MÉTODOS ECONOMICAMENTE EFICIENTES PARA MONITORIZAÇÃO DE ÁGUAS DE TRANSIÇÃO

COST-EFFECTIVE METHODS FOR MONITORING ENVIRONMENTAL CONTAMINATION IN TRANSITIONAL WATERS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Amadeu M.V.M. Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e o Prof. Doutor Trevor C. Telfer, Senior Lecturer do Institute of Aquaculture, University of Stirling, Scotland, United Kingdom.

Apoio financeiro do POCTI no âmbito do III Quadro Comunitário de Apoio
Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de bolsa de Doutoramento SFRH/BD/4778/2001
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Nunca fácil, agradecer. Porque a memória (traçoeira), o tempo (esse assassino silencioso), a rotina (o mais antigo dos soporíferos), a distração (um mal moderno) ou a insensibilidade (que todos negamos possuir) laboram para que assim seja. Além do mais, sendo um espaço contextualizado, faz soar estrangeiras gratidões que por vezes nos atrevermos a expressar e que apesar do aspecto alienígera para terceiros, a nós tanto disseram ou dizem. Mas porque será que é, serão a primeira, uma das primeiras secções onde um qualquer folheador de teses se dirige? (outra questão será entender porque me perco sempre nestes considerandos...). Talvez pelo tangível, pela necessidade de perspectivação, de enquadramento do não racional da mente que subjaz à pressuposta cientificidade do resto texto. Será sempre este o referencial: o humano.

O de quem lê e o de quem escreve.

Assim, com antecipadas desculpas aos ausentes e pela parcimônia das particularizações (quando presentes, porque me sinto na necessidade de a todos agradecer em igual medida), a minha gratidão vai para as pessoas, locais, objectos e eventos que tiveram a sua influência no percurso que o texto ora presente representa.

São eles:
Amadeu Soares (orientador), Trevor Telfer (co-orientador), Donald Baird (ex-orientador).

Phil Ross (na memória jaz a eternidade...) , António Nogueira (corrente mentor, futuro orientador).

LabEcos e LabEcas (a vida seria muito mais cinzenta sem vocês...).

Pessoal do LETAL, funcionários do Departamento de Biologia (em particular o Sr. Rui pelas conversas e nunca ter frío... e às “meninas” da Secretaria... por serem meninas...).

Billy Struthers, Andy Sheen, Gundmundur Helgasón e Halldór Haldorsson.

México, Escócia e Islândia (a minha alma por lá ficou...).

A minha cadeira (por todo o apoio e suporte prestados durante a escrita), Bill Gates (e os patudos da Microsoft)... 

Alentejo.

TODA a (minha) família.

Mãe, Pai (...palavras não bastam...).

D. Maria Pia (pela ternura, e personalidade... e quase me levar à loucura...).

Caty (...tu sabes...).
palavras-chave

Ecotoxicologia; bioensaios; metais; *Echinogammarus marinus*; gradiente latitudinal; anorexia pós-exposição.

resumo

A avaliação de ecossistemas aquáticos, tradicionalmente realizada pela análise de parâmetros físicos e químicos tem-se revelado manifestamente insuficiente. Por norma as concentrações de poluentes variam rápida e amplamente e a medição destas parametrías representa unicamente uma pequena janela temporal de uma realidade contínua e complexa. O aparecimento de índices bióticos veio preencher parcialmente esta lacuna dado que os organismos aquáticos actuam como monitores a tempo inteiro da qualidade ambiental, fornecendo informação de difícil acesso por outros meios. A necessidade do estabelecimento de relações mecanísticas entre tóxicos e efeitos observados conduziu à tentativa de ligação entre os perfis de toxicidade obtidos em ensaios laboratoriais e os obtidos no terreno. Considerando a saúde dos seres vivos como um resultado de interacções sinergísticas ou antagonísticas de uma combinação de agentes poluentes e/ou de stress, extrapolações relativas ao estado de “saúde” dos ecossistemas eles próprios podem ser obtidas. Um reduzido número de organismos pode assim ser utilizado com este propósito, a efectividade da correlação estabelecida depende da sua relevância ecológica e do realismo das concentrações dos tóxicos utilizados. Neste estudo o apuramento das concentrações de metais pesados na Ria de Aveiro (uma lagoa costeira de hidrografia complexa e diversas fontes de emissão destes elementos) foi realizado por meio de amostragem de sedimentos de fundo e o anfípode *Echinogammarus marinus* foi usado como organismo-teste para determinação de efeitos biológicos. Os invertebrados, em particular os crustáceos, são frequentemente seleccionados para este efeito dada a sua relevância nas cadeias alimentares, facilidade de manutenção laboratorial e grande dispersão. Bioensaios in situ, devidamente suportados por testes laboratoriais com metais em misturas, foram realizados utilizando como parâmetro a anorexia de pós-exposição. Esta avaliação foi estendida a toda a distribuição conhecida para a espécie em termos latitudinais. A influência exercida por factores como o estágio de desenvolvimento, gênero e estado nutricional anterior à exposição foram avaliados. Foi ainda estudada a existência de uma possível correlação entre a presença de metais e a incidência de intersexo.

*E. marinus* revelou ser um biomonitor sensível do potencial tóxico de metais pesados em concentrações ambientais ecologicamente relevantes, que pode ser utilizado em toda a abrangência biogeográfica do organismo. Um bioensaio in situ, simples e custo-eficiente é apresentado. O bioensaio destina-se à monitorização de águas salobras e salgadas para a presença de metais, permitindo o apoio à decisão na alocação de recursos humanos e financeiros por norma limitados.
The traditional assessment of aquatic ecosystems, based upon the analysis of physical and chemical parameters has been proven insufficient. Concentrations of pollutants usually fluctuate widely and rapidly and those analyses are but a freeze frame of a continuous complex reality. The use of biotic indexes partly covered this shortcoming since aquatic organisms act as full-time monitors of environmental quality providing otherwise hardly accessible information. The need for the establishment of mechanistic correspondences between toxicants and detected effects lead to attempt of coupling toxic profiles obtained in laboratory with the ones observed in the field. By considering the condition or health of organisms as a response to synergistic and antagonistic effects of combined pollutants and/or stressors, extrapolations towards the health of the ecosystem itself can be drawn. A restrict number of organisms can be used for this purpose with the effectiveness of the connection relying mainly on their ecological relevancy and in the use of realistic toxicant concentrations. In our study a survey was performed upon the sediments of Ria de Aveiro (a coastal lagoon with complex hydrography and varied heavy metal sources) as a means to determine heavy metal concentrations, and an amphipod, *Echinogammarus marinus* was used as test organism. Invertebrates, and particularly crustaceans, have been highly favored as organisms of choice due to their relevancy in food chains, easiness of culture and ubiquitousness. In situ bioassays, duly backed by ex situ toxicity tests using metal mixtures with post exposure feeding inhibition as endpoint were performed. This evaluation was extended to encompass the entire latitudinal distribution of the organism. Furthermore the influence exerted by life stage, gender and pre-exposure nutritional state upon metal toxicity were investigated. A correlation between metal contamination and the incidence of Intersex was attempted. We conclude that *Echinogammarus marinus* is a sensitive biomonitor of the toxic potential of metals at environmental realistic concentrations, suitable to be used at the entire breadth of its biogeographic distribution. A simple, cost effective in situ bioassay is presented, destined to screen brackish and saltwater bodies for metals presence, thus helping decision making in the correct allocation of usually limited financial and human resources.
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CHAPTER 1

General Introduction
1. GENERAL INTRODUCTION

“Change is [...] norm...” (McManus 1998)

1.1. Estuaries: physicochemical and biological profile

An estuary could intuitively be defined based in its primary variable: salinity. An area where fresh water enters saline water and where the salinity is, at least during some period of time, neither truly saline (>30 g/l) nor truly fresh (0 g/l) (however, there are areas that fit this definition and are not estuaries - e.g., anthropogenic point-source discharges, groundwater flow into the sea) (Chapman and Wang 2001). A more “formal” definition could be “An estuary is a semi-enclosed and tidal coastal body of water which has a free connection with the open ocean and within which sea water is measurably diluted with fresh water derived from land drainage.” (Pritchard 1967). As a reflex, the definition for estuarine sediments could be presented as “sediments whose interstitial salinities are neither truly fresh nor truly saline; that is they range above 1 and below 30 g/l.” (Chapman and Wang 2001).

Estuaries possess a group of associated characteristics that grant them a unique physicochemical and biological profile:

- Deposited sediments can come from inland and/or from the sea (Guilcher 1967);
- Presence of strong gradients for salinity, temperature, pH, dissolved oxygen, redox potential, nutrients, and amount and composition of particles (with marked variations on the temporal and spatial scales) (Chapman and Wang 2001);
- Represent areas of great productivity and important feeding, migration and rearing (never ceasing, being transitional areas, to challenge both residents and immigrants) (Herman et al 1999);
- Ecologically are zones of reduced interspecific (but not intraespecific) competition (Herman et al 1999);
- Faunal distributions are primarily controlled by salinity (leading to the “brackish water paradox”) and secondarily by factors such as substrate, temperature, dissolved oxygen and anthropogenic pollution (a complex correlation is sometimes established between salinity and temperature that can modify biological tolerance to the other factors) (Berger et al. 1995; Boesch 1977; Kline 1966; Wolff 1983).

(Note: is never enough to reinforce the idea that the majority of these phenomena are salinity driven, with vertical, horizontal and time variations both in overlying and interstitial waters) (Chapman and Wang 2001).

As a result of such constraints organisms can only survive in estuaries by one or a combination of the following strategies: avoidance (of estuarine conditions), reduced contact with inimical environments, adaptation or acclimation. However not all organisms living in estuaries live under optimal conditions, which results in natural bioenergetic stress to those organisms (Chapman and Brinkhurst 1981). This could be translated in more (or less) susceptibility to anthropogenic stress.

1.2. Coastal areas, estuaries and pollution

Privileged places of human settlement, recipients of river discharge, repositories of anthropogenic waste disposal, “the septic tank of the megalopolis”, coastal areas and estuaries are silent witnesses on the history of manmade contamination (Shahidul Islam and Tanaka 2004). Contaminants enter estuarine and marine waters via several key pathways, specifically direct pipeline discharges from coastal communities, discharges and dumping from ships, riverine output, atmospheric deposition, and nonpoint source runoff from land (Kennish 1992; Windom 1992). The most common anthropogenic wastes disposed in the coastal zone are industrial and municipal wastes, sewage sludge, and dredged material. Pollutants typically associated with these wastes include heavy metals, synthetic organic compounds (xenobiotics), organic carbon, nutrient elements and pathogens (Liss 1976).
The controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters is, unlike pH in freshwater, salinity (Luoma 1983; Schuel and Kennedy 1984). High ionic strengths in these waters can salt out hydrophobic organic chemicals from water to the sediment phase. In addition salinity enhances the removal of dissolved organic matter from the water to the sediment phase and the formed particulate organic matter can effectively sorb hydrophobic chemicals (Brunk et al. 1997). As a result, an increase in salinity generally results in an increase of the partitioning coefficient ($K_d$ - ratio of a contaminant concentration in the sediment to that dissolved in the underlying or interstitial water) for the sediment for hydrophobic chemicals (Brunk et al. 1997). In contrast, $K_d$s for metals may decrease (e.g., Cd, Zn), increase (e.g., Fe), or be constant (e.g., Ir) when salinity increases (Turner and Millward 1994), depending on the relative importance of the two counteractive processes: (1) desorption due to increasing complexation with seawater anions (Cl$^-$ and SO$_4^{2-}$) and/or increasing competition for particle sorption sites with seawater cations (Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$) and (2) coagulation, flocculation and precipitation. One exception to desorption has to be pointed: with increasing salinity this process can increase metal concentrations in water, but concurrent increases in Ca$^{2+}$ and Cl$^-$ can decrease water bioavailability (Chapman and Wang 2001). According to Sharp and Culbertson (1982) all physicochemical considerations made regarding salinity are equally applicable to estuarine and the coastal environments, since seawater can be looked upon as undiluted “brackish” water. Since it affects partition of contaminants between sediment and overlying and interstitial waters, salinity also affects bioavailability of these contaminants in the three compartments. Partitioning to particles favors uptake by sediment feeders (Forbes et al. 1998), whereas desorption to the water (overlying or interstitial) favors uptake via dermal exchange surfaces such as gills (Fisher and Reinfelder 1995; Forbes et al. 1998). Nevertheless it should be noted that concentrations of heavy metals in sediments quite trivially exceed those of the overlying water by between three and five orders of magnitude (Bryan and Langston 1992). Two implications ensue from this fact: the bioavailability of even a small fraction of the total metal in sediment assumes considerable importance and; environmental degradation by
metals can occur in areas where water quality criteria are not exceeded, yet organisms living inside, in direct dependence or near sediments can experience adverse effects (Salomons and Forstner 1984). Nevertheless this situation could be subverted when the system suffers disturbance. Fine-grained bottom sediments partly prevent the occurrence of toxicity towards organisms living in the water column, but are simultaneously latent metals reservoirs. Changes in sediment chemistry, due to seabed disturbance, can result in metal remobilization and consequent transformation into more bioavailable or toxic chemical forms with the concomitant effects (Zhuang et al. 1994).

But chemistry isn’t the sole process controlling bioavailability. Biological (e.g., microbial degradation and bioturbation) and physical (e.g., currents, river flow, and tidal exchange) processes can also influence amounts of pollutants available for uptake (Rasmussen et al. 2000).

In the specific case of estuaries, these water bodies act as reaction vessels where chemically very different fresh (the main carrier of contaminants) and salt water are drastically mixed. This mixture is often far beyond a simple dilution, behaving non-conservatively (Liss 1976). As pointed before, sediments can act both as a sink or a source in response to salinity, constituting, for many contaminants, a filter between land and sea. Only metals forming very strong complexes and organic chemicals that are less hydrophobic may be transported out of estuaries to the ocean. This natural mechanism renders estuaries more susceptible to contamination (Turekian 1977). From these facts becomes evident that the fate and effects of pollutants in brackish and saltwater must be a priority in ecotoxicology, with a special emphasis in the associated sediment compartment.

1.3. Grain size effects in sediment metal distribution

The grain size distribution is the most important factor controlling sediment metal concentrations, commonly existing correlations between decreasing grain size and increasing metal concentration ( Förstner 1989; Horowitz 1991). This causes different sediments to have
significantly different capacities for collecting metals (minor modulating factors are grain surface area and coatings). In consequence the correct interpretation and reporting of sediment metal concentrations can only be correctly performed if these concentrations are normalized to grain-size. Nonetheless, physical separation methods present problems of efficiency and feasibility (particularly in small samples), rendering mathematical normalizations as the best way for grain size effect correction (Horowitz and Elrich 1988). Several elements have been used as surrogate parameter to obtain the desired normalizing effect (e.g. Potassium, Lithium, Scandium, Gallium, Zirconium, Thallium, Iron and Aluminum) (Loring 1991). Ratios of metal/normalizing parameter are fairly constant in the earth’s crust returning robust regressions, allowing identification of deviations in individual samples, corresponding to enrichment or depletion of natural backgrounds. Other sediment constituent recurrently pointed as normalizing agent is organic carbon (OC) (Di Toro et al. 2001; Soto-Jiménez and Páez-Osuna 2001; Summers et al. 1996). Despite being the leading factor controlling hydrophobic organic chemicals in sediments (Di Toro et al. 2001) organic carbon is also an important partitioning phase for metals in sediment and in water because of the tendency for positively charged metal ions to bind to negatively charged sites that are associated with natural organic matter (Di Toro et al. 2001).

1.4. Sediment Quality Guidelines for the evaluation of risk in sediment associated metals

Ideally, site-relevant or regional Sediment Quality Guidelines (SQGs) should be developed wherever possible, to take into account site-specific conditions (e.g. bioavailability, sensitivity of indigenous organisms, exposure pathways) (Wenning and Ingersoll 2002). But limited resources do not always permit this. Fortunately there is a great wealth of SQGs being used by various regions, nations and organizations. When choosing what SQG to apply it is important to note that even if included in the same category (e.g. “background based”; “no effects”, “probable effects”, “extreme effects”) they differ in many ways. These differences include purpose, calculation methods, applicability, individual and groups of Contaminants Of
Potential Concern (COPCs) covered, analytical methods, and absolute values (Apitz et al. 2007).

It should be noted that every author, nation, and organization deriving and using SQGs always states with more or less emphasis that SQGs should be only one (initial) part of a decision framework, basically used for sediment ranking and for flagging of COPCs (e.g. Apitz et al. 2007; Moffat et al. 2004; Wenning et al. 2005; WFD 2000). The rationale being that transcendence of a determined pre-set value isn’t an obligatory synonymous of toxic capabilities of the sample.

1.5. Ecological Risk Assessment of metals under the Water Framework Directive

Among the available instruments for evaluation of risk, the ones set in the Water Framework Directive ("Directive 2000/60/EC of the European Parliament establishing a framework for the Community action in the field of water policy", commonly designated “WFD”), are necessarily among the most pertinent, being Portugal one of the European Union member states (and thus bound to its application).

The entering in force of the WFD on 22 December 2000, rationalized the pre-existing EU (and EEC before that) water legislation and developed the concept of ecological quality status (EcoQ)(2000/60/EC). The EcoQ is based upon the status of biological, hydromorphological and physico-chemical quality elements. Special emphasis is bestowed in the biological component, while being supported by the others (TGD 2003). For a “good status” to be achieved it establishes a “combined approach” of emission limit values and Environmental Quality Standards (EQSs).

As a result of this process, EQSs for Priority Substances and Priority Hazardous Substances (Annex X to the WFD) are contained in an EU Daughter Directive that underwent formal negotiations (2455/2001/EC) and the aim to “meet environmental objectives” by 2015 was assumed by European partners. On the other hand, setting of EQSs for “Main Pollutants”
(listed in Annex VIII of the WFD) and substances not yet included in this list but “liable to cause pollution” are under initiative of member states. Should they deem it necessary standards could be set for water, sediment or biota.

The derivation of EQSs for metals can be based on one of two approaches: added risk or total risk. The added risk approach accounts for natural background concentrations (C_b) of metals, simultaneously preventing environmental standards to be set at values below that background and considering that those concentrations have contributed to ecosystems biodiversity and/or fulfill the needs for micronutrients of species in nature (Klepper et al. 1998). Toxicity tests results are used to derive a Maximum Permissible Addition (MPA) quantity of metal to the environment without causing toxic effects (Lepper 2005). According to the amount and adequacy of data available, Assessment Factors (AFs) are applied compensating eventual weaknesses. The sum of the background concentration with the MPA derives a Maximum Permissible Concentration which constitutes the Environmental Quality Standard (MPC = EQS) (Lepper 2005).

The focus of the total risk approach is the bioavailable fraction of metal in the medium despite of provenience (natural background or anthropogenic input). In this case the Environmental Quality Standard equals the Maximum Permissible Addition (EQS = MPA) (CSTEE 2004).

The differing regional geological nature dismissed original intentions (and some initial work) on establishing a “default background concentration” (CSTEE 2004). In a context of environmental assessments it is essential that regional and even local background levels be derived (e.g. Carral et al. 1995). The concept of being able to define a “global background” is impracticable (Reimann et al. 2005).

At the present moment there are no defined EQSs for sediment. In the opinion of Lepper (2005) this gap occurs due to working economy that lead to the derivation of an EQS for each priority substance only for water by default. The Scientific Comitee on Toxicity, Ecotoxicity and the Environment (CSTEE) advances two reasons for the Commission not to consider
presenting specific Quality Standards for sediment and biota: data on toxic effects on benthic organisms and biota is of limited availability, is provenient from different types of sediment matrices (with the corresponding implications for registered effects) and different contaminant levels in sediment are of varying importance according to location (CSTEE 2004). Furthermore it is stated that uncertainties regarding monitoring points, sampling and analytical methods, would make compliance checking in sediment and biota difficult for the purpose of implementation of Community legislation.

Thus Priority Substances (WFD - Annex X, e.g. lead and cadmium) had their EQSs derived only for the water compartment the same happening for Annex VIII substances (amongst which #7: "metals and its compounds", e.g. chromium, copper and zinc). TGD (2003) delineates a strategy were SQGs for "New and Existing Chemicals" can be derived according to emergent necessities from basin management plans.

1.6. Biomonitoring the aquatic environment

The monitoring of physical and chemical components in water and sediment cannot be regarded as the sole assessment approach upon aquatic ecosystems. Concentrations of pollutants usually fluctuate widely and rapidly and those analyses are but a freeze frame of a continuous complex reality. Aquatic organisms can provide useful information on the water quality over periods of time, especially when chemicals are present in extremely low concentrations (Chapman and Brinkhurst 1981). Organisms in the field integrate responses in time scales reacting to synergistic and antagonistic effects of combined pollutants and/or stressors. As such, they can be considered full-time monitors of environmental quality.

Biomonitoring was introduced on the assumption that the measurement of the condition or health of organisms can in turn be used to assess the health of the ecosystem itself. The initial advance was under the form of bioassessments: the evaluation of the biological condition of a waterbody using biological surveys and other direct measurements upon biota (usually in surface waters) (Gibson et al. 2000). It was a field “passive” exercise involving the
collection of organisms, known as “bioindicators”, “sentinel organisms” or “biomonitor”, from their natural habitat (Chapman 1996) and contrast them with benchmarks (biocriteria) (USEPA 1990). The necessity for manipulative approaches simultaneously with the adoption and adaptation of the pharmacological concept of bioassay (a laboratory test in which the strength of a substance is measured by the response of living organisms or living systems) created a whole new set of experimental approaches and designs in ecotoxicology (e.g. mono-species bioassays, test-batteries, analyses of benthic communities and bioaccumulation studies). It was acknowledged that close coupling between laboratory and analogous field experiments was, more than useful, essential to allow extrapolations regarding natural populations or community patterns in nature (Swartz et al. 1986).

Organisms selected for biomonitoring purposes generally differed from those used in standardized laboratory experiments. The latter were often selected for their sensitivity, whereas the former due to the capability to endure a relatively wide range of pollutant conditions. The choice of organism has a determining influence on the relevance, success and interpretation of the test results. Ideally species to be chosen as modern biomonitor should fulfill several criteria (Kelly & Harwell 1989; Kramer and Botterweg 1991; Chapman 1996; Mayer et al. 1992; Forbes and Forbes 1994; Lau et al. 1998): be abundant, sedentary, easy to collect (throughout the year), easy to identify and handle, relatively long-lived, reasonably sized to provide adequate tissue samples for (individual) analyses, easily cultivated and maintained in the laboratory and resistant to handling stress, accumulate the pollutant of interest at levels present in the environment without lethal toxic effects and be relatively tolerant to pollutant exposure, and show rapid response to an early exposure of various pollutants. According to the study design and objectives some of these criteria could be prioritized acquiring higher importance, some others could be totally overlooked and/or new ones could be demanded.

So it was that in situ toxicity tests (the preferred name in environmental work for bioassays) duly backed by ex situ assays, became inextricable steps on risk assessment with
invertebrates, and particularly crustaceans, being highly favored as test organisms of choice (Lenihan et al. 1995).

1.7. Amphipods in toxicity testing

Both the number of different morphological, physiological and behavioral adaptations and the immense variation in life history patterns and reproductive traits have facilitated the evolutionary success of crustaceans (Sastry 1983). Due to their sensitivity and ecological relevance, crustaceans can provide means for deriving powerful indicators of environmental stress. The specific biological attributes demonstrated, where life history strategies pontify, endow them with great advantages when compared to other invertebrate groups, as for example, bivalves or gastropods (Marsden and Rainbow 2004).

