect on relative growth rate, while the others caused an accentuated inhibition after 24 h. The highest concentration was toxic to algae causing its death before 48h. Under controlled conditions, the *U. lactuca* high and fast ability for mercury accumulation could be useful for phytoremediation and for industrial wastewaters treatment.

Keywords

Mercury · kinetic of accumulation · relative growth rate · *Ulva lactuca.*

Introduction

At the moment, several areas of knowledge have been observing the increasing number of pollutants in the world with great interest. Discharge of industrial wastewaters and untreated sewage causes a serious risk to the coastal and estuarine areas and consequently to human populations. Among the contaminants, metals are of utmost concern due to the potential impact of contaminated foodstuffs consumption on humans (Commission Regulation EC 466/2001) and consequent health hazard. Despite their natural sources, human activity is largely responsible for their abnormal release and accumulation in the environment. Several industries use metals due to their technological importance and applications. In order to control metal levels in the environment, the treatment of the contaminated effluents is highly important since they can accumulate on organisms with severe toxic effects (Boening 2000; Coelho et al. 2008; Elumalai et al. 2005; Hebel et al. 1997). The most commonly used treatment processes are physicochemical based, but in the past few years, a great deal of research has been undertaken to develop alternatives like biosorption (Wang and Chen 2009). Simultaneously, remediation skills have also being assessed for a possible use in contaminated areas. The aim is to find techniques that present efficiency, simplicity in the execution and lower cost. Several bioremediation methodologies have been studied and the phytoremediation, defined as the use of plants to remove pollutants from the environment or to render them harmless (Raskin et al. 1997), can achieve a predominant role. This biotechnology recently has become a subject of intense public discussion and scientific interest and also a theme for some reviews (Gerhardt et al. 2009; Lasat 2002) but little research about the use of macroalgae as phytoremediator for aquatic ecosystems could be found in literature (Fereshteh et al. 2007). Nevertheless, there are some studies focusing the importance of non-living algae biomass as biosorbents, due to their heavy metal uptake capacity, as reviewed by Wang and Chen (2009). Some authors studied the accumulation of diverse metals by live macroalgae (Amado Filho et al. 1997; Lee and Wang 2001; Masakorala et al. 2008), but only few recent studies focused attention on the kinetics of mercury accumulation (Turner et al. 2008; Vasconcelos and Leal 2001). Moreover, the research on metals effects on algae, at a laboratory scale, was focused in metals such as Cd, Cu, Zn and Pb (Han and Choi 2005; Han et al. 2008) but no study dealt with the effects of

mercury on macroalgae growth rate. The awareness on that point is important because macroalgae, as primary producers, are very important to most aquatic ecosystems and, consequently, changes in their density and composition can affect the chemical and biological quality of the habitat (Klaine et al. 2003).

Since mercury persists in the environment and often creates long-term contamination problems (Miretzky and Cirelli 2009*),* its influence on biota is important to address. This metal has important anthropogenic sources like chloralkali production, mercury mining, smelting, fossil fuel burning, and waste incinerators (Boening 2000). In the last decades, there has been an increase awareness of mercury contamination in various aquatic ecosystems being highlighted in international regulations (e.g. Water Framework Directive-UE 2000). Many studies have examined sources, environmental transport, accumulation, and biological effects of this metal (e.g. Boening 2000; Coelho et al. 2008; Laport et al. 1997). Despite this progress, many significant questions remain concerning its exposure and toxicological effects. Its phytotoxicity must be measured because is an essential component of any ecological risk assessment.

Macroalgae could be considered an important and abundant bioindicator of metal contamination in near shore coastal environments. They are able to concentrate essential and non-essential metals from water by several orders of magnitude (Turner et al. 2008). A green macroalgae often employed is *Ulva* sp. (Ho 1990; Villares et al. 2001). Its thin and sheet-like thallus results in large absorptive area to volume ratio and its cells are structurally uniform and physiologically active. These features, coupled with its cosmopolitan nature and ability to grow in eutrophic coastal waters and of moderate pollution, indicate that it may be a good bioindicator species of metal pollution (Ho 1990). It is tolerant to a wide range of salinity levels, and grows well in estuaries where salinity levels change with the tides. *Ulva* can grow at salinity levels both higher and lower than normal seawater (Yamashita et al. 2009). Furthermore, macroalgae are of economical importance, because some species are used for alimentary, agricultural or medicinal purposes.

The present work focused on the kinetics of accumulation of mercury in *Ulva lactuca* at low (5 $\mu g.L^{-1}$), medium (50 $\mu g.L^{-1}$) and very high (500 $\mu g.L^{-1}$) metal level, at two exposure conditions (static and renewal) and at two salinities (15 and 35). The aims were to assess the capacity of *U. lactuca* to remove mercury from water, to

evaluate the speed of this process and the way the salinity could affect the process. The salinity is a factor naturally unstable in estuaries, and the discharges from industries could also contribute to modify it. Since the effects of mercury on *Ulva* growth are unknown in the literature, influence of this contaminant on the relative growth rate was also assessed. The *U. lactuca* live biomass potential to remove mercury from aqueous solution, using them as possible phytoremediator of contaminated waters or in treatment of industrial wastewater, was furthermore discussed.

Material and Methods

Materials and reagents

All material used in the experiments and for the storage, processing and analysis of samples was washed in Derquim 3%, rinsed in Milli-Q water (MQW), soaked in 25% HNO_3 for at least 24 hours and subsequently rinsed with MQW. Mercury standard solution (analytical grade), containing 1001±2 mg L^{-1} of mercury (II) as $Hg(NO_3)_2.H_2O$, in nitric acid 0,5 mol.L^{-1}, was purchased from Merck. Working solutions were prepared by diluting the standard solution with MQW.

