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FACULTAD DE BIOLOGÍA

Chronotoxicity of Contaminants (etanol and heavy metales) in a Freshwater Teleost Dario rerio (Hamilton, 1822) and Marine Amphipods Gammarus aequicauda (Martynov, 1931) and Gammarus chevreuxi (Sexton, 1913).

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2015

Memoria de la Tesis Doctoral presentada por Dña. Carolina Bello Marín para optar al grado de Doctor en Biología por la Universidad de Murcia

TABLA DE CONTENIDOS

Summary

Introduction

1. Toxicology

1.1.1 The interdependence between human health and healthy ecosystems

Human health and natural environment are unavoidably linked: the health of human population depends on quality of environments directly and, in particular, of aquatic ecosystems (rivers, lagoons, estuaries, and rias) which underpin human economic, social and environmental wellbeing. From an anthropocentric point of view, healthy ecosystems are in which reside the capacity to provide goods and services for human populations(Maller et al., 2006).

Transitional watersare important ecosystems for their high productivity but also for their provision of ecosystem services to human welfare, supporting many major cities,ports and industrial development. The exploitations of those valuable ecosystems by human activities release contaminants from land-based sources via rivers and the atmosphere to the water bodies where they may accumulate and recycle in sediments and organisms. These contaminants such as heavy metals are considered as pollutants and hazardous substances with harmful effects in the ecosystems. Some of these effects are well known due tothe biomagnifying effects of chemicals in marine food web. This accumulation of substances is toxic to aquatic organisms and also presents a health risk to humans (van der Oost et al., 2003). The consequences of other chemicals as Persistent Organics Pollutants (POPs); fungicides, insecticides, which cause endocrine-disrupting effects are maybe less evident because their effects appear in the long term(Colborn, 1995; Guillette, L.J, 2005)

Another important consideration is the effect of **climate change**. It has potential to alter the environmental distribution andbiological effects of those chemical toxicants (Stocker, T.F. et al., 2013).Furthermore, there is an intimate, inseparable, and immediate connection between human activities and the environment in which they live. Therefore the study of pollutants effects should be addressed in a multidisciplinary way and take into account as much variables or drivers as possible. The predictedchanges in environmental parameters such as temperature, precipitation and salinity due to climate change will have an effect on the contaminants distribution, concentration, remobilization and deposition. For instance, PCB areexpected to increased the volatility and atmospheric concentrations andthis fact will intensify the bioavailability of dangerous pollutants(Noyes et al., 2009). On the other hand the increases of temperature will affect also to organism metabolism and niche distribution(Carere et al., 2011).Consequently our studies had also addressed the rise of temperature in our case study areas where there is presence of contaminant sediments in order to raise a better understanding of the possible effects of heavy metals in these ecosystems.

In this thesis, we address whether contaminants exceed or notan effects threshold somewhere in the ocean, and thus become pollutants: both exposure and susceptibility should be taken in account in order to determine more accurately the effects of pollutants and try to forecast the ecological death edges(Morillo-Velarde et al., 2011).

1.1.2Chronobiology and chronotoxicology

Integrating physiological insights intoecology pattern

Hundred of years ago it was hard to distinguish the line between physiology and ecology studies. Nonetheless the development of these two disciplines has created an invisible barrier between them. Whilst physiology is increasingly focusedon thestudy of mechanisms atlower levels such as organ systems, cells or biomolecules, ecolgoy gaze is focused on populations and species interactions(Chown & Gaston, 2008). Nevertheless it is evident that physiological knowledge is required to understand organism response, and to address environmental changes issues (Austin, 2007).For this reason, integrative studies focus on bottom-up models of species responses to environmental changes, namely on how physiological responses and abiotic factors might interact to determine species distribution and abundances (Ghalambor et al., 2006). In fact, this need of integration has resulted in the emergence of hybrid disciplines such as molecular ecology, functional ecology, conservation physiology and ecophysiology(Mouquet et al., 2012; Cooke et al., 2013)

Chronobiology is the study of biological rhythms and the mechanisms of biological timekeeping. Cyclic environmental events such as light-dark (resulting from the regular rotation of the earth around its central axis) and temperature cylces influence all living organisms. Plants, insects and animals including humans possess the ability to anticipate these daily changes, wich provides the organisms with important adaptative and survival advantages allowing them to optimize their adaptation to their ecological niches.Biological rhythms can be classified according to their period (T): ultadian (T<24h; e.g. heart beeping), circadian (T~24h; e.g. activity-rest rhythm, food intake) and infradian (T>24h, e.g. menstruation, breeding, tidal) (Kulczykowska et al., 2010).Those fluctuations of their rhythms have endogenous nature.

Chronobiology is a field of biology with a multidisciplinary approach which study those rhythms and the different features of the organisms taking into account the timeof the day.Chronobiological studies embrace different disciplines, includinganatomy, physiology, molecular biology and behaviour of organisms within biological rhythms mechanics(Patricia J. Decoursey, 2003).But as mentionedabove it also studies aspects that include reproduction and ecology.

Behavioural rhythms are one of the most evident rhythms in animals, specifically the activity-rest rhythm andfood intake behaviour. Along this thesis activity-rest rhythm has been usedto characterise the normal behaviour of the studied organisms and as the main bioindicatorto evaluate the effect of xenobiotics and heavy metals and the influence of temperatureon theses effects.

Chronotoxicology refers to time-dependent differences in appearance and severity of xenobioticharmful effects to the organisms(Pszczolkowski & Dobrowolski, 1999).The concepts of chronobiology andchronotoxicology have an important application in the fields of occupational medicine and industrial medicine (Smolensky & Peppas, 2007) as shift and night workers are likely to be exposed to potential harmful workplace contaminants at different circadian times. Some industrial processes with risk of exposure to ethanol are: leather tanning and processing, painting solvents, silk (screen printing) or the use of disinfectants andbiocides.

Furthermore many laboratory animal studies show that the deleterious effects of numerous potential noxious chemical and physical agents varies sometimes dramatically according to the circadian time of exposure (Cui et al., 2004; Rebuelto et al., 2004). Recent researches in chronotoxicity of common anaesthetics used in aquaculture revealed differences of toxicity and effectivenessin commercial species such as gilthead seabream (*Sparus aurata*)(Vera et al., 2010) and zebrafish (Sánchez-Vázquez et al., 2011).Similar differenceshave been observedin the application of pesticides in weed control(Miller et al., 2003). This chronobiological approach to the effects of pollutants on living organisms can lead toa maximal outcome in terms of contaminant control by increasing cost and time-effectiveness ratio. In addition, the application of these strategies would be safer for the environment.

Due to those evidences we considered interesting to dig deeper in daily rhythms of toxicity of pollutants in order to assess how they affect to key species, the time-dependent differences in their effectsand the possible implications in the organisms’populations. Moreover we would like to raise the importance of consider the time of day in experimental toxicity setups, choosing the appropriate time to get more accurate results.

Given all the exposed above, this thesis aims at combiningmultiple methodologies, from the classical ones used in toxicology to an innovative approach tophysiological behaviour andtranscriptomerhythms, to explore the effects of several water pollutants in model and key species inhabiting transitional waters bodies. This new information aims atcontributing to a better understanding of toxicantseffects in aquatic organisms and their possible consequences.

1.2. From fundamental research to environmental approach: Pollutants in water

1.2.1. Ethanol

Alcoholism represents a serious problem for society as alcohol abuse can cause fetal alcohol syndrome or FAS, alcohol-related birth defects (ARBD) or alcohol-related neurodevelopmental disorder (ARND)(Bilotta et al., 2002). Current treatments are limited and inefficient(Fuller & Hiller-Sturmhofel, 1999; Vengeliene et al., 2008) and there is a need to better understand alcohol (EtOH or ethyl alcohol) effects. Due to this, intense research is being conducted from many different points of view, to characterise both acute and chronic effects of alcohol, including development anomalies, behavioral effects as well as the alcohol mechanisms of action(Carvan, Loucks, Weber, & Williams, 2004; Chen, Wang, & Wu, 2011; Gerlai, Ahmad, & Prajapati, 2008). However, the problem is that alcohol has been found to act through a large number of biochemical pathways(Vengeliene et al., 2008), and its effects are also subjected to variations depending on chronobiological variations in its metabolism(Danel & Touitou, 2004).

The current thesis contributes to this growing research by providing new findings by applying a chronobiological perspective totraditional acute toxicity testsandcomputerized methodsto evaluate behavioral effects of ethanol. Moreover we also explored the genetic mechanisms underlying thesebehavioural changes. For this, we analysed the expression of the main enzymesinvolved in ethanol metabolism.

We do not claim that by studying the above topics we are proving mechanistic details on the actions of ethanol, but we hope that these findings will raise the importance of taking into account circadian rhythms when designing future studies focusing on alcohol toxicology, from a fundamental or environmental perspective, since this will allow to produce predictable and reproducible responses.

1.2.2. Heavy metals: cadmium and mercury

Metals are naturally present in the environment, with a variety of concentrations among different regions. While organic pollutants can be degraded to less harmful components by different processes, metals are deem as non-degradable pollutants. Mercury and cadmium are considered to be the most toxic metals to aquatic organisms ,according to Abel (1989)who tried to established a tentative order of metals depending on their toxicity: - mercury (Hg), cadmium (Cd), copper (Cu), zinc(Zn), nickel (Ni), lead(Pb), aluminium (Al), and cobalt (Co).Theseheavy metals are one of the most abundant, persistent and toxic contaminants that we can find in transitional waters. These areas are highly exploited by anthropogenic activities which release toxic chemicals from different sources. The effects of metal pollution on local environments and organisms can be long lasting in terms of time. Thesecontaminants can result in deleterious effects onwildlife habitats, degradation of ecosystems and result harmful for humans health due to the consumption of contaminated seafood(Ip et al., 2004; Pan & Wang, 2012).