Since the early days of ecotoxicity testing with invertebrates that amphipods have been repeatedly selected amongst the crustaceans (Lenihan et al. 1995; Moore et al. 1991; Rainbow et al. 1989; Swartz 1979, 1984, 1985; Weeks 1992). A good example of amphipod potential is females carrying eggs or juveniles in specialized structures on the body (the marsupium). Being this reproductive behavior easily assayed in laboratory and semi-field experiments as well as in biological field surveys, it can be regarded as a true ecological endpoint for disturbance. Moreover it allows predictions of the potential quantitative and qualitative impairment of the studied population development (e.g. Lawrence and Poulter 2001; Sundelin and Eriksson 1998). Moreover not only reproduction but also other amphipods responses, e.g. behavioral, such as changes in feeding (Maltby et al. 2000), swimming (Lawrence and Poulter 2001) or precopula behavior (Pascoe et al. 1994) have provided sensitive endpoints with respect to bioindication. In addition amphipods are often the main food source for predatory fish and birds (Beare and Moore 1997; Bocher et al. 2001) generating considerable potential for accumulated contaminants (e.g. metals) to be transferred along marine food chains (Wang 2002).
1.8. Amphipods and metals

Metal taken up by amphipods (a sum from dissolved and food sources, with sediment-associated metals also playing a role when present) will initially be available to bind with metabolites in receiving cells, and to be transported anywhere in the body via the haemolymph (Rainbow 2002). This metal will participate in the metabolism of the receiving organism either fulfilling an essential function (e.g. as constituent of an enzyme) or generating toxicity by binding to the wrong place at the wrong time, a feature common to both essential and non-essential trace metals in excess (Marsden and Rainbow 2004). To avoid toxic effect the incoming metal must either be detoxified or excreted (Rainbow 2002). The detoxification process requires the metal to be bound (in soluble or insoluble form) with such high affinity that it is unavailable to be bound to other metabolites, thereby preventing these latter from completing their metabolic role. As long as the combination of detoxification and excretion exceed the overall rate of uptake, incoming metal will not cause toxic effect upon the organism. If the opposite is to happen, then concentrations of metabolically available metal will increase to the point where thresholds are transposed and sublethal and finally lethal toxic effects ensue (Rainbow 2002). There are quite diverse patterns of accumulation according to the strategies used by organisms. If detoxification is the dominant strategy an individual can accumulate large amounts of detoxified metal without effects if the rate of uptake does not transcend the detoxification capability (Rainbow 1998). If excretion is the followed path then the rate of excretion is concomitant with the uptake and toxic effects will only appear once the latter rises above the former.

Bioaccumulation is then the result of the net difference between summated uptake from all sources and excretion. When excretion is insignificant then accumulation is synonymous with uptake.

Usually in amphipods the rates of uptake of trace metals from solution are directly proportional to the dissolved concentration of the metal under unchanged physicochemical conditions (Rainbow and White 1989, 1990; Rainbow 1998), being intermediate between
those of decapods and barnacles, with the exception of Zn, that can be, in some species, regulated to levels similar to those of palaemonid decapods.

The total accumulated content resulting from uptake can then be divided into two components — metabolically available metal and metal that has been detoxified for temporary or permanent storage (Rainbow 2002). Thus the total accumulated metal concentration does not reach a threshold that is toxic, except in the special case of regulators that store no detoxified metal (Rainbow 2002). These strategies are adopted by different species and may even coexist according to the metal being uptaken. But metal uptake ratios can be modulated by both external (typically physicochemical) and internal (physiological) factors.

External factors include: the dissolved concentration of metals (Rainbow and White 1989, 1990); the physicochemistry of the individual metals (generically the available amount of the corresponding free ion) (Rainbow 1997); the interaction between the different metals in presence (literature on this subject is eminently confusing and sometimes contradictory, but interactions are nonetheless proven) (Amiard-Triquet and Amiard 1998); the presence of sediment in the exposure environment (Bryan and Langston 1992); type of diet and mode of feeding (Oakden et al. 1984); habitat location (Moore et al. 1991) and adaptation (species exposed over many generations have evolved biochemical and physiological mechanisms leading to the reduction of potential harmful effects of metals) (Luoma 1977).

Internal factors include: individual variability (Marsden et al. 2003); body size (White and Rainbow 1987); development stage (with the juveniles being normally more sensitive) (Ritterhof and Zauke 1997); gender (some species exhibit marked differences between males and females) (Moore and Rainbow 1987; Marsden et al. 2003); breeding condition (Bondgaard et al. 2000); brooding (Marsden et al. 2003); molting and growth (White and Rainbow 1985); and finally, behavior (e.g. burrowing, swimming, pre-copulatory guarding, crawling, prey capture and drifting) (Fialkowski et al. 2003).
All these factors constitute sources of overlapping variability that can become extremely difficult to monitor (especially in field studies) but simultaneously creates a quite unique set of endpoints and points of intervention for manipulative studies with amphipods, rendering these organisms quite appealing in ecotoxicology.

1.9. Characterization of the species

The gammarid amphipod *Echinogammarus marinus* (Leach, 1815) (=*Chaetogammarus*; =*Marinogammarus*; =*Eullimnogammarus*) (Clarke et al. 1985; Pinkster 1993) inhabits estuaries and marine intertidal areas of north-eastern Atlantic coastlines from Norway and Iceland to Portugal including the British Isles (Lincoln 1979). Quite frequently reaches high densities (Lawrence and Poulter 2001; Maranhão and Marques 2003) being found on sandy or slightly muddy substrata, secluded under stones and algae (usually fucoids like *Fucus vesiculosus*) particularly in the more sheltered areas of the ecosystem (Platvoet and Pinkster 1995). Regarding diet, aside from foraging on plant material (with algae being used as shelter but also as feeding ground), *E. marinus* has proven the ability to actively capture and prey upon other community members, such as isopods (e.g. *Jaera norhamni*) and oligochaetes (e.g. *Tubificoides benedii*), thus varying foodstuffs to maximize fitness (Dick et al. 2005).

![Figure 1.1 - Scientific illustration of the gammarid amphipod Echinogammarus marinus. Adapted from Lincoln (1979).](image-url)
Under laboratory conditions growth of individuals is continuous throughout life and reproduction is rapid, taking 17 days at 20°C for an embryo to be released from the brood pouch as a juvenile (Maranhão and Marques 2003). Added advantages are culture maintenance which necessitates very modest resources and organisms being easy to manipulate. The organisms demonstrate high resilience, surviving for long periods in hypotonic sea waters and even for a number of days in distilled water (Hunter 1949) due to its strong euryhaline character (Pinkster and Broodbakker 1980). Nevertheless, field observations, laboratory tests and modeling have shown that low temperatures combined with low salinities cause negative impacts upon the species (Maranhão et al. 2001; Maranhão and Marques 2003; Martins et al. 2002; Pinkster and Broodbaker 1980).

When in contact with metals in solution E. marinus, as amphipods in general, will show a net accumulation strategy, an important pre-condition for its use as biomonitor. When working with E. marinus, Clason et al. (2003) was unable to establish a toxicokinetic model for zinc, reaching the conclusion that, similarly to what happens for paleamonid shrimps, this metal was being regulated.

1.10. Site selection

Field work was undertaken at three locations geographically distributed so as to encompass the entire latitudinal distribution (Lincoln 1979) of Echinogammarus marinus: three coastal sites in south-west Iceland (Reykjanes Peninsula, 64°02’N, 22°42’W), two sea Lochs and one estuarine in Scotland (generically 56°10’N, 5°05’W) (Lcch Long, Loch Fyne and Forth estuary) and four estuarine sites in Portugal (Ria de Aveiro, a coastal lagoon in the north-western coast, 40°38’N, 8°44’W) (Figure 1.2). The supporting laboratory tests for each location were performed at local institutions (Sandgerði Marine Center, Sandgerði, Iceland; Institute of Aquaculture, University of Stirling, Scotland and; Department of Biology, University of Aveiro, Portugal). Selection was eminently presided by three criteria: adequacy of the location for field deployment of environmental chambers; availability of credible information regarding
presence or absence of metals in water and; relative proximity to the respective support institution.

Figure 1.2 - Map of Western Europe with location of the field sites involved in this study.

1.11. Aims

The rationale for the present thesis is embodied by a series of actions contributing for the acquirement of information regarding the toxic potential of metals, individually and in mixture, towards the amphipod *Echinogammarus marinus*, throughout the geographic distribution of the species, as follows: 1) survey of a brackish water body for the metal concentrations in bottom sediments, bearing in mind that the amounts in presence determined the maximum possible loads in the aqueous phase; 2) use of environmental realistic concentrations of metals (determined by action #1) in mixtures to evaluate toxic responses of a cosmopolitan amphipod species in the laboratory using simple easy-to-
quantify endpoints; 3) elaboration of predictive models; 4) validation of the models recurring to field data through organism deployment in environmental chambers; 5) ex situ investigation of unclear points sprouting from the metal/organisms interaction.

The proposed objectives are to: i) obtain a description of the species sensitivity in localized populations when in a context of environmental exposure to metals; ii) validate the species as a sensitive biomonitor in situ and ex situ; iii) develop a simple, cost effective in situ bioassay, destined to screen brackish and saltwater bodies for the presence of metals (delivering rough estimates of quantity and quality), thus helping decision making in the correct allocation of, usually limited, financial and human resources.

This manuscript is organized by chapters formatted as journal articles.

Chapter 1, introduction, compiles information considered relevant for the understanding and framing of the developed work.

Chapter 2 reports a survey performed in bottom sediments of the Ria de Aveiro coastal lagoon to identify environmental concentrations of metals (aluminum, cadmium, chromium, copper, zinc and lead). The values obtained were used as reference in the choice of laboratory exposure concentrations.

Chapter 3 reports the development and results of the laboratory and field assays in the three locations of the study: Iceland, Scotland and Portugal.

Chapter 4 reports the investigation of intersex incidence in the three populations of the test organism (Iceland, Scotland and Portugal).

Chapter 5 reports the proposed clarification of differing patterns in metal uptake according to life stage and gender in *E. marinus*, the effect of pre-exposure diet in uptake, and also the quantification of metal sequestration by the carapace.

Chapter 6 is a summation of the attained conclusions on the previous chapters and their integration with the starting objectives.
REFERENCES


(http://europa.eu.int/com/n/environment/water/water-dangersub/pri_substances.htm)


An evaluation of trace metal distribution, enrichment factors and potential risk in sediments of a coastal lagoon
2. AN EVALUATION OF TRACE METAL DISTRIBUTION, ENRICHMENT FACTORS AND POTENTIAL RISK IN SEDIMENTS OF A COASTAL LAGOON

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ABSTRACT

Historically, estuaries have served as corridors for the transit of land metal-rich materials to the sea, being thus for millions of years the main contributor to coastal metal loads. But recently (in a geological timescale) human activities have altered the geochemical metal cycle causing sediments to become enriched well above natural background levels. Among sediment characteristics influencing metal adsorption, the most important is surface area being clay particles the ones that offer the most favorable volume to area ratio. Since clay content varies from sample to sample a normalization procedure had to be applied and aluminum, due to its high correlations with naturally occurring metals and sparse anthropogenic sources, was the chosen element. To account for organic scavengers in the system organic carbon was also chosen as normalizer. The application of Sediment Quality Guidelines (SQG) of varied provenance revealed insufficient characterization capability, especially when compared to Enrichment Factors calculated from regression methods. These pointed a mildly enriched system with localized “hot spot” areas, mainly identified by aluminum normalization. The characteristics of the system lead to a fast flushing of contaminants as the main sources (rivers Caster, Antuã and Vouga) are located in areas of medium residence time. Zinc is the only metal presenting enrichment in all the four main channels and there is strong evidence for chromium being exported from the adjacent continental shelf. Vouga river has been a poorly studied source of metals within this system. Added research is necessary to fully understand its contribution in terms of metal loads.

2.1. INTRODUCTION

Bottom sediments in water bodies accumulate contaminants and act (secondarily) as sources of contaminants to the ecosystems they are connected with (Burton 2002). Metals and estuarine sediments can be cited as a perfect illustration of this phenomenon. Metal loads derived from riverine transport, atmospheric deposition or direct anthropogenic dumping reach estuaries (Kennish 1992; Windom 1992) and due to their “filtration capacity” are
stored in sediments only to be remobilized by reversion of the complex set of chemical, biological and hydrodynamic factors that initially led to their accumulation (Shulkin and Bogdanova 2003).

Historically, estuaries have served as corridors for the transit of land metal-rich materials (originated from rock or mineral deposit weathering) to the sea, being thus for millions of years the main contributor to coastal metal loads (Sharp et al. 1986). With the advent of the industrial era, and the associated demographic boom, anthropogenic sources of these materials have been equal or higher than those originated by the action of natural agents (Summers 1996; Rae 1997). To obtain a clear picture of the amount of metal input in a specific ecosystem and hence extrapolate the impact being generated, it is critical to differentiate natural backgrounds from technogenic enrichment.

It is well known that sediment trace metal content is often to a large extent a function of its chemical and mineralogical characteristics. The key characteristic is particle size surface area, being trace metal concentration mainly correlated with the particles that offer the greatest adsorption area – the clay fraction (Mayer and Fink 1980). Since the percentage of this fraction varies from sample to sample, the correct interpretation and reporting of sediment metal concentrations can only be correctly performed if these concentrations are normalized to grain-size. This granulometric approach, however, requires a separation step (only a specific size fraction is used to correct for natural variability, usually <63um) and concentrations in the analyzed size fraction may not reflect the concentration in the total sediment, a limitation that lead to the adoption of geochemical-based grain-size proxies (Covelli and Fontolan 1997; Loring and Rantala 1992; Schropp et al. 1990; Summers 1996; Wang and Chen 2000). Besides compensating for the granular variability, the use of these proxies offers the added advantage of mineralogical variability normalization (Loring 1991). Several elements have been used as a substitute parameter to obtain the desired normalizing effect (e.g. potassium, lithium, scandium, gallium, zirconium and thallium) but special incidence was put upon iron (Fe) and aluminum (Al). They are among the most abundant crustal elements. Ratios of metal/Fe or metal/Al are fairly constant in the earth’s crust and
technogenic point sources can be considered very sparse and easily identifiable. Despite their similarity there are advantages in the use of aluminum over iron as normalization factor. The former is more tightly associated with the alumirosilicate fraction which is the dominant metal-bearing phase of the sediment (the already mentioned clays), and a fraction of the latter may quite frequently represent anthropogenically induced iron sulfides and oxides (Summers 1996). These facts allied to aluminum yielding significant linear regressions with the majority of co-occurring metals over wide geographical areas lead to its usage as normalizing agent in a vast number of studies (e.g. Armstrong et al. 2005; Schropp et al. 1990; Summers 1996; Trimble et al. 1999; Wang and Chen 2000).

Other sediment constituent recurrently pointed as normalizing agent is organic carbon (OC) (Di Toro et al. 2001; Soto-Jiménez and Páez-Osuna 2001; Summers 1996; Villares et al. 2003). Despite being the primary sediment phase accounting for sorption of neutral organic chemicals (Di Toro et al. 1991) organic carbon is also an important partitioning phase for metals in sediment and in water because of the tendency for positively charged metal ions to bind to negatively charged sites that are associated with natural organic matter (Di Toro et al. 2001).

Human activities have greatly altered the natural geochemical cycle of trace metals, resulting in their accumulation in sediments and the consequent widespread environmental contamination (Nriagu and Pacyna 1988). This unbalance can affect water quality and bioassimilation and bioaccumulation in aquatic organisms, with potential long-term implications on human health and ecosystems (Burton 2002). It is therefore important to understand the mechanisms of accumulation and geochemical distribution of trace metals in sediments to develop pollution control strategies and approaches to water quality management (Nriagu and Pacyna 1988).

Ria de Aveiro is a coastal lagoon (also mentioned in literature as a “bar built estuary”) 45km long and 10 Km wide (Figure 2.1), located in the northwestern (40°38’N, 8°44’W) coast of Portugal (Dias et al. 2001). The four main constituting channels (S. Jacinto, Espinheiro, Mira
and Îlhavo), mostly very narrow, concatenate a highly irregular and complex geometry. Due to being directly connected to the ocean through the lagoon mouth, the most important are S. Jacinto and Espinheiro channels. In consequence they register the strongest currents, reaching values of about 2 m s⁻¹ at the oceanic end during spring tides and flooding period. The other channels are dominated by extensive intertidal zones, namely mud flats and salt marshes characterized by a very irregular geometry that contributes to a strong damping of the currents and an increase of the phase delay of the tidal wave (Lopes et al. 2007).

Due to their unique characteristics each of them may be regarded as an independent estuary connected to a common inlet. Their individual hydrology is determined by a complex interaction of a specific tidal prism, freshwater input and wind forcing (the latter mainly in shallow, wider areas) (Vaz et al. 2008). Even though generally considered vertically homogeneous, some channels may reveal characteristics of a partially mixed estuary, depending on the freshwater input, deriving from two major rivers, the Vouga and the Antuã, which flow into the east side of the lagoon (Lopes et al. 2001).

The area encircling this system is inhabited by over 300,000 people, and besides intensive agriculture, highly polluting chemical, metallurgic, ceramics, tannery and pulp milling industries are installed in the vicinity, draining their effluents (partial collection and pre-treatment being performed only in recent years) into the lagoon, be it directly or via the two major rivers (Delgado et al. 2000).

This paper describes a sediment survey undertaken to unravel patterns of distribution and dispersion of trace metals in the Ria de Aveiro system. Cadmium, a “priority hazardous substance” (2455/2001/EC) and List I metal (2006/11/EC)¹, lead, a “priority substance” (under review for identification as possible "priority hazardous substance") and List II metal (2006/11/EC)¹ and three other “main pollutants” (Annex VIII, Water framework Directive,

¹ This Directive, a re-codification of the Dangerous Substances Directive - 76/464/EEC for the benefit of clarification, does not see its provisions affected or changed until its full repeal in 2013.
2000/60/EC) and List II metals (2006/11/EC), namely chromium, copper and zinc, were scrutinized.

Several national and international numerical Sediment Quality Guidelines (SQGs) were used to detect the occurrence of enrichment and the associated potential risk to biota.

Geochemical normalization was performed to obtain Enrichment Factors (EF) which allow estimates on the relative importance of anthropogenic contributions to the metal loads of the studied sediments. Additionally, the geoaccumulation index ($I_{geo}$) is used as criterion to indicate metal-polluted critical areas in relation to global baseline levels. As a result an overview of metal distribution in the Ria de Aveiro system is generated with the associated potential risk towards biota, and due to the importance of captured species (mainly mollusks) in local and other economies, to human health.

2.2. MATERIAL and METHODS

2.2.1. Sediment collection

A total of 47 bottom sediment samples were collected in Ria de Aveiro (Figure 2.1), a coastal lagoon in the NW Portuguese coast, using a van Veen type grab sampler. The two top cm of sediment from each grab (3 grabs per site) were gathered from the centre of the device with an acid cleaned plastic scoop and stored in metal-free polyethylene bags (Birch et al. 2001). After transport on ice into the laboratory were frozen at -20°C until analysis to preclude microbial activity (Mudroch and Anzwe 1995). Recently deposited sediment was preferentially sampled to obtain contemporaneous material and areas of bank collapse, land fill, landscaping and slumping were avoided (Birch et al. 2001). Spatial variation in sediment metal composition was investigated by sampling in transepts (Birch et al. 2001) at Canal de Mira which offered the most favorable conditions to perform this type of evaluation.
2.2.2. Sediment analysis

Extraction of metals from sediments was carried out using nitric acid – hydrogen peroxide digestion. Approximately 2g wet weight (ww) of sediment was dried at 110°C for 24-hours and ground to a fine powder. A 500mg sub-sample was added to a 20ml Teflon screw top digestion vessel. 5ml of concentrated nitric acid (69%, Aristar, BDH, 106 U.K.) was added and the sample was heated to 110°C for 24-hours. Once cooled, 3ml of hydrogen peroxide (Aristar, BDH, U.K.) was added in 1 ml steps until the sample became totally clear and ceased effervescing. Higher metal recoveries can be obtained if more robust digestion methods are
used (e.g. perchloric or hydrofluoric acids) however, such digestion will bias results for environmental assessment as they extract metals belonging to the aluminosilicate matrix, which are not direct interveniens in environmentally relevant geochemical processes (Hill et al. 2004). Samples were re-heated at 110° C for a further 2 hours, allowed to cool and made up to 15ml with distilled water and centrifuged at 2000 rpm for 15 minutes. A 1ml sub- aliquot was analyzed by atomic absorption spectrophotometry using a Unicam 939QZ Atomic Absorption Spectroscopy with coupled Unicam GF90 Graphite Furnace (GF-AAS) and Zeeman® correction. All glassware was soaked overnight in 5% hydrochloric acid (Aristar, BDH, U.K.) and then triple rinsed with Millipore water prior to use.

For carbon and nitrogen contents determination, three replicate sub-samples of each sample were dried at 110° C for 48 hours. The analysis was carried out using a Perkin Elmer series II 2400 HNS/O elemental analyzer in the triplicate sub-samples. To correct for inorganic carbon, a different set of sub-samples were combusted at 550° C for four hours and then processed along with un-combusted samples.

2.2.3. Statistical methods

2.2.3.1. Correlation coefficients
The Kolmogorov-Smirnov test was applied to ascertain the normality of distribution of the data set, an assumption which was not verified even after log transformation. Consequently a non-parametric method (Spearman’s rank correlation coefficient) was used to determine the correlation between the analyzed elements (including organic carbon). The software package SigmaStat 5.1 was used to perform all necessary statistics.

2.2.3.2. Multivariate statistics
Many studies offer algorithms of different multivariate statistical approaches. For instance, cluster analysis is carried out to reveal specific linkage between sampling sites being an indication of similarities or dissimilarities between their trace metal contamination. Further,
Principal Component Analysis (PCA) is applied to detect the “hidden” structure of the data set, trying to explain the influence of latent factors on the data distribution (Simeonov et al. 2000). Several pretreatments can be applied and it is known that this can have a profound effect on the resulting PCA plots. Varimax rotation (Kaiser 1958) is a method for rotating the axes of a plot such that the eigenvectors remain orthogonal throughout the rotation ending in a position in which the sum of the variances of the loadings is the maximum possible, thus increasing easiness of interpretation. In the present study Ward’s and single linkage clustering methods (Euclidean distance as dissimilarity measure) and PCA on autoscaled data (Esbensen et al. 1994) with varimax rotation were used.

2.2.3.3. Normalization procedures

To account for natural mineralogical variations and provide baseline relationships with which to assess metal enrichment, aluminum and organic carbon (OC) were tested as normalizing agents.

To find the baseline relationship between the trace metal of interest and the normalizer (under the form of regression plots representing the expectation, or ‘prediction’, of naturally occurring concentrations for the element in the area of the study) a method based upon the work of Schiff and Weisberg (1999) was applied. It consists in the identification of outliers using regression analysis. To meet the requirements of this criterion, regressions were developed between each metal and aluminum and each metal and OC, followed by the analysis of the residuals from the regression for normality. Normality was assessed using the Kolmogorov–Smirnov test. If a normal distribution was not achieved, sites with residuals greater than two standard deviations (positive or negative) were eliminated and the regression was recalculated. This process was repeated the necessary number of times until a normal distribution was achieved for each element. Once the baseline regressions are obtained a calculation (a ratio of ratios) between the ratios of the quantified element ($X_{\text{sample}}$) and the normalizer and the predicted value for that element ($X_{\text{predicted}}$) and the normalizer,
can be performed leading to an indicative enrichment factor (EF) (Borrego et al. 2002; Covelli and Fontolan 1997; Di Toro et al. 2001; van den Weijden 2002; Woitke 2003).

\[
\text{EF} = (X_{\text{sample}}/\text{normalizing agent}) / (X_{\text{predicted}}/\text{normalizing agent})
\]

An estimation of anthropogenic contribution of a given trace element to the sample is generated, EFs ultimately representing the anthropogenic inputs to the sampled site metal budget.

Similarly based in a comparison between metal concentration in samples and metal baseline levels (in average shales) the Geoaccumulation Index \( I_{\text{geo}} \), was created by Müller (1979) as criterion to indicate metal-polluted critical areas. It’s calculated according to the expression:

\[
I_{\text{geo}} = \log_{2} \left[ \frac{C_{m}}{1.5 \times B_{m}} \right]^{1}
\]

where \( C_{m} \) is the measured content of element “m”, and \( B_{m} \) is the background content of the species in average shale. The factor 1.5 is used for the possible variations of the background data due to lithological discrepancies (Fürstner and Salomons 1980). The six classes considered are described in Table 2.1.

<table>
<thead>
<tr>
<th>( I_{\text{geo}} ) value</th>
<th>( I_{\text{geo}} ) Class</th>
<th>Pollution intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 5</td>
<td>6</td>
<td>very strongly polluted</td>
</tr>
<tr>
<td>4 - 5</td>
<td>5</td>
<td>strongly to very strongly polluted</td>
</tr>
<tr>
<td>3 - 4</td>
<td>4</td>
<td>strongly polluted</td>
</tr>
<tr>
<td>2 - 3</td>
<td>3</td>
<td>moderately to strongly polluted</td>
</tr>
<tr>
<td>1 - 2</td>
<td>2</td>
<td>moderately polluted</td>
</tr>
<tr>
<td>0 - 1</td>
<td>1</td>
<td>unpolluted to moderately polluted</td>
</tr>
</tbody>
</table>

There are several advantages in the use of this classification: 1) it has a high intrinsic power and added-value by allowing comparisons between studies in diverse geographical locations; 2) There are no established background levels for Ria de Aveiro for metals in sediments, and the comparison to average shales creates a bypass to this situation; 3) shales are part of the "signature" of the mineralogical composition of the soils and rocks outcropping in the hydrographic basins being drained to the lagoon (Abrantes et al. 2005)
2.2.4. Selection of SQGs and considerations upon their use

Ideally, site-relevant or regional Sediment Quality Guidelines (SQGs) should be developed wherever possible, to take into account site-specific conditions (e.g. bioavailability, sensitivity of indigenous organisms, exposure pathways) (Wenning and Ingersoll 2002). But resources do not always permit this. Fortunately there is a great wealth of SQGs being used in different ways and to attain different objectives by various regions, nations and organizations. When choosing what SQG to apply it is important to note that even if included in the same categories (e.g. “background based”; “no effects”, “probable effects”, “extreme effects”) they differ in many ways. These differences include purpose, methods of calculation, applicability, individual and groups of Contaminants Of Potential Concern (COPCs) covered, the analytical methods, and the absolute values (Apitz et al. 2007). Among other things this means, in practical terms, that the selection of several criteria within the same group can produce added information when analyzing the same dataset. This was the approach attempted in this work and a set of “no effect” criteria were selected to submit metal levels in Ria de Aveiro sediments to scrutiny. The choice fell upon Pollution Index (DR 1995), Environmental Quality Standards (EQS, WFD-2000/60/EC), Environmental Assessment Criteria - low (Lower-AEC, Moffat et al. 2004) and Effects Range Low (ERL, Long et al. 1995).