Collection and maintenance of algae and water samples

Macroalgae *U. lactuca* was collected in the Mondego estuary (Figueira da Foz, Portugal, 40°08`N, 8°50`W). The algae were transported to the laboratory in isothermal plastic bags with some local water, and subsequently rinsed with seawater to remove epibionts and debris. The algae were then transferred to 40 L clear glass tanks filled with aerated filtered seawater enriched with PES- Provasoli Enriched Seawater (modified of L. Provasoli 1963 in Bold and Wynne 1978) and maintained one week before beginning the experiment with 80 µmol photons m^{-2} s^{-1} of white fluorescent light, 14L:10D photoperiod, temperature 24±2°C and salinity 15 and 35. A sample of the field collected algae (initial reference algae) was analysed for mercury content through the methodology explained below and was considered the initial level of the contaminant in the algae used in the experiment. Seawater was collected at Buarcos beach (8 Km North from the estuary), filtered through 0.45 µm pore size filters and stored at 4 ºC until further use.

Experimental set-up

Due to the morphological and physiological characteristics of *Ulva* similar in all thalli (Ho, 1990) we used only a portion of it (disks). Algal disks (Ø18 mm) were cut from the healthy thalli of the macroalgae and placed in erlenmeyers with 50 mL of filtered natural seawater enriched with PES and appropriated amount of mercury (solutions prepared using the mercury standard solutions ($Hg(NO_3)_2.H_2O$)). Nominal concentrations were 5 µg L^{-1}, 50 µg L^{-1} and 500 µg L^{-1}. The control group consisted of enriched seawater medium without addition of mercury. Cultures were undergone to same room conditions previously mentioned but were not aerated, although maintained under constant stirring. Two conditions of exposure were performed: static (algae were exposed to the culture medium without changing the water during the duration of the test), and renewal (culture medium was replaced periodically - every 12 hours) at two salinities (15 and 35). There were three replicates per treatment and the experiments were run for 72 h. The amount of mercury accumulated by the algae was determined at 15, 45 and 90 minutes and 4, 8, 12, 16, 24, 48 and 72 h. At each sampling time algae were removed from water, well washed in clean seawater and freeze dried for later mercury analysis. Additionally, at 24, 48 and 72 h, the algal disks were harvested to determine changes in its area.

The concentrations tested were selected to simulate different scenarios. One was the contamination caused by industries, in which effluents cannot contain more than 50 µg L^{-1} (maximum limit admissible by legislation chloralkali electrolysis industry effluents - Directive 82/176/EEC). Other scenario was to simulate an accidental discharge of non treated effluent (500 µg L^{-1}). Simultaneously, this concentration was used to evaluate the potential capacity of *Ulva lactuca* to remove mercury from highly contaminated industrial wastewaters, and use them for its treatment. The lower concentration tested (5 µg L^{-1}) was chosen to simulate the effluent after in-house partial treatment. Simultaneously, the static and renewal conditions were tested to simulate a punctual discharged (static conditions) or intermittent discharged (renewal conditions), respectively.

Analytical procedures

Algae disks were analysed for total mercury by thermal decomposition atomic absorption spectrometry with gold amalgamation, using a LECO AMA-254. Accuracy of the results was assessed by using the certified reference material (CRM) BCR60 (*Lagarosiphon major*). The average of daily recovery percentage of the CRM analyses was 103±1% (average±standard error, *n*=31).

Data analysis

For the mercury accumulation kinetics patterns, were applied the equations that had better adjustment to the data. They were based on the substrate inhibition equation, Michaelis–Menten equation and linear regression.

Relative growth rates (RGR) were calculated from the differences between initial (0) and final (t) area (A) over the time (t), in days, using the equation: RGR (% day^{-1}) = ((ln A_t – ln A_0) /t) x 100%, where A_0 and A_t are the areas of disks at time zero and after t days, respectively (Han et al. 2008).

The significance of observed differences of mercury accumulated by *U. lactuca* between the two salinities till 12 h (common to both exposure conditions), were assessed by the Mann-Whitney Rank Sum test. The differences after 16 h were tested performing two-way analysis of variance (ANOVA). The analysis considered salinity and conditions of exposure in the variation of mercury accumulated by algae. Three-way ANOVAs were used to test differences in growth rates across salinity, conditions of exposure and initial mercury concentration. When a significant effect of the response occurred, Tukey-adjusted pairwise comparisons amongst groups were computed. Prior to statistical analyses, normality and homogeneity of variance were checked and, when necessary, logarithm transformations were performed.

Results

Mercury accumulation in algae

The kinetics of mercury accumulation by the algae in the two conditions of exposure and for the two salinities tested is shown in the Fig. 1. For the highest concentration (500 μgL^{-1}) there are only results for the first 24 h, because afterwards

the algae were discarded due the accentuated colourless indicating its decay or sporulation (Han et al. 2005), at the two conditions of exposure, and for both salinities. The disks from the other concentrations did not show colour change.