Cadmium

Cadmium (Cd) is a scarce **element** in the earth's crust. Its ores are hard to find and usually in small amounts. Anthropogenic sources producing an increased release of cadmium are the combustion of oils and coals, and incineration plants. Other major sources related to specific uses are fertilizers obtained from sediments or rocks rich in cadmium, batteries and electric batteries, electrolytic coatings and pigments(López-Artíguez, M. & Repetto, M., 1995; Moreno-Graw, M.D., 2003)

Cadmium is among the heavy metals with greater impact on health(Repetto, M. & Sanz, P., 2008). It has been demonstrated to be a highly toxic metal to wildlife and to possess teratogenic and carcinogenic effects to humans (Ronald, 2012).It is a non-essential metal for biological systems except for marine diatoms(Lane & Morel, 2000), whilstit is present as a contaminant in food, water or air, being ingestion and inhalation the major routes of exposure to cadmium(López-Artíguez, M. & Repetto, M., 1995). Furthermore several studies have shown cadmium **bioaccumulation** and biomagnification in the food chains of various marine ecosystems, accumulating primarily on fish and arthropods (Bustamante et al., 2003; Croteau et al., 2005; Pan & Wang, 2008).

One of the major pathways for the **uptake** of trace metals by aquatic invertebrates isdirectly from the waterthrough permeable surfaces including the gills.Cadmium canalsobe **absorbed** through the respiratory and digestive tract (ventral caeca) during food intake and burring with the sediments.Infish cadmium uptake occurs through the gills and the main storage organs are liver, kidney and bones (Glynn, 2001; Hattink et al., 2005).

Mercury

Mercury (Hg) is a heavy metalwhichnaturalsources of mercury are mainly from the evaporation of the element from the surface of the oceans, land surface and inland waters, as well as emissions from the biosphere and volcanic emissions. This element has been used for centuries in medicine-poison duality and that, at present, is used with a variety of commercial and industrial purposes(Broussard et al., 2002). But nowadays the anthropogenicsources has increases the emissions level of about 1930 tons, (Pacyna et al., 2010). This sources are primary type (mercury mobilized geological origin and introduced into the environment unintentionally product) and secondary (intentional use of mercury). Among the primary highlight the mining and production of iron, steel and nonferrous metals (chromium, lead and zinc), extraction and burning of fossil fuels (coal and gasoline) and cement production. Among the secondary industrial processes (production of vinyl chloride monomer and chlor-alkali industries), manufacture of certain products (batteries, thermometers, barometers, manometers, fluorescent lighting, high intensity discharge, pesticides, fungicides, paintings, etc.)(Pacyna et al., 2010).

In recent years has greatly increased concern for the problems of environmental exposure that causes its high persistence in the environment. Most of the mercury in the environment, except the atmosphere, is in the form of inorganic mercuric salts and organomercury compounds. The latter are the most relevant environmental level bioaccumulation and its great potential for **biomagnification** in food chains, mainly in aquatic environments(Hintelmann, 2010).The flow between the natural reserves of mercury (air, land, water and biosphere) forms the so-called global mercury cycle, since the industrial era is clearly influenced by anthropogenic activity (Fig. X). The oceans are the largest reserves of mercury (1017g) followed by soils and sediments (1013g). The biota represents around 1011g, the atmosphere 108g and 107g inland waters.

Contamination in the aquatic environment by mercury (Hg) has been recognized as a potential environmental and public health problem for over half of a century. The concentration of Hg in rivers, lakes and lagoons has increased by 90ng L^{-1} per year (Benoit et al., 1998).

Taking in to account the impact of exposure to heavy metals in sediments in gammarids and how the effect of climate change can affect them. This thesis tried to throw some light in the risk of heavy metals contamination of lagoons and their impact.

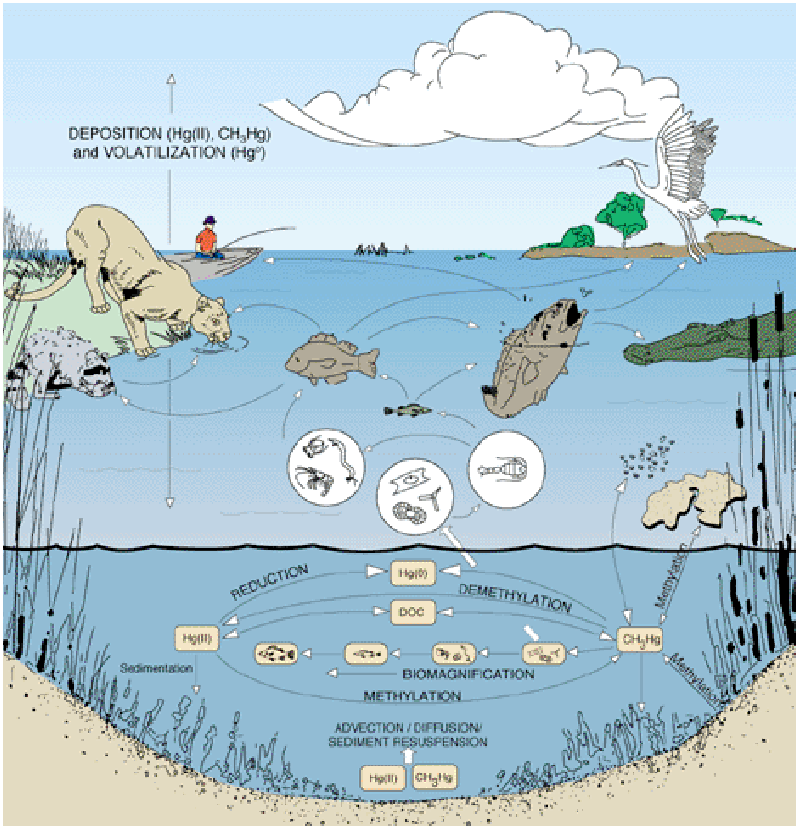


Fig.1. Mercury cicle.

1.3. Case study areas

1.3.1. Mar Menor (Spain)

The Mar Menor is a hypersaline coastal lagoon **located** in a semi-arid region of southeast Spain (Figure 1). The lagoon occupies a surface of approximately 135 km^2 and a total volume of 610x103 m$^{3.}$ Maximum depth in the lagoon reaches 6.5 m with an average depth of 3.6 m. **Geomorphologically** Mar Menor is isolated from the Mediterranean Sea by a 22 km long and 100 to 900 m wide sandy bar (La Manga) crossed by three shallow channels (Marchamalo, Encañizadas delVentorillo y La Torre and El Estacio). In the early 1970s, one of these channels (El Estacio)was dredged and widened to make it navigable. Since then, it has become the lagoon's mainconnection with the sea. The enlargement of El Estacio channel led to a substantial increase ofwater renewal rates from the Mediterranean, as well as subsequent changes in watertemperatures and salinities. These changes favoured the colonization of the lagoon bynumerous marine species as lagoonal temperatures and salinities reached less extreme values. Nowadays, salinity ranges from 42 to 47 and temperatures are lessextreme ranging from 10º C in winter to 30º C during the summer.(Perez-Ruzafa et al., 1991)

The lagoon is situated at the end of a watershed delimitated by a group of mountain ranges(Escalona, Algarrobo, Cartagena) that surround the Campo de Cartagena, an extends plain ofabout 1,440 km^2. Freshwater inputs into the lagoon are restricted to six ephemeralwatercourses called ‘wadis’ or ‘ramblas’.Due to the semi-arid climate ofSE Spain those watercourses remain dry for a 5–10 yearperiod, and fresh water does not reach the lagoon unless sporadicand torrential rainfall occurszotero(Marin-Guirao et al., 2005).Hence this wide, shallow gullies are generally inactive,but can carry great quantities of water and sediment during flood episodes. The torrentialnature of the supplies is aggravated by the impermeable soils and scarce vegetation cover ofthe watershed areas (Figure 2).

El Albujón wadi is the principal watercourse responsible for major inputs of organic andinorganic nutrients that flow into the lagoon (Velasco et al., 2006; Garcia-Pintado et al., 2007). It drains a surface of 441 km^2, about one third of the total surface of the adjacent agriculturalarea (Campo de Cartagena). The principal source is drainage from irrigated crops, butsometimes waste-water treatment plants located in the watershed area discharge largeamounts of untreated or insufficiently treated water into the channel.

Moreover this watercourses has brought to the lagoon drainage and sedimentation wastes from abandoned mined lands. These sediments derived from mountains located in the southern part of the lagoon, where mining activity was the most substantial in Spain during the last two centuries. Even mining activities stopped in the 90's the **metals** of mine tailings released into the lagoon during the floods episodes (Gundersen & Steinnes, 2001). Subsequent studies carried out in the area confirms the levels of metals (Pb, Cd, Zn, Mn, Fe and Cu) in sediments from severalstations, finding the maximum concentrations in the S-SW lagoon area, near the mouths of wadi. The study attributed to mining waste high concentrations of Pb, Zn, Cd, Mn, Fe (Marin-Guirao et al., 2005).