The pollution index established under Portuguese law (DR 1995) (Table 2.2) is the closest to site-local or regional SQGs. Based in local data is consequently, the one that most closely mirrors particularities of Portuguese ecosystems. Originally tailored by the legislator to classify dredged materials and orientate their disposal (a different concept from the one underlying a quality guideline), the development of the index was solely based in physico-chemical parameters (density, percent of solids, total organic carbon, concentration of organic pollutants and metals) with no ecotoxicological data inputs.
Table 2.2 - Classification under Portuguese law (Pollution Index) of dredged materials in coastal zones (DR 1995)

<table>
<thead>
<tr>
<th>Class</th>
<th>Pollution index</th>
<th>Cd</th>
<th>Cu</th>
<th>Cr</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>Clean dredged material</td>
<td>&lt;1</td>
<td>&lt;35</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Class 2</td>
<td>Trace contaminated dredged material</td>
<td>1-3</td>
<td>35-150</td>
<td>50-100</td>
<td>50-150</td>
<td>100-600</td>
</tr>
<tr>
<td>Class 3</td>
<td>Lightly contaminated dredged material</td>
<td>3-5</td>
<td>150-300</td>
<td>100-400</td>
<td>150-500</td>
<td>600-1500</td>
</tr>
<tr>
<td>Class 4</td>
<td>Contaminated dredged material</td>
<td>5-10</td>
<td>300-500</td>
<td>400-1000</td>
<td>500-1000</td>
<td>1500-5000</td>
</tr>
<tr>
<td>Class 5</td>
<td>Highly contaminated dredged material</td>
<td>&gt;10</td>
<td>&gt;500</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

The derivation of Environmental Quality Standards (EQSs) for metals under the Water Framework Directive (WFD - 2000/60/EC) offers the possibility of these being based either on the added risk or total risk approach. The added risk approach is a methodology that takes natural background concentrations ($C_b$) of naturally occurring substances, such as metals, into account and prevents the setting of environmental standards below that background. The total risk approach just considers the bioavailable fraction in the medium despite of its provenience (background or anthropogenic influence).

A Maximum Permissible Addition (MPA - maximum concentration of the substance to be added to its background concentration without causing deleterious effects (Lepper 2005)) is derived from data on the added metal concentration in toxicity tests. The obtained data is modeled by Assessment Factors (AFs) to compensate for eventual scarcity (e.g. insufficient number of species in different trophic levels) or low-adequacy (e.g. freshwater species in a context of saltwater) of that data (Table 2.4).

In the case of the added risk (the followed approach in this work), the sum of the background concentration ($C_b$) with the MPA derives a Maximum Permissible Concentration (MPC = EQS) (Lepper 2005):

$$MPC = C_b + MPA$$

Operatively MPC can also be represented by the PNEC (Predicted No-Effect Concentration) of the substance for a certain compartment (TGD 2003).
Contrary to original thinking and some tentative work on establishing a “default background concentration” (CSTEE 2004), there is convenience of deriving local or regional background levels due to the geological variety of the different regions, especially if they are necessary for environmental assessments (e.g. Carral et al. 1995). In fact, for large areas, the concept of being able to define just “one background” is at the least illusive (Reimann et al. 2005). As previously stated there is, to the best of the author’s knowledge, no established background concentrations for the Ria de Aveiro hydrographic basin. In the face of this, background concentration values (Table 2.3) used to derive the MPCs in this work were obtained from the geometric mean of regression function predictions (Aluminum vs metal of interest).

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Cr</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_b$</td>
<td>0.14</td>
<td>5.99</td>
<td>3.97</td>
<td>4.97</td>
<td>55.63</td>
</tr>
</tbody>
</table>

The same lack of information affects MPAs related to transitional waters and sediment; both on local and international scales: a database search only returned one MPA (lead and its compounds) specifically derived for metals in these compartments. The other elements in this study had to be evaluated based in freshwater sediment MPAs (cadmium, chromium, copper and zinc). This means that presently there are no EQSs (or more precisely, MPAs usable in their calculation) that can be used to scrutinize our dataset. Under these constraints Table 2.4 presents MPAs adopted for this study.
Table 2.4 - Maximum Permissible Additions used in this study. dw: dry weight; ww: wet weight.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Compartment</th>
<th>MPA</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>Freshwater sediment</td>
<td>2.3 mg/kg dw</td>
<td>Only indicative. Derived with no data for marine species.</td>
<td>European Union Risk Assessment Report – Cadmium metal, 2007</td>
</tr>
<tr>
<td>Cu</td>
<td>Sediment</td>
<td>92.7 mg/kg dw</td>
<td>Only indicative. The value was derived for this study using the EqP method[1]</td>
<td>Crane et al. 2007a; TGD 2003; Admiral et al. 1995; Chen et al. 2007; Lijzen et al. 2001; de Zwart et al. 2006</td>
</tr>
<tr>
<td>Pb</td>
<td>Saltwater sediment</td>
<td>53.4 mg/kg dw</td>
<td>This is the only MPA derived for sediments in salt/transition waters (Rhine dataset)</td>
<td>Environmental Quality Standards (EQS) Substance Data Sheet - Lead and its Compound, 2005</td>
</tr>
<tr>
<td>Zn</td>
<td>Freshwater sediment</td>
<td>49 mg/kg dw</td>
<td>Only indicative. Derived with no data for marine species.</td>
<td>Bodar 2007</td>
</tr>
</tbody>
</table>

[1] The Equilibrium Partitioning method was used to calculate the Maximum Permissible Addition (MPA) for copper in sediment as laid down in the TGD (2003): \( MPA_{\text{Cu}} = MPA_{\text{water}} \times K_{\text{Cu,water}} \times 10^{-3} \).

MPA is the MPA for the water compartment; \( K_{\text{Cu,water}} \) is the coefficient of partition of copper between water and Suspended Particulate Matter (SPM). This coefficient is used instead of the one between water and sediment to “protect new sediments” (TGD 2003). Values used: \( MPA_{\text{water}} = 8.2 \mu g/L \) (Crane et al. 2007a); \( K_{\text{Cu,water}} = 11307 \) L/kg (geometric mean of data from Admiral 1995; Chen et al. 2007; Lijzen et al. 2001; de Zwart et al. 2006).

[2] Available PNECs for chromium were relative to freshwater sediment and for the two stable Cr valences: Chromium (VI): 1.5 mg/kg (acidic conditions), 0.15 mg/kg (alkaline conditions); Chromium (III): 31 mg/kg (acid conditions), 307 mg/kg (alkaline conditions) in European Union risk assessment report – chromium trioxide, sodium chromate, sodium dichromate, ammonium dichromate and potassium dichromate. Considering that: (i) Of the two valences, the hexavalent (VI) is about 100-fold more toxic than the trivalent (III), being the main responsible for mutagenicity, carcinogenicity, and teratogenicity (Nakayama et al. 1981); (ii) The calculated \( K_{\text{d}} \) for oxidation of Cr(III) to Cr(VI) predicts that Cr(VI) is the predominant species under normal marine conditions (Nakayama et al. 1981); (iii) The average pH of the water overlying the sediment in the area were samples were taken was 8.26 (information not reproduced). The adopted value was: 0.15 mg/kg w.w.

The non-uniformity of the EQSs derived from these values (more that the intrinsic validity of the guideline in itself) deserve some criticism. Nevertheless, eventual shortcomings can be put into perspective if the following is considered:

1) Similar methodology as been already used in several studies. For instance a report from the study group implementing the WFD for the Dutch Environmental Agency (Bodar 2007) states that “pragmatically, the freshwater PNEC for sediment was also used in some local risk assessments where emissions to the marine environment occurred”.

2) Continued concerns raised by the inexistence of specific EQSs for sediment (and biota) were answered by the issue of the proposal for a “Directive on environmental quality standards in the field of water policy and amending Directive 2000/60/EC”
(2006/0129 (COD)) of 21 June 2007, creating a somewhat late insight that sediments must not be neglected in this context (Förstner, 2007). It gives the member states the possibility of opting to establish and apply EQS for sediment and/or biota instead of those laid down in Annex I, Part A as long as these offer at least the same level of protection as the EQS for water set out in the same annex.

Ecotoxicological Assessment Criteria agreed between the members of the Oslo and Paris Conventions for the Prevention of Marine Pollution (OSPAR) were for almost a decade the pan-European standard in sediment contamination evaluation (OSPAR 1997). Their change of definition to Environmental Assessment Criteria (Moffat et al. 2004) reflected a convergence to EU legislation, particularly the WFD. The former EACs are no longer endorsed, but replaced by Lower-EAC and Upper-EAC values that have defined ecotoxicological meaning.

In this study only Lower-AEC ("derived for protection of all marine species from chronic effects, including the most sensitive species" (Moffat et al. 2004)) in their new framing are of interest. The values presented in Table 2.5 were derived (Moffat et al. 2004) re-using the databases supporting the values issuing from the 1996 workshop (OSPAR 1997). For this reason they are considered demonstrative. Other than this reason, no explanations are advanced by Moffat et al. (2004) for the strangely low value for lead EAC.

Originally developed by Long and Morgan (1990) for copper and reviewed to a wider set of other substances by Long et al. (1995), the concept of Effects Range Low (ERL) was defined as "minimal effect range that intends to estimate conditions in which effects would be rarely observed". It corresponds to the lower 10th percentile among concentrations found to co-occur with a biological effect. Since 1995, the predictive powers of ERLs have been tested against large datasets obtained by US-EPA (United States Environmental Protection Agency) and NOAA (National Oceanic and Atmospheric Administration) monitoring programs containing simultaneously measured chemical and toxicological parameters (O’Connor 2004).

It should be noted that every author, nation, and organization deriving and using SQGs always states with more or less emphasis that SQGs should be only one (initial) part of a
decision framework, basically used for sediment ranking and for flagging of Contaminants Of Potential Concern, COPCs (e.g. Apitz et al. 2007, Moffat et al. 2004, Wenning et al. 2006, WFD 2000). The transcendence of a determined pre-set value isn’t an obligatory synonym of toxic capabilities of a sample.

| Table 2.5 - Summary of International Criteria used to evaluate sediments sampled in Ria de Aveiro. QS- Quality Standard (derived under WFD (2000/60/EC); Lower EAC- Environmental Assessment Criteria (Moffat 2004); ERL- Effects Range Low (Long et al. 1995). All values in ug/g. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                | Cd     | Cu    | Cr     | Pb     | Zn    |
| QS                             | 2.44(1) | 98.7 (1) | 4.12(1) | 58.4(1) | 105(1) |
| EAC Lower                      | 0.67G(2) | 18.7(2) | 52.3(2) | 0.00293(2) | 124(2) |
| ERL                            | 1.2 | 34 | 81 | 46.7 | 150 |

(1) QSs were derived as specifically laid down by the TGD (2003), Leger (2005) and CSTEE (2004); EQS= Cb + MPA. Cb = Background Concentration for the specific metal (Table 2.3); MPA= Maximum Permissible Addition for the specific metal (Table 2.4).

(2) Calculated Lower EAC. Calculated from the 1996 workshop data using WFD methods as set in the TGD (2003), these values are here cited just as an illustration of the change of approach (Moffat 2004).

All adopted SQGs are above the background levels (Table 2.3) with the exception of EAC for lead.

2.3. RESULTS

2.3.1. Trace metal concentrations in Ria de Aveiro sediments

Table 2.6 presents the results of metal concentration in surface sediments of Ria de Aveiro, alongside with the respective organic carbon (OC) contents.

<p>| Table 2.6 - Metal concentrations in Ria de Aveiro sediment samples. All values in ug/g (dw). Aluminum and organic carbon (OC) in %. |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Station Code</th>
<th>Cd</th>
<th>Cu</th>
<th>Cr</th>
<th>Pb</th>
<th>Zn</th>
<th>Al</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>42.8</td>
<td>19.9</td>
<td>12.1</td>
<td>772.8</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>6.4</td>
<td>25.0</td>
<td>0.1</td>
<td>2.7</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>25.8</td>
<td>4.9</td>
<td>15.9</td>
<td>0.5</td>
<td>56.4</td>
<td>25.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>5.4</td>
<td>15.9</td>
<td>1.3</td>
<td>12.8</td>
<td>15.9</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>5.4</td>
<td>9.5</td>
<td>2.2</td>
<td>17.2</td>
<td>9.5</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>682.3</td>
<td>166.5</td>
<td>169.5</td>
<td>50.6</td>
<td>595.1</td>
<td>510.1</td>
<td>134.2</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>0.7</td>
<td>1.9</td>
<td>0.2</td>
<td>1.3</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>1.1</td>
<td>2.8</td>
<td>0.3</td>
<td>2.7</td>
<td>2.4</td>
<td>2.7</td>
</tr>
</tbody>
</table>
2.3.2. Comparison with sediment quality guidelines

Portuguese legislation criteria (Table 2.2) indicated a majorly uncontaminated system with only two samples (4.3%) being considered as “lightly contaminated” by zinc and without any sample for chromium transcending the “clean” status (Table 2.7).

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Cr</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>1.7</td>
<td>62.5</td>
<td>9.3</td>
<td>59.0</td>
<td>366.0</td>
</tr>
<tr>
<td>34</td>
<td>0.3</td>
<td>9.0</td>
<td>1.4</td>
<td>7.6</td>
<td>284.2</td>
</tr>
</tbody>
</table>

Table 2.7 - Summary of samples (n=47) transcending each of the considered guidelines. P Index: Pollution Index for dredged materials under Portuguese law (DR 1995); Lower-EAC: Lower Environmental Assessment Criteria (Moffat et al. 2004); QS: Quality Standard developed under the Water Framework Directive (2000/60/EC); ERL: Effects Range Low (Long et al. 1995).

<table>
<thead>
<tr>
<th>P Index</th>
<th>Class 2</th>
<th>Class 3</th>
<th>&gt; QS</th>
<th>&gt; EAC lower</th>
<th>&gt; ERL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cd</td>
<td>Cu</td>
<td>Cr</td>
<td>Pb</td>
<td>Zn</td>
</tr>
<tr>
<td>P Index</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Class 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>&gt; QS</td>
<td>2</td>
<td>0</td>
<td>26</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>&gt; EAC lower</td>
<td>6</td>
<td>10</td>
<td>0</td>
<td>47</td>
<td>17</td>
</tr>
<tr>
<td>&gt; ERL</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>14</td>
</tr>
</tbody>
</table>

The comparison with the calculated Quality Standards (Table 2.5) indicates 4.3% of samples above the standard for cadmium, 55% for chromium, 2.1% for lead and 34% for zinc. Copper didn’t register any transcendance of the QS (Table 2.7). An absence of chromium contamination, 13% of samples for cadmium, 21% for copper, 100% for lead and 36% for zinc (Table 2.7) were the results obtained by comparison of the dataset to OSPAR’s Lower-EAC (Moffat et al. 2004) (Table 2.5). Comparison with Effects Range Low (Long et al. 1995) (Table 2.5) indicated contamination for 11% of cadmium samples, 11% for copper, 2.1% for lead and 30% for zinc. Once again no samples transcended the value for chromium (Table 2.7).
2.3.3. Comparison with the Geochemical Index ($I_{geo}$)

Results from the comparison of the dataset with the Geochemical Index are presented in Table 2.8.

<table>
<thead>
<tr>
<th>Geochemical Index ($I_{geo}$) classes</th>
<th>Zn</th>
<th>Pb</th>
<th>Cr</th>
<th>Cu</th>
<th>Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 0, unpolluted</td>
<td>32</td>
<td>46</td>
<td>47</td>
<td>47</td>
<td>38</td>
</tr>
<tr>
<td>Class 1, unpolluted to moderately polluted</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Class 2, moderately polluted</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Class 3, moderately to strongly polluted</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Samples for chromium and copper were 100% included in the “unpolluted” class (class 0), whilst zinc registered 68%, lead 98% and cadmium 81% for the same class. Class 1 (“unpolluted to moderately polluted”) was indicated for 17% of zinc samples, 2.1% of lead and 6.4% for cadmium. Class 2 (“moderately polluted”) was awarded to 8.5% of zinc samples, the same as cadmium. Only for zinc and cadmium “moderately to strongly polluted” samples were registered with 6.4% and 4.3%, respectively.

2.3.4. Relationship between analytes

The evaluation of the influence from inorganic (aluminum) and organic (organic carbon) scavengers on metal mobility and distribution within sediments was performed using Spearman’s rank coefficient matrices (Table 2.9). Using literature based consensus (e.g. Cox and Predat 2005, Wilcox and Muska 2002), $r$ values in the correlation matrix (Table 2.9) can be considered demonstrative of overall strong positive correlations between all the elements (all $r>0.68$). When comparing the two normalizing agents, Al demonstrated higher coefficients towards all elements (with an unimpressive exception for Zinc: $r=0.808$ against $r=0.810$ for this element normalized by OC) proving to be the most suitable normalizer.
**Table 2.9 -** Spearman’s rank correlation matrix for superficial sediments from Ria de Aveiro (Values presented: Spearman correlation $r$, significance level $p$, number of samples $n$, respectively)

<table>
<thead>
<tr>
<th></th>
<th>Pb</th>
<th>Cr</th>
<th>Cu</th>
<th>Cd</th>
<th>Al</th>
<th>Org C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>0.875</td>
<td>0.782</td>
<td>0.848</td>
<td>0.891</td>
<td>0.808</td>
<td>0.810</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Pb</td>
<td>0.787</td>
<td>0.809</td>
<td>0.81</td>
<td>0.817</td>
<td>0.727</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Cr</td>
<td>0.812</td>
<td>0.871</td>
<td>0.931</td>
<td>0.680</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Cu</td>
<td>0.843</td>
<td>0.838</td>
<td>0.731</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Cd</td>
<td>0.867</td>
<td>0.749</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Al</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.702</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>47</td>
</tr>
</tbody>
</table>

The interpretation of a correlation coefficient depends on context and purpose. In the geochemical sphere a certain degree of caution should be used since it is recurrent that data sets do not possess a normal or log-normal distribution (as is the case) derived to the abundance of rather small values along with few very large ones (Grünfeld 2005). These large values (outliers) can be caused by sampling or quantification errors or by contamination and should be dealt with in either of two ways: removal of the abnormal samples from analysis or proceed to a change of their values (Grünfeld 2005). Hence the presence of outliers was detected and dealt with according with the methodology described in 2.2.3.3. The results are presented in Table 2.10.

Nevertheless, it became a preoccupation for the authors that the procedure followed, focused in the need for normal distribution of the data set, could be producing a biased
interpretation by solely excluding outliers and not accounting for influential cases. To provide a method for comparison, it was decided to parallelly adopt the procedure followed by Summers et al. (1996). Outliers and influential cases analysis (using studentized residuals for the former and Cook’s distance and DFFITS for the latter) led to the removal of the identified points falling above the potential regression line from further analysis. This approach was taken despite the risk of generating calculations of confidence intervals too wide or too narrow if the error distribution proved to be significantly non-normal (Kilgour 1997). Reassurance was found in the comparative purpose of the exercise. A regression plot of each metal versus aluminum was then generated, which allowed the calculation of predicted values and associated 95% Confidence Limits. The indications of a better “performance” of aluminum as a more robust normalizer (Table 2.10) lead the application of this alternative methodology only to that element. The differences between the two methods were minor; with the first method producing slightly better values of $R^2$ for zinc, lead and copper whilst the values for chromium and cadmium being discreetly more robust in the second method (Table 2.10).

Table 2.10 - Linear regression models for elements relative to Al for Ria de Aveiro sediments. In sequence: the original data set, outliers removed (adapted from Schiff and Weisberg 1999) and outliers and influential cases removed (adapted from Summers et al. 1996). Correlations between metals and Al were all significant at the $p<0.001$ level.

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cr</th>
<th>Cu</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original dataset vs Al</td>
<td>47</td>
<td>0.334</td>
<td>4.0E-05</td>
<td>-0.01</td>
<td>47</td>
</tr>
<tr>
<td>Outliers out</td>
<td>42</td>
<td>0.816</td>
<td>3.0E-05</td>
<td>-0.04</td>
<td>43</td>
</tr>
<tr>
<td>Outliers &amp; Inf. Cases out</td>
<td>41</td>
<td>0.845</td>
<td>3.0E-05</td>
<td>-0.04</td>
<td>36</td>
</tr>
</tbody>
</table>

2.3.5. Enrichment factors

Enrichment factors (EFs) of metal (vs. aluminum) were calculated for the two statistical procedures considering the very close power of the regressions. The dataset was more conservatively evaluated by the method based in Summers et al. (1996) for all metals. The
present context advises the use of the values obtained from this statistical method (presented in Figure 2.2) for interpretation of the dataset.

Due to natural mineralogical differences of the sediments and analytical uncertainty, only sediments with an EF greater than 2 were considered as enriched (e.g. Aloupi and Angelidis 2001).

![Figure 2.2 - Enrichment factors (EFs) calculated based on aluminum normalization of the data set, after removal of outliers and influential cases.](image)

Despite aluminum constituting a more suitable normalizer (Table 2.9), correlations for OC vs metals were also strong. The respective regression lines (Table 2.11) were plotted to obtain information regarding the influence of organic scavengers in Ria de Aveiro. The calculated EFs are presented in Figure 2.3.

### Table 2.11 - Linear regression models for elements relative to organic carbon (OC) for Ria de Aveiro sediments.

In sequence: the entire data set and dataset with outliers removed (adapted from Schiff and Weisberg 1999). Correlations between metals and OC were all significant at the $p<0.001$ level.

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original dataset vs OC</td>
<td>47</td>
<td>0.905</td>
<td>0.046</td>
<td>0.000</td>
</tr>
<tr>
<td>Outliers out</td>
<td>22</td>
<td>0.991</td>
<td>0.144</td>
<td>0.002</td>
</tr>
</tbody>
</table>

55
As could be expected, the identification of enriched samples differs from the one obtained by Al normalization. Organics indicated a lower number of contaminated samples (hence stations). Curiously there are a large number of EF values between 1.5 and 2 (not depicted), hence insufficient to be considered as enriched, according to the used criteria: 22 samples, being 7 between 1.9 and 2.

2.3.6. Multivariate statistics
The cluster analysis results of the sampling sites as objects are shown in Figure 2.4. Three clusters could be identified: in general terms the first (bigger weight, 68%) corresponds to stations in the channels of the south part of the lagoon (Mira and Ílhavo), the second (medium weight, 21%) to stations in the initial portion of the channels confluence in the central area (Mira, Ílhavo, S. Jacinto and Espinho) and third (the smallest in weight, 11%) to stations in the northern part of the Ria (Ovar channel and Laranjo bay). Every group presents exceptions to this general distribution and is clearly divided in two subgroups each. The most heterogeneous is the third were measures of dissimilarity are stronger.
The application of PCA grouped the chemical components by the loadings plots (Figure 2.5) and the sites by the scores plots (Figure 2.6). The factor loading matrix is listed in Table 2.12.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>0.602</td>
<td>0.742</td>
</tr>
<tr>
<td>Pb</td>
<td>0.672</td>
<td>0.624</td>
</tr>
<tr>
<td>Cr</td>
<td>0.896</td>
<td>0.358</td>
</tr>
<tr>
<td>Cu</td>
<td>0.719</td>
<td>0.571</td>
</tr>
<tr>
<td>Cd</td>
<td>0.762</td>
<td>0.568</td>
</tr>
<tr>
<td>Al</td>
<td>0.879</td>
<td>0.403</td>
</tr>
<tr>
<td>Org C</td>
<td>0.342</td>
<td>0.895</td>
</tr>
<tr>
<td>% Exp. Var.</td>
<td>51.507</td>
<td>38.281</td>
</tr>
<tr>
<td>% Cum. Var</td>
<td>51.507</td>
<td>89.788</td>
</tr>
</tbody>
</table>
PCA reduced the seven original variables to two principal components that accounted for almost 90% of the total variance of the system (PC1 51.5% and PC2 next 38.3%, Table 2.12). It can be concluded that PC1 is mainly associated with aluminum and chromium and that PC2 is strongly associated with OC. In a lesser extent copper and cadmium are associated to PC1 and zinc to PC2. Lead presents even representation in both axis.