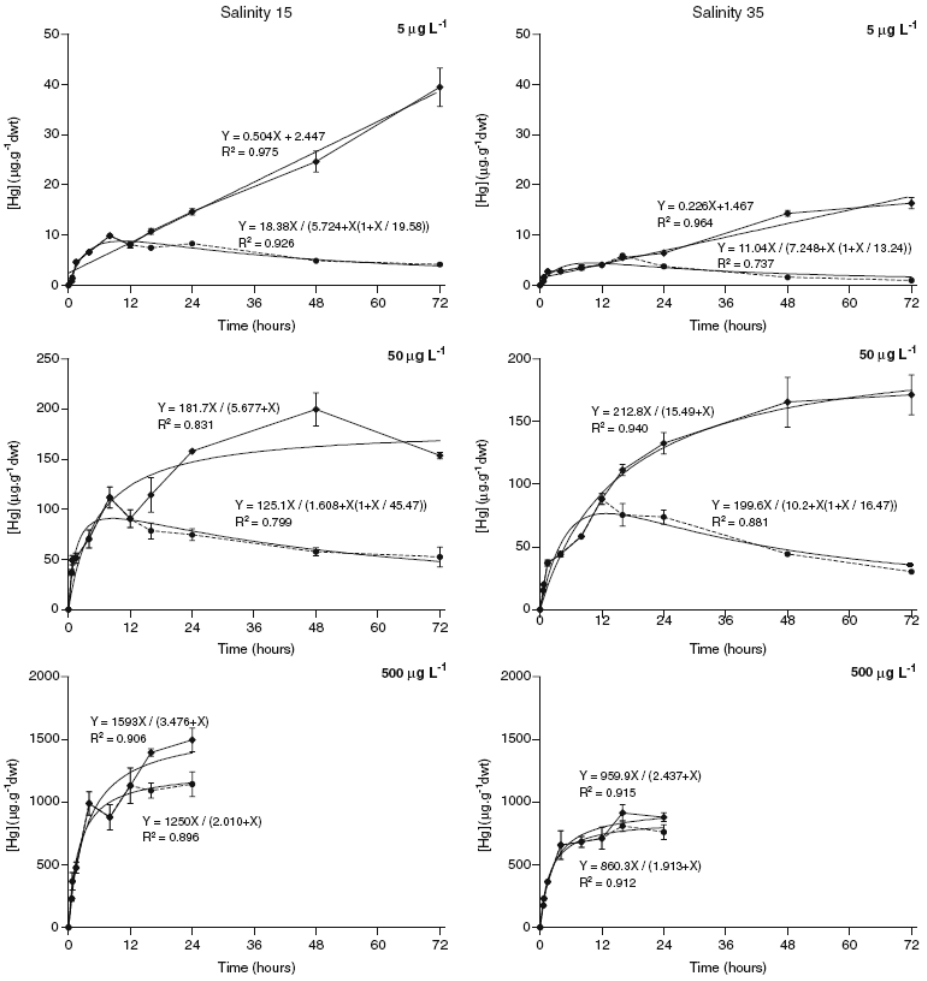

Fig. 1. Mercury accumulated by *Ulva lactuca* (µg g^{-1} dry weight) during the experimental period (72 h), for static and renewal test. The results are expressed as the mean ± standard error (n=3).

In all conditions there was a fast boost in the algae mercury concentrations from the beginning. A dose-dependent accumulation was evident, with mercury levels on the algae increasing with the increase of the initial water mercury concentration. For the static test, and salinity of 15, the mercury algae content ranged between a minimum of 0.016±0.003 $\mu g.g^{-1}$, in the initial reference algae (algae analysed just after

to be collected in the field), and a maximum of 0.11±0.02, 8.3±0.3, 111±10 and 1142±98 μg.g^{-1}, respectively for control, 5, 50 and 500 μg.L^{-1}. For salinity 35, the mercury achieved a maximum of 0.032±0.009, 5.9±0.4, 88±4 and 80±43μg g^{-1}, respectively, for control, 5, 50 and 500 μg L^{-1}. For the renewal test, the highest mercury algae content, at salinity 15, was 0.11±0.01, 39±4, 199±16 and 1495±94 μg g^{-1}, respectively for control, 5, 5 and 500 μg.L^{-1}, while at salinity 35 was 0.092±0.040, 16±1, 171±15 and 914±35 μg.g^{-1}.

Concerning the total mercury accumulated by the algae during the experimental period, three different kinetic patterns it could be observed, as a function of the exposure time (Fig. 1). At static conditions after an increase during the first hours, a steeply decrease till 72 h was recorded. For the accumulation rate a model based on a substrate inhibition equation could be applied for the two lowest concentrations (5 μg.L^{-1} - salinity 15, R^2=0.926, salinity 35, R^2=0.737; 50 μg.L^{-1} - salinity 15, R^2=0.800, salinity 35, R^2=0.881). At the renewal test, on the contrary, a continuous raise from the first minutes till the end of experiment was registered, except at 72 h for 50 μgL^{-1}, salinity 15 and at 24 h for 500 μg.L^{-1}, salinity 35, where was observed a slight decline. For 50 and 500 μgL^{-1}, the accumulation rate was fast in the first hours followed by a slower phase, presenting a model based on a Michaelis-Menten equation (50 μg.L^{-1} - salinity 15, R^2=0.831, salinity 35, R^2=0.904; 500 μg.L^{-1} - salinity 15, R^2= 0.906, salinity 35, R^2=0.912). For the concentration 500 μg.L^{-1}, in static conditions, could also be applied the Michaelis-Menten model for the first 24 h (salinity 15, R^2=0.8960; salinity 35, R^2=0.9155). For 5 μg.L^{-1}, at renewal conditions, algae mercury increased all over the experimental period, with a linear trend (salinity 15, R^2=0.975; salinity 35, R^2= 0.964).

When differences were found between salinities the amount of mercury accumulated was superior at lower salinity (15). For the first 12 h, the differences were significant for 5 and 50 μg L^{-1} (Mann-Whitney Rank Sum Test, F=396, p<0.05; F=415, p<0.05), but for 500 μg L^{-1}, besides the highest values at 15, the differences were not significant (Mann-Whitney Rank Sum Test, F=106, p>0.05). Between 16 and 72 h two-way ANOVA (Table 1) showed significant differences for the factor salinity at 5 and 500 μg L^{-1} (p<0.001). For the concentration 50 μg.L^{-1} the results were only statistically different at 48 hours (p<0.05). For the factor exposure conditions, as expected, the

mercury accumulated was higher at renewal than static conditions, presenting significant differences for all concentrations tested (p<0.05) (Table 1).

Table 1 - Results of factorial two-way ANOVA to evaluate significant differences in mercury accumulated by *U. lactuca*, after 16, 24, 48 and 72 h, to salinity (S), exposure system (E) and their interactions.

Time (h)	Concentration (µg L^{-1})	Salinity		Exposure condition		SxE
		F-value	Tukey test	F-value	Tukey test	F-value
16	5	51.60***	15>35	11.25*	R>S	18.21**
	50	0.02		12.13**	R>S	0.018
	500	32.35***	15>35	7.85*	R>S	1.17
24	5	279.23**	15>35	140.76***	R>S	23.26***
	50	5.00		147.54***	R>S	4.39
	500	42.89***	15>35	9.59*	R>S	2.36
48	5	38.37***	15>35	219.62***	R>S	10.12*
	50	7.60*	15>35	242.61***	R>S	0.13
72	5	217.74***	15>35	1050.53***	R>S	12.51**
	50	0.057		161.22***	R>S	4.41

Salinity:15 and 35; Exposure condition: S, static test; R, renewal test.
*p<0.05, **p<0.01, ***p<0.001

Effects of mercury on algae growth

The effect of mercury accumulated by *U. lactuca* on its relative growth rate (RGR), expressed as %.day^{-1}, is presented in the Fig. 2. For 500 µg.L^{-1} RGRs were only calculated for 24 hours due to the signs of decay presented by algae after that sampling time, as previously mentioned.