1.3.2. Ria de Aveiro (Portugal)

This coastal lagoon is located on the North west coast of Portugal (Fig. X). It is connected with the Atlantic Ocean through an artificial inlet. The lagoons occupies a surface approximately 45km long and 10 km wide, influence from Atlantic tides (Dias et al., 2000). The system can be subdivided into channels, and complex network of branches, bays and narrow channels with different characteristics connected to a common outlet. In general, the common typology is intertidal zones called mud flats and salt marches, except in the central area of the lagoon. Freshwater supply comes from four major rivers converging tothe Ria: Vouga and Antuã from the East, Caster from theNorth and Boco from the Southeast(Dias et al., 2001). The influence of the rivers is higher in the north and internal parts of the lagoon. The water circulation inside the lagoon is mainly tide-dominated (Dias et al., 2001). But his volume of seawater entering to the lagoon during flood events is far larger than the freshwater. This shallow lagoon is generally less than 3m depth except the area close to the connection with the Atlantic Ocean where the water depths reach 20m.(Lopes & Dias, 2007). Taken as a whole, the system acts as a mixing zone for water andsediments from two end members: marine and freshwater.

The surrounding population is gathered in municipalities and the number of inhabitants in the entire watershed are approximately one million people(INE, 2011). The activities of this region are agriculture, cattle rearing, industries (metallurgic, ceramics, chemical tannery and pulp milling), fish and shell fish capture are the main activities inside the lagoon. Themain municipality (Aveiro town) is located 15 km south from an industrial complex locatedin Estarreja, in

Laranjo bay (Pereira et al., 1998)that includes a chlor-alkali plant that have discharged mercury from the 1950s until themid-1990s. The discharges resulted in an accumulation of about 33t of Hg in the lagoon, which is sediment-associated in Laranjo bay. Nowadays the mercury discharge decrease being inside regulatory level (50 ug 1 -1, limit value for discharges form chlor-alkali in accordance with Directive 82/176/EEC, 1982). Nonetheless, mercury concentrations in the sediments surface still high due to the historical loads(Coelho et al., 2005).

Fig. 2 Study areas, Iberic peninsul.

1.4. Species in focus

1.4.1Why we choose these species?

In regulatory purposes and risk assessment, mortality, growth and reproduction are the most commonly used endpoints, as they are not affected by subjectivity of the operator.

The study of toxics can be approachedas **basic research** where the focus is on the mechanism of action of a target organ or process, genetic toxicology, etc. In this field acute mortality test andother conventional bioassays are commonly used in toxicology studies (Silbergeld & Mattison, 1987).But nowadays toxicology has become an important discipline in **occupational and environmental health** to evaluate hazards. So this short-term assays (e.g. acute test) used to apply unrealistic concentrations froman environmental point of view (Cheung et al., 2002; Scott & Sloman, 2004). Aquatic ecosystems are usually exposed to low concentrations of toxicants(Gerhardt et al., 2002). Therefore in this thesis we want to combine these traditional assays with sensitive ones to low concentrations. A possible approach is to assess the effect of pollutants on behavioural endpoints. Behaviour is normally affected before death, growth and reproduction, being a suitable ecological warning system(Pestana et al., 2007). Behavioural endpoints are useful tools in ecotoxicology studies since chemical-induced behavioural effects are generally quick, sensitive and easily detected. Moreover, their characterization involves non-invasive methods and possess ecological relevance(Gerhardt, 2007). Behavioural changes may be used as indicators for ecosystems health, because they reflect changes in biochemical processes, and also reveal the fitness of the organisms and the potential consequences at the population level.

Eather for basic research or evironmental health, there are several key species use to study of toxics effects. As we are studing aquatic environments and the effect of toxics present on them, we chose three key species in aquatic toxicology research, namely zebrafish (*Daniro rerio*) and two species of gammarids family (*Gammarus aequicauda* and *Gammarus chevreuxi*). Having in mind these methodologies of work and type of assays metioned above, we consider zebrafish as an excelent vertabrate model (Shin et al., 2002). This small but robust fish is used as model in human disease investigations (Guyon et al., 2007), and is also wide used in chronobiology research where is a useful tool to study molecular bases of circadian clock (Cahill, 2002) and also behavioral rhythms (Hurd et al., 1998; Carvalho et al., 2006). All these features makes him a very appropiate sp. four our experiments. Whereas gammarids family is considered as an excellent biomarker to study ecosystems and the effect of contaminants in them(Subida et al., 2005). Currently used in ecotoxicological test due to its wide distribution, this invertebrate is listed as the most sensitive to a range of stresses. This together with other features make them a very suitable invertebrate to study the effects of the heavy metals in sediments from a behavioural point of view.

1.4.2. Zebrafish, *Danio rerio* (Hamilton 1822)

This teleostspecies is native and distributed over the Indian subcontinent. Its common habitats are slow-moving or standing water bodies and ditches adjacent to rice fields and rivers(Spence et al., 2008). Its taxonomic hierarchy is as follows(Fang, 2003)

Indeed, zebrafish has many features that make it an ideal model system. Adults have a small size which allows keeping themeasily in captivity in large numbers. Its generation time is short and most importantly a single spawning can produce hundreds of embryos(Westerfield, 2000). It is also a tractable species for behavioural experiments, readily acclimatizing to new environments and being little disturbed by the presence of observers(Spence et al., 2008). Furthermore, zebrafish embryos has a rapid *ex utero* development and they are transparent, which allows direct microscopic observation.

In factzebrafish has become an excellent **vertebrate animal model** system that isbeing used as an effective alternative to mammalian animal testing to study mechanism of toxicity(Carvalho et al., 2006) and genetics studies (Tierney, 2011), and lately, for human disease and the screening of therapeutic

Phylum: Chordata

Subphylum:Vertebrata

Superclass: Osteichthyes

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: Danio

Species: ***Danio rerio*** (Hamilton, 1822)

Danior rerio

drugs(Penberthy et al., 2002; Sumanas & Lin, 2004). Given all these facts, experiments in zebrafish are significantly easier, faster and cheaper comparing with other experimental animals, therefore is one of the main model systems for drug target discovery and validation.

Zebrafish has been described as a diurnal species(Cahill et al., 1998). This circadian rhythm has been observed since larval development (5 days post fertilization)(Cavallari et al., 2011),being their behaviour and development well described by many authors.Consequently we have usedthis behavioral rhythm as a biomarker to assess toxicants effects in this species..

1.4.3. *Gammarus*

Gammarids are present in a wide variety of **habitats** as freshwater, brackish and marine habitats, playing a key role in the structure and function of aquatic communities(Costa et al., 2009).There is about 100 *Gammarus*species in the

Northern hemisphere.Theyrepresent an important keystone family in aquatic **ecosystems**with a broad role along the food chain as shredders, detritus feeders, carnivorous, and carrion. This foraging plasticity being also herbivores, predators and cannibalists might contribute to their ecological success in colonizing ecosystems(Gerhardt, 2011)and allows an ecological niche being holdby different species (Sanz-Lázaro & Marín, 2009).Its taxonomic hierarchy is as follows:

This invertebrate has many features that make them an ideal test organism to be used both in and ex situ applicationbecause ofseveral reasons: possess high sensitivity to contaminants associated with sediment; short time over

Phylum: Arthropoda

Subphylum:Crustacea

Superclass: Osteichthyes

Class: Malacostraca

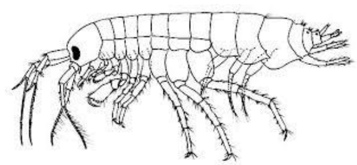
Order: Amphidoda

Family: Gammaridae

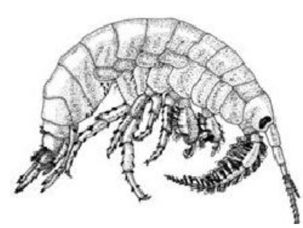
Genus: *Gammarus*

Species: ***aequicauda*** (Martynov, 1931)

Species: ***chevreuxi*** (Sexton, 1913)



Gammarus aequicauda



Gammarus chevreuxi

generations; easy to handling in the laboratory; tolerance to a wide range of physicochemical characteristics, and live directly in contact with the sediment(Cesar et al., 2004).Therefore due to their widespread distribution, importance in the food web, and sensitivity to a large range of pollutants, they are consider as standard test species in ecotoxicity testing in the USA and Europe for testing acute toxicity of sediments(Cincinnati, 1996) and also an important bioindicator for water quality assessment.

G. aequicauda (Martynov, 1931)

Gammarus aequicauda (Martynov, 1931) is one of the most common and abundant amphipods in lagoons and brackish environments Mediterranean and Black Sea.*G. aequicauda* isan euryhaline species, being very resistant isolates sea habitats with a very large salinity range(Prato & Biandolino, 2005). This species has an important trophic role in the transport of energy to higher consumer and superbly feeding activities contribute to the fragmentation of macrophytes, thus improving the microbial colonization and decomposition of macrophytes(Prato & Biandolino, 2005).*G. aequicauda* live from the intertidal zone, up to 20 meters deep, but is most abundant in surface waters between macroalgae as *Chaetomorphalinum*, Ulva spp. which provides food and protection from predators. It also feeds the sediment where remains of other living beings, bits of organic material or by scraping accumulate on the surface of mineral particles. In loamy sediments, *G. aequicauda* digs in sediments surface(Prato & Biandolino, 2005).

Gammarus chevreuxi (Sexton, 1913)

This epibenthic amphipod is common in the upper reaches of estuaries in the West of Europe and North Africa. The specie is stenohaline being restricted to low-salinity environments. They rich high densities of population in habitats under 10 psu(Cunha & Moreira, 1995). *G. chevreuxi* live between macrophytes on rocky, sandy and muddy bottoms. It can be found in intertidal habitats of the Iberian Peninsula and, Portugal in particular in estuaries, lagoons and rias along the coast (Cunha & Moreira, 1995).