Figure 2.5 - PCA loading plots (PC1 vs. PC2) for the 7 chemical components (varimax normalized). Cumulative explained variation: 89.79%.

Figure 2.6 depicts the scores plot (PC1 vs PC2) with the delimitation of the three groups obtained by the hierarquical dendogram of Figure 2.4.
Figure 2.6 - Bivariate PCA scores plot (PC1 vs. PC2) for the 47 sampling sites (varimax normalized). The groups indicated by the hierarchical dendogram are delimited. Cumulative explained variation: 89.79%. G1: Group 1; G2: Group 2; G3: Group 3.

2.4. DISCUSSION
There has been some criticism on the *per se* use of “standardized” values such as Sediment Quality Guidelines (SQGs) and geochemically based criteria (such as $l_{geo}$) in the evaluation of environmental samples. While some European nations and regions, including Portugal, currently use SQGs as absolute criteria, there is growing international consensus that in almost all cases, SQGs should be used only as screening tools (Wenning et al. 2006). In this perspective decisions regarding sediment management should be supported by the application of SQGs, in a context of risk-based, tiered assessment. Simultaneously the use of $l_{geo}$ for evaluation of pollution status should be based in normalized values of local relevance (Covelli and Fontolan 1997). But even very site-specific SQGs (or $l_{geo}$) should be validated with a weight of evidence approach (Wenning and Ingersoll 2002).

In this study, SQGs from several sources were used as weight factor to compare the metals under study, and obtain preliminary information on the quality of sediments from Ria de Aveiro. In general terms, the application of SQGs (independently of its origin and derivation) to the dataset lead us to conclude that we are in presence of slightly contaminated sediment...
(Table 2.7) given the generally low percentages of contaminated samples, despite of a few exceptions in individualized sampling sites. Regardless of this general conclusion, when analyzed individually, the quantified metals are divisible by three groups: chromium and lead with a very small number (lead) or none at all (chromium) samples transgressing SQGs; cadmium and copper with a moderate number of samples in transgression (around 10-15%); and finally zinc which was consistently indicated by all criteria as having around 30% of the samples with toxic capability. Two of the results could lead to the rejection of these general inferences: 55% of contaminated samples for chromium indicated by the EQS and 100% for lead indicated by the EAC. But they are purely circumstantial and readily dismissible: the chromium Maximum Permissible Addition (MPA) used to derive the respective EQS was for Cr(VI). If the Cr(III) value (310.97 mg/Kg) had been used the number of contaminated samples would be zero; the lead percentage of contaminated samples is obtained due to the very low value of EAC (0.0029 mg/Kg) proposed by Moffat et al. (2004) in the recalculation of these standards.

The selection of the used SQGs is arguable given already pointed limitations. It was based on criteria of proximity, i.e., national and international criteria that could frame Ria de Aveiro more appropriately both in geochemical and ecological terms. If compared (information not depicted) to other group of differently derived “No Effect” SQGs (empirically derived, ANZEC ISQG-Low (Babut et al. 2003); equilibrium partitioning based, Netherlands Target (Babut et al. 2003); consensus-based, US TEC, (Wenning et al. 2005) the dataset returns quite similar results. This corroborates the conclusion achieved by Wenning et al. (2006) that despite some continuing controversy about exactly which method for generating SQGs is the “best” one, there is no fundamental difference in their “efficacy” (i.e. the ability of a given type SQG to predict toxicity or non-toxicity), provided that SQGs from the same class but different risk-based derivation methods are applied to the same sediments.

Geoaccumulation Index ($I_{geo}$) came as a reinforcement of this conclusion: in its majority sediments sampled in Ria de Aveiro are “unpolluted”: 100% of samples for chromium and copper, 98% for lead, 81% for cadmium. Once again zinc is confirmed as element of concern.
It scored the lowest value of “unpolluted” samples among the metals analyzed (68%), being 6% of them “moderately to strongly polluted”. For cadmium 4% of samples registered a similar class indicating the existence of contaminated sampling sites.

As previously stated the application of these “screening tools” implies they should be used as such. In addition the description of metal dynamics in Ria de Aveiro would be most incomplete if it was to rely solely in the information generated by SQG application (e.g. in what regards the origin of sediment metal contents).

Multivariate statistical approaches deliver more substantial information on links between sampling sites, pollutant concentrations, and latent factors responsible for the data set structure and pollutant sources apportioning (Simeonov et al. 2000). As stated by Einax et al. (1997) and Einax and Sodt (1999), the complexity and the large variance of environmental sets limit the use of common statistical or standardized geochemical methods for the assessment of the state of pollution, so the application of geostatistical and multivariate statistical methods is recommendable. The three clusters of stations originated by the cluster analysis delimitates three generic areas: (1) the western and southern part of the lagoon (comprising the majority of S. Jacinto Mira and Ílhavo channels); (2) the central area corresponding to the confluence of the Ílhavo and Espinheiro channels and terminal section of Mira channel and; (3) the upper northern and eastern reaches of the system (Ovar channel and the mouths of the rivers Antuã and Vouga). Figure 2.7 presents the sampling stations shape-coded according to the cluster they were attributed to: Group 1 – triangle; Group 2-circle ; Group 3- diamond . Differing shades upon the same shape indicate the major sub-clusters according to dissimilarity scores. Double icons in the same station (only in Canal de Mira) represent transept samples that were included in different clusters.
Figure 2.7 – Map of Ria de Aveiro depicting results from the hierarchical dendrogram presented in Figure 2.4. The three shapes (triangle, circle and diamond) represent the three groups (G1, G2 and G3, respectively). Shades upon the same shape represent subgroups of stations. In Mira channel more than one icon represents transect samples included in different subgroups.

The interpretation of such distribution was aided by the application of PCA. According to the SQGs and $I_{geo}$ evaluation, chromium does not present levels indicating contamination and aluminum traditionally is considered without anthropogenic contribution. The dominance of aluminum and chromium, could award PC1 the designation of “natural” (as representative of
the lithological input) and clear indicative of the preponderance of inorganic scavengers. PC2 is dominated by organic carbon (and in smaller measure by zinc) consequently the influence of organic scavengers is represented by stations that are under the influence of this axis. The covariation of metals with aluminum plays an important role at this stage of our analysis. The Enrichment Factors (EFs) one-dimensional approach complements the multivariate one by allowing sediment metal concentrations to be partitioned into natural and anthropogenic fractions.

According to Abrantes et al. (2005) who mapped the bed composition of Ria de Aveiro, the area of Group 1 stations (Figure 2.7) is mainly constituted by fine/medium/coarse sands with sparse finer grained sediment patches. This implicates low background values for Al (poor clay contents) and also the exclusion of high organic contents. Our data reflects this composition since in median terms stations in Group 1 are the poorest in organics (median 0.27%) and aluminum contents (median 2404 ug/g). The identity of this group is clearly defined by low levels of the metals under study (leading to none of the SQGs being transcended, with the exception of station 19A for Cu, accounting for its dissimilarity regarding the remainder stations of the cluster). Surprisingly enough, the calculation of enrichment factors pointed, in a considerable number of samples in several stations, towards another reality. Despite station 19A dissimilarity, it’s the most compact of all main sub-clusters (dissimilarity <2.3). This value drops drastically if only the rest of the stations is considered (dissimilarity <0.55). The occurrence of unrelated enrichment of Cu could be explained by the registered occurrence of pyrite among constituents of the bottom sediments of all channels (Abrantes 2005). Authigenic pyrite formation controls the solubility of many trace metals, in particular chalcophillic metals as the ones in this study (Cooper and Morse 1999) among which copper.

In generic terms neither of the PCA axis exerts a determinant influence (Figure 2.6) reflecting the low levels of both normalizers. Nevertheless at the two sub-cluster level, influences are more readily identifiable. The first comprises stations all situated in the western side of the lagoon (Figure 2.7). Station 19A is singled out with an EF value of 22.5 (47.8 for the EFs
calculated with OC normalization). Metal background levels calculated for this work are generally low (Table 2.3) and this result is a reflection of the particularly low levels of the normalizer. Even small anthropogenic sources and emissions imply a multiplication of the background values, as seems to be the case since this station is located under the influence of a small sewage drain belonging to a camping facility.

The presence of enrichment for at least one of the metals (Figures 2.2 and 2.3) serves as basis for stations 9 to 24C (Figure 2.4) being a part of a subgroup. The first three (9, 5, 24B) present the heavier enrichment (all metals except lead) and, not surprisingly, are under the influence of PC1, whilst the others have unclear influence. Station 16 coincides with the coastal fishing harbor and is the only one that presents lead enrichment. The remaining stations register lesser enrichment situations, being PC2 the main influence, hence better detected by the OC normalization.

The second sub-cluster comprises stations (3 to 15, Figure 2.4) with higher median contents of aluminum and OC, transcending levels of Zn for some stations and of Cr in all stations for QS (except Sr. 30). The level of dissimilarity from stations 3 to 21B is fairly low (<0.8) being much bigger for stations 10 to 15 (around 2.9). The separation is due to lower OC levels (Table 2.6) and cases of anthropogenic enrichment on the first group, particularly for station 24A (Figures 2.2 and 2.3). The fact of occupying different areas (the first sub-sub-cluster is fully located in the southern channels -Mira and Ílhavo- whilst the second is in the central area) also contributes to this difference. The central area possesses lower residence time (particularly the stations nearer to the mouth), and a net seaward residual transport (Lopes et al. 2007) which has a lithological impact, since it promotes the export of sediment to the shelf area, especially the finer particles, with which metals are more intimately associated.

Station 10 has overall low metal concentrations, despite of the highest amount of OC of the entire group (4.7%, Table 2.6). Lopes et al. (2006) indicated this area as being subject to erosion due to a turbidity maximum that travels longitudinally in the S. Jacinto channel. The close association of metals to the finer fractions being exported explains the low
concentrations but not the high OC contents, a component of the fine fraction. A clue emerges from the C:N ratio calculation, since the obtained value of 46.6 clearly indicates a terrestrial origin of the organic matter. Bianchi et al. (2007) reported extensive amounts of sand-sized woody plants material (“coffee-grinds”) in sandy sediments accumulated as a result of hydrodynamic sorting (a critical mechanism in the distribution of terrestrially derived organic matter). In the case of station 10, the wooden fragments could have been provided by any of the two main rivers discharging into the lagoon (Antuã and Vouga) since there is a connection between Laranjo Bay and Espinheiro Channel (where the mouth of the rivers is located, respectively) and the area where the station is located (Dias et al. 2001). The coarse vegetal materials collected in this sample were eliminated from metal analysis but not from OC quantification. The connection between areas also accounts for the clustering of Station 10, 34, 12 and 15.

Groups 2 and 3 represent different branches of the same cluster. This cluster is characterized by higher contents in aluminum and OC in comparison with the cluster representing Group 1. Group 2 stations possess lower levels of organics (median 2.57%) and higher levels of aluminum (median 18519 µg/g) than Group 3. Zinc levels systematically transgressed SQG and Igeo values. Transgressions for other metals are registered as well but not in coincidence for the same stations nor the same SQG. EFs totally disconfirm the indication of potential contamination by not detecting any widespread enrichment for this metal or others. When values are bigger than 2 is only for specific metals in specific stations. These results are somewhat surprising as this is the area of implantation of a commercial and chemical sea port terminal, a coastal fishing harbor, an offward fishing harbor and shipyards. Metal enrichment was to be expected. The central sector of the lagoon where the stations are located includes the first half of Mira and Ílhavo channels and the confluence of S. Jacinto and Espinheiro channels. A mighty influence of the sea waters influx-reflux (strong hydrodynamism), high depths (maintained by regular dredging to allow navigability) and relatively small residence times (2 to 6 days) are rule in the area (Lopes and Dias 2007). As a result there is a net sediment and water export, and consequently the mineralogical
signatures of detrital inputs coming from north, east (from the Vouga watershed soils) and south are vanished (Abrantes et al. 2005). This set of conditions could account for the inexistence of metal enrichment due to flushing. Curiously enough, anthropogenic enrichment is indicated for lead in station 14, a recess area of low hydrodynamics (were consequently deposition occurs) near the lagoon entrance, connecting with Mira and S. Jacinto channels and the navigation inlet channel. The relevance of this fact resides in the high affinity of this metal towards clay minerals and organic matter forming high stability complexes (Jørgensen and Jensen 1984), a proof on the efficient fine material export by tides. Another token indicative of the interface characteristics of the area is the display of one of the highest contents in calcite, a mineral typical of marine depositional environments (Abrantes 2005).

Among the clusters, Group 3 presents the highest organic carbon contents. The no-effects threshold is systematically surpassed by zinc and cadmium and more intermittently by copper. Enrichment factors corroborate this preliminary scrutiny and even enlarge the contamination scenario to include lead and chromium. Indisputably this group includes the most contaminated stations in this survey. In terms of granulometry, high amounts of aluminum (median 15702 μg/g) and the highest OC contents (median 3.54%) of all three main groups attest the preponderance of fine particles. Despite being included in the same cluster, the two sub-clusters show a high level of dissimilarity (>22), even higher than between stations of the much heavier Group 1. This is mainly due to differences in OC and Cd contents.

Levels of metals in Stations 1, 2, 6 and 7 reflect the direct influence of industrial areas aided by the presence of clays, silty clays and silts deposited due to weak currents (Abrantes 2005). For the same reason these areas have a prevalence of high residence times, an adding factor for metal enrichment of these sediments (Lopes and Dias 2007).

The densely populated northern limits of Ria de Aveiro are drained by a profuse set of small waterways of which the Caster river is the more important (despite possessing average flows
lower than 1 m³/s) (Dias et al. 2001). Aside from the strong organic influence characteristic of densely urbanized areas and cattle rearing activities (Silva et al. 2002), industries (chemical, pulp mills, metal plating) exert their impacts in this waterway. Station 2 is located in its area of influence and the respective sediment samples clearly attest the contaminant loads being deposited: zinc, copper and cadmium are enriched 2.3, 4.9 and 4.9 times, respectively. Castro et al. (2006) used Corophium multisetosum to evaluate these sediments ex situ and they were, on the entire system, the only ones causing mortality both in chronic and acute tests.

Station 1 presents zinc enrichment (as well as Cd for OC normalization). Clays and silty clays are deposited in the far reaches of Ovar channel due to weak tidal currents (there is a "strangulation" derived from a very shallow area were station 3 is located) and freshwater inputs are insignificant (Lopes and Dias 2007). In consequence only more mobile metals, such as Zn, are transported enriching this area (Linnick and Zubenko 2000).

Metal-wise, the Laranjo Bay area (stations 6, 7 and 8) is normally pointed as the most polluted location of the lagoon (e.g. Pacheco et al. 2005; Monterroso et al. 2007). Discharges of fifty years of industrial activities in adjacent areas (Estarreja industrial park) reaching the area through artificial channels (Costa and Jesus-Rydin, 2001), plus the Antuã river (the most prominent freshwater source, receiving important quantities of metal-rich industrial effluents and running through heavily populated areas) (Silva et al. 2002) have contributed to severe contamination of its bottom sediments. Our study confirms this status and produces evidence of metal export. The growing value of EFs from stations 8 to 6 (distributed from the bay to the S. Jacinto channel) and the enrichment of neighboring station 5 thus attest (Table 2.3). Underlying this process is the conjunction of the bay shallowness and the narrowness of the channel leading to it. Flood tides will cause enhanced bottom erosion re-suspending sediments and carrying them back to the main body of the lagoon with the ebb (Lopes et al. 2001). From there they are distributed by the northern areas towards Ovar channel and southwards towards S. Jacinto channel (and hence to the sea). There is also evidence of suspended sediment circulation originated from this area through Espinheiro channel (Dias et al. 2001). The identification of Laranjo Bay as the major source of metals in Ria de Aveiro
system lead to a devaluation of the importance of other potential areas of concern, like Vouga river. The Vouga is the third major river flowing exclusively within Portugal and the main freshwater contributor to the lagoon. Despite being considered “relatively clean” in terms of nutrient inputs (Silva et al. 2002), receives into its course (directly or via tributaries) impacts from agriculture, cattle farming, poultry production, untreated urban effluents, discharges from several industrial parks (including ceramics, metal processing plants, tannery, wine cellars, pulp mills) (Cerqueira et al. 2005; Sobral et al. 1985). Seven abandoned mines (mainly lead and copper extraction) are located in its watershed (Comans et al. 1987). It has been reported that waters flowing through the Braçal – Talhadas mine (the biggest of the seven) were being enriched with Cu, Pb and Zn (da Silva et al. 1995). Absolute metal values obtained from station 33 (the river mouth) sediments are high (Table 2.6) and transcend several SQGs for Zn, Pb, Cu and Cd, as well as $I_{geo}$ for Zn, Pb and Cd. The OC content is also very high (4.59% - the highest in the group and second only to station 10). EFs indicate enrichment for Zn (3.7), Pb (8.5), Cu (8.0) and Cd (7.4). This is demonstrative of the fact that, despite Ria de Aveiro being considered an overall well mixed system (Dias et al. 2001), under certain conditions (e.g. low freshwater discharge concurrent with flood tide) the water column in this channel is mainly filled with salt water from the ocean (Vaz and Dias 2008). This water will, at least twice a day, exert a “salting out” effect on metals transported in suspended and dissolved forms, accumulating these elements in the sediment (Turner 2003). Results from upriver station 34 corroborate this conclusion, by not presenting any indication of enrichment, a testimony of the lack of influence of the salt wedge in the area (Vaz and Dias 2008). In terms of sediment composition there is a prevalence of medium/coarse sands mixed with a small percentage of finer materials (clays and silts) giving the indication that metals must be adsorbed to organic materials (PCA reinforces this idea by identifying PC2 as the axis notoriously influencing this station), being readily exported given the low residence times (approximately a day) for this area (Lopes and Dias 2007). Further study is needed to better understand the metal load contributions of Vouga river. The remaining stations of this cluster are unclear in terms of association to any of the two axis,
with the exception of station 7 which is clearly under PC1 (the prevalence in this depositional area of fine cohesive particles is determinant).

A sampling campaign such as the one performed in this study is a freeze frame of a system at a certain moment in time. A highly dynamic system like Ria de Aveiro possess a myriad of factors in simultaneous action. The collectible sediment at a given location (the one to be analyzed in the laboratory) is there as result of the ultimate interaction of these factors. Several studies (e.g. Abrantes et al. 2005; Dias et al. 2001; Lopes and Dias 2007; Silva et al. 2002) were performed investigating the ruling factors in sediment transport dynamics for Ria de Aveiro. Tidal currents are the primordial factor, modulated by a set of the lagoon’s intrinsic characteristics: shallowness, very complex network structure, division in several subsystems, tidal asymmetries between and within channels (different areas of ebb and flood dominance), and weak gravitational circulation due to its well-mixed structure (Lopes and Dias 2007). Other factors such as wind driven residual circulation and river runoff are also important (Abrantes et al. 2005). The obtained clusters are a fine illustration of the interaction of these factors, when in colligation with metal sources. Enrichment factors distribution analysis clearly pinpoints some of these sources towards the system. The rivers Caster, Antuã and Vouga are contributors of Zn, Cu and Cd. Antuã and Vouga rivers are contributing Pb as well. Furthermore the Spearman rank correlations between metals were strong and this proof of mixed associations is coherent with the typology of these sources.

Mira channel does not fit into this profile since it possesses no clear sources for the verified enrichments. Freshwater discharge in this channel is almost insignificant (drainage ditches flowing from a lagoon), the population density is much lower that the northern reaches of the lagoon and it has no direct influence from industries. To understand the metal distribution a set of small scale, local factors have to be considered: the regular traffic of recreational and small fishing boats, which create bottom waves in the shallower areas (Schoellhamer 1996); the presence of groundwater upwelling (Dias et al. 1999) occurring in the middle and upper reaches (station 21 onwards). This phenomenon is pointed as one of the generally overlooked localized contamination factors (Crane et al. 2007b); the regular use
of the channel shores as recreational area and amateur fishing abodes; the more than probable existence of seeping biogenic methane reservoirs as Duarte et al. (2007) reported exist in deep sediments of other Ria areas; and finally the capture of fresh bait that mobilizes, according to Cunha et al. (2005), at least 150 collectors daily, plus unaccounted cockle and clam collectors, which create a metal remobilization dynamic from the anoxic layers of sediment. Nevertheless in their majority these factors contribute to the redistribution and reworking of superficial sediments and are not sources in themselves. A strong possibility is that this channel is receiving direct influence from the adjacent continental shelf via the artificial lagoon inlet. Several facts point in this direction: each Ria channel can be regarded as a separate estuary (being this channel pointed as the best example (Dias et al. 1999)) in direct contact with the adjacent platform with which dynamic exchanges (import and export) of particulate materials occur (Lopes and Dias 2007; Vaz et al. 2008); the presence of Cr enrichment occurs nowhere else in the system (aside from station 9 located in an area of direct tidal influence as well); the enrichment (OC normalization) in Cr, Cu and Cd that is maintained throughout the major part of the stations, which has no parallel in the remaining channels, revealing continuity and stability of the hydrodynamic factors affecting circulation (e.g. tidal forcing) and; the high levels of calcite (a tracer of biogenic marine contribution) and quartz (characteristic of marine deposits) in the sediment matrix (Abrantes 2005, Bobos and Rocha 2006). The continuous enrichment in Cd is of particular importance in reinforcing this last fact, since the substitution of Ca by Cd in the mineral lattice is recurrent (Bottcher and Gehlken 1997).

Strengthening this possibility is the report that sediments of the shelf area outside the Ria’s mouth possess enrichment of Cr, Zn and Pb above background levels due to anthropogenic influence (Abrantes 2005). This enrichment can be allocated to the direct contributions from the lagoon and to metal rich sediments originated from Douro River (located to the north), brought into the area by littoral drift or advective currents (Abrantes 2005).

Metal contribution of the lagoon to the adjacent platform can be considered substantial due to an efficient flushing of sediments delivered by inner sources. As previously referred the
conglomerate of channels constituting Ria de Aveiro are hydrologically considered separate, independent estuaries, but some exceptions with relevancy to contaminant dispersion and flushing exist. Dias (2001) stated that despite virtually no particles transference between channels exists, exceptionally that can occur over small time scales. The most relevant of these exceptions is the wind induced residual circulation directed along the main S. Jacinto and Espinheiro channels, which tend to form a closed loop between these two channels (Dias et al. 2003; Lopes and Dias 2007), mixing sediment loads being delivered by two of the lagoons’ main metal contributors Laranjo Bay and Vouga river) and subsequently, in a very short time scale, exporting them to the adjacent platform.

Another external contributor to the metal loads entering Ria de Aveiro (aside from Douro river sediments) can possibly be found in the effluents (urban and industrial) discharged by a sewage outfall located 3km out of S. Jacinto. Despite in 2002 da Silva et al. stating that discharges from the new regional sewerage system would potentially contribute to an increase in the particle fluxes entering the lagoon through the inlet channel, no evaluation of its influence upon the lagoon was performed since the beginning of the discharges (2005).