Different RGR patterns of responses to the experimental conditions were observed. Three-way ANOVA revealed significant differences between salinities at the three times sampled (p<0.001) (Table 2), being higher at 35. The lower growth rates at low salinity corresponded to the highest mercury accumulation by algae. Static tests also resulted in high RGR, but only significant at 24 hours (p<0.001), between control and the highest concentrations (Tukey test, Q=5.005, p<0.001; Q=17.698, p<0.01, respectively for 50 and 500 µg.L^{-1}). At salinity 15, the mean RGR of 50 µg.L^{-1} (8±1 %.day^{-1}) was reduced to 38% of the control (13±3 %.day^{-1}) and at 500 µg.L^{-1} the RGR was negative (-7.0±0.4 %.day^{-1}). For salinity 35 the significant inhibition was 39% from the control (18±1 %.day^{-1}) at 50 (11±2 %.day^{-1}) and RGR was negative at 500 µg L^{-1} (-4±2 %.day^{-1}). The negative RGR is a sign of a shrinkage or margin decomposition of the disks, aspects precursors of the algae death.

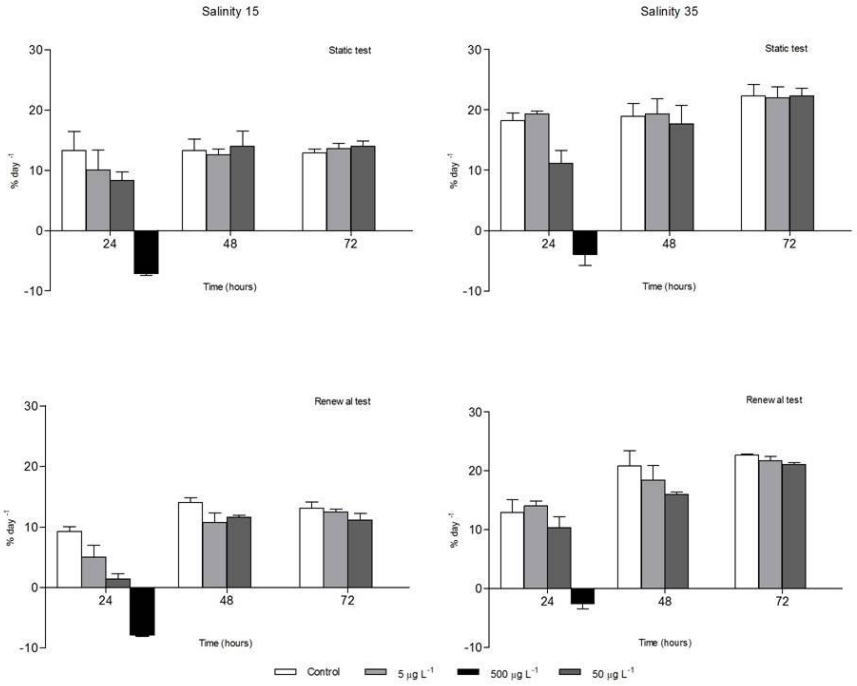

Fig. 2.- Relative growth rate (% day^{-1}) of *Ulva lactuca* exposed to 5 µg L^{-1}, 50 µg L^{-1} and 500 µg L^{-1} Hg, at 24, 48 and 72 hours. Results are expressed as the mean ± standard error (n=3).

Table 2 - Results of factorial three-way ANOVA to evaluate significant differences in relative growth rate of *U. lactuca*, after 24, 48 and 72 h, to salinity (S), exposure system (E), concentration (C) and their interactions.

Time (h)	Salinity		Exposure condition		Concentration (µg L^{-1})	
	F-value	Tukey test	F-value	Tukey test	F-value	Tukey test
24	47.47*	35>15	15.96*	S>R	102.24*	Ctl, 5>50>500
48	25.58*	35>15	0.42		1.01	
72	219.41*	35>15	1.76		0.29	

Time (h)	SxE	SxC	ExC	SxExC
	F-value	F-value	F-value	F-value
24	0.99	1.81	2.12	0.90
48	0.17	0.65	0.80	0.00
72	0.52	0.10	1.21	0.12

Salinity: 15 and 35; Exposure condition: S, static test; R, renewal test; Concentration: Ctl, control; 5, 5 µg L^{-1}; 50, 50 µg L^{-1}; 500, 500 µg L^{-1}

*p<0.001

On the renewal tests, similar patterns were also found with statistical differences at 24 h (between control and 50 µg L^{-1}, Q=4.409, p<0.001; between control and 500 µg L^{-1}, Q=13.638, p<0.05). At 48 h, differences on RGR were observed between control and the concentration 50 µg.L^{-1}, but were not significant (Tukey test, Q=1.010, p≥0.05). For salinity 15, the significant inhibition was 85% from the control (9 ±0.7 %.day^{-1}) at 50 µg L^{-1} (3±1 %.day^{-1}) and at 500 µg L^{-1} the RGR was negative (-7.9±0.1 %.day^{-1}). For the salinity 35, the inhibition was 21% from the control (13±3 %.day^{-1}) at 50 µg L^{-1} (10±2 %.day^{-1}) and RGR was negative at 500 µg L^{-1} (-2.6±0.9 %.day^{-1}). On renewal conditions, independently of the differences statistically found, there was a tendency for a lower *U. lactuca* RGR with increasing mercury concentration in the medium. Those differences are more noticeable than in the static tests and tend to decrease during the time, reaching a minimum at 72 h. The lowest concentration tested (5 µg.L^{-1}) did not cause any significant effect on RGR (Tukey test, 24 hours, Q=1.589, p≥0.05; 48 h, Q=1.509, p≥0.05 and 72 h, Q=0.523, p≥0.05).