2. Objectives

This thesis aims at investigating the effects of Ethanol and heay metals (cadmium and mercury) on the daily patterns of two key aquatic species: zebrafish (*Dario rerio*) and amphipod gammarus (*G. aequicauda* and *G. chevreuxi*). The research was conducted in two organizative levels: Physiology behavior and molecular.With this purpose we have established four particularobjectives:

1. To investigate time-dependenteffects of ethanolexposure inzebrafish.

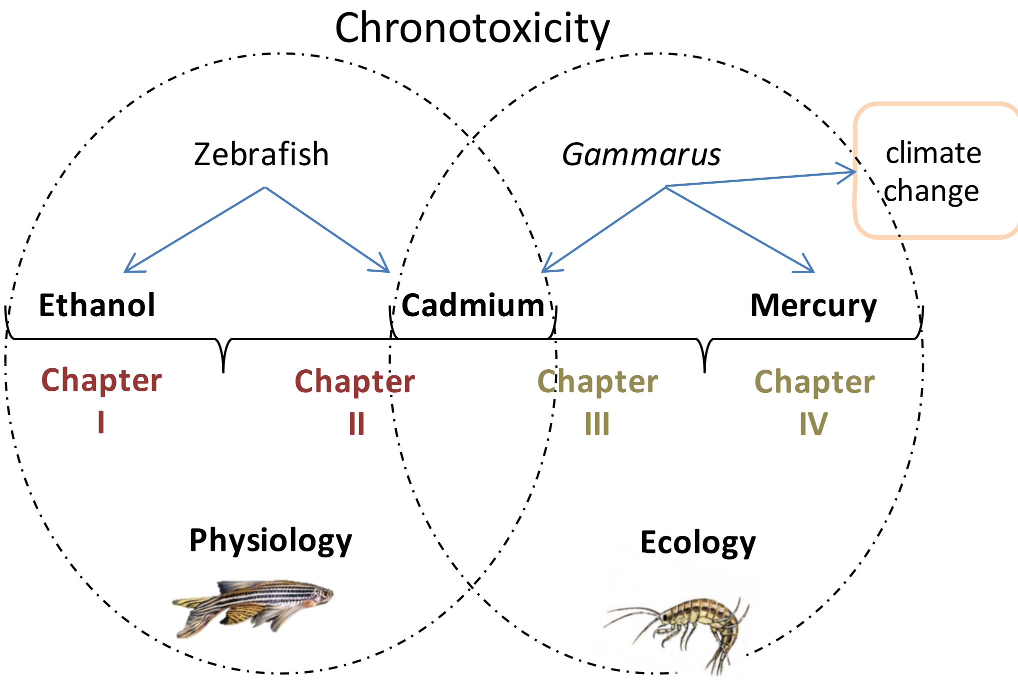
2. To determine the existence of a daily rhythm of cadmium toxicity in zebrafish.

3. To characterisethe effects of locomotor activity to mercury-contaminated sediments in *Gammarus chevreuxi.*

4. To evaluate the environmentalquality of the soft-bottoms from the southern basin ofthe Mar Menor lagoon influenced by historical miningactivities.

On the basis of the above this thesis is aiming at combining traditional studies with new approaches in behavioral assessments and molecular techniques to explore some of the main effects that xenobiotics and pollutant shave on the animal species used as experimental models.

Hence, this new data intends to point out the importance of applying a multidisciplinary approach to toxicology studies, and also raise the importance of the time experimental setting of toxicity test.


Chronotoxicity
Zebrafish
Gammarus
climate change
Ethanol
Cadmium
Mercury
Chapter I
Chapter II
Chapter III
Chapter IV
Physiology
Ecology

Chapter I

Ethanol toxicity differs depending on the time of day:

a chronobiological study in zebrafish (*Danio rerio*).

Introduction

Chronotoxicology is a modern discipline which study temporal variations in the presence and severity of adverse effects of drugs and other chemicals when administered to an organism at different times of the day (Bruguerolle, 1998). Over the past four decades, circadian variation in drug absorption, distribution, metabolism and excretion has been extensively investigated in mammals (Lemmer, 1989; Reinberg, 1989; Cambar & Pons, 1997; Smolensky & Peppas, 2007). Assessments conducted in experimental animals under constant lighting conditions have demonstrated that many of these toxicity rhythms persist, indicating its endogenous nature. In fish, however, the chronotoxicity of xenobiotics and time-dependent differences in the effectiveness of drugs are still largely unknown.

In the case of ethanol (alcohol or ethyl alcohol), an early work in humans by Wilson et al. (1956) first questioned the previously held view that alcohol metabolism remained constant throughout the day. These authors administered small amounts of alcohol hourly to human subjects, reporting daily variations in salivary and blood alcohol levels, which, curiously enough, were reversed in a nurse on night shift work. More recently several studies have suggested reciprocal interactions between the circadian system and alcohol intake at both physiological and molecular-genetic levels (Cambar & Pons, 1997; Rosenwasser, 2001; Danel & Touitou, 2004; Spanagel et al., 2005), finding that numerous functions exhibit a circadian variation in their sensitivity to ethanol (chronesthesy). In humans, the morning consumption of alcohol results in the greatest peak of blood alcohol concentrations due to daily rhythmicity in the rate of alcohol metabolism (Wilson et al., 1956). Ethanol is primarily metabolized in the liver in a two-step process by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In this route ethanol is converted first into acetaldehyde and then into acetic acid, the accumulation of these products being responsible for the physiological and behavioural effects of alcohol (Ramchandani et al., 2001). Current evidence suggests that ethanol detoxification in zebrafish follows the same pathway as in humans (Reimers et al., 2004; Lassen et al., 2005; Bencan & Levin, 2008). Therefore, it has been argued that results of ethanol studies carried out in zebrafish could be extrapolated to mammals (Gerlai, 2003; Tropepe & Sive, 2003). Zebrafish (*Danio rerio*) has become a widely recognized model organism in chronobiological, pharmacological and toxicological research. Moreover, in fish hydrophilic substances such as ethanol can easily be taken up from the water through the gills and the whole body surface. Zebrafish larvae provide an excellent experimental model due to their particular features: they are free living after postembryonic day 5, have a well-established nervous system and patterns of behaviour (Cahill et al., 1998), and express *adh* and *aldh* genes from day 3 onwards (Dasmahapatra et al., 2001; Lockwood et al., 2004). Therefore zebrafish has been proposed as a useful model to study ethanol effects on animal behaviour (Gerlai et al., 2006; Kurta & Palestis, 2010). Nevertheless, nothing is known about alcohol effects at different times of the day in this experimental model. For these reasons the present research assesses daily rhythms in ethanol toxicity and detoxification mechanisms in zebrafish. To this end, we investigated the (1) daily variations in mortality of zebrafish larvae exposed to ethanol, (2) daily rhythms in the effect of sublethal concentrations on locomotor activity and (3) day/night differences in basal gene expression of alcohol and aldehyde metabolizing enzymes (*adh8a* and *aldh2*).

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Materials and Methods

Animals and housing

Larval zebrafish of heterogeneous wild-type stock (standard short-fin phenotype) were raised according to standard methods (Nüsslein-Volhard & Dahm, 2002). Fertilized eggs were collected within 2 h of laying, and aliquots of 30 eggs were transferred into sterile petri-dishes (85x10 mm) filled with embryo medium (Nüsslein-Volhard & Dahm, 2002). Pairs of petri dishes (N = 60) were incubated for 5 days in 9 L, thermostat-controlled aquaria (28.5 °C).

For behavioural trials, 14 adult (7 males and 7 females) of 0.4 g mean body weight and 3.4 cm length were used in the experiment. Wild type zebrafish were purchased from a local provider (Jumipez SL, Murcia, Spain). The experiments were carried out in the Chronobiology Laboratory at the Faculty of Biology of the University of Murcia, where the zebrafish were housed in glass aquaria (60cm x 40cm x 30cm) equipped with filter pumps. The aquaria were placed in a chamber with the temperature kept constant at 26.0 ±0.5 °C and a photoperiod of 12:12 h LD (light: dark). Lights were turned on at 9:00 h and off at 21:00 h. Light was provided by fluorescent tubes (F15W/GRO, Sylvania Gro-Lux, Germany), reaching an intensity of about 700 lux at the water surface. Fish were fed once a day at random times with a trademark diet (Nutron Hi-Fi; Prodac, Italy). Locomotor activity during the acclimation period was monitored to check the existence of a daily rhythm and its synchronization to the LD cycle. To this end an infrared photocell (E3Z-D67, OMRON, Japan) was placed in each tank. The photocells were connected to a computer, and every time a fish interrupted the infrared light beam it produced an output signal that was recorded and stored in 10 min bins using specialized software (DIO968USB, University of Murcia, Spain).The experiments performed in the present research followed Spanish legislation on Animal Welfare and Laboratory Practices and was approved by both the National Committee on Animal Welfare and the Bioethics Committee of the University of Murcia.