Thus tidal “feeding” of the metal loads together with the already pointed peculiar specificities of this channel can account for the enrichment distribution in canal de Mira. This enrichment presents an illustrative case: the mutual exclusion of zinc and lead. Zinc is confined to the upper reaches (stations 25 and 24) reflecting the higher mobility of this metal. Higher residence times prevailing in the area cause sedimentation, and undisturbed depositional areas allow persistence and enrichment (Linnik and Zubenko 2000). Lead is enhanced in the sediments of the lower reaches (stations 14 and 16) reflecting a lower mobility of the metal and shorter residence times, with a very fast flushing of finer materials (Linnik and Zubenko 2000; Lopes and Dias 2007). Moreover, the upper reaches have much higher organic contents. These compounds have a much stronger exchange capacity with water for Pb than for Zn, since divalent ions are preferred (Jørgensen and Jensen 1984).
Some of the more subtle patterns of distribution inside this channel could be explained by the already alluded existence of pyrite in the sediments, being the more preeminent case the one of Cr in station 9. Ilhavo was the only channel that did not present noticeable metal elevations despite of the existence of a porcelain factory that has been reported to cause metal enrichment upon sediments (Delgado et al. 2000). This author categorized this area as the second more important source of zinc to the system. In the context of our study only OC normalization indicates Zn enrichment for station 27 (a tangential 1.9) which is more easily attributable to the shipyards located in the area. The sample obtained in a site located opposite to the factory only returned enrichment (also tangential at 1.9) for Cu, and this despite this channel being the one with higher amount of clays and silts in the entire system (Abrantes 2005). This could be due to absence of relevant discharges or to an efficient export of metals. The hydrodynamics depend heavily in the tidal circulation since River Boco, the freshwater source associated to this channel, has very limited freshwater discharge and the beginning of the channel is very shallow if compared to the mouth of the lagoon. This causes the propagation of the tide not to suffer appreciable delays (unlike the rest of the system) and, indeed to have an accelerated initial ebb flow into the channel, creating, simultaneously, a small but effective gradient of salinity (and temperature) in the water column (Dias et al. 1999). The final result is that more mobile metals like Zn (Linnik and Zubenko 2000) are transported to adjacent areas (as station 29 attests). According to OC normalization there is small enrichment (1.9) of lead in station 30, probably because of the proximity to a shooting range. The transects sampled in Mira channel returned high horizontal gradients of metal concentrations in very small spatial scales. A growing process of “channelization” corresponding to changes in channel’s geometry and bathymetry has been described for Ria de Aveiro, with particular incidence in the southern channels: mud flat and salt marsh erosion with a simultaneous deepening of the more dynamic areas were freshwater flows and the tidal excursion primarily advances and recesses (Araújo et al. 2008; da Silva and Duck 2001; Vaz and Dias 2008). Depositional environments within each channel have thus been
suffering a continuous, gradative process of change. Station 24 presents good illustration: from the heavy enrichment in the sample collected by the bank (24A were Zn, Pb, Cr, Cu and Cd have EFs of 3.3, 1.7, 7.8, 17.9 and 12.9, respectively) to no enrichment at all in sample 24E, situated in the deeper, major flow area, with a decreasing enrichment gradation in between. It should be noted that reed beds bordering the channel most certainly have responsibility for the enrichment in station 24A. du Laing et al. (2008) indicted the decay of plant debris as one of the metals major sources in Scheldt estuary sediments. The validity of the obtained results is not put at risk by such observations, since for high energy systems small scale metal distribution discrepancies are common, and as long as normalization procedures are applied, the effect field variance has upon the analysis will drop significantly (Birch et al. 2001).

Overall, sediment metal contents normalization revealed a not entirely different reality to the one presented by the scrutiny using SQGs and $I_{geo}$ criteria: as a whole the system is not enriched, despite a few areas (specially under the influence of the rivers caster, Antuã and Vouga and Mira channel) presenting severely enhanced metal concentrations, several times above calculated background levels.

Despite the general agreement, our study reinforces the preliminary approach character currently awarded to SQGs (Wenning and Ingersoll 2002; Wenning et al. 2006). These standards were almost invariably over or under predictive. Only in 6.3% of the cases did the prediction of contamination totally coincided with the one cast by enrichment. In all other cases there was either prediction of contamination were no enrichment existed or no prediction of effectively enriched metal levels.

When comparing the two normalizing agents, Al demonstrated higher coefficients than OC towards all elements proving to be the most suitable normalizer and simultaneously indicating a prevalence of inorganic scavengers as dominant factors controlling trace metal distribution in the catchment. Nevertheless the use of OC normalization can be valuable
especially in areas were the organic component is of importance, despite this normalization having no geochemical basis and organics being, themselves, pollutants (Kersten et al. 1994).

2.5. CONCLUSIONS

The application of “standardized” methods like SQGs are insufficient to characterize a sediment metal dataset, since they don’t have regard for local backgrounds, existing high possibilities of under or overestimation of sediment contamination.

Ria de Aveiro is in general a mildly contaminated system, with localized areas of enrichment (with the exception of one channel were enrichment is felt almost throughout its entire extent— Mira). The characteristics of the system lead to a fast flushing of contaminants as the main sources (rivers Caster, Antuã and Vouga) are located in areas of low to medium residence times. Zinc is the only metal presenting enrichment in all the four main channels. There is strong evidence for chromium being exported from the adjacent continental shelf where sediments from the much more contaminated Douro river are being deposited and a sewage outfall discharging industrial and urban effluents recently entered into activity. This influence is probably applicable for all metals in areas of tidal influence, particularly lead in the initial portion of canal de Mira.

The other metals under appreciation (copper, cadmium) appear to be dispersed from point sources, with the wind driven loop including S. Jacinto and Espinheiro channels contributing for metal exchange. Strong Spearman rank correlations between metals give indication of mixed associations and are coherent with the existence of consistent sources within Ria de Aveiro. Vouga river has been a poorly studied source of metals within this system. Added research is necessary to fully understand its contribution in terms of metal loads.

Mira channel presents a very complex metal enrichment distribution due to the strong tidal influence and the interaction of a set of particular circumstances. Since it is one of the main sites of bivalve and bait catching, concerns are raised towards its consumption and potential subsequent biomagnification.
Of the two normalizing agents tested, aluminum returned higher coefficients than OC. This is indicative of the prevalence of inorganic scavengers as potential controlling factors in metal mobility throughout the system, being organic scavengers confined to specific areas. The normalization based enrichment factors proved extremely handy in the identification of trace metal hotspots that SQGs will not detect or in the dismissal of predictions of contamination of those standards. This identification allows focusing of investigational efforts and a more rational and effective allocation of (sometimes limited) resources.

Acknowledgements

FCT – Fundação para a Ciência e Tecnologia for funding this research through PhD Grant SFRH/BD/4778/2001. The authors express their deep gratitude to Mr. Rui Marques for all the help with the collection of the sediments. We are also grateful to Mr. Ricardo without whose rowboat a van Veen grabber would have become a submarine.

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Chapter 2 – Trace metal distribution in Ria de Aveiro
How tough is my neighbor? - A feeding inhibition based prediction of toxic effect of metal mixtures upon *Echinogammarus marinus* at field relevant concentrations in a latitudinal gradient
CHAPTER 3. HOW TOUGH IS MY NEIGHBOR? - A FEEDING INHIBITION BASED PREDICTION OF TOXIC EFFECT OF METAL MIXTURES UPON ECHINOGAMMARUS MARINUS AT FIELD RELEVANT CONCENTRATIONS IN A LATITUDINAL GRADIENT

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ABSTRACT

The allocation of individual toxic responsibilities inside a complex mixture is intricate. Not only metallic species, when in a context of mixture, have the possibility of exerting toxicity at concentrations well below their individual sub-lethal values, but also could in this process be the sole responsible for the felt toxic effect. Assessment of metals in nature is mainly performed with approaches based in single element toxicity evaluations, a highly inadequate framework since in the environment seemingly infinite amounts of substances are mixed. The development of models for the prediction of combination effects on the basis of the concentration–response relationships of individual mixture components emerged as an answer to the unfeasibility of testing all possible combinations of a given set of chemicals. Of the two general models: Concentration Addiction and Independent Action, the first one was used to predict post-exposure feeding impairment in Echinogammarus marinus, a widely distributed gammaridean amphipod. Field data was obtained in a latitudinal gradient which allowed comparisons between the behavior of the species in the presence of metal contamination.

3.1. INTRODUCTION

In spite of international efforts to reduce emissions to the environment, evidence that the levels of heavy metals are increasing throughout ecosystems worldwide proliferates (e.g. Dessus et al. 1994; García-Rico et al. 2004). Beghin and Poitier (1997) while prospectively hypothesizing about the effects of trade liberalization upon the environment identified several ‘hot spots’ of expanding pollution caused by intensive manufacturing activities. Newly industrialized countries in Asia and even OECD countries (particularly Canada and Mexico) were expected to increase their metals output.
Metals in the aquatic environment, either from anthropogenic or natural sources recurrently occur in complex mixtures (Barata et al. 2006) in which context they may exert effects upon aquatic organisms at concentrations well below their individual sublethal values (Enserink et al. 1991; Verslycke et al. 2003). But aside from this “group effect” it has been proven that a sole metal species could be responsible for the entirety of felt toxic effects (Förstner & Prosi 1979). The effective exposure of organisms is not determined by the total metal bulk concentration in a particular environment, but by the concentrations and speciation state of the individual metal forms (Roesijadi and Robinson 1994; Tack and Verloo 1995). Nevertheless since assessments of chemical risks to aquatic life rely on toxicological data that in its vast majority relates to single pure substances, the allocation of individual toxic responsibilities inside a complex mixture is intricate.

The development of models for the prediction of combination effects (Berenbaum 1985) on the basis of the concentration–response relationships of individual mixture components emerged as an answer to the unfeasibility of testing all possible combinations of a given set of chemicals. Two reference concepts are on the base of such models, concentration addition (CA) and independent action (IA) (EIFAC 1987; Bodecker et al. 1992). A shared common target site and similar action mechanism are CA assumptions, whilst different target sites and dissimilar mechanisms of action of all components in the mixture underlie IA (Barata et al. 2006). For a more detailed discussion see Faust et al. (2001) and literature cited therein.

In the present work observed mixture toxicity of four metals (cadmium, zinc, copper and nickel) to the amphipod *Echinogammarus marinus* was compared to predictions, calculated from the concentration–response data of the individual toxicants by applying the CA concept and validated by field deployments of the gammarid. Amphipods have been growingly used in laboratory and field studies to evaluate metal contamination being feeding inhibition one of the most consistent endpoints used (Maltby et al. 1990; Maltby and Crane 1994). Sublethal effects on food acquisition influence production rates (i.e. growth and reproduction) and other life traits in several species, among which amphipods (Barata and Baird 2000; McWilliam and Baird 2002; Macedo Sousa et al. 2007; Pestana et al. 2007). In this
study a particular variant of this endpoint was used: post-exposure feeding depression (Barata and Baird 2000). Post exposure anorexia has been known to be caused by metals (Barata and Baird 2000; McWilliam and Baird 2002) and when used in field studies (in situ toxicity tests) supported by laboratory exposures, allows linkage of physiological individual-level responses to populations and even communities (Maltby et al. 2000; McWilliam and Baird 2002). This linkage has been frequently barred by the use of unrealistic metal concentrations in toxicity evaluations, which undermine the possibility of laboratory-field extrapolation by lack of ecological relevance (Janssen et al. 2000). The need to provide regulators with sound data upon which to base guidelines have emphasized the necessity of environmental realistic formats in toxicity tests notably in what regards to concentrations of used chemicals (Bindesbøl et al. 2005; Chapman 2008).

The cosmopolitan characteristics (sensu Rainbow 1995) of E. marinus allowed the evaluation to be performed in the entire breadth of latitudes of the species distribution, encompassing Iceland, Scotland and Portugal.

3.2. MATERIAL and METHODS

Unless otherwise specified, described methodologies and procedures are common to all three laboratories/field locations.

3.2.1. Experimental animals

Test organisms were captured from local populations in Iceland (South of Sandgerði, Reikjanes Peninsula 64°02′N, 22°42′W), Scotland (Loch Fyne, 56°10′N, 5°05′W), and Portugal (Mondego estuary, 40°07′N, 8°49′W) (Figure 3.1.) and taken to neighboring laboratories (Sandgerði Marine Center, Institute of Aquaculture; University of Stirling and Department of Biology, University of Aveiro, respectively) in 50L plastic buckets filled with local water and brown algae. Organisms were left to acclimate and depurate (Clason and Zauke 2000) for one
month (Portugal and Scotland) or three weeks (Iceland) in plastic containers (40 x 20 cm) filled with 4 l of continuously aerated saltwater (see below). Twice a week water was changed and dry *Fucus vesiculosus* (obtained from local clean sites) was supplied *ad libitum* as food. To provide shelter and mimic the organism’s habitat, small black polyethylene sheet rectangles were placed in the tank (Maranhão and Marques 2003). Artificial seawater was used in Scotland and Portugal (SERA PREMIUM® in deionized water) and in Iceland natural seawater (taken from a 50-m deep drilling hole, and free from any contaminants (Svavarsson, unpublished data)). Salinity was set at 30% using deionized water. Acclimation and test temperatures were maintained at 10±1°C in Iceland, 15±1°C in Scotland and 20±1°C in Portugal mirroring the typical average water temperatures of each region at the time of organisms collection (Hafrannsóknastofnunin 2008; SEPA 2006; Martins et al. 2005). Photoperiod regimes were 12h light /12h dark.

3.2.2. Toxicity tests

3.2.2.1. Laboratory

Stock solutions of four metals (zinc, cadmium, copper and nickel) were prepared from salts (ZnSO₄·7H₂O, CdCl₂·2H₂O, CuCl₂·2H₂O, NiCl₂·6H₂O, Sigma-Aldrich) in ultra-pure Milli-Q water. Five nominal concentrations per metal (Table 3.1.) were obtained adding appropriated aliquots of the stock solution to saltwater (see 3.2.1.).

The choice of concentrations was based on values obtained for Ria de Aveiro sediments (Chapter 2). Individual and equitoxic mixtures were tested (96h static exposure) using five replicates per treatment (ten organisms of approximate size per chamber). All material (including the plastic test chambers) was acid washed and pre-soaked in the appropriate test medium for 24h to saturate all adsorption sites (Rainbow et al. 2004).
No food was presented to the organisms during experiments. Immediately after exposure time was spent, individuals were transferred to new chambers with clean seawater and feeding was measured for 24h.

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### 3.2.2.2. Field

Environmental chambers were constructed from clear polyvinyl chloride cylindrical piping as described in McWilliam and Baird (2002). Twenty organisms (from the same laboratory culture as the ones used in the toxicity assays) of approximate size were introduced in each chamber and deployed in the field for a period of 96h. After time elapsed, chambers were taken back to the laboratory (< 2h driving in all cases) in local water. There organisms were carefully retrieved from the chambers and immediately allocated to the feeding assays.

#### 3.2.2.2.1. Field Sites

Figure 3.1. generically indicates de location of the field sites used in this study.
3.2.2.2.1.1. Iceland

Three sites were used reflecting local circulation conditions (a strong costal drift circulating clock-wise around the entire island) (Egilsson et al. 1999), with Sandgerði harbour as central point (first location) and two other sites, chosen by their sheltered conditions, at approximately 5km to the North (Hafurbjarnastaður) and South (Hvalsnes), respectively. Despite these shores being considered pristine environments (Sarà et al. 2007) there is indication of heavy metal background values being higher than in equally remote areas, being volcanic activity regarded as the main probable cause (IME 2001) alongside point sources coincident with the (sparse) human settling such is the case of Sandgerði (Leung et al. 2005).

3.2.2.2.2. Scotland

Strachur (Loch Fyne), Ardentinny (Loch Long) and Hound Point (Forth estuary) were used as deployment sites. Loch Fyne is the longest, deepest fiordic sea loch of Scotland’s Western
coast, being part of the Firth of Clyde watershed, traditionally considered one of the country’s most contaminated (Leung et al. 2001). Loch Long, equally located in the West coast, is the second most brackish sea loch in Scotland and is considered as a remote, mostly undisturbed system where intensive aquaculture is maintained (SNH 2006). The Forth estuary (in the East coast) receives large amounts of industrial effluents (Chemical, pulp mill, sewerage) (EPER, 2004) and in its watershed the influence of highly populated areas is felt (e.g. Stirling and Edinburgh). It possesses a traditional record of metal pollution (Balls et al. 1997) that, nevertheless, has been strongly abated in recent times (SEPA 2006).

3.2.2.2.3. Portugal

Four sites were selected inside Ria de Aveiro (a coastal lagoon in the SW coast of the Iberian peninsula): Areão, S. Jacinto, Bico da Murtosa and Ovar marina. A complete description of these locations including sediment metal profiles can be found in Chapter 2.

3.2.3. Feeding assays

Pre-dried (60°C, 24h) and weighed discs of *F. vesiculosus* were offered to individuals in clean seawater (artificial or natural, see above) right after the exposures and amphipods allowed to feed for 24h. Remaining algae were collected from the chambers, dried (same conditions) and reweighed. Feeding ratio (in mg per individual) was obtained from the amount of food consumed (initial *Fucus* mass minus final mass) divided by the number of individuals feeding.

3.2.4. Metal analysis

Metals were analyzed in acidified samples of field and test waters by atomic absorption spectrophotometry by a Unicam 939QZ Atomic Absorption Spectroscope with coupled Unicam GF90 Graphite Furnace (GF-AAS) and Zeeman® correction. All glassware used in the
analysis was soaked overnight in 5% hydrochloric acid (Aristar, BDH, U.K.) and then triple rinsed with Millipore water prior to use.

3.2.5. Data analysis

The rate theory (Eyring, 1935) can be used to describe the inhibition ($I$) of a biological process (like feeding) as a function of toxicant concentration, with observed values ranging from the control values to zero,

$$I = I_o \times \frac{EC_{50}^k}{EC_{50}^k + [C]^k} \quad (1)$$

Where $I$ is the measured value of the biological process, $I_o$ the maximum value measured for the biological process (i.e. the average response in the control), $EC_{50}$ is the half saturation constant (i.e. concentration that causes an inhibition of 50% in the biological process), and $k$ is the decay index. Rearranging the equation we get

$$\frac{I}{I_o} = \frac{EC_{50}^k}{EC_{50}^k + [C]^k} \quad (2)$$

and

$$\frac{I_o - I}{I} = \frac{[C]^k}{EC_{50}^k} \quad (3)$$
Applying logarithms to both sides of the equation a linear equation is obtained (i.e. $Y = mX + b$)

$$\log\left(\frac{I_{0}-I}{I}\right) = k \log([C]) - k \log(EC_{50}) \quad (4)$$

Thus, if we use feeding ($F$) as the biological process in equation (3) we have

$$\frac{1-F}{F} = \left[\frac{C}{EC_{50}}\right]^k, \quad \text{or} \quad \frac{1-F}{F} = TU^k \quad (5)$$

where $TU = \left[\frac{C}{EC_{50}}\right]$ refers to the toxic units of an individual chemical as defined by Sprague (1970). Solving equation (5) in relation to feeding ($F$) we get

$$F = \frac{1}{1 + TU^k} \quad (6)$$

Considering a mixture of $n$ chemicals, where each chemical contributes to the overall toxicity proportionally to concentration (expressed as $TU$) of each chemical, the expected feeding for the mixture ($F_{mix}$) can be calculated as

$$F_{mix} = \frac{1}{1 + \left(\sum_{i=1}^{n} TU_i\right)^k} \quad (7)$$
Where $\chi' = \sqrt[n]{\prod_{i=1}^{n} k_i^{TU_i}}$, and $z = \sum_{i=1}^{n} TU_i$ \hfill (8)

i.e. $TU = \frac{C}{EC_{50}}$ is the weighted geometric mean of the $k_i$ obtained for each chemical in the mixture.

_Echinogammarus marinus_ feeding under exposure to toxicants does not follow this general pattern (i.e. feeding rate decaying to zero with increasing exposure of the toxicant), instead feeding decays to a minimum value for increasing concentrations of the toxicant (Figure 3.2).

![Figure 3.2 - Theoretical sigmoid function (with an offset) describing _E. marinus_ feeding decay, and the 50% feeding decay (FDC50) derivation.](image)

Thus, for this species, equation (6) must be rewritten as

$$F = (1 - Min) \times \frac{1}{1 + TU^k}$$ \hfill (9)
and consequently equation (7) becomes

\[ F_{\text{mec}} = \left(1 - M\text{in}\right) \times \frac{1}{1 + \left(\sum_{i=1}^{n}TU_i\right)^k} \] (10)

Similarly, since some of the original concepts have been adapted (EC\textsubscript{50} and TU), to avoid misinterpretations it is suggested that this particular EC\textsubscript{50} is referred as FD\textsubscript{50}, and the TU are referred as fTU

\[ fTU = \frac{C}{FD\textsubscript{50}} \] (11)

### 3.3. RESULTS

#### 3.3.1. Chemical analysis

Measured concentrations of the test solutions for single and mixture metals were within 10% of nominal concentrations; hence responses to all metals were based on nominal concentrations (Table 3.1). Analysis of field water samples are presented in Table 3.2.

<p>| Table 3.2 - Concentration of metals in field samples taken at the in situ deployment sites. |
|-------------------------------------------------|--------|------|------|------|
|        | Zn     | Cd   | Cu   | Ni   |
| ICELAND|        |      |      |      |
| Hafurbjarnastaður | 589.3 | 5.264| 35.2 | 242.1|
| Sandgerði Harbor    | 717.5 | 0.593| 35.9 | 75.56|
| Hvalsnes            | 22.4  | 0.557| 22.4 | 30.4 |</p>
<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>Concentration</th>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCOTLAND</td>
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<td>7.863</td>
<td>0.009</td>
<td>6.455</td>
</tr>
<tr>
<td></td>
<td>Strachur</td>
<td>5.045</td>
<td>0.018</td>
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<tr>
<td></td>
<td>Ardentinny</td>
<td>6.493</td>
<td>0.026</td>
<td>5.392</td>
</tr>
<tr>
<td>PORTUGAL</td>
<td>Ovar Marina</td>
<td>324.45</td>
<td>1.02</td>
<td>21.93</td>
</tr>
<tr>
<td></td>
<td>B. Murtosa</td>
<td>245.98</td>
<td>2.3</td>
<td>34.09</td>
</tr>
<tr>
<td></td>
<td>S. Jacinto</td>
<td>12.34</td>
<td>0.06</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>Areão</td>
<td>11.43</td>
<td>0.01</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Metal concentration in Icelandic water samples was unexpectedly high, particularly for Hafurbjarnastaður. This site is almost the northernmost tip of the Reykjanes peninsula where rock outcrops form an area of shallow waters abating the currents. Thus the circulation patterns of the area are quite different from the other two stations were a strong northwards current is felt, which could partly explain the higher metal concentrations due to settlement of the particulate fraction. On the contrary Scottish samples were low. The intensive plan of emission reduction set in motion by the Scottish Environmental Protection Agency (SEPA) is contributing determinately for this state of affairs. Nevertheless even for the historically polluted Forth estuary reported values of metals in waters were always low (SEPA 1998) being the turbid nature of the estuary pointed as a significant factor controlling the concentration of dissolved metals in the water column by enhancing particle/water interactions in detriment of the latter. Portuguese stations presented a very diverse scenario (stations with generalized enhancement, with overall low levels or just two enriched metals – Zn and Cu for Ovar marina) mirroring the complex conditions inside the lagoon.

3.3.2. Single metal toxicity
In all performed exposures no mortality occurred either in controls or treatments. The expected sigmoid decay curves describing the proportional feeding were rather flat but
verified the model for all metals at all three locations, with the exception of copper and zinc in the experiments conducted in Portugal. In all cases residuals of the regression model obtained were normally distributed (Kolmogorov-Smirnov test at P<0.05). Toxicity varied according to metal and location.

### 3.3.2.1. Iceland

Reproducibility between controls and individual metals was good. High sensitivity for all metals was observed. Low values of $F_{dC_{50}}$ (73.43 ug/l for Zn, 0.21 ug/l for Cd, 1.78 ug/l for Cu and 10.8 ug/l for Ni) corroborate this diagnosis, being copper, and to a lesser extent, cadmium the metals registering the strongest feeding inhibition (Figure 3.3).

![Graphs showing concentration response curves for E. marinus toxicity upon post-exposure feeding behavior of the four studied mixture components in Icelandic individuals.](image)

**Figure 3.3** - Individual concentration response curves for *E. marinus* toxicity upon post-exposure feeding behavior of the four studied mixture components in Icelandic individuals.
3.3.2.2. Scotland

Excellent reproducibility between controls and individual metals was obtained. Scottish individuals showed high sensitivity to all metals (low $F_d C_{50}$) but zinc (Figure 3.4) towards which was shown no sensitivity. As in Iceland copper and cadmium were the metals reaching higher inhibitory effects. Calculated $F_d C_{50}$ were 0.28 ug/l for Cd, 2.02 ug/l for Cu ug/l and 8.39 ug/l for Ni.

![Graphs showing toxicity of metals](image)

*Figure 3.4 - Individual concentration response curves for E. marinus toxicity upon post-exposure feeding behavior of the four studied mixture components in Scottish individuals.*

3.3.2.3. Portugal

There was good reproducibility between controls and individual metals. The general agreement patented by Icelandic and Scottish sensitivity results had no correspondence for the organisms tested in Portugal except for cadmium ($F_d C_{50}$ 0.87 ug/l). At the used concentrations no toxicity was detected for zinc and copper (Figure 3.5). Sensitivity for nickel was much lower than for cadmium. The range of concentrations tested was evidently low.
(Figure 3.5), leading to an estimation of $FdC_{50}$ at 44.96 µg/l, almost the highest used concentration (50 µg/l). Nevertheless, in comparison with Zn and Cu, it can be said that sensitivity for both metals exists.

![Graphs showing concentration response curves for E. marinus toxicity upon post-exposure feeding behavior of the four studied mixture components in Portuguese individuals.]

**Figure 3.5 -** Individual concentration response curves for *E. marinus* toxicity upon post-exposure feeding behavior of the four studied mixture components in Portuguese individuals.

### 3.3.3. Mixture and field toxicity

Comparisons of the predicted and observed quaternary mixture toxicities with those of the individual components and field data are depicted in Figures 3.6., 3.7. and 3.8. for Iceland, Scotland and Portugal, respectively.