Discussion

The results obtained corroborate previous knowledge that marine macroalgae accumulate dissolved metals in their tissues at high rates (Andrade et al. 2006; Rainbow 1995) due to the density of surface functional sites and the binding capacity of its intracellular ligands (Turner et al. 2008). Nevertheless, in situations of exception (i.e. discharge of non-treated effluents), simulated by an extremely high mercury concentration (500 µg.L^{-1}) the algae showed signs of decay before 48 hours, independently of both exposure conditions and salinity. It was observed that high mercury concentrations in water result in high values reached by algae and in fast accumulation. Thus, the levels of mercury in the algae tissue reflect the relative amounts of metal in the surrounding medium.

The pattern of mercury accumulation kinetics was the same within each concentration independently of the salinity. The different models found have distinct performances. On the renewal test, mercury stayed always available due to renovation of the medium, shown by the larger levels of mercury found on algae. In these conditions, the lower concentration showed a linear pattern similar to the one found

by Lee and Wang (2001), which exposed *Ulva pertusa* to Cd, Cr and Zn for a period of 8 h. Similar results were found by Wang and Dei (1999) that recorded for *U. lactuca* exposed to Cd, Cr, Se and Zn a linear uptake over 47 h of test to the lower concentrations, but at the highest concentration, they recorded an initial rapid adsorption of metals within the first 2 h, followed by a linear pattern of metal uptake between 2 and 47 h. Metals are generally taken up into cells by membrane transport proteins that have receptor sites to bind metals (Sunda and Huntsman 1998). When the majority of the metal transport sites are available the uptake rate should be directly proportional to either the concentration of free metal in solution or to the metal transport sites (Andrade et al. 2006; Sunda and Huntsman 1998). This could explain the linear regression pattern of the accumulation rate at the lower concentration, 5 $\mu g.L^{-1}$, at renewal conditions. However, in waters with high dissolved mercury levels, the active sites of the algal surface become saturated and the intracellular metal becomes independent of metal concentration (Andrade et al. 2006). For 50 and 500 $\mu g.L^{-1}$ the pattern was similar to the one found by Vasconcelos and Leal (2001) that exposed *Porphyra* and *Enteromorpha* to 1 $\mu mol.L^{-1}$ mercury for 24 h. The results are in agreement with studies that postulate that algal uptake mechanisms leading to an enrichment of the metal within the cells involve a combination of adsorption of the metal ions to the surface of the algae and intracellular uptake (Andrade et al. 2006; Vasconcelos and Leal 2001). After some initial lag time, equilibrium is attained between the metal in solution and the metal adsorbed to active sites on the cell surface (Andrade et al. 2006; Sunda and Huntsman 1998). Under such steady state conditions, the flux of metal into the cells follows the Michaelis-Menten kinetics for facilitated or active transport (Andrade et al. 2006), as observed in the present research for the highest concentrations at renewal conditions. In this way, the initial rapid accumulation phase observed may correspond to an extracellular adsorption and/or to passive intracellular uptake (metabolism-independent) involving cell surface adsorption and simple diffusion into the cells or intercellular spaces (Vasconcelos and Leal 2001). Sorption of metals on algal cell walls has been treated in terms of two models: adsorption, in which the metal becomes bounded to one of several unoccupied sites with no additional changes at that site, and ion exchange, in which a metal displaces another ion in the sorption process (Crist et al. 1994). The

slower phase will correspond to metabolism-dependent incorporation in the cell body (Vasconcelos and Leal 2001).

Under a chemical approach, the biotic phase (algae) of the present laboratorial experiment can be seen as an inanimate volume of material that is approaching thermodynamic equilibrium with its aqueous medium (Mackay 1982). Once this equilibrium has been attained, no further change in the quantities of the components will occur as long as the system remains undisturbed, although, according with Le Chatelier`s principle, the chemical equilibrium could be disturbed tending to reach new one. This happened in the renewal conditions, because mercury was added every 12 h, which displaced the equilibrium to compensate the additions resulting in mercury accumulation by the algae.

At static conditions, after the initial fast accumulation, a decreasing phase occurred, which could be justified due to the "dilution effect". Mercury availability in the water decreased over time due to absorption and metabolism of the test organisms, volatilization and adsorption to the walls of the flasks (Walker et al. 2008), which associated with the algae growth, diluted the accumulated metal and resulted in less mercury by dry weight of algae.

The performed experiment demonstrated that salinity had significant influence in the mercury accumulation, being higher at lower salinity and corresponding to the lower RGRs calculated. These results are in agreement with findings of other authors (Laport et al. 1997, Wang and Dei 1999) which reported that low salinities favour metal accumulation. At lower salinity there are fewer ions in solution reducing the possibility of formation of metal chloro-complexes and increasing the abundance of free metal ion, the most bioavailable form, to be accumulated (Verslycke et al. 2003). Also, algal cell walls can act as cation-exchange systems and bind toxic metals as a result of different reactions between the metal ion and link sites on the cell. The cell wall in *U. lactuca* is a structure consisting of negatively charged polysaccharides which present a number of potential metal binding sites with different affinities for metals. The functional significance of negatively charged polysaccharides in the cell walls is that binding of cations allows the ionic environment of the alga to be regulated during the tidal cycle (Webster et al., 1996). In this way, interactions and/or competitions between the mercury and sodium (Na^+) cations could happen. In situation of low

salinity there are less Na^+ in solution and bounded to algae surface. So, mercury has more sites available to bind. That could explain the highest mercury accumulation at low salinity.