Experimental design:

Daily rhythm of ethanol toxicity in zebrafish larvae

Zebrafish larvae at 5 days post-fertilization (dpf) were exposed to 4% ethanol (v/v) in fresh embryo medium for an hour at different "zeitgeber times"(ZT) 2, ZT6, ZT10, ZT14, ZT18 or ZT22 (lights onset at ZT0). At each sampling point 3 independent petri dishes containing 5 larvae (n=15/time point) were used to carry out the ethanol exposure test. Petri dishes were kept in a 60 L thermostat-controlled aquarium at 28.5 ºC. At the end of each exposure, larval mortality rate was assessed. To this end, the presence or absence of heartbeat was determined with a Leica EZ4D magnifying glass. At 5dpf zebrafish larvae are transparent and therefore their heart rate can be easily observed and measured.

Effect of sublethal ethanol concentrations on locomotor activity of adult zebrafish

To investigate the existence of daily rhythmicity in the effect of ethanol on zebrafish activity, adult fish (n=14) were exposed to 1% ethanol (v/v) in dechlorinated water for 15 min at ZT2, ZT6, ZT10, ZT14, ZT18 or ZT22. Both ethanol concentration and the duration of exposure were chosen based on previous trials and published data (Gerlai et al., 2000; Dlugos & Rabin, 2003). To evaluate the effect of ethanol on locomotor activity, fish were filmed prior to, during, and after exposure. For this, two 8L aquaria were divided into 7 individual compartments with methacrylate separators that were pierced to allow water circulation. The experimental conditions were the same and the experiment was run in both aquaria at the same time. Activity was recorded during the 15 min before exposure (basal activity), 15 min during ethanol exposure and 30 min after exposure to record the recovery phase, for which fish were removed from the glass aquarium and placed into a new one containing clean water.

Water temperature was kept constant throughout the trials at 26 °C. Fish were fasted for 24 h prior to the experiment and access to the experimental laboratory was restricted during the course of the experiment to avoid fish disturbance. Fish were filmed with webcams (Webcam C250, M/N: V-U0003, Logitech, Switzerland) which were adapted for infrared recording at night by removing the UV filter located in front of the lens. During the day, light was provided with a fluorescent bulb (F15W/GRO, Sylvania Gro-Lux, Germany), whereas at night infrared LED lamps were used (LEDs monocolor, mod. L-53F3BT, 5 mm). These lamps were not perceived by fish but allowed video-filming.

The video-recordings were analyzed using the specialized software Fish Tracker (Vera et al., 2010) to measure locomotor activity levels and determine fish position in the water column. This software tracks each fish position during the experiment and generates a file that can be exported to Microsoft Excel for further analysis.

Gene expression of alcohol and aldehyde metabolizing enzymes

To examine day/night differences in *adh*8*aaldh*2 expression, a total of 40 zebrafish were reared under a 12:12 cycle for 10 days. Then, after checking that fish activity was synchronized to the LD cycle, zebrafish were divided into two groups (n=20). Zebrafish were euthanised with an overdose of anesthetic and liver samples, were taken and immediately frozen and stored at -80 ºC until further analysis. For gene expression analyses, tissue samples were homogenized in Trizol reagent (Invitrogen) using a tissue homogenizer (POLYTRON, PT1200; Kinematica,

Gene	Accession number	F/R	Primer sequence (5'- 3')
adh8a	NM_001001946.4	F	CCCTCTTCCTCTCTCAGTGTG
		R	CTTGTAGGTTCAGCCATAATGTTAT
aldh2	AF260121.1	F	GAGTTGGGCGAGTATGGACT
		R	TTAACGTGGCAATTCGTGACT
β-actin	AY222742.1	F	AAGCAGGAGTACGATGAGTC
		R	TGGAGTCCTCAGATGCATTG

Table 1. Abbreviations: *adh8a*–alcohol dehydrogenase; *aldh2*–aldehyde dehydrogennase; *β-actin*–Beta actin.

Lucerne, Switzerland). Total RNA concentration was determined by spectrometry (Nanodrop ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA), and 1 µg was treated with DNase I amplification grade (1 unit/µg RNA; Invitrogen) to prevent genomic DNA contamination. cDNA synthesis was carried out with Superscript III reverse transcriptase (Invitrogen) and Oligo (dT)18 (Invitrogen) in a 20-µL reaction volume. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and ABI Prism 7500 apparatus (Applied Biosystems). The ABI Sequence Detection System 7000 software (Applied Biosystems) was programmed for *adh8a* with the following cycling conditions: 95ºC for 10 min, followed by 39 cycles at 94ºC for 10 s, then 56ºC during 1 min and 72ºC for 14 s. And for *aldh2* the conditions were: 95ºC for 10 min, followed by 40 cycles at 95ºC for 15s, then 60ºC during 1 min and 72ºC for 30 s. The final volume of the PCR reaction was 20 µl: 5 µl of cDNA, 10 µl of the qPCR Master Mix, and 5 µl of forward and reverse primers with a concentration of 100 nM for *aldh2*, 700 nM for *adh8a* and 500nM for *β-actin,* the reference gen. All samples were run in duplicate. Negative-control reactions containing water instead of cDNA were included in the PCR reactions. The amplification efficiency, specificity of primers and the quantity of cDNA per sample were tested by the standard curve method. Moreover, melting curves were analyzed to verify PCR specificity. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$method (Livak & Schmittgen, 2001). The primers used and their Accession numbers are indicated in Table 1.

Statistical analysis

Data from 10 days of toxicity tests, locomotor activity tests and physicochemical parameters were analysed for normality and homogeneity of variances with Shapiro-Wilk's test and Hartley's test, respectively. Once data passed these tests, they were subjected to a parametric analysis of variance (one-way or two-way ANOVA, $p< 0.05$), followed by *post hoc* Tukey´s test. Data from the toxicity tests was expressed as percentage of amphipod survival. Differences between light and dark periods of locomotor activity were analysed using the t-Student test for comparisons of means. Univariate analyses were carried out with the statistical package SPSS V.19.

Results

Daily rhythm of ethanol toxicity in zebrafish larvae

The mortality rate of zebrafish larvae exposed to 4% ethanol showed a significant daily variation: the highest mortality rate (80%) was observed at ZT2, at the beginning of the photophase, whereas at ZT22 all larvae survived ethanol exposure (Figure 1). Cosinor

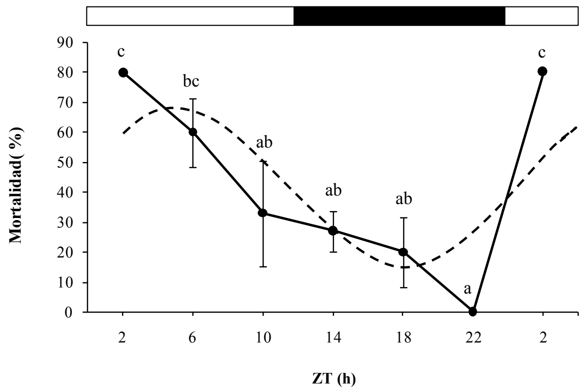

Figure 1. Daily rhythm mortality rate in larvae exposed to 4% ethanol. White and black bars at the top of the graph indicate the light and dark periods, respectively. Data are shown as the mean ±SEM (n= 5). Dotted line represents the sinusoidal function determined by Cosinor analysis. Superscript letters indicate the existence of significant differences between groups (ANOVA I, p>0.05)

analysis revealed a significant daily rhythm of mortality with the acrophase around ML (at ZT = 5:13 h).

Effect of sublethal ethanol concentrations on locomotor activity of adult zebrafish.

The effect of exposure to 1% ethanol on locomotor activity showed daily rhythmicity. Thus, during the light phase the effects were more severe than during the dark phase. At night no effect at all was observed (Fig. 2). At ZT2 fish significantly reduced their activity during 1% ethanol exposure (*t*-Student related samples, p<0.05) (Table 2). Once ethanol exposure had finished and the

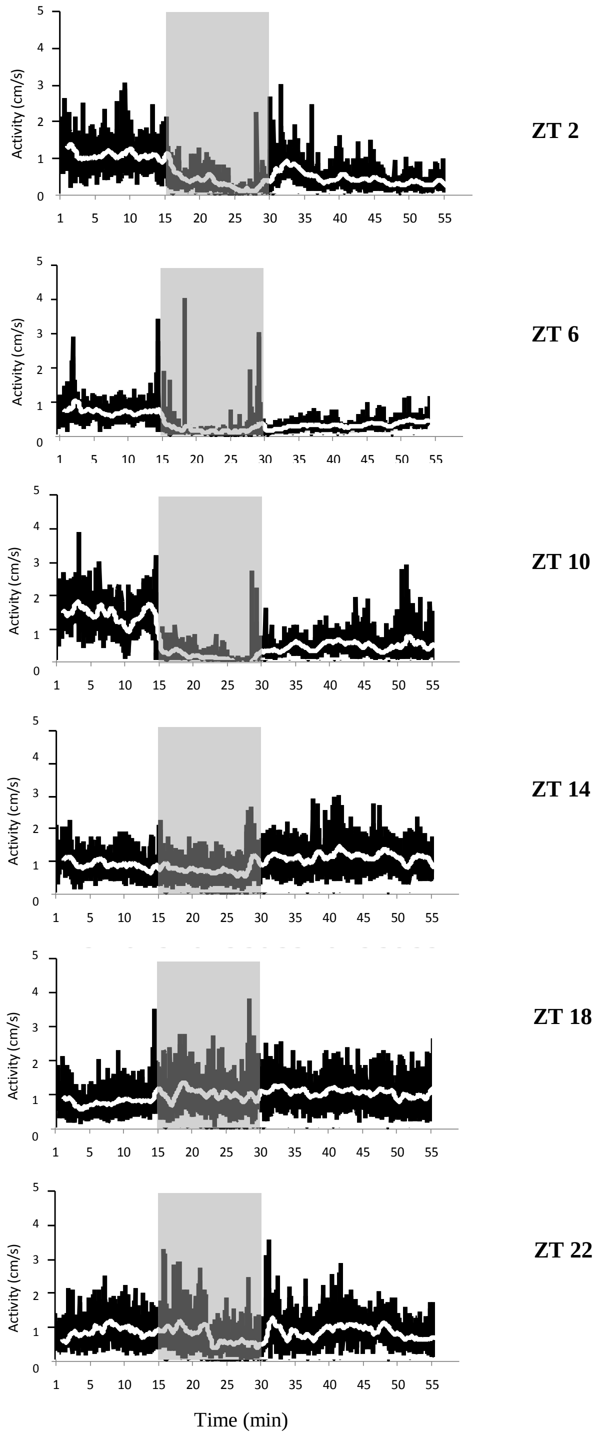

Figure 2. Mean locomotor activity (n=14) before, during and after exposure to 1% ethanol during a 24-h cycle (exposures at ZT2, ZT6, ZT10, ZT14, ZT18, ZT22). Grey boxes indicate the 15 min exposures to ethanol. Black lines indicate the displacement (cm/s) of fish throughout the experiment, whereas the white line represents the moving average of the original data every 60 seconds