#### 3.3.3.1. Iceland

Isolated or in mixture the compounds toxicity is always high, both in the laboratory exposures and in the field. For this reason the predictions are all situated in the vicinity of the
minimum values. The percentage of observations falling between ±25% of the predicted value are, control 93%, single metals 88%, mixtures 60%, and field data 33%.

Figure 3.6 - Predicted and observed joint action relationships of a quaternary mixture (Zn, Cd, Cu and Ni - dots), individual constituents (diamonds) and field collected data (triangles) towards Echinogammarus marinus feeding inhibition in the Icelandic population. The identity line (solid line) and 25% deviation intervals (dotted lines) are also depicted. Each data point corresponds to a single observation.

3.3.3.2. Scotland

The mixture toxicity predictions based on laboratory data are reasonably good (67% within ±25% of the predicted value) with a tendency towards over prediction. The remaining percentages of observations falling in the same interval of the predicted value were 100% for Control, 88% for single metals and 45% for the field.
3.3.3. Portugal

The Portuguese dataset produced the most consistent predictive scenario of the three locations. Laboratory based mixture toxicity prediction is reasonably good (68% within ±25% of the predicted value) and good agreement was found for the field data (75% for the same interval). The remaining percentages were control 88%, and single metals 80% (±25% of the predicted value). The high density of single metals data at the maximum predictive value is due to the use of a high number of control observations.
Figure 3.8 - Predicted and observed joint action relationships of a quaternary mixture (Zn, Cd, Cu and Ni – dots), individual constituents (diamonds) and field collected data (triangles) towards *Echinogammarus marinus* feeding inhibition in the Portuguese population. The identity line (solid line) and 25% deviation intervals (dotted lines) are also depicted. Each data point corresponds to a single observation.

### 3.4. DISCUSSION

Cairns and co-workers (Cairns et al. 1975) noted that “Although rather extensive bibliographies give the impression that there is a vast amount of literature on the effects of temperature on aquatic organisms, when one tries to apply this information to specific interactions, such as the effects of temperature changes on chemical toxicity to aquatic organisms, often very little of the evidence is applicable.” More recently Chapman et al. (2006) stated “It has been hypothesized that toxicity to chemical contaminants may increase from polar to temperate (...) species based on (...) increased temperatures. (...) However, such a clear, universal pattern has not been supported by the few comparative studies conducted to date.” From the several inferences one could drawn from these statements
three are relevant for the present study, given its contours: there is an historical scarcity of ecotoxicological studies with species occupying high latitude; doubts subsist regarding the role of temperature in toxicity mechanisms in nature; and available data is insufficient to pronounce temperate species as more sensitive than boreal ones. The last deduction can be readily dismissed as motive of concern, since the species tested at the various latitudes is the same, hence any detectable differences cannot be imputable to differing phylogenies. The second assertion was carefully pondered. Should we opt in our experimental design for ecological relevance and vary test temperatures according to location or should we follow a standardized protocol which would facilitate comparisons but would open way for speculation regarding temperature stress? The option for environmental realism prevailed as it did also in the choice of test concentrations.

Temperature can influence the sensitivity of different organisms to toxicants (among many other factors). Almost every biological rate is affected by temperature (e.g. biochemical reaction rates, metabolic rates) having its activity increased exponentially (Chapman et al. 2006), and inevitably so are the metabolic pathways involved in sequestration and secretion of toxic substances. If only temperature is to be considered, the obtained results for individual metals agree by being quite diverse for the three locations. Nevertheless they seem to disconfirm the general accepted rule of higher temperature = higher toxicity (Castillo et al. 1997; Peters et al. 1997).

For the used concentrations, a transition was observable accompanying the latitude decrease: Icelandic organisms were highly sensitive to all metals, Scottish organisms were equally sensitive, but possessed insensitivity for zinc, and finally Portuguese individuals were insensitive for zinc and copper and the calculated \( FeC_{50} \) for the other two metals was much higher (four fold). Given the fact that different temperatures were used in the tests, simulating average temperatures found in the field at the corresponding latitude, it can be concluded that, for \( E. marinus \), temperature is not a major influence in metal toxicity and other factors must be at work.
What can then be the underlying mechanisms explaining differing toxicities for metals administered to the same organisms in similar concentrations? There has been growing attention drawn upon the modulation previous exposure history has in the uptake of metals by organisms (Blackmore & Wang 2002; Rainbow et al. 2004; Wang and Rainbow 2005). Mechanisms of tolerance have been awarded extensive research (Chapman 2008) and it has been proven that when concentrations are sufficiently elevated, from either anthropogenic or natural sources, selection for metals-resistant populations can occur, with resultant inheritable genetic adaptations (Vidal and Horne 2003; Zaldibar et al. 2006). Nevertheless, genetically determined metal resistance to one metal does not endow resistance to all metals nor to other stressors (Lopes et al. 2005).

In alternative, mechanisms not involving cross-generation changes may occur. Reduced metal uptake or accumulation and detoxification mechanisms may suffice to deal with metals without the necessity of drastic selection of metal-tolerant populations (Zhou et al. 2008), with the concomitant loss of genetic variability. But the former are energetically costly metabolic processes, and given organisms energetic constraints, it is not surprising that metal tolerance can disappear with relative quickness once metal contamination is removed (Tsui and Wang 2007).

We believe this framing fits our data. Icelandic organisms live in an energetically more demanding environment were savings in maintenance costs (e.g. detoxification) set free energy for growth, and natural selection favors individuals for energy efficiency and maximized growth (Pörtner et al. 2005) not for ability to deal with contaminants. In addiction the main source of metal contamination in Iceland is volcanism (IME 2005) which exerts its influence in pulses, corresponding to an intermittent scenario of exposure. On the opposite side of the spectrum we have the Portuguese community. Organisms used in the laboratory experiments were captured in the south arm of the Mondego estuary, in an area that has been, for a long time, suffering eutrophication processes, mainly due to the discharges of nutrient rich waters from rice fields. Effects have been reported upon macrobenthic communities of total biomass losses and species richness decrease (Cardoso et al. 2004).
Dolbeth et al. (2003) noted that "eutrophication constitutes a major threat to the sustainability of the estuarine ecosystem". But aside from these generalized effects there are more subtle mechanisms at work. The amount of nutrients being supplied to this area by the lower valley farmlands are accompanied by considerable amounts of heavy metals, present in pesticides and fertilizers used in the crops (Andreu and Gimeno-García 1999) (with the further contribution of the loads from the upper reaches of the watershed). Furthermore, eutrophication enhances heavy metal retention, by several possible mechanisms (increased carbon levels from algal blooms; reduction in decomposition rates under reducing conditions; increased sulfide formation as a result of anoxia; and physical stabilization of fine particles by algal mats) (Zago et al. 2001). These circumstances are consentaneous with a continuous exposure to metals at sub-lethal levels (according to Luoma (1977) one of the necessary pre-requisites for selection mechanisms to act) and together with a lower environment constriction, create conditions for the emergence of a genetically selected metal tolerant specimens profile. Additional evidence is present in the body burdens of these organisms (unpublished data). Portuguese individuals possess much higher body burdens of the metals they were insensitive to (and indeed Scottish individuals for zinc as well, constituting this prove that the resistance to one metal is not extensible to the others) than Icelandic individuals, which, once again, is characteristic of metal tolerant species.

From the amphipod’s perspective, being captured and depurated in the laboratory is physiologically regarded as a period of non-exposure in the field. When the bioassays start, suddenly metabolic capabilities are again solicited to be fully online. Those who possess metal tolerance cope with toxicity with easiness; those who have waged in detoxification strategies face, once again, added difficulties.

The application of the CA model returned modest predictions for mixtures (60 and 70% of the predictions falling between ±25% of the predicted value for the three locations).

The existence of different strategies towards metals, between metals, and between individuals (Wang and Rainbow 2005) could account for these results. Besides organism
associate variability, one of the assumptions of this model (similar action mechanism) has been recently challenged in what regards its application to metals. Poynton et al. (2007) used gene expression profiles to show that Cu, Cd and Zn had distinct modes of action to *Daphnia magna* when up till now the contrary was believed to be true. In addition, it has been considered as a good worse-case scenario in metal toxicity due to overestimated predictions (Lock and Janssen 2002).

At this moment, aside from some sporadic works in specific species (see above) it’s not possible to accurately predict interactions among metals, let alone interactions between metals and other contaminants (as is the case of field exposures), without site-specific testing (Norwood et al. 2003). Less than additive and more than additive responses for metal-metal interactions are about as likely as strictly additive responses (Norwood et al. 2003); multiple metal effects are not always additive (Hagopian-Schlekat et al. 2001) and metal uptake is influenced by the specific metals and their ratios in a mixture.

For Iceland and Scotland the agreement between observations and prediction for the field data was fairly low (33% and 45% between ±25% of the predicted value). These results compromise the possibility of use of the in situ bioassay with *E. marinus* as a screening tool for metals in saline and Brackish waters. Nevertheless it is our opinion that the low predictive power is due to an inadequate choice of the concentrations used in the laboratory toxicity tests, not reflecting local exposure levels in the field. As previously explained, the choice was made in accordance with the values obtained by the survey upon Ria de Aveiro sediments, reported in Chapter 2. An argument in favor of this is the comparatively high agreement between observations and predictions for the field data on that system: 75% between ±25% of the predicted value. Another factor to be considered that could be of importance is that water might not be the primary exposure route for metal uptake in *E. marinus*.

If natural local populations sensitivity to each metal are taken into account (and even Portuguese organisms need more adequacy for Ni) quite possibly this tool will become more sensitive, robust and, as a corollary, ecologically relevant, aiding in the identification of hot
spots of metal contamination and pollution, and maximizing allocation of resources in an Environmental Risk Assessment context.

Acknowledgements

Work at the SMC was supported by the programme “Improving Human Potential: Access to Research Infrastructures” (IHP/ARI) provided by the European Community under Marie Curie Actions - EC-IHP Transnational Access Grant. Thanks are also due to Billy Struthers and remaining staff at Water Quality Laboratory, Institute of Aquaculture, University of Stirling.

FCT- Fundação para a Ciência e Tecnologia, for funding this research through PhD Grant SFRH/BD/4778/2001.

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CHAPTER 4

Amphipod Intersex, Metals and Latitude: a Perspective
4. AMPHIPOD INTERSEX, METALS AND LATITUDE: A PERSPECTIVE

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ABSTRACT

Intersexuality (the simultaneous occurrence of secondary sexual characteristics in the same individual) has been reported in crustaceans, among which a large number of amphipod species. The full significance and costs of intersexuality for amphipods are still unknown. Several mechanisms are directly or indirectly held responsible for its occurrence amongst which pollution. Aside from the classical case study of organotins induced imposex and intersex in gastropods no mechanistic relationship between metals and the occurrence of intersex in crustaceans has ever been established. Nevertheless the study of metals potential to cause intersexuality is enveloped in high relevance due to the ubiquitous presence of metals in the environment and in metabolic processes. Also the incidence of intersex in populations of the same invertebrate species in a latitudinal gradient has never been studied so far. Three populations (from Iceland, Scotland and Portugal) of the gammarid amphipod \textit{Echinogammarus marinus} were scrutinized. Intersex females from Iceland registered the highest fecundity loss (a predicted effect for intersexes): two fold decrease when compared to Portuguese individuals (48\% versus 22\%) and four-fold when compared to Scottish individuals (48\% versus 12\%). Only in Scottish samples females with two genital papillae and males with only one genital papillae were observable. Nevertheless samples from environmental compartments (water, biota and sediment) pointed to equivalent metal levels in the three environments and in consonance the prevalence of intersex was not significantly different between locations. An unequivocal relationship between metal presence and intersex induction cannot be presented, but our results advocate the potential role of metals as a direct cause of intersexuality in \textit{E. marinus}.

4.1. INTRODUCTION

Intersexuality, the condition whereby otherwise normal gonochoristic species possess both male and female characteristics, has been reported throughout the animal kingdom (Reinboth 1975). This phenomenon has been widely identified in crustaceans, among which a
large number of amphipod species (e.g. Dunn et al. 1990; Ford and Fernandes 2005; Ladewig et al. 2003; Sars 1895; Sexton and Huxley 1921).

Several mechanisms are directly or indirectly held responsible for its occurrence including parasitism (Bulnheim 1977); bacterial infection (Rigaud and Juchault 1998); genetic control (Lebederf 1939); the ablation or implantation of androgenic glands, from/into males and females, respectively (Charniaux-Cotton 1958); environmental sex determination (ESD) (Dunn et al. 1993) and pollution (Ford et al. 2006; Moore and Stevenson 1991). Recently it has been demonstrated to be induced chemically by juvenile hormone mimics (Olmstead and LeBlanc 2007).

The full significance and costs of intersexuality for amphipods are still unknown. Some studies report lower fecundity and fertility, reduced pairing success and delayed maturation of Echinogammarus marinus (Ford et al. 2003; Ford and Fernandes 2005). Dunn et al. (1993) also reported reduced pairing success for Gammarus duebeni and Kelly et al. (2004) found lower fecundity in intersex females of the same species. An investigation on the costs of intersex upon precopulatory guarding by Plaistow et al. (2003) on Gammarus pulex, indicated that these were correlated with female size. Consequently pair forming with larger intersex females (which grow larger than the normal counterpart), will lead not only to large energy costs but also reduced reproductive success. Barbeau and Grecian (2003) noted reduced fertility in intersex male Corophium volutator, a finding confirmed for the same species by McCurdy et al. (2004) which registered smaller broods issuing from the pairing of intersex males with normal females when comparisons to matings with normal males were performed.

A model simulation for an Echinogammarus marinus population run by Ford et al. (2007) to evaluate the reproductive costs associated with intersexuality revealed that intersexes can have dramatic effects: population extinction within approximately 6 years if 5% of normal females are replaced by intersex females. Nevertheless in behavioral terms no differences
appear to occur with intersex females and intersex males acting as do normal females and males, respectively (Ford et al. 2007).

Aside from the classical case study of organotins induced imposex and intersex in gastropods (Matthiessen and Gibbs 1998), continued studies of metals endocrine disrupting effects in invertebrates are scarce, leading to inconsistent knowledge on the subject. More recently a growing body of work has been trying to bridge this gap of knowledge (e.g. Bondgaard and Bjerregaard 2005; Corrêa et al. 2005; Ford et al. 2006; Medesani et al. 2004), as the study of metals as endocrine disruptors can be considered highly relevant if we bear in mind their ubiquitous presence in the environment (from both natural and anthropogenic sources) and in metabolic processes. In the authors best knowledge no mechanistic relationship between endocrine disruptive capabilities of metals and the occurrence of intersex in crustaceans has ever been established. The incidence of intersex in populations of the same invertebrate species in a latitudinal gradient is also unknown.

*Echinogammarus marinus* (Leach, 1815) (=*Chaetogammarus*; =*Marinogammarus*) is a highly abundant amphipod species of the marine and estuarine intertidal north-eastern Atlantic coastlines (Lincoln 1979), with documented intersex prevalence (Ford et al. 2003). *E. marinus* occurs in close association with *Fucus* spp. (Phaeophyta, Fucales), which is used by the amphipod as source of food and shelter to live and breed (Schreider et al. 2003).

This study aims to ascertain the incidence of intersexuality in independent populations of *E. marinus* (encompassing the entire breadth of its distribution) while at the same time attempts to unveil possible correlations with the presence of heavy metals.

### 4.2. MATERIAL and METHODS

#### 4.2.1. Study sites

The organisms used in this study to evaluate intersex variation in latitudinal terms were collected in Iceland, Scotland and Portugal (Figure 4.1). A literature survey was performed to
choose specific sites where described metal levels were of approximately similar magnitude and *Echinogammarus marinus* presence was registered.

Icelandic individuals were captured in Reikjanes Peninsula (64°02'N, 22°42'W). Despite these shores being considered pristine environments (Sarà et al. 2007) there is indication of heavy metal background values being higher than in equally remote areas. Volcanic activity is regarded as the probable cause (IME 2001), mainly because of the substantial increase of metals concentration in Icelandic rivers (due to eruptions). The redistribution of these metals is efficiently performed by the unique hydrography of the Icelandic coastal current, which is initiated in the northeast, moves clockwise and ends at the mid-north coastal areas (Egilsson et al. 1999). The closest human settling from the collection site is the small town of Sandgerði (1700 inhabitants) which is one of the most important fishing harbours in Iceland, were fish processing facilities are installed. Olafsson (1986) used *Mytilus edulis* to run a
“mussel watch”-type of survey on the south-western coast to identify anthropogenically enhanced metal concentrations. The conclusion was that localized spots existed, being the Sandgerði area one of them. This verdict was confirmed by Leung et al. (2005) who alludes to Sandgerði as a “polluted site”.

Loch Fyne is the longest, deepest fiordic sea loch of Scotland, being part of the Firth of Clyde watershed (one of the country’s most contaminated). In spite of this, it has been expected to be relatively free from metal contamination due to serving mainly forestry, extensive grazing, aquaculture and recreation purposes (SEPA 2005). Leung et al. (2001) presented data from Nucella lapillus tissues indicating higher metal contents in individuals from this area than in individuals collected in areas traditionally described as metal polluted. The organisms for the present study were collected in the vicinity of Strachur (56°10’N, 5°05’W) located a few km to the south of a “Marine Consultation Area” (NCC, 1986).

The Mondego Estuary is located in the Portuguese Atlantic coast. It comprises two arms (north and south) with very different characteristics. North arm is highly hydrodynamic, possesses navigational capabilities and it is pointed as having pristine conditions in terms of heavy metals presence (Vale et al. 2002). South arm dynamics are much weaker, depending partly of the tidal excursion and of the artificially controlled discharges (according to the necessities of the extensive lower valley rice crops) of a small river – the Pranto. This leads to extended residence times and fine particles deposition. The Mondego possesses a sizeable watershed (6670 km²) upon which the impact of urban areas summing up to 695.000 inhabitants is felt (INAG, 2004). Few industries are to be noted but deactivated uranium mines are located approximately 100km from the estuary and increases of metals, particularly Zn, Mn, Fe, U, Pb and Ra (Pinto et al. 2004) are registered in the areas directly receiving the mine effluents. The organisms were collected in the south arm (40°07’N, 8°49’W) in the confluence with Pranto river. Castro et al. (2007) noted an increase of Pb in sediment profiles from this area since the 1960’s, but emphasized that, when compared to other European estuaries, the Mondego was not example of a heavily contaminated one.
Pereira et al. (2005) indicated enrichment of the fine fraction of the south arm, when compared to the remainder of the estuary, in Hg, Cu, Cd, Cr, Mg, Zn and Fe pointing the Pranto river as the presumable source.

4.2.2. Sampling

Echinogammarus marinus specimens were collected by gently scraping the surface of intertidal rocks with attached algae. Upon capture organisms were readily preserved in 75% ethanol. Simultaneously samples of brown algae (Fucus spp.), water and sediment from the collection sites were gathered to perform analysis of metal contents. No sediment was collected in Iceland due to the rocky nature of the substrate at the sampling site.

4.2.3. Intersex analysis

Seven hundred and five adult specimens were separated (Iceland =230, Scotland =254, and Portugal =221), sexed (females - presence of 4 pairs of brood plates – oostegites – in the thoracic region; males – presence of 2 genital papillae (GP) between the last pair of pleopods), observed for external intersex characteristics (presence of both structures) and measured (distance between the base of the first antenna to the base of the telson) (Ford et al. 2003).

In ovigerous females eggs were removed from the brood pouch (formed by the oostegites – brood plates) and counted.

4.2.4. Metal analysis

4.2.4.1. Sediment

Extraction of metals from sediments was carried out using a nitric acid–hydrogen peroxide digestion. Approximately 2g wet weight (ww) of sediment was dried at 110°C for 24-hours and ground to a fine powder. A 500mg sub-sample was added to a 20ml Teflon screw top
digestion vessel. 5ml of concentrated nitric acid (69%, Aristar, BDH, 106 U.K.) was added and the sample was heated to 110°C for 24-hours. Once cooled 3ml of hydrogen peroxide (Aristar, BDH, U.K.) was added in 1 ml steps until the sample became totally clear and ceased effervescing. Samples were re-heated at 110°C for a further 2 hours, allowed to cool and made up to 15ml with distilled water and centrifuged at 2000 rpm for 15 minutes. Quantification was performed using a THERMO™ ICP - Mass Spectrophotometer.

4.2.4.2 *Fucus spp* and *E. marinus* tissues
The algae were washed with deionised water to remove adhering sediment and the bladders and tips were removed by tearing (Dobson 2000).

Tissues (of amphipods and algae) were digested and subsequently analyzed using the methods for sediment metals analysis described previously (section 4.2.4.1).

4.2.5. Statistical analysis
One-way Analysis of Variance (ANOVA) was used to evaluate the existence of significant differences between groups of the different stations and between sampled compartments among stations. When data proved to have a non-normal distribution (by application of the Kolmogorov-Smirnov test) the Kruskal-Wallis test (ANOVA on ranks) was used. Dunn’s multiple comparison procedure helped elucidated further the differences within groups of the same station. All Statistical analysis was performed using SIGMASTAT (Version 3.1) statistical software.

4.3. RESULTS

4.3.1. Organism size
For all three stations the size of the organisms was variable and within each station decreased in the following order: intersex males, normal males, intersex females and normal
females (Figure 4.1). All identified groups were significantly different ($P<0.001$) apart from normal males and intersex females ($P>0.05$). Intersex specimens were consistently bigger than the normal counterparts (Figure 4.2).

![Graph showing organism length in three locations with standard deviations](image)

*Figure 4.2 - Results for the organism's length (in mm) in the three locations (with standard deviations).*

### 4.3.2. Intersex incidence

In general terms, intersex incidence was higher in Scotland (18.5%), followed by Portugal (14.3%) and Iceland (10.0%). Females registered higher intersex rates, once again with Scotland leading with 13.0%, followed by Portugal (9.5%) and Iceland (6.7%) (Table 4.1). Only in Scottish samples was possible to find females with two genital papillae (3.7% of the total individuals analysed, Table 4.1).

Regarding males, the general pattern was once again repeated, though with more discreet differences with 5.6%, 4.8% and 3.3% for Scotland, Portugal and Iceland, respectively (Table 4.1). Only in Scotland males with only one genital papillae were observed (1.9%, Table 4.1).
### Table 4.1- Incidence of intersex for males and females in the three stations (in %) and average number of eggs (avg egg) inside females brood pouch. GP- Genital Papillae.

<table>
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<tbody>
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<td>Scotland</td>
<td>Portugal</td>
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<td>Scotland</td>
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</tr>
<tr>
<td>Normal</td>
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<td>29.6</td>
<td>33.3</td>
<td>56.7</td>
<td>51.9</td>
<td>52.4</td>
</tr>
<tr>
<td>Avg egg</td>
<td>15.3</td>
<td>15.0</td>
<td>16.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intersex</td>
<td>6.7</td>
<td>13.0</td>
<td>9.5</td>
<td>3.3</td>
<td>5.6</td>
<td>4.8</td>
</tr>
<tr>
<td>Avg egg</td>
<td>7.9</td>
<td>13.2</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 GP</td>
<td>6.7</td>
<td>9.3</td>
<td>9.5</td>
<td>0</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>2 GP</td>
<td>0</td>
<td>3.7</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 4.3.3. Female fecundity

Normal ovigerous female’s fecundity (measurable by the number of eggs present in the brood cavity) showed, on average, little variation between the three locations (15.0; 16.7 and 15.3 for Scotland, Portugal and Iceland respectively) (Table 4.1). Intersex specimens registered a generalized drop in these values: 13.2 for Scotland, 13.0 for Portugal and a pronounced decrease for Icelandic organisms, with 7.9 eggs/female (Table 4.1).

#### 4.3.4. Metal Analysis

Figures 4.2 presents the values obtained from the analysis of water, sediment (except Iceland), *Fucus spp.* tissue and *E. Marinus* tissue of samples collected at the three sites, respectively.
Figure 4.3 - Metal values obtained from the analysis of water [A], sediments [B], Fucus spp. tissue[C] and E. marinus tissue [D] collected from the sites in Iceland, Scotland and Portugal (in ug/l).

One way Analysis of Variance (ANOVA) showed no significant differences for any of the analysed compartments among the three sites to whom data showed a normal distribution (water, Fucus spp tissue and E. marinus tissue, all P>0.05). The same happened with sediment (no normal distribution) for which Kruskal-Wallis test returned a statistically non-significant difference (P>0.05). Despite these results Scotland leaves the consistent idea of systematically presenting the most elevated metal values for all compartments (with the exception of water in Iceland).