When a constraint is exerted by environment, plants responses usually take the form of changes in the rate and/or pattern of growth. For several factors, including chemicals, there is a range that allows growth of all individuals without harmful effects and other that does not allow growth of any individuals (Fitter and Hay 2002). In the present study, the control was assumed as the range that allowed health algae growth and 500 $\mu g.L^{-1}$ that caused algae death. Metals can operate as stress factors causing physiological reaction changes and reducing vitality, or in the extreme, totally inhibit plant growth (Levitt 1980). Transport systems that take up metals into the cells are never entirely specific for a single "intended" nutrient metal. These systems often bind non-nutritive or toxic metals, resulting in inhibition of uptake of the nutrient metal and intracellular transport of the competing toxic one (Sunda and Huntsman 1998). These competitions can influence growth rates by two ways: inducing nutrient metal deficiency or direct metal toxicity. This was particularly clear at the highest concentration tested since the algae showed signs of decay.

A plant is considered "sensitive" when the stress that it is subjected results in injury or death, and "resistant" if the plant is able to survive and reproduce, contributing genetically to the next generation (Baker 1987). Resistance to metals can be achieved by either one of two strategies: a) avoidance, by which a plant is protected externally from the influences of the stress and b) tolerance, whereby a plant survives the effects of internal stress. Tolerance is therefore conferred by the possession of specific physiological mechanisms which collectively enable it to function normally, even in the presence of high concentrations of potentially toxic elements (Baker 1987). In the present study *U. lactuca* was sensitive to 500 $\mu g\ L^{-1}$, since decay was observed but resistant below that concentration tolerating mercury. Plant growth rate is a crucial factor for showing tolerance of the test plant to contaminants. In this experiment, besides the presence of the metal on *U. lactuca* tissues, its effects on growth were small. Only at 24 h, 50 and 500 $\mu g.L^{-1}$ concentrations caused significant reduction on RGR. As well, 5 $\mu g.L^{-1}$ had any measurable significant effect on growth over time. Shiber and Washburn (1978), based on field studies, also proposed that

lower mercury concentrations in algae (0-8 $\mu g.L^{-1}$) do not appear to inhibit the overall growth of *U. lactuca* or be toxic to it. Probably, as reported by Válega et al. (2009) to the salt marsh plant *Halimione portulacoides,* the biggest part of mercury was immobilized in the cell walls of algae which could function as a protection barrier by reducing the metal concentration in the cytoplasm.

At 72 h the RGR of the control and the two lower concentrations, at the static conditions, was similar probably because the amount of mercury decreased in the water and the algae was no longer under high metal toxicity as in the beginning of the experiment (24 h). This allowed algae to recover and to grow enough to achieve the area of the control ones. On the renewal test, due to renovation of water, a new input of mercury was added periodically being in that way constantly to the algae. This could justify, the tendency to lower 50 $\mu g\ L^{-1}$ RGR over time, nevertheless without statistical significance. Those differences were decreasing during the exposure period and at 72 h the RGRs were very similar. These could be indicative of adaptation of *U. lactuca* to the presence of mercury in the water by some physiological process. Moreover, we can also deduce that more severe effects on RGR will probably be observed within the range of 50 and 500 $\mu g\ L^{-1}$, since the first caused a growth reduction (despite being slighter at the end of the experiment), but the last one was toxic enough to cause algae death.

From the results, we could infer that salinity 35 allows higher growth independently of the presence of mercury. Also, Yamashita et al. (2009) found the highest growth rates when salinity was similar to seawater. We could also infer that renovation of medium caused, probably, stress on algae at first hours, as proved by lower RGR compared with static test. Overall, we could suppose that the principal factor that influences *U. lactuca* growth at long term (at least 72 h) is salinity when mercury concentrations are low or equal to 50 $\mu g.L^{-1}$. On the contrary, the mercury (below 50 $\mu g.L^{-1}$) and renovation of medium only influenced the growth at short time (24 h).

The extension in which the accumulation happens in the organisms could be used as an essential parameter in the ecotoxicological evaluation. The accumulation of a potential harmful substance by algae could make it, at least temporally, unavailable to the other organisms, reducing the harmful substance in the aquatic environment. For

the other groups (e.g. fishes, invertebrates) the accumulation by algae implies greater impact resistance. In this way, algae could be considered “protecting species” in the aquatic systems (Vidotti and Rollemberg 2004). In another way, accumulation by algae could be an important factor to the biomagnification of contaminants, since as primary producer represent an important link in the food web, constituting the food source for the higher trophic levels. In that case, the toxic metal can be transferred up through the estuarine food webs and be incorporated in economically important species (Coelho et al. 2005). Its human use in many forms (food, agricultural, and industrial purposes) may also result in health risks. Moreover, these floating algae could play a key role in mercury transport from contaminated to non-contaminated areas.

This work provides some evidences that the maximum limit admissible by legislation to chloralkali electrolysis industry effluents, 50 μg L^{-1} (Directive 82/176/EEC), seems to be appropriated when taking into account the survival and growth of *U. lactuca* at 72 h. Additionally, some considerations about the potential of *U. lactuca*, as phytomediator can be made. Phytoremediation often involves high contaminant concentrations that may cause stress or toxicity beyond natural or background levels (Medina et al. 2003). Therefore, in order to use effectively plants to remove metals from the environment, firstly they must be able to tolerate and then accumulate (Stearns et al. 2008). Regarding the conclusions of that study we can affirm that *U. lactuca* presents these two criteria. The high ability to take up large amounts of mercury from water, as proved by kinetic patterns, associated with the fact that its growth at 48 and 72 h was not significantly affected by the two lower concentrations, which suggests that *U. lactuca* live biomass has potential for phytoremediation. That technology could be ideal to clean up low to moderate levels of some contaminants over extensive areas and to restore sites to more pristine conditions. The use of this species as a phytoremediator and also for binding and removing mercury from industrial wastewaters takes a prominence place due to its efficiency, low cost and abundance.

The present work allows assessing the kinetics of mercury accumulation in *U. lactuca* and its growth effects. The mercury accumulated by *U. lactuca* reflects its occurrence in water and its kinetic pattern is different according to the exposure conditions and metal concentration, but is always a fast process. Additionally, we can

conclude that low mercury levels do not have effects on RGR and that high salinities favour the macroalgae growth. Instead, low salinities favour mercury accumulation. Overall, this work has provided evidence of the potentialities of *U. lactuca* to remove mercury from contaminated waters, but more studies and evaluations of the interactions among algae, water, pollutants and physical and chemical parameters are necessary. The study of the specific biochemical processes behind mercury kinetic and tolerance of *U. lactuca* is an interesting research to follow.