fish were placed in clean water, there was an increase of activity during the first 3 min of the recovery period (*t*-Student related samples, $p<0.05$), but then activity levels decreased again andremained low until the end of the trial. At ZT6 and ZT10 fish showed a significant decrease of activity after ~4 and 2 min of exposure, respectively. No recovery was observed when zebrafish were transferred to clean water (*t*-Student related samples $p<0.05$) (Fig. 2).

In contrast, at ZT14 activity levels remained similar to those registered before exposure, whereas at ZT18 fish increased their activity levels during both the exposure and recovery periods (*t*-Student related samples, $p<0.05$). Finally, at ZT 22 fish decreased their activity significantly after 8 min of exposure to ethanol (*t*-Student related samples, p <0.05).Regarding the effect of ethanol exposure on the vertical

Time (min.)					
ZT 2	ZT6	ZT10	ZT14	ZT18	ZT22
3.1 ± 0.6	3.9 ± 0.8	1.9 ± 0.3	–	–	8.0 ± 1.3

Table 2. Time required for the reduction of locomotor activity in zebrafish (min) during ethanol exposure over a 24 h period. Data are expressed as mean ±SEM. Dash symbol (-) indicates no effect on fish activity levels during ethanol exposure.

position of fish, in the water column, at ZT2, 6 and 10, zebrafish swam between 6-10 cm from the bottom of the aquarium prior to ethanol exposure. During exposure movements up and down in the water column were reduced and the fish remained between 5-6 cm above the bottom of the aquarium. After exposure, once locomotor activity had recovered fish resumed swimming up and down. At ZT14,ZT18 and ZT22 (corresponding to the hours of darkness) zebrafish went down to the lower part of the water column during the exposure to ethanol. However, once in clean water, fish return to their initial position, closer to the surface (data not showed).

Gene expression of alcohol and aldehyde metabolizing enzymes

In zebrafish liver the expression of *adh8a* did not statistically differ between ML and MD. However, *aldh2* expression showed statistical differences, expression at MD being almost three-fold higher than that at ML (*t*-student, $p<0.05$)(Figure 3). This test was performed three times in order to confirm the results, and the same differences were obtained each time.

Discussion

Zebrafish has been widely used to investigate ethanol effects at different physiological levels, including developmental, behavioural and toxicological studies. Hence, there is ample evidence that ethanol causes both teratogenic and neurobehavioural effects (Arenzana et al., 2006; Hallare et al., 2006). However, reports focusing on time-dependent effects of xenobiotics are scarce in fish, and current knowledge is mostly based on results obtained at single and arbitrary time points. The present investigation provides the first evidence of daily differences in mortality and behavioural effects of ethanol in zebrafish.

Furthermore, these results are in accordance with those about day/night variations in the expression of genes involved in ethanol detoxification. In zebrafish larvae, our results showed that ethanol exposure resulted in large differences in the mortality rate depending on the time of the day, i.e. exposure to 4% ethanol for 1 h killed 80% of larvae at the beginning of the light phase (ZT2), but none died at the end of the dark phase (ZT22). Similar time-dependent effects were previously observed in toxicity tests carried out with anaesthetics (MS-222 and eugenol) in adult zebrafish. For both compounds, higher mortality rates were reported when fish were exposed during the day than at night (Sánchez-Vázquez et al., 2011). Likewise, in gilthead sea bream (*Sparus aurata*), a marine teleost, MS-222 also showed higher toxicity during daytime (Vera et al., 2010).

Previous studies have shown that acute ethanol exposure alters a number of behavioral responses in adult zebrafish including locomotor activity and

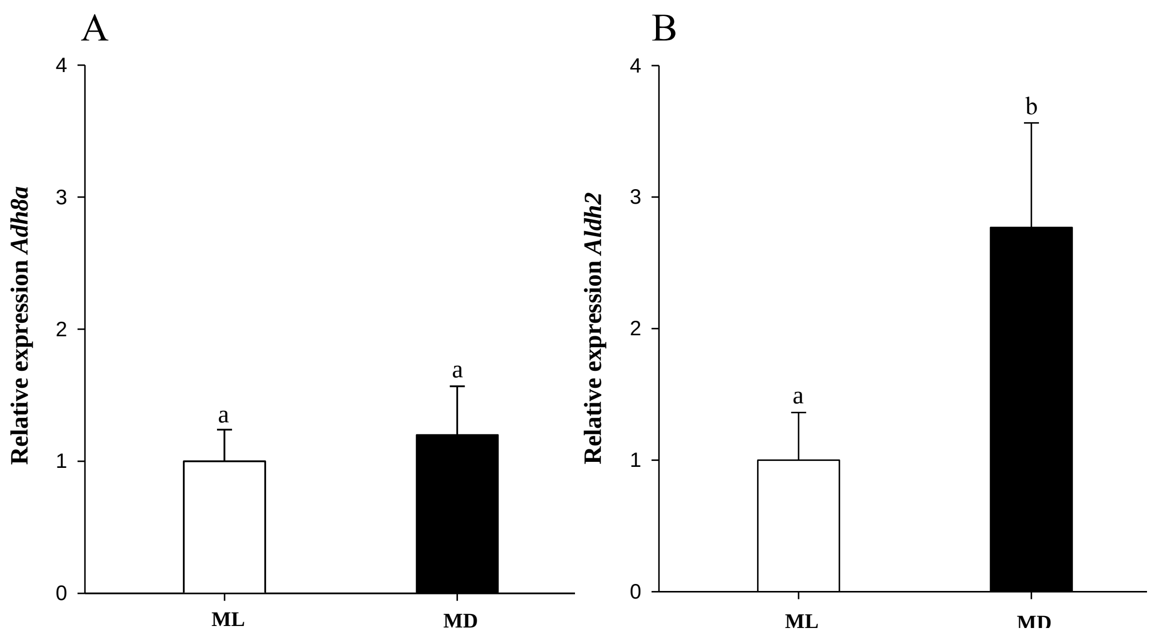

Figure 3. Relative gene expression of *adh8a* (A) and *aldh2* (B) in zebrafish liver at ML and MD. Data are shown as the mean ±SEM (n = 14). Superscript letters indicate significant statistical differences (T-Student, $p<0.05$).