4.4. DISCUSSION

The relationship between pollution and the occurrence of intersex in crustaceans was never irrefutably established. The available literature provides several examples of studies that failed to prove this connection (in spite of some of them producing evidence that other
endocrine disruptive effects were occurring). Marine copepod communities (Paramphiascella hyperborean, Stenhelia gibba and Halectinosoma sp.) associated with sewage outfalls registered elevated numbers of intersex individuals (Moore and Stevenson 1991). The simultaneous occurrence in several species of this phenomenon indicated the involvement of a common environmental factor. However, no clear relationship could be established between exposure to sewage effluent and incidence of intersex (Moore and Stevenson 1991). Gross et al. (2001) surveyed Gammarus pulex populations living in an area were sewage effluent was being discharged but failed to reveal any relationship between intersex incidence and the exposure, in spite of a highly significant number of females having oocytes containing a reduced number of yolk bodies and lipid globules (a suggestion of reduced vitellogenesis). In a study using populations of the amphipod Gammarus fossarum (Jungmann et al. 2004) the incidence of intersex increased among gammarids transplanted from a location with a low incidence of intersex to a location having a high incidence of intersex, suggesting that some environmental factor was responsible for the high incidence of intersex at some localities. Sites deemed unaffected by this factor had incidences of intersex less than 1%. Yet no discernible relationship between intersex incidence and pollution could be established (Jungmann et al. 2004).

The interpretation of the results obtained with our study could point in a different direction. The differences obtained among locations for all the four sampled compartments (water, sediment, Fucus spp. tissues and E. Marinus tissues) were non-significant. This means that, from a metal contamination point of view, the initial literature based selection of sampling sites, which aimed to select locations with a profile of limited disturbance where a moderate presence of metals above natural backgrounds existed was proper. A similar setting for all three sampled populations was thus confirmed. Coincidently the incidence of intersex in the three sampling sites is not significantly different either (one way ANOVA, P>0.05). Seemingly then, the statistics based conclusion to this study is that when exposed to approximately the same environmental metal concentrations, populations of E. marinus will show similar incidences of intersex. However we are aware that despite being statistic inference a
fundamental tool, it should not be the sole contributor to the interpretation of a data set. Discreet differences can be otherwise observable, allowing the observer to retain palpable information regarding the capability of a certain location of exerting a more marked influence upon organisms than others judged (e.g. by statistics) similar. Nevertheless an “influence ranking” of the three sites is hard to establish. Chemical stressors, like heavy metals, tend to be discreetly present in the water column both in time and space (Hall et al. 1998). Periodical disturbances may occur and these pulse events (of anthropogenic and natural origin) when ended can leave the physical and chemical environment similar to pre-disturbance conditions (Bender et al. 1984). If sampling occurs in these intermediate periods, erroneous conclusions could be drawn in terms of frequency and levels of perturbations.

Icelandic organisms are likely to be more frequently influenced by natural occurring pulse-like perturbation whilst Scottish and Portuguese organisms more liable to frequently receive anthropogenically originated influences. These, due to their diverse and complex nature (e.g. accidental spills, urban runoff, licensed periodic discharges) represent added stress upon these organisms (atop natural occurring events). The differences between sites in our results for intersex female fecundity could represent a token of this influence. Charnov (1982) predicted that intermediates (=intersexes) should incur in reduced fitness. Our data confirmed that overall prediction but Icelandic intersex females had a two-fold decrease in fecundity when compared to Portuguese individuals (48% versus 22%) and four-fold when compared to Scottish individuals (48% versus 12%). *E. marinus* eggs are relatively large for amphipods (Clarke et al. 1985) and as expected in the arcto-boreal zone they become even larger (Skadsheim 1984). This means that intersex females from Iceland will have more difficulties in maintaining their brood inside the marsupium, especially if we consider that one of the effects of intersex is the partial or total loss of the setae closing the brood pouch (Ladewig et al. 2003).

In a scenario of pollutant caused intersex, intermediates from populations that have been inhabiting for numerous generations environments were recurrent episodes of stress occur,
hence subjected to selection mechanisms (Luoma 1977), would be more prone to cope with that fecundity loss, when compared with individuals living in areas where those episodes are more sparse. This idea is reinforced by given proof of a genetic basis for metal tolerance in crustaceans (Barata et al. 1998; Ross et al. 2002). Besides this gradation regarding fecundity, another fact reinforcing a discrepancy between locations is that only in Scottish samples females with two genital papillae and males with only one genital papillae were present. Despite a certain similarity in the observed conditions this occurrence allows a differentiation between the intensity of the mechanisms affecting the Scottish population and the ones affecting the Portuguese population.

Recent work by Ford et al. (2006) confirmed a consistent higher level of intersexuality in *Echinogammarus marinus* throughout the year at sites receiving industrial contaminants (metal rich pulp mill effluent) when compared with reference sites. In this context, one can envisage environmental constraints modulating pollution (possibly metal pollution) derived intersex, keeping the former an open door to make intermediates viable. Furthermore one should expect that this situation would only occur in sites with marginal and not heavy pollution. A plausible scenario for *E. marinus* intersex as it was here observed.

In this study specific focus was put upon the environmental occurrence of metals and their possible relationship with intersexuality. An unequivocal relationship between the two factors, such is for instance the case of organotin caused intersex in gastropods (Matthiessen and Gibbs 1998) cannot be presented, but our results advocate the potential role of metals as a direct cause of intersexuality in *E. marinus*.

Acknowledgements

We are very grateful to Dr. Gudmundur V. Helgason and Dr. Halldór P. Halldórson for arrangements and hospitality at the Sandgerði Marine Centre, Iceland (SMC). Work at the SMC was supported by the programme “Improving Human Potential: Access to Research Infrastructures” (IHP/ARI) provided by the European Community under Marie Curie Actions - EC-IHP Transnational Access Grant.

FCT – Fundação para a Ciência e Tecnologia, for funding this research through PhD Grant SFRH/BD/4778/2001.
REFERENCES


Chapter 5 – Amphipod susceptibility to metals

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5. AMPHIPOD SUSCEPTIBILITY TO METALS: CAUTIONARY TALES

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ABSTRACT

The amounts of metals bioaccumulated by crustaceans in environmental studies are usually evaluated in a whole body burden perspective. Furthermore toxicity testing is performed without separation of genders and using only one life stage. From a mechanistic point of view erroneous conclusions upon dose-response relationships can be drawn by not discerning, for instance, percentages of metal sequestered in the cuticle and consequently not liable to cause toxicity, or, in a context of ecological risk assessment, pronouncing that a certain concentration of a certain metal in a certain compartment is “safe” for a species disregarding higher sensitivities for earlier life stages, could lead to population loss. Another frequently neglected aspect is the evaluation of the effect of nutritional state previous to metal exposure. The disregard for these issues may lead to inaccuracy in such fields as metal budgeting in organisms/populations, evaluation of trophic transfers and ecodynamic extrapolation. This study evaluated the influences of life stage, gender and a priori nutritional state in the uptake of an essential (Zn) and a non essential (Cd) metal. Furthermore, acute exposures of both metals to determine percentages adsorbed to the exoskeleton were also performed. Results are discussed under the light of metabolic, enzymatic and biochemical arguments.

5.1. INTRODUCTION

Great amount of effort has been put into metals investigation using amphipods, but several aspects of currently used protocols have been successively disregarded. These aspects have been object of consideration by a limited number of studies but their conclusions are seldom reflected in a modification of investigative practices. The differential distribution of uptaken metals between organs targeted by these chemicals is one of them (Rainbow 2007; Weeks et al. 1992). A particular aspect in this thematic is the amount of metal embedded on and coating exoskeletons (Viarengo and Nott 1993). From a mechanistic point of view erroneous
conclusions upon dose-response relationships can be drawn if we bear in mind that routine
evaluation of bioaccumulation rates are based in whole body quantifications, therefore not
discerning percentages of metal sequestered in the cuticle and consequently not available for
metabolic processes (Rainbow 1993; Rainbow 2007).

A limited number of studies account for differences between different life stages
(males/females/juveniles and neonates) (Marsden 2002). Data regarding variations
introduced by gender on metal levels is still patchy and inconsistent, with results being
frequently contradictory (e.g. Fialkowski et al. 2003; Marsden 2002). Hence, usually only one
life stage is used and when adults are selected gender separation is not performed
(Fialkowski et al. 2003). As recently Zhou et al. (2008) stated “There are overwhelming needs
for the study of the gender-related differences in metal bioaccumulation”.

Furthermore, studies evaluating the effect of the nutrition state previous to metal exposure
are also scarce. The disregard for these issues may lead to errors in such fields as metal
budgeting in organisms/populations, evaluation of trophic transfers and ecodynamic
extrapolation. From an ecological point of view, the maintenance of such practices and
uncertainties, besides leading to biased data, may prove disastrous. If, for instance, the
mentioned limitations are to play a role in a context of ecological risk assessment and in its
outcome is concluded that a certain concentration of a certain metal in a certain
compartment (e.g. water, sediment or tissue) is “safe” for a species, this misconception could
lead to population loss.

The ubiquitous marine littoral gammarid amphipod *Echinogammarus marinus* was chosen as
test organism due to a set of favourable characteristics: wide distribution, reported from
Norway and Iceland to Portugal (Lincoln 1979); frequent occurrence of high abundances with
important trophic role (Dick et al. 2005; Maranhão and Marques 2003); ability to act both as
mesograzer and predator (Dick et al. 2005); easiness of culture, manipulation and rapid
reproduction (17 days at 20°C for an embryo to be released from the brood pouch as a
juvenile (Maranhão and Marques 2003)).
The aim of this work was to answer specific questions regarding metal uptake of the different life stages, influence of gender, influence of diet and amounts of metal retained in the exoskeleton of *Echinogammarus marinus* when in presence of available essential and non-essential metals.

**5.2. MATERIAL and METHODS**

**5.2.1. Experimental layout**
Organisms were captured in Mondego estuary (40°08'N, 8°50'W) alongside with *Fucus vesiculosus*. In the laboratory, organisms were placed in plastic tanks (40 x 20cm) filled with 4l of continuously aerated artificial seawater (SERA PREMIUM®) changed thrice a week and under a 12h dark/12h light regime. The use of artificial seawater ensures that physico-chemical conditions affecting trace metal uptake are reproducible (Rainbow 1997). Organisms were fed a diet of *F. vesiculosus* being conditions thus maintained for one month to acclimate individuals to test conditions and allow them to depurate (Clason and Zauke 2000).

All experiments consisted of 96h static exposures to 1mg/l of Cadmium (CdCl₂·2H₂O, Sigma-Aldrich) or Zinc (ZnSO₄·7H₂O, Sigma-Aldrich) with five replicates (ten organisms per chamber) per experiment. No food was presented to the organisms during experiments. All used material (including experimental vessels) was acid washed and pre-soaked in the appropriate test medium for 24h to saturate all adsorption sites (Rainbow et al. 2004).

To evaluate differences in metal bioaccumulation between development stage adults, juveniles and neonates were exposed in separate chambers to the solutions of the two metals. Adults were separated by gender and exposed in separated chambers.

The influence of pre-exposure diet in metal uptake was evaluated by feeding two types of foods to two groups of males, females and juveniles kept in separated chambers. *F. vesiculosus* collected in a site with oceanic conditions was fed to one of the groups and to the
other *Artemia salina* (brine shrimp) hatched from commercially obtained cysts (Sanders Brine Shrimp Co.) was offered. Each of the foods was the sole diet for one month prior to water metal exposure. Metal amounts present in *F. vesiculosus* and *A. salina* used as nutrition were determined.

The evaluation of the amount of metal adsorbed into the cuticle was performed using males and females in 1:1 proportion. At the end of the exposure individuals were frozen at -80°C then thawed on ice and the exoskeleton stripped from the remaining tissues under a dissecting microscope. Soft and exoskeletal tissues were separated for quantification of the respective metal burdens.

With the exception of one of the groups of the pre-feeding experiment all tested individuals were kept in a *F. vesiculosus* diet prior to exposure.

### 5.2.2. Metal analysis

Metal extraction and quantification of amphipod body burdens and cuticles, *F. vesiculosus* and *A. salina* was carried out using the same protocol followed in Chapter 2 (Section 2.2.2.).

### 5.2.3. Statistical analysis

One-way Analysis of Variance (ANOVA) was used in Log 10 transformed data to evaluate the existence of significant differences between life stages and different treatments. When data proved to have a non-normal distribution (by application of the Kolmogorov-Smirnov test) the Kruskal-Wallis test (ANOVA on ranks) was used. Student-Newman-Keuls Method was applied as pairwise multiple comparison procedure to further elucidate differences between groups/treatments. When pre-requisites were not satisfied and ANOVA on ranks was applied, Dunn’s was the used post-hoc test.

All Statistical analysis was performed using SIGMASTAT (Version 3.1) statistical software.
5.3. RESULTS

5.3.1. Life stage metal accumulation

Figure 5.1 present the quantification of metal body burdens for females, males, juveniles and neonates after exposure to test solutions of 1 mg/l Cd and 1mg/l Zn, respectively.

![Figure 5.1 - Whole body burdens (+St Dev) of Cd and Zn in females, males, juveniles and neonates of E. marinus after 96h exposure to 1mg/l. (*) indicates statistical significant difference from Control (at P<0.05).](image)

Different life stages of *E. marinus* have differential bioaccumulation rates of cadmium and zinc. Results from Student-Newman-Keuls multiple comparison method indicate that mean responses of treatment groups are significantly different for both metals (P<0.001, except juveniles vs females and females vs males that registered a lower significance level at P<0.05). For cadmium the order of growing bioaccumulation rates is: Neonates >>Juveniles > Females = males whereas for zinc adults present gender differentiation, with females accumulating significantly higher amounts of the metal being the order of accumulation rates Neonates >>Juveniles > Females > males.

5.3.2. Pre - feeding influence on metal accumulation

The analysis of metal contents of *F. vesiculosus* and *A. salina* used in the pre-feeding phase are presented in table 5.1.
Table 5.1 - Mean metal contents (μg/g ± St Dev) of *F. vesiculosus* and *A. salina* used in to feed the two experimental groups of *E. marinus*.

<table>
<thead>
<tr>
<th></th>
<th>Cadmium</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. salina</em></td>
<td>0.025±0.0061</td>
<td>179.4±21.35</td>
</tr>
<tr>
<td><em>F. vesiculosus</em></td>
<td>0.28±0.047</td>
<td>14.9±1.12</td>
</tr>
</tbody>
</table>

Figures 5.2 and 5.3 present the values for metal body burdens for organisms exposed to 1 mg/l of cadmium or zinc, respectively, for 96h after being previously fed differing diets.

![Graph showing metal body burdens](image)

**Figure 5.2 -** Whole body burdens (+St Dev) of diet differenced *E. marinus* males, females and juveniles exposure to Cadmium (96h, 1mg/l). Differences between the median values significantly different at P < 0.05, Kruskal-Wallis One Way ANOVA on Ranks. (*) indicates statistical significant difference from the respective Control.
Our results suggest that different diets do not affect water mediated cadmium uptake in *E. marinus*. The application of Dunn’s multiple comparison method returned a non significantly difference (P>0.05) between the mean responses of similar life stages offered different foods, implying that different diets do not affect water mediated cadmium uptake in *E. marinus*. To the contrary, with zinc the Dunn’s multiple comparison method indicated that mean responses of the same life stage under dissimilar nutrition were significantly different (P<0.001). This implies that different diets affect water mediated zinc uptake in *E. marinus*.

![Graph showing zinc uptake](image)

*Figure 5.3 - Whole body burdens (+St Dev) of diet differed *E. marinus* Males, Females and Juveniles exposure to Zinc (96h, 1mg/l). Differences between the median values significantly different at P<0.001, one way ANOVA. (*) Indicates statistical significant difference from the respective Control.*

5.3.3. Percentages of metal coating the exoskeleton

Results of the analysis of cadmium and zinc coating the exoskeleton of *E. marinus* are presented in Table 5.2.
Table 5.2 - Average percentages (± St Dev) of metal coating E. Marinus exoskeleton. Whole body burden quantifications performed after exposure to 1mg/l of cadmium and 1 mg/l of Zn (separate exposures) for 96h.

<table>
<thead>
<tr>
<th></th>
<th>Cadmium</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.68±1.5</td>
<td>23.57±1.16</td>
</tr>
<tr>
<td>Exposed</td>
<td>26.21±1.75</td>
<td>34.05±1.47</td>
</tr>
</tbody>
</table>

For both metals there was a statistically significant difference (P<0.05, one way ANOVA) in metal contents between exoskeletons of exposed individuals and the exoskeleton of control organisms.

5.4. DISCUSSION

Amphipod crustaceans are net accumulators of trace metals both from solution and diet, and the accumulated metal levels can be regarded as integrated measures of metals taken up by an amphipod over recent periods (Fialkowski et al. 2003; Rainbow 1997). Once uptaken, essential and non-essential metals are concurrently dealt accordingly but always with the potential to be accumulated to high internal concentrations, given the large number of potential binding-sites internally (Rainbow 1990). These binding sites are intimately related to detoxification processes since even being essential, the excessive accumulation of a given metal can be lethal (Hopkin 1990). Aside from a few studies regarding metallothioneins (MT), which can have both metal-detoxication and antioxidant functions, little is known about defense mechanisms in amphipods against oxidative stress and anti-oxidant defenses (Correia et al. 2003). Antioxidant enzymes are an important protective mechanism and, like many other biochemical systems, their effectiveness may vary with the stage of development and other physiological aspects of the organism (Halliwell and Gutteridge, 1999; Livingstone, 2001). Our study illustrates this reality by attaining significant statistical differences between
life stages both for cadmium and zinc uptake. Neonates accumulated much higher amounts of these metals than juveniles (threefold for Cd and approximately twofold for Zn) but especially high if compared to adults (fivelfold for Cd and 2.5 times for Zn, approximately). The observation that smaller animals within a species tend to bioaccumulate higher levels of toxicants has been well documented in a variety of aquatic organisms and with a multitude of compounds (Rand 1995). Rainbow and Moore (1986) put forward considerations that crustacean organisms under certain weight (1.5mg) should not be used for metal assessment, because weight highly influences metal accumulations in the youngest organisms. In nuclear terms the explanation of the results resides in the differing metabolic profiles existing between early life and adult stages. These age-related differences have clear translation in antioxidant enzymes that during development from neonates to adults progressively decrease (between 70% and 90% depending on the enzyme) their activities (Correia et al. 2003). Higher levels of enzymes are synonym of higher detoxification capability. Thus a greater amount of metals can be sequestered inside organisms without deleterious effects. Besides harmful effects prevention, there are enhanced advantages for early life stages (neonates and juveniles) to maintain an enlarged non-toxic zinc pool available for the synthesis of metalloenzymes, allowing the homeostasis of many cellular processes (Amiard et al. 2006) especially those related with cell division and proliferation (McDonald 2000) characteristically high at these life stages (Sutcliffe 1984). Similar results were obtained for the Gammarid amphipod Gammarus locusta by Correia et al. (2004) with no significant differences among the groups for Zn body-burdens, except in juveniles that were indicated to be the best accumulators of this metal. A similar process occurs for Cd with the difference that the entire budget of accumulated metal will remain bound by metallothioneins at specific cells whilst Zn will be remobilized when necessary. Such is for example the case of Orchestia gammarellus where Zn appeared in lysosomes degrading metallothionein, whilst Cd remained MT-bound in the cytosol of the ventral caecal cells (Amiard et al. 2006). Furthermore young stages molt more frequently due to higher growth rates (Pöckl 1995; Neuparth et al. 2002) and display an increased surface area to volume
ratio and thinner body covering (Rand 1995) contributing these characteristics for enhanced metal uptake. In the context of acute tests (96h) molting frequency has been particularly highlighted as an important factor generating higher differences in metal uptake between life stages, as they represent episodes of higher susceptibility to metals (McGee et al. 1998).

Adding to the decline in antioxidant enzyme activities adults possess increased levels of polyunsaturated fatty acids (PUFA) rendering ageing animals more susceptible to growing lipid peroxidation and oxidative stress (Kawashima et al. 1999). PUFA account for a significant percentage of membrane phospholipids in amphipods and their peroxidation is promoted by metals (Correia et al. 2002; Roméo et al. 2000).

Saccharomyces cerevisiae yeast cells exposed to similar concentrations of cadmium and copper as the ones used in our study, led to the observation of high levels of peroxidation in PUFA enriched membranes and to their consequent loss of permeability (Howlett and Avery 1997). Copper and cadmium were injected in Dicentrarchus labrax, with cadmium showing less toxicity to the kidney lysosomal membrane than copper, but greater uptake rate (Roméo et al. 2000). In adult specimens of G. locusta exposed to 4 ug/l water-borne copper over 10 days, lipid peroxidation increased after 1 day and peaked at day 4 before returning to control values by day 6. Simultaneously high concentrations of MT were also observed but only at days 6 and 10 concomitantly with the decrease of lipid peroxidation (Correia et al. 2002). The previous experiments seem to point in the same direction: due to higher contents in PUFA, the peroxidative capability of metals (at least at high concentrations) causes an initial disruption on adult’s cellular membrane organization, a mechanism that could temporarily block metal uptake. This fact could help explain further discrepancies between early and adult life stages, but the need for further research is obvious. There is indication in G. locusta that males possess higher levels of PUFA (but not for overall lipids) and lower levels of MT (Correia et al. 2003; 2004) which could account for our differences between genders. Marsden et al. (2003) while investigating exposure in talitrid amphipods also found higher
cadmium levels in females but the amounts of zinc were equal, allocating the explanation to the dissimilar reproductive specificities of males and females.

Despite using concentrations of the same magnitude (1 mg/l) results obtained for the uptake of the two metals were diverse. Zinc is an essential trace metal with the potential to vary in tissue concentration as a result of physiological requirements between amphipods of different development stage, gender and reproductive state, as well as bioavailability. Cadmium is a nonessential metal with accumulated concentrations expected to vary only in line with bioavailability differences (Marsden et al. 2003). That is the reason why for a given molar concentration, the molar uptake of dissolved zinc by marine invertebrates is greater than that of cadmium (Rainbow and White 1989).

The nutritional state of an individual is regarded as a non contaminant source of stress (Wolfe 1992). Heugens et al. (2001) noted while reviewing the literature that a negative correlation of approximately 80% exists between toxicity decrease and increasing values of nutritional state. The fact that the majority of experiments leading to such conclusions are performed by means of a comparison of sensitivity between fed and starved animals (e.g. Chandini 1988, 1989; McGee et al. 1998) has raised criticism regarding for instance the influence higher food levels cause upon metabolic rate, and consequently toxicokinetics. Information upon the effects of different diets is rare. In our experiment uptake was diversely affected by the two foods. *Fucus vesiculosus* provides shelter and food for *E. marinus* creating overall good fitness conditions (Maranhão and Marques 2003). *Artemia salina* when used as sole food has been known to cause low fitness, low egg production rates and low survival rates (Cruz-Rivera and Hay, 2000). Regarding cadmium no statistical significant differences were obtained for the uptake between individuals of the same development stage fed with *Artemia salina* and *Fucus vesiculosus*. That is to say that, for instance that a female (or juvenile, or male) *Artemia* fed will uptake similar amounts of cadmium as a female grazing on *Fucus*. Nevertheless dietary exposure organisms were object to has to be considered. Cadmium contents in of the two offered foods is quite different,
being the amount in _Fucus_ (0.28 ug/g) one order of magnitude higher than the one for _Artemia_ (0.025 ug/g). Under these conditions it is somewhat surprising that after the water exposure the amounts of uptaked metal are approximately the same, especially being cadmium a non-essential, non-regulated metal (Marsden et al. 2003). Plausible explanation resides in two factors: firstly the high concentration of the water exposure that precipitates mechanisms of peroxidation (see above). Being herbivore diets lower in nutrients than the tissues they must build (Cruz-Rivera and Hay, 2000) storage of reserves (lipids) is reduced, the opposite happening with animal tissue. Thus individuals feeding carnivorous will be more exposed to oxidative stress. In an experiment conducted with carnivore and herbivore amphipods decreased levels of antioxidant enzymes were measured in the former (occurring peroxidation reactions in a large extent) whilst in the latter optimal levels were detected (no oxidative stress occurrence), a fact the authors attributed to the algal based diet (Obermüller et al. 2005). Secondly, algae are rich in indigestible materials, thus being the metals in these matrices inaccessible for the predator (Cruz-Rivera and Hay 2000).