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In this last section is addressed an integrative approach of all data obtained from the previous chapters. It is discussed its contribution to the increase knowledge about the effects of environmental variations and of the mercury in the studied estuarine species. As well, an overview of its consequences to the ecosystems is also presented.

Ecosystems and biota: pressures and responses

Facing the progressive degradation of the ecosystems due to anthropogenic pressure and the increasing natural drastic changes, severe impacts are expected on biota. Chemical contaminants are of great interest to humankind due to their toxic effects on the organisms. Nevertheless, they represent only one type of stressor in the environment. Others include for instance: nutrients, turbidity, temperature, oxygen, salinity, hydrological regimes, food quality and quantity. Some stressors can be anthropogenically induced but their natural variation can also produce stress. Consequently, it is not easy to distinguish the effects of anthropogenic from natural stress (Elliot and Quintino 2007).

The ability to withstand/tolerate/adjust/adapt to stressors being it at the levol of the individual, population, community or ecosystem is known as homeostasis (Elliot and Quintino 2007). Thus, the natural systems have capacity to deal with perturbations within some limits. In this way, when a perturbation occurs, the responses are in order to achieve a stable state but if the perturbation is severe, the system may not be able to compensate it. The perturbations will force the individuals, populations and communities to respond. The responses coul be diverse and, in many situations, are unpredictable.

The stressors acting on the species could impact them through single, cumulative or synergistic processes (Adams 2005). Additionally, the studies of multiple stressors suffer difficulty in establishing cause and effect (Adams 2005) because the incorporation of all interactions is not straight forward. Species responses are complex and are influenced by the history of previous stress or disturbance to which a

population or a community has been exposed (Thrush et al. 2008). As well, density effects and species location relative to optimal niche requirements affects the responses (Thrush et al. 2008). Additionally, individuals or populations that exist in sub-optimal conditions, in terms of habitat requirements or physical disturbance regime, may well be more sensitive to additional contaminant stresses (Scott-Fordsmand and Deplege 1993; Dissanayake et al., 2008; Thrush et al. 2008). The stressors can impair health and fitness of biota leading to changes in its reproduction, growth, abundance, diversity, population dynamics, production, mortality and geographical range of distribution (Drinkwater et al. 2010; Primo et al. 2011; Adams 2005; Cardoso et al. 2004; Dolbeth et al. 2007). Due to the complexity and variability of marine systems, multiple lines of evidence are needed to understand relationships between stressors and effects on marine resources (Adams 2005). Some species are more tolerant than others, but all are affected by changes in the surrounding environment. The responses of biota depend on their sensitivity, adaptive capacity and vulnerability (Houghton 2005) and are an integrated result of both direct and indirect processes (Adams 2005). Consequently, different groups of organisms may respond differently to changes depending, for instance, on the ecology and feeding habits of the species.

It is very important trying to understand the causal relationships and the processes between environmental conditions and/or contaminations, and its potential effects in order to improve the effective management and restoration decisions of damaged ecosystems. Some effort has been made towards this through the classification of chemical contaminants and the type of biochemical response expected. Also, the concern about protection of water resources has been highlighted in international reports and legislation (e.g European Union Water Framework Directive). However, ecosystems are difficult to restore in full, since they have many components and support very complex interactions (Cardoso 2005).

There is no doubt that Earth is experiencing progressive global warning resulting in diverse and serious costs for our planet. The climate change, and its consequent related stressors, is disrupting ecosystem services like fisheries, water quality and shoreline protection that are fundamental to society (Chapin et al. 2000, Worm et al. 2006). The frequency and intensity of extreme events induces changes in the physical

environment resulting in significant proximate and emergent responses in the biosphere (Christopher et al. 2006). At local and global scales, climate change has been responsible for changing the distributions, population size and subsequent genetic drift, seasonal life cycles and biota interactions (Christopher et al. 2006; Alheit 2009; Kraberg et al. 2011). It can reorganize marine communities and the trophic relationships inducing readjustments in the dominant species. Currently, patterns related with latitudinal distribution of species could be influenced by climate change for instance by a northward displacement of appropriate habitats due to the increase in seawater temperature. The succession of different extreme weather events could have a synergistic effect on the global stability of the ecosystems, delaying its recovery (Grilo et al. 2011).

C. maenas responses to the environmental conditions

The decapoda crustacean studied in the present thesis is known to tolerate a wide range of external conditions. Even though, despite interanual variations, it was possible to assess some effects of drought events on *C. maenas* population structure and abundance. On the first chapter we recorded that the response to the drought conditions was an increasing in the *C. maenas* abundance determined mostly by the larger recruitment. Various causes could impact the recruitment. Salinity, temperature, river discharge variations, currents, coastal wind speed and direction may be major environmental driving forces acting in the recruitment success and in the population development of various estuarine species (van der Veer et al. 2000; Attrill and Power. 2002; Martinho et al. 2009). Nevertheless, the presence of first life stages predators, the cannibalism and the availability of food are other density-dependent important factors to consider (Mosknes et al. 1998; van der Veer et al. 2000; MacDonald et al. 2001; Amaral et al. 2009). For some species, like for *C. maenas,* which some planktonic life stages are developed in the coastal waters, the connectivity between estuarine and coastal areas are of great importance. In the green crab some blockage in the zoeae I exportation to the sea or in the reinvasion of the estuary by the megalopae stage could decrease sharply the recruitment. Mortality rates operating over the eggs and larval stages could be capable of generating wide variations in recruitment. In general,

changes in the abundance of key species in the ecosystems could have severe consequences for its dynamics. It will impact the species relationships including the food webs. Accordingly, the implications of increasing abundance of *C. maenas* could be critical to the estuarine and coastal ecosystems. One of the consequences of distributional shifts of individual species is the possibility of changes in the composition of ecosystem assemblages (Drinkwater et al. 2010). Several studies have confirmed that crab predation is an important factor structuring marine benthic communities (Raffaelli et al. 1989; Yamada and Bouling 1996; Grosholz et al. 2000). The predation by the European green crabs can influence the abundance and distribution of commercially important bivalve species (Raffaelli et al. 1989; Richards et al. 1999).