preference for the bottom (Tran and Gerlai, 2013; Pannia et al., 2014). In our behavioral test, adult zebrafish (male and female) were exposed to 1% ethanol for 15 minutes to allow absorption through the fish gills and skin (Dlugos & Rabin, 2003; Gebauer et al., 2011). The results showed that ethanol exposure during the day had a strong effect on zebrafish, which significantly reduced their activity levels. In contrast, during the night no effect was observed on swimming activity. This inhibition of zebrafish activity during ethanol exposure was previously observed by Gerlai and colleagues (2000) and could be due to the sedative effects of ethanol, which causes general slowing and impaired coordination and swimming (Nutt & Peters, 1994) by affecting the central nervous system mechanisms (Gerlai et al., 2000). In addition, fish position in the water column during ethanol exposure was also affected: fish moved downwards and stayed closer to the bottom of the aquarium. Once ethanol exposure ended, the original upper position was resumed, which occurred quicker during the dark phase. These findings agree with previous studies showing daily variations in the behavioral response of zebrafish and gilthead seabream exposed to sublethal concentrations of anaesthetics. In these cases, the greater effects (shorter induction time of anesthesia and longer recovery time) were also observed during the day, coinciding with the active phase of the animals (Vera et al., 2010; Sánchez-Vázquez et al., 2011). The fact that in zebrafish both ethanol and anaesthetics toxicity was higher during the active phase of fish suggests a link between the daily rhythms of activity and toxicity. Furthermore, a recent paper reported that feeding time (a single daily meal provided at ML or MD) can shift the behavior of fish and their daily rhythms of anesthetics toxicity, so that fish fed at MD became nocturnal and had larger effects when exposed at night (Vera et al. 2013). Similarly, in mammals, toxicity also appears to be higher during the active phase of the animal (i.e. at night in nocturnal rodents) (Spanagel et al., 2005). Taken altogether, the daily differences in toxicity reported in the present study might be the result of the existence of daily rhythmicity at different physiological levels- from the absorption, distribution and excretion mechanisms to the metabolizing pathways (Hooven et al., 2009). In zebrafish liver the ethanol-metabolizing machinery is similar to the system described in mammals involving the enzymes ADH and ALDH (Dasmahapatra et al., 2001; Tsedensodnom et al., 2013). As recently suggested by Tran et al. (2015), behavioral response to ethanol may be accompanied by changes in enzyme activity and gene expression in the zebrafish organs (Rico et al., 2008; Rosemberg et al., 2010). However, despite ethanol metabolism has been widely investigated, the existence of temporal variations in the enzymes involved in this process had not been considered so far, although there is evidence of circadian rhythms in several antioxidant enzymes in mammals, such as glutathione S-transferase (GST), glutathione peroxidase (GP) and superoxide dismutase (SOD) (Inoue et al., 1999; Cao et al., 2015). In the present study we have observed day/night differences in the expression of *aldh2*, an enzyme involved in the metabolism of ethanol, but not in *adh8a* (Fig. 3). This fact might be related to the temporal availability of enzymes involved in catalytic oxidation. Hence, the levels of *adh8a* gene expression did not show differences between ML and MD, whereas the expression of *aldh2* was around three-fold higher in MD. These results support the lighter effects of ethanol during the dark phase, as observed in the mortality and behavioural tests. Actually, in zebrafish embryos the product of ethanol metabolism by alcohol dehydrogenase (acetaldehyde) is more lethal than ethanol itself (Reimers et al., 2004). Acetaldehyde is metabolized by aldehyde dehydrogenases, a superfamily of $NAD(P)^+$ dependent enzymes that catalyze the oxidation of a wide variety of endogenous and exogenous aldehydes to their corresponding carboxylic acids (Vasiliou et al., 1999). It is well accepted that acetaldehyde mediates ethanol effects (Israel et al., 1994; Deitrich, 2004). In fact, it is possible that acetaldehyde may potentiate rather than diminish some of the effects of ethanol. In humans, *aldh2* plays a key role in acetaldehyde metabolism (Ramchandani et al., 2001). Thus, a low-activity of *aldh2* leads to alcohol sensitivity reaction. Moreover, a recent study in zebrafish has shown that acute exposure to 1% ethanol decreased *aldh2* activity in liver (Tran et al., 2015). Our gene expression results support the hypothesis that acetaldehyde may be responsible for some of the noxious effects observed in the mortality and behavioural tests, since *adh8a* expression did not differ between day and night but the effects of ethanol exposure were more marked during the day.

In conclusion, this study revealed the existence of a daily rhythm of ethanol toxicity in zebrafish, which is reflected by higher mortality rates and behavioural effects during the day than at night. This fact should be considered to determine the dose–response relationship when calculating ethanol LD50 and other mortality indexes in this species. Furthermore, these results should also be taken into account when designing new neurobehavioural studies. Not only the time of exposure should be controlled but also the light regime under laboratory conditions, in order to produce predictable and reproducible responses, which can be key when assessing developmental toxicity endpoints in the study of deleterious effects of ethanol.

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CHAPTER II

Cadmium chronotoxicity in zebrafish *(Danio rerio)*: mortality rate, behavioral responses, Cd accumulation and detoxification depends on time-of-day.

Introduction

Cadmium (Cd) is a non-essential metal with a wide distribution. This metal is an extremely toxic element of rising concern due to the continuous increase of its levels in the environment as a consequence of the anthropogenic mobilization(Goering et al., 1995; Nriagu and Pacyna, 1988). In the aquatic environment, Cd is a pollutant with a wide range of ecological and physiological effects (WHO, 1992). When exposed to sublethal levels of Cd, aquatic animals show haematological alterations, impaired calcium homeostasis as well as histological and morphological changes in ion-regulating tissues such a kidney, gills and intestine (Besirovic et al., 2010). In fish, Cd is slowly accumulated and its main target organs are kidney and liver, where several enzyme systems areaffected (Wright &Welbourn, 1994), being these effects severe even when fish are exposed to low concentrations.The effect of Cd on fish gill, as well as its sensitivity to metal, differs from species to species and probably from organ to organ (De Smet et al., 2001).

In fish, daily variations in Cd toxicity have not been investigated, although previous observations in mice models suggested that Cd was better tolerated in the evening hours (Cambar et al., 1983).Actually in mammals, several studies reported the existence of diurnal oscillations in xenobiotics absorption, distribution, metabolism, and excretion (Paschos et al., 2010). Even in insect, some studies have also provided evidence that the effects of organophosphate, organochlorine, and pyrethroid pesticides in various species vary significantly with the time of day at which they are applied with concentrations resulting in 50% of mortality differing significantly during the day (Sullivan et al., 1970; N.M. EESA, 1995; Pszczolkowski&Dobrowolski, 1999; Hooven et al., 2009). Since fish can be considered rhythmic physiological systems, it is not surprising that their responses to xenobiotic exposure at different times of the day are equally rhythmic and therefore, contaminants toxicity for a given concentration might be time-dependent.In fact, previous studies on anaesthetics chronotoxicity in gilthead seabream and zebrafish have showed that both toxicity and effectiveness are significantly higher when fish are exposed during the day than at night (Vera et al., 2010; Sánchez-Vázquez et al., 2011). Such day/night variations seem to be related to greater uptake of anesthetics during the day, coinciding with the active phase of fish (Vera et al. 2013).

Metallothioneins (MTs) are metal-binding proteins which play an essential biological role in metal homeostasis, detoxification and cytoprotection(Kagi& Schaffer, 1988). Two isoforms have been characterized in zebrafish, MT1 and MT2. They are present in most tissues (Gonzalez et al., 2006) and are particularly sensitive to Cd (Kagi& Schaffer, 1988). However, the existence of daily variations in the expression of these enzymes has not been investigated either.

Zebrafish (*Danio rerio*) has become a popular model in aquatic toxicology (Nagel, 2002; Hill et al., 2005) and has been used as a model to study Cd effects on gene expression (Long et al., 2011). In this research we investigated the existence of daily variations of Cd toxicity in zebrafish. To achieve this purpose we studied the existence of day-night differences in (1) the acute response of zebrafish to a lethal concentration of Cd (100 mg/l), (2) the effect of a sublethal concentration of Cd (40 mg/l) on locomotor activity, (3) the accumulation of Cd in liver and gills and (4) the daily expression rhythms of metallothionein genes (*mt1* and *mt2*).

Material and methods

Animals and housing

Adult wild-type zebrafish (standard short-fin phenotype) of 38±2 mm body length were used for this study. The

Gene	Accession number	F/R	Primer sequence (5'- 3')
mt1	X97278	F	CGTCTAACAAAGGCTAAAGAGGGA
		R	GCAGCAGTACAAATCAGTGCATC
mt2	AY305851	F	GGAGGAGGGTCAGAGGAACC
		R	AGCAACTGAAGCTCCATCCG
β-actin	AY222742.1	F	AAGCAGGAGTACGATGAGTC
		R	TGGAGTCCTCAGATGCATTG

Table 1. Accession numbers and specific primer pairs for the *D. rerio* genes used in our study. Abbreviations: *mt1* – alcohol dehydrogenase; *mt2*– aldehyde dehydrogennase; *β-actin* – Beta actin. F: Forward primer, R: Upstream primer.

experimental fish were purchased from a local provider (Jumipez SL, Murcia, Spain). The experiments were carried out in the Chronobiology Laboratory at the Faculty of Biology of the University of Murcia. Fish were kept in two 60 L glass aquaria whichwere filled with chlorine-free water that was continuously oxygenated and filtered. The animals were isolated from the external environmental conditions in a chronolab chamber and subjected to a 12 h light: 12 h darkness (LD) photoperiod and controlled temperature (28 ± 0.5 ºC) throughout the experiment. Illumination was provided by fluorescent tubes(F15W/GRO, Sylvania Gro-Lux, Germany), reaching an intensity of approximately 700 lux at the water surface. Fish were fed ad libitum once a day at random times with a commercial food (PRODAC,Tropical fish flake. Casone, Parma (Italy)). The experiments performed in the present research followed Spanish legislation on Animal Welfare and Laboratory Practices and was approved by both the National Committee on Animal Welfare and the Bioethics Committee of the University of Murcia.

Experimental design

First of all, the existence of a daily activity rhythm was checked. To this end, locomotor activity of the experimental fish was recorded by infrared (IR) photocells that were connected to a computer. Every time a fish interrupted the IR light beam it produced an output signal that was recorded and stored in 10 min bins, for which specialised software was used (DIO98USB, University of Murcia, Spain). The following 4 trials were designed to assess chronotoxicity of Cd:

Exp. 1.Acute toxicity test: mortality rate.

In this experiment, the mortality rate caused by $CdCl_2$ (100 mg/l) at mid-light (ML) or mid-darkness (MD) was investigated. Fish were placed into a 1 L glass jar (n=8) containing chlorine-free water plus the corresponding amount of Cd.Exposures to Cd were carried out for 3 h, according to Hirt and Domitrovic protocol (2002).Water was aerated during the test and temperature kept constant at 26ºC. After exposure, fish were transferred to new jars containing clean water. The mortality rate was recorded immediately after exposure (0

h) and 24 h later (+ 24 h). A control test was also performed keeping fish in the same conditions but without Cd.

Exp. 2.Behavioral response.