Contrary to cadmium, zinc uptake registered significant statistical differences between individuals fed different diets. Enhanced amounts of zinc were uptaken by the _Artemia_ fed group. The brine shrimp zinc contents has to be taken into account since it is one order of magnitude higher than that of _Fucus_ (179.4 ug/g versus 14.94 ug/g) which could mean that the metal was accumulated via food exposure. Nevertheless, objections could be raised due to the rate of uptake being very small which causes the net effect on Zn uptake to be similar to that of regulation (Rainbow and White 1989). In fact, Clason et al. (2003) working with _Chaetogammarus_ (=_Echinogammarus_) _marinus_ was unable to determine a toxicokinetic model for the metal, which inevitably led to the conclusion that the species is able to regulate zinc. Our data indicates otherwise due to the significant differences between the groups (even adults) and control. The explanation resides in the concentrations used by the authors: two orders of magnitude smaller than the ones in the present study. This implies that in our case the uptake rate (which is a direct function of the concentration of aqueous metal) transcended the excretion rate and net accumulation occurred (Marsden and
Rainbow 2004). These results provide an interesting insight into the gender dietary preferences of *E. marinus*. Dick et al. (2005) noted that gut contents from field females indicated they consumed 'animals only' significantly more frequently than males, the same pattern being verified in laboratory feeding preference assays. The advanced explanation was they would more often exploit different microhabitats, due to smaller size, and there forage for high protein foodstuffs to invest in egg production. Under the auspices of our results could be prognosed that females will possess higher metal body burdens, in accordance with food preferences, a reality indeed observed in field amphipods (Marsden et al. 2003)

The results obtained for the experiments to evaluate percentages of metal into the carapace returned values within the range indicated for other crustaceans. Table 5.3 presents a comparison of the calculated percentage of cadmium and zinc bound to exoskeletons of crustaceans in several studies.

**Table 5.3** - Obtained amounts of zinc and cadmium (in % + Std Dev) bound to crustaceans exoskeletons in different studies.

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>% Zn</th>
<th>% Cd</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acartia spp.</em></td>
<td>98.5˚</td>
<td>97.4˚</td>
<td>Reinfelder and Fisher 1994</td>
</tr>
<tr>
<td><em>Temora longicornis</em></td>
<td>65.0 (2.0)</td>
<td>17.0 (1.0)</td>
<td>Wang and Fisher 1998</td>
</tr>
<tr>
<td><em>Palaemon elegans</em></td>
<td>48.4 (4.0)</td>
<td>np</td>
<td>White and Rainbow 1984</td>
</tr>
<tr>
<td><em>Penaeus indicus</em></td>
<td>np</td>
<td>27.5 (11.0)</td>
<td>Nuñes-Nogueira and Rainbow 2005a</td>
</tr>
<tr>
<td><em>Penaeus indicus</em></td>
<td>41.0 (8.0)</td>
<td>np</td>
<td>Nuñes-Nogueira and Rainbow 2005b</td>
</tr>
<tr>
<td><em>Orchestia gammarellus</em></td>
<td>9.0 (3.0)</td>
<td>np</td>
<td>Mouneyrac et al. 2002</td>
</tr>
<tr>
<td><em>Echinogammarus marinus</em></td>
<td>34.1 (1.5)</td>
<td>26.2 (1.8)</td>
<td>This study</td>
</tr>
</tbody>
</table>

(˚) – the authors did not present standard deviation values; np – quantification of the metal was not performed in the study
Unsurprisingly *E. marinus* exoskeleton shows percentages of metal adsorption more similar to other Malacostraca (*Palaemon elegans* and *Penaeus indicus*) than to Maxillipoda (*Acartia spp.* and *Temora longicornis*). More surprising is the discrepancy between our study and the one on *O. gammarelius* (Table 5.3). References regarding metal contents in amphiphods exoskeletons are rare limiting the possible range of comparisons. Mouneyrac et al. (2002) calculated the Zn percentage by means of desorption and not of direct analytical quantification, which could account for the low level reported (9±3%). The two metals followed diverging trends. Cadmium decreased the relative amounts in the exoskeleton form the control organisms to the exposed ones. As inferable by the observation of Table 6.3., cadmium possesses a lower affinity towards the exoskeleton. This constitutes a token of the increased soft tissue contents and the internal storage processes the metal undergone (Amiard et al. 2006). Furthermore Wright (1980) demonstrated using radiolabelling techniques that most cadmium uptake in amphipods may be internal as opposed to adsorption to the body surface, and that the exoskeleton is a major site of Cd storage. Contrarily Zn registered an increase of exoskeletal percentages which indicates higher uptakes from exposure (Table 5.3) and possible underlying mechanisms of storage for future remobilization according to needs. Wang and Fisher (1998) noted that Zn can appreciably bind (when compared to other metals among which cadmium) to the exoskeleton if uptake is mostly from the dissolved phase. Nuñes-Nogueira and Rainbow (2005b) found that in *P. indicus* newly accumulated zinc is distributed to all organs with the highest proportions of body content being deposited in the exoskeleton.

Our results provided clear demonstration of the influences several parameters connected to individuals (sex, age) and external parameters (types of foods offered) exert upon metal uptake experiments. The need to include different stages of development and both genders of amphipods in acute exposure experiments (and chronic if pre-feeding is looked upon as exposure), as well as amounts of metals bound to the exoskeleton, was emphasized as indispensable procedure if a clear evaluation of metal-biota associated issues is to be
undertaken. Furthermore our study reinforces the amenability and high adequacy of *E. marinus* as test species.

**Acknowledgements**

FCT – Fundação para a Ciência e Tecnologia for funding this research through PhD Grant SFRH/BD/4778/2001.

**REFERENCES**


Chapter 5 – Amphipod susceptibility to metals


CHAPTER 6

General Discussion
6. GENERAL DISCUSSION

Environmentally, metals pose added problems by being quite unique chemical substances. Their natural occurrence, essentiality (for some) and speciation, demand the development of specific ways of assessing their occurrence and effects (Chapman et al. 2008). They require the determination of background levels to separate human from natural contributions, but sometimes, ironically, the major contributor to contamination and pollution is nature itself (Zaldibar et al. 2006). Independently from source, total metal concentrations, in any of the compartments they are distributed in (air, water, soil and sediment) usually are not synonym of total availability to biota. Nevertheless it is a well established fact that when considering the aquatic systems sediments will always be the most concentrated physical reservoir of metals (Thomson et al. 1984). By determining the amounts of metal present in this compartment at a certain site, an upper threshold of available metal to be uptaken by biota is identified. Sediment Quality Guidelines (SQG) derived from toxicological data can then be applied and pass a preliminary judgment upon the chances of organisms living at that site being negatively affected. But as already mentioned total amounts of metal are not solely composed of bioavailable forms which have, in regular terms, a direct reflection in the metal body burden of exposed organisms (Chapman 1985). In marine and transitional environments salinity is the determinant factor controlling metal speciation, thus bioaccumulation opportunities (Pierron et al. 2007). However, there are no simplistic truths regarding these issues. The intention of deriving explicative models for speciation in sediments (and indeed in the other compartments) do collapse before the all natural truth: there are no static variables. Steady state is a rarity in the environment (Chapman et al. 2008). In addition, a combination of difficulties and criticism has been enveloping SQGs. The practical use of measurements on sediments to provide the basis of environmental control is barred by several complexities such as the ones inherent to monitoring of high spatial variability (in addiction a costly process), and the calculation of the needed controls to secure compliance with the standards. This stance reflects the limited availability of toxicological
data and the consequent uncertainty in standards (CSTEE 2004). In addition, concerns have been raised regarding the suitability of the partitioning theory as an approach to standard setting (UKTAG 2008). This state of affairs ultimately led to the current non-adoption of Sediment Quality Guidelines for the priority list substances in the undergoing implementation of the Water Framework Directive (WFD - 2000/60/EC) by European Union (EU) countries, which is necessarily a transitory situation if the 2015 objectives set in the document are to be achieved.

The use of ecologically relevant concentrations of tested pollutants has been for long pointed as of paramount importance in toxicology (Crane et al. 1995). Sediments were collected throughout Ria de Aveiro system (here used as a study model, given its varied geomorphologic characteristics together with uneven patterns of metal distribution) and metal contents determined to achieve a notion of realistic concentrations. The aim was the projection of laboratory toxicity test results to the field, while simultaneously evaluating the suitability of *Echinogammarus marinus* as toxicity testing organism. A simple stepwise approach was implemented including laboratory bioassays using concentrations suggested by the sediment analysis followed by field bioassays at sites with metal concentrations reflecting the ones previously used. In the latter phase and despite the above-mentioned considerations, SQGs were applied to the sediment metal contents dataset with the purpose of identifying sites with adequate potential to be recipients of the in situ bioassays. This procedure was seconded by the determination of enrichment factors (EF) for each sampling site, and a short list was elaborated. The selection of this shortlist also took into account logistic constraints in terms of number and location of the sites. The outcome of this process was the selection of Marina de Ovar, Bico da Murtosa, S. Jacinto and Areão. The first two presented transcendence of several SQGs differing in the EFs, with Bico da Murtosa presenting enrichment for all metals analyzed except chromium, whereas Marina de Ovar only for zinc and higher carbon contents. The other two did not transcended any of the used SQGs being the difference in terms of carbon contents (much higher for S. Jacinto) and Areão registering zinc enrichment.
The selection of a suitable endpoint that can accurately reflect the presence of single and mixtures of metals in laboratory and field exposures is of extreme importance. The used endpoint was post-exposure feeding inhibition. By tapping directly into the energy budget of the test organism this tool is, under appropriate application, a very powerful and robust extrapolator from the individual level to the population and community levels.

The deployment of field chambers containing *E. marinus* was performed in the water column of the selected sites where, as expected (Cross et al. 1970; Thomson et al. 1984), metal concentrations were lower than the ones registered in the sediment. These concentrations were scrutinized using ecological targets for surface waters set by the Water Framework Directive (WFD - 2000/60/EC). The values used for comparison were environmental quality standards (EQSs), that are indicated in the “common position” adopted by the Council (2006/0129) for dangerous substances. For specific pollutants UK values (UKTAG 2008) were used since to the best of our knowledge no EQSs have been set by the Portuguese Government. Despite the lower water concentrations the record obtained from the comparison of sediment values with SQG was repeated when comparing water values with EU EQSs (Table 6.1) for the dangerous substances Cd and Ni, and the specific pollutants Cu and Zn.

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ovar marina</strong></td>
<td>1.02</td>
<td>21.53</td>
<td>6.34</td>
<td>324.45</td>
</tr>
<tr>
<td><strong>B. Murtaosa</strong></td>
<td>2.3</td>
<td>34.69</td>
<td>9.43</td>
<td>245.98</td>
</tr>
<tr>
<td><strong>S. Jacinto</strong></td>
<td>0.06</td>
<td>2.87</td>
<td>1.73</td>
<td>12.34</td>
</tr>
<tr>
<td><strong>Areia</strong></td>
<td>0.01</td>
<td>1.45</td>
<td>2.76</td>
<td>11.43</td>
</tr>
<tr>
<td><strong>EU EQS</strong></td>
<td>0.2</td>
<td>5*</td>
<td>20</td>
<td>40*</td>
</tr>
</tbody>
</table>

- *Values proposed by the UKTAG (2008) for “specific pollutants” in brackish and saltwaters.*
The response obtained for post exposure feeding inhibition after 96h of exposure (Figure 6.1) was consistent with water metal concentrations (Table 6.1.). That is to say that the sites registering transcendence of the EQSs were the ones were anorexia was more marked: Marina de Ovar and Bico da Murtosa, particularly the latter, with significant differences between themselves and also the other stations (P<0.05). S. Jacinto and Areão did not present significant differences between them.

**Figure 6.1** - Feeding ratios registered by *Echinogammarus marinus* after 96h exposure at the field in Ria de Aveiro, Portugal.

A simple method to clarify the relation between metal effect and post-exposure feeding performance in the field is to separately award scores to individual metals according to the concentration and to the feeding amounts in a universe encompassing the entire dataset for each parameter. The obtained scores by each station are summed. This unsophisticated yet expedite method, allows the establishment of two rankings: one for “contamination” (enhanced amounts of metal) and another for “pollution” (effect upon organisms)\(^2\).

\(^2\) contamination and pollution are here used *latu sensu* and not as textbook definitions, and should be regarded as such henceforth.
Table 6.2 - Ranking of sites used in Ria de Aveiro according to “contamination” (enhanced amounts of metal) and “pollution” (effect upon organisms) scoring (see text for explanation).

<table>
<thead>
<tr>
<th></th>
<th>Ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contamination</td>
</tr>
<tr>
<td>Ovar marina</td>
<td>2</td>
</tr>
<tr>
<td>B. Muriosa</td>
<td>1</td>
</tr>
<tr>
<td>S. Jacinto</td>
<td>3</td>
</tr>
<tr>
<td>Areão</td>
<td>4</td>
</tr>
</tbody>
</table>

The overlapping is complete (Table 6.2), indicating that stations possessing overall higher metal concentrations in the water column are the ones exerting higher levels of biological effects (feeding inhibition) upon *E. marinus*.

With these results in hand, widening the characterization of metal exposure response of *Echinogammarus marinus* to the entire breadth of distribution of the species seemed challenging and relevant. Thus bioassays were performed with organisms from two other biogeographic areas: Iceland (one of the northernmost limits of the species) and Scotland (intermediated when Portugal being the southernmost location is considered). Due to logistic imperatives laboratory test concentrations were the same as the ones used in the test performed in Portugal.

It has been postulated that mainly due to the effect of temperature upon metabolism, boreal species would be more susceptible to the effects generated by toxicants (Castilho et al. 1997; Peters et al. 1997), but comparative studies showed no directly identifiable correlation between geographic location of a species and its susceptibility to chemicals (Hobbs et al. 2004; Hose and Van den Brink 2004; Leung et al. 2008). In laboratory bioassays, when single metals were tested (Table 6.3) a transition accompanied latitude decrease: Icelandic organisms were sensitive to all metals, Scottish organisms were equally sensitive,
but demonstrated insensitivity for zinc, and Portuguese organisms did not show significant feeding inhibition for zinc and copper.

### Table 6.3 - Nominal single and mixture tested metal concentrations (ug/l). $M_{m}$-mixture

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.2</td>
<td>2.25</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>M2</td>
<td>0.38</td>
<td>4.5</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>M3</td>
<td>0.6</td>
<td>9</td>
<td>20</td>
<td>75</td>
</tr>
<tr>
<td>M4</td>
<td>1</td>
<td>18</td>
<td>35</td>
<td>150</td>
</tr>
<tr>
<td>M5</td>
<td>1.75</td>
<td>36</td>
<td>50</td>
<td>300</td>
</tr>
</tbody>
</table>

Nevertheless, when the same metals are tested in mixtures of the same concentrations (Table 6.3) discrepancies are abated and surprisingly Icelandic organisms perform rather well, behaving less sensitively than organisms from the other locations, despite a certain lack of consistency rendered by high standard deviations. This lower sensitivity could be a result of metal interaction during uptake. Despite having demonstrated sensitivity to all metals, Icelandic organisms were particularly sensitive to copper. Daka and Hawkins (2006) demonstrated an (not continuous) antagonistic effect between these two metals for the gastropod *Littorina saxatilis*. It is a possibility that during mixture exposure uptake of Cu was diminished by competition with Zn, thus reducing overall toxic effects. Furthermore the unstable nature of the two metals antagonistic interaction could be represented in the high standard deviations observed.

Differences between the three locations in terms of the post-exposure feeding inhibition is minor in each of the mixtures (Figure 6.2). Given the fact that temperatures used in the tests mimicked the average temperatures that organisms are exposed to in the field at each location, one can conclude that for *E. marinus* temperature is not a major influence in metal toxicity. Indeed for the tested concentrations, *E. marinus* is a sensitive organism independently of the biogeographic location of the populations.
Mechanistic understanding of how specific metals cause toxicity in different species is increasing (Lacroix and Hontela 2006), but is far from complete. Similarly, much remains to be understood regarding differences between the toxicity of different metals. Toxicity tests, run under tightly controlled laboratory conditions, are useful but normally there is no direct translation to field populations. The extrapolation of hazard in the laboratory to risk of damage upon the environment is complex, being most of the obtained data of dubious ecological relevance (Crane et al. 1995). This is mainly due to laboratory single species tests not adequately reflecting the range of responses to toxicity of individuals, populations and communities in natural environments (Cairns and Niederlehner 1987; Crane et al. 1995) and exposure conditions not reflecting true exposure conditions in the field (Graney et al. 1989). Traditionally, field biomonitoring was performed through chemical quantifications and macroinvertebrate monitoring techniques (e.g. Crane et al. 1995), to which Nebeker et al. (1984) proposed the addition of in situ test chambers as a more appropriate mean to overcome such difficulties. The strong development of this last approach, duly supported by the other two, has been helping bridge the gap between toxicity test results in the laboratory and probable effect in the field. Thus effects both in the laboratory and the field need to be considered, particularly effects of contaminant interactions. Contaminated environments
typically comprise a mixture of substances, some of which are contaminants whereas others (e.g., organic carbon) modify contaminant bioavailability and toxicity. Contaminant interactions are complex but need to be considered to guarantee ecological relevance in ecotoxicological evaluation. It is presently not possible to accurately predict interactions among metals, let alone interactions between metals and other contaminants (the natural field reality), without site-specific testing and laboratory bioassays back up using concentrations near to those found in situ (Norwood et al. 2003).

After field bioassays were concluded at the additional locations, the application of the same rationale to the field data obtained (Table 6.4. and 6.5., and Figure 6.3) showed similar findings as the ones achieved for Portugal. All values for metal concentrations in water in Icelandic field samples were above EQSs (with the exception of zinc at Hvalsnes) but there is a North-South gradient (slight from Hafurbjarnastaður to Sandgerði harbor and much more marked towards Hvalsnes) with a steady decrease to the South. On the contrary Scottish samples, except for Cu, were all well within SQG limits (Table 6.4) and possessed uncanny similarity in terms of concentrations.

Table 6.4. Concentration of metals (µg/l) in field samples taken at the in situ deployment sites at Iceland and Scotland. EU EQS – Quality standards adopted for transitional and saltwaters by the European Union for "dangerous substances". * Values proposed by the UKTAG (2008) for "specific pollutants" in brackish and saltwaters.

<table>
<thead>
<tr>
<th>ICELAND</th>
<th>Cd</th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hafurbjarnastaður</td>
<td>5.264</td>
<td>35.2</td>
<td>242.1</td>
<td>589.3</td>
</tr>
<tr>
<td>Sandgerði Harbor</td>
<td>0.593</td>
<td>35.9</td>
<td>75.56</td>
<td>717.5</td>
</tr>
<tr>
<td>Hvalsnes</td>
<td>0.557</td>
<td>22.4</td>
<td>30.4</td>
<td>22.4</td>
</tr>
<tr>
<td>SCOTLAND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hound Point</td>
<td>0.009</td>
<td>6.5</td>
<td>4.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Strachur</td>
<td>0.018</td>
<td>6.2</td>
<td>3.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Ardentinny</td>
<td>0.026</td>
<td>5.4</td>
<td>3.5</td>
<td>6.5</td>
</tr>
<tr>
<td>EU EQS</td>
<td>0.2</td>
<td>5*</td>
<td>20</td>
<td>40*</td>
</tr>
</tbody>
</table>
As can be observed in Figure 6.3 feeding behavior at the two locations followed quite different patterns. Differences between station in Iceland were significant (P<0.05) whilst in Scotland no significant differences were obtained between stations.

![Figure 6.3 - Feeding ratios registered by Echinogammarus marinus after 96h exposure at the field in Iceland and Scotland, respectively.](image)

The stations' contamination and pollution ranks for the two locations are presented in Table 6.5. For Iceland there is full concordance between the two rankings, mirroring the dichotomy higher metal concentrations - higher toxic effects. The case of Scotland is an interesting one. The similarity between field concentrations at the three sites was totally unexpected. Remediation plans have been in place for a long time and notoriously their outcome is plain to see. Particularly surprising are the low levels for the Forth estuary that traditionally was considered one of the most polluted in the UK (Dobson 2000). The ranks obtained by Strachur and Ardentinny (Table 6.5) were similar in terms of the metal amounts but differences were so slight (5 units difference between the highest and lowest score, while, for instance, at Iceland that difference was 25) that there was no direct translation in terms of feeding effect. Nevertheless these results demonstrate a crucial point: when exposed to similar (low) metal concentrations at different field sites, feeding behavior in *E. marinus* will be highly consistent.
Table 6.5 - Ranking of sites used in Iceland and Scotland according to "contamination" (enhanced amounts of metal) and "pollution" (effect upon organisms) scoring (see text for explanation).

<table>
<thead>
<tr>
<th></th>
<th>Iceland</th>
<th>Scotland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contamination</td>
<td>Pollution</td>
</tr>
<tr>
<td>Iceland</td>
<td>Hafurbjarnastaður</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sandgerði Harbor</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hvalsnes</td>
<td>3</td>
</tr>
<tr>
<td>Scotland</td>
<td>Hound Point</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Strachur</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ardentinny</td>
<td>2</td>
</tr>
</tbody>
</table>

At a given dissolved concentration, metals in aqueous form are often more toxic in the laboratory if compared to the field, because laboratory dilution water does not include metal-binding ligands found in natural waters (Stanley et al. 2005). This was the case of our laboratory data that despite being slightly over predictive related rather well to field results. Using as a referential the range of tested mixtures and their corresponding feeding inhibition effects it became evident from Figures 6.1 through 6.3 and Tables 6.1 through 6.5 that field responses were in accordance.

Our development of models with the intent to predict the simultaneous effects of the four tested metals towards E. marinus feeding inhibition, creating a surrogate reality, was partially marred by the selection of the tested concentrations. This selection was based in the Ria de Aveiro system and the transposition of the ecologically relevant field concentrations was only moderately successful. As a result the predictive power within the normally considered ±25% of confidence was good for Portugal (75%) but poor for Scotland (45%) and very poor for Iceland (33%). A better calibration taking into account natural occurring metal concentrations is necessary. Also de fact that only Concentration Aciditon (CA) was considered could have contributed to these results under the light of already pointed constraints.
Even if not always explicitly, up till now data has been discussed mainly under the consideration of one factor: speciation. Despite metal bioaccumulation primarily depending on speciation, by now it has become evident that this is not the sole modulator of that relentless process. Consequently other aspects influencing metal bioaccumulation in *E. marinus* need to be pointed out. Once bioavailable (i.e. are in the “correct” speciation state for that phenomena to occur), metals are uptaken by living organisms, that is to say are able to enter the internal medium of an organism. Despite this work being in it majority focusing uptake via respiratory surfaces (gills), dermal contact or ingestion are also portholes for metals to penetrate internal mediums (Chapman 2008). But once inside, the simple presence of metals in cells is not a necessary synonym of harmful effects (as the feeding enhancement caused by some of the tested metals in this study testify). Unless there is sensitive portions retention in amounts that transcend the binding capacity of internal sequestering ligands, no toxic effects are expressed (Rainbow 2007). Bioreactive metals (capable of exerting toxicity) are also metabolically dynamic, fulfilling functions like transporting respiratory gases or cofactoring enzymes. The way of handling these agents in the context of a certain species will vary from metal to metal, from individual to individual and from organ to organ (Rainbow 1997). The main problem regarding bioreactive fractions is the complexity inherent to their precise determination. In the words of Rainbow (2007) “toxicity is related to a threshold concentration of metabolically available metal and not to the total accumulated metal concentrations”. Metals bound to inducible metal-binding proteins such as metallothionein (MT) or precipitated into insoluble concretions consisting of metal-rich granules comprise biologically detoxified metal, whereas metals in metal-sensitive fractions such as organelles and heat-sensitive proteins can be metabolically active; the higher the proportion of metal-sensitive fraction, the greater the vulnerability to metals toxicity and consequently more chances of effects to occur (Rainbow 1997). Our experiments have determined that for *E. marinus* all these factors are modeled according to gender, life stage, physiological state (dependent on the pre-exposure diet), and exposure history of a specific organism towards a
specific metal. Moreover a percentage of what normally are considered body burdens is in reality adsorbed to the exoskeleton.

Exposure history deserves further comment as our data seems to point to a division of the tested populations into two different groups: Scotland and Portugal present genetically selected metal tolerant specimens whilst Icelandic organisms are favored by natural selection for energy efficiency and maximized growth. This is the reflex of a continuous sublethal chronic exposure in the first group, whilst in the second group, pulse exposures and a more energetically demanding environment lead to plasticity.

There is also a much disregarded and obscure detail upon the toxicological evaluation of crustaceans: the occurrence of intersex. *E. marinus* Icelandic populations were the ones registering the highest incidences and curiously enough were also the ones that possessed highest variations in the feeding assays (despite Scottish organisms being the ones patenting cases with characteristics denouncing a stronger drive towards this condition). To what extent intersex interferes with metal uptake and individuals’ susceptibility; could metals cause higher prevalence of these individuals? Regarding the latter our findings apparently indicate that a chronic exposure to moderate amounts of metals in natural populations could induce higher prevalence of intersex. Nevertheless these are matters needing further study.

The hereby presented work validates *Echinogammarus marinus* as a suitable organism for ecotoxicity testing. Its sensitivity, amenability to laboratory conditions and wide geographic distribution vehemently advise its usage in screening in situ bioassays, constituting a cost-effective tool for preliminary metal hot-spot identification, allowing support on decision making regarding allocation of often limited resources.

REFERENCES