Implications of parasitism in the scope of climatic changes

Another factor that could regulate populations of free-living organisms is the parasitism. Accelerated perturbation in global ecosystems can increase pathogen development and its survival rates, disease transmission, and host susceptibility (Harvell et al. 2002). The knowledge about the responses of parasite processes during episodes of climate change is important to research. To follow this, it is fundamental to first know the host-parasite interactions and its impacts on host fitness. The effects of global warming on the assemblages of hosts, parasites and pathogens can be numerical, functional or microevolutionary, and can involve cascading changes in ecosystems, as reviewed by Brookes et al. (2007).

The external form of the studied parasite *S. carcini*, increased more sharply in winter, when the temperature was lower. We can suppose that a change in the environmental temperature could displace the parasite presence and manifestation to other seasons. It was also confirmed that the parasite`s pressure on *C. maenas* is exerted at various levels. The infected crab usually does not moult and its behaviour is greatly influenced by the *S. carcini* presence resulting in consequences to the *C. maenas* demography.

The climatic changes could have direct effects on the *C. maenas* population, as presented in the first chapter, but also indirectly through the probable changes in the life cycle of its parasites.

Dealing with mercury

The performed studies presented in this thesis not only found stressors responses at the level of *C. maenas* population dynamics but also at the physiological level in respect to the crab mercury accumulation. *C. maenas* seems to be tolerant to an acute mercury contamination because during the hours of mercury exposure no deaths were recorded. Nevertheless, the effects of stressors are not restricted to death and many organism functions could be affected. Through the performed experimental tests some evidences emerged from the differential genders, morphotypes and tissues patterns of mercury aacumulation which were recorded in the thirth chapter. Either, distinct patterns of mercury accumulation, depending on the preferential uptake pathway, were observed. The variability in the way the shore crab hold mercury may to be directly related to its physiological condition, which could be also related with previous exposure to other stressors that were impossible to determine in our studies. The toxicity of mercury of aquatic organisms is affected by both abiotic and biotic factors, including the form of mercury, environmental conditions (temperature, salinity and pH), the individual tolerance of each organism and the sensitivity of each species and life stages (Tchounwou et al. 2003). The toxic effects of mercury are also dependent on dose and duration of exposure. It is important to note that both the concentration in the tissues, the time and the exposure route are critical factors in the toxic symptoms to aquatic organisms. When evaluating the environmental hazards of mercury it is necessary to extrapolate from laboratory experiments to ecosystems. This must be done with extreme caution because in natural systems many factors interfere with mercury behaviour. Overall, it is interesting the ability of *C. maneas* to tolerate the adverse and variable conditions within an estuary (in the current case the mercury presence and the drought hidrological conditions) having advantages over other less tolerant species, achieving thus high population densities.

Relatively to the experiment with the primary producer *U. lactuca,* it contributed to the better understanding of the influence of water renovation and salinity in the toxicity of mercury. Moreover, it gave some indications about the effects of the mercury and its dependence on the natural stressor salinity. The accumulation by the macroalgae reflected its occurrence in water and its kinetics pattern was different according to the exposure conditions. It seemed that low mercury levels do not have

effects on its growth rate evidencing the ability of *U. lactuca* to deal with low levels of mercury. This study was important because *U. lactuca* could be a pathway for mercury entering food webs. It is actively grazed upon by several herbivore species (Geertz-Hansen 1993; Kamermans et al. 2002). However, from another point of view the approached detailed in this thesis could have important utility to understand the potentialities of *U. lactuca* to remove mercury from contaminated waters using it as phytoremediator. Phytoremediation is widely viewed as the ecologically responsible alternative to the environmentally destructive physical remediation methods currently practiced. Significant progress has been made in recent years in developing native or genetically modified plants for the remediation of environmental contaminants (Meagher, 2000). When contaminants are in low concentration, phytoremediation alone may be the most economical and effective remediation strategy (Jones, 1991). Indeed, this is an important subject of research because over the time, the quantity of xenobiotic compounds in ecosystems has increased considerably. In the particular case of mercury besides the several global strategies intending to reduce its uses and emissions, the transport and fate of this contaminant will remain a key issue of environmental concern (Martins 2007).

We are rapidly changing the earth`s environment but we not yet fully understand the consequences of these changes. The appropriated management of the ecosystems, and of its associated resources, depends how well we could detect variations in the environment and predict the responses of organisms to them. However, the multiplicity of natural and anthropogenic stressors endangering the key systems, including the estuarine ones, is high and could compromise its functions. They might not support future generations unless major measures are implemented to protect them.

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FINAL REMARKS

From the studies presented in this thesis some questions emerged indicating future potential research directions.

Since the increase in frequency of extreme weather events is expected, the estuarine fauna will face new challenges. The impacts will be not only ecological, but also economical. So, predict the responses of species and ecosystems to the climate are essential. In face of this, prolonging monitoring programs should be encouraged, in order to address the long-term effects of climatic changes in the various ecosystems. Several other processes and relationships (e.g. migration, predation, cannibalism) relevant to explain the species dynamics should be included in further studies. Yet, further research must be focused in the potential ramifications of climate change for parasites and consequently for its hosts.

Because the stressors did not act single, future work must focus in the determination of the stressors interactions and processes. So, this more evaluations of the relations among species, pollutants and physico-chemical parameters are need. For instance, the interaction of mercury with other contaminants may produce synergistic or antagonistic effects. Moreover, the abiotic factors determine the chemical speciation of mercury compounds in aquatic media making it more or less bioavailable to uptake by the organisms. Thus, the study of the biochemical processes behind mercury bioaccumulation and biomagnifications dynamics in the aquatic ecosystems is an interesting and essential subject to research. As well the use of other methodologies of research to understand the flux of mercury from one trophic level to the next is a key subject in the next future.

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