To study the response of fish to sublethal concentrations during the day or at night, locomotor activity was recorded in zebrafish exposed to 40 mg/l of Cd at ML and MD. To this end, a 6L aquarium was divided into 7 compartments with methacrylate panels which were perforated to allow water circulation. This aquarium was placed inside another 10 L glass tank (30cm x 20cm x 18cm). At ML, illumination was provided by a fluorescent tube (F15W/GRO, Sylvania Gro-Lux, Germany), whereas at MD infrared LED lamps were used (LED monochrome, L - 53F3BT, 5 mm), which allowed the video recording while fish did not perceive any light. Locomotor activity of fish during the assays was recorded with a video camera provided with a "Nightshot Plus" option, which enables to record at night (SONY, Handycam, DCR-SR55).

Exp.3. Cd accumulation in zebrafish.

Fish were exposed to 10 mg/l, 20mg/l and 80 mg/l of $CdCl_2$ during 3 h at ML and MD, following the same protocol than for the acute toxicity test.A control group (not exposed to Cd) was also included in the experiment. At the end of each exposure, fish were transferred to another jar containing clean water. Zebrafish were then euthanized with an overdose of anaesthetic and dissected on ice. The surgical material was cleaned with nitric acid (2%) and then rinsed with distilled water, before being used and between individuals. The accumulation of Cd was measured in the whole fish using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES), which was performed by a local laboratory (Fitosoil©, Murcia, Spain), according to (CE) Nº 333/2007. The analyses were done in triplicate on each sample and the mean values calculated. Two acid blanks were also run along with each batch of samples. Blank solutions were prepared in the same way as the samples.

Exp.4 Daily rhythm of metallothionein expression (mt1 and mt2).

To examine the daily rhythm of two metallothionein genes involved in Cd detoxification (*mt1* and *mt2*), a total of

36 zebrafish were reared under an LD cycle for 10 days. Then, after checking that fish activity was synchronized to the LD cycle, zebrafish were fasted for one day and then euthanized and gills samples taken every 4 h during a 24 h period time, at "Zeitgeber Times" ZT2 (2 h after lights onset), 6, 10, 14, 18 and 22. For gene expression analyses, tissue samples were homogenized in Trizol reagent (Invitrogen) using a tissue homogenizer (POLYTRON®, PT1200; Kinematica, Lucerne, Switzerland). Total RNA concentration was determined by spectrometry (Nanodrop® ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA), and 1 µg was treated with DNase I amplification grade (1 unit/µg RNA; Invitrogen) to prevent genomic DNA contamination. cDNA synthesis was carried out with Superscript III reverse transcriptase (Invitrogen) and Oligo (dT)18 (Invitrogen) in a 20µL reaction volume. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and ABI Prism 7500 apparatus (Applied Biosystems). The ABI Sequence Detection System 7000 software (Applied Biosystems) was programmed for *mt1*and *mt2* with the following cycling conditions setup: 95ºC for 3 min, followed by 40 cycles at 95ºC for 15 s, then 60ºC during 45 s and 72ºC for 30 s. The final volume of the PCR reaction was 20 µl: 5 µl of cDNA, 10 µl of the qPCR Master Mix, and 5 µl of forward and reverse primers with a concentration of 500nM for *mt1* and *mt2* and 200nM for *β-actin* (Table 1). All samples were run in duplicate. Negative control reactions containing water instead of cDNA were included in the PCR reactions. The amplification efficiency, specificity of primers and the quantity of cDNA per sample were tested by the standard curve method. Moreover, melting curves were analyzed to verify PCR specificity. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$method (Livak&Schmittgen, 2001) using *β-actin* as the endogenous reference.

Data analysis.

Locomotor activity displayed by fish before the experiments and recorded by IR photocells was analysed by a Chronobiology software (El Temps® v. 179; Prof.Díez-Noguera, Barcelona).A two-way ANOVA (ANOVA II) was used to determine the existence of significant differences in the mortality rate, being the fixed factors the "time of observation" (T0 -at the end of the exposure- and T24 -24 h later) and the "time of day" in which the exposure was carried out (ML or MD), followed by t-test independent samples to compare mortality between ML and MD in both T0 and T24. In addition, differences in ML between T0 and T24 and in MD again between T0 and T24 were checked with a paired-samples-test.The video-recordings were analysed with specialised software (Fish Tracker; G. Ros-Sánchez, University of Murcia). This software tracked the swimming activity of fish and provided their position (X, Y) every second, generating a file that was exported to Excel for further analysis and graphics creation. To compare locomotor activity levels of fish before and during Cd exposures, a paired-samples *t*-test was performed. In addition, differences between average activity levels at ML and MD were checked with a t-test independent samples.

A t-Student test for independent samples was performed to determine the existence of significant differences in the Cd accumulation between fish exposed at ML and MD. Statistical differences in metallothionein gene expression levels between different sampling times were analyzed by a one-way ANOVA, followed by Tukey's post hoc test. Data analysis and statistical tests were carried out with Microsoft Excel and SPSS v16.0 programme (SPSS Inc., USA), respectively. The statistical threshold was set at p values < 0.05 in all tests.

Results

In the present study zebrafish showed diurnal behaviour, with 76 % of their locomotor activity being displayed during the day. Fish started their activity once lights were on and remained active during the day until lights off, when their activity decreased sharply. The activity level remained high during the first part of the day and decreased gradually towards the end of the day(data not shown).

Exp. 1. Acute toxicity test

At both ML and MD, the mortality rate after 24 hours (T24) of exposure to Cd was significantly higher (paired-samples t-test, $p<0.05$) than the mortality rate observed at the end of the exposure (T0). Furthermore, immediately after the exposure to Cd, no significant differences were found between the mortality rates recorded at ML and MD (~ 3-5%), whereas at +24h this rate was significantly higher when the fish had been exposed to Cd at ML (77.1 ± 7.5%) than at MD (33.3 ± 8.3%) (Fig.1).

Exp.2. Behavioural response

Video-recording under control conditions at ML and MD showed that mean levels of activity were significantly higher during the day (0.55 ± 0.03 cm/s) than at night (0.22±0.02cm/s) (t-Student independent samples, $p<0.05$).

At ML, a significant peak of activity (1.40 cm/s) was observed immediately following Cd exposure, being these levels significantly higher than basal activity observed before the assay. Then, during the first hour of exposure, fish activity decreased significantly to values around 0.10 cm/s (paired-samples t-test $p<0.05$) (Fig. 2A). Regarding fish position, at ML fish swam constantly changing their position (8-14 cm depth)

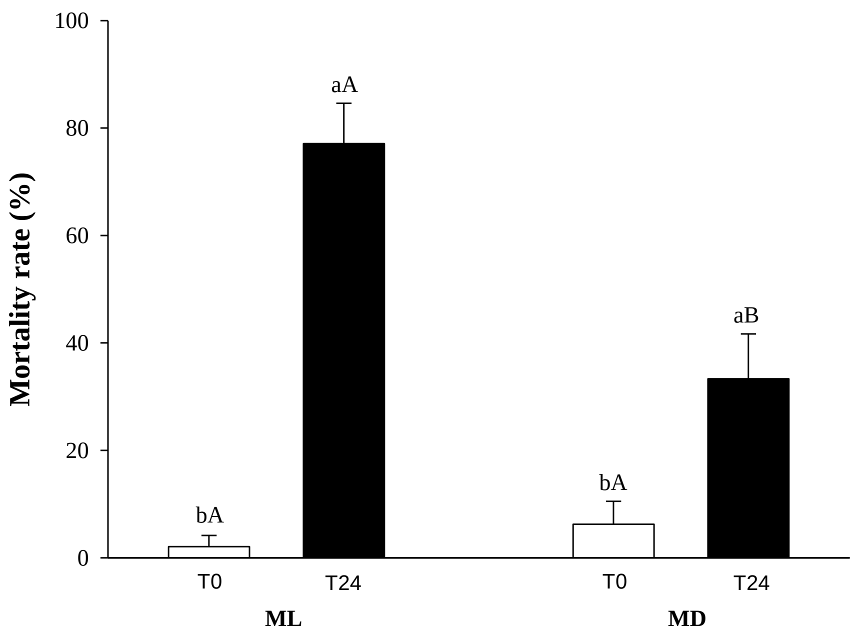

Fig. 1. Mortality rate (%) at ML and MD at the end of Cd exposure (T0, white bars) and 24 hours later (T24, black bars). Superscript lower case letters indicate significant differences between times of observation for the same experimental group (ML or MD) (paired-samples t-test $p<0.05$). Capital letters indicate significant differences between experimental groups for the same observation time (T0 and T24) (t-test independent samples, $p<0.05$).

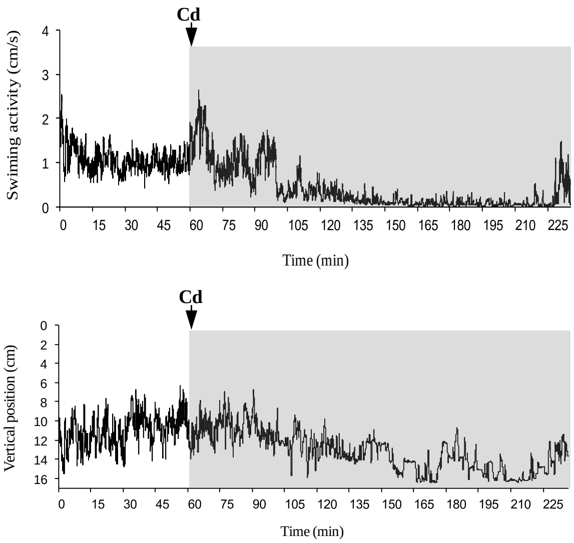

Fig 2. Average locomotor activity (A) and water column position (B) of zebrafish exposed to Cd and filmed at ML (n=7). The black lines represent the mobile average of the original data .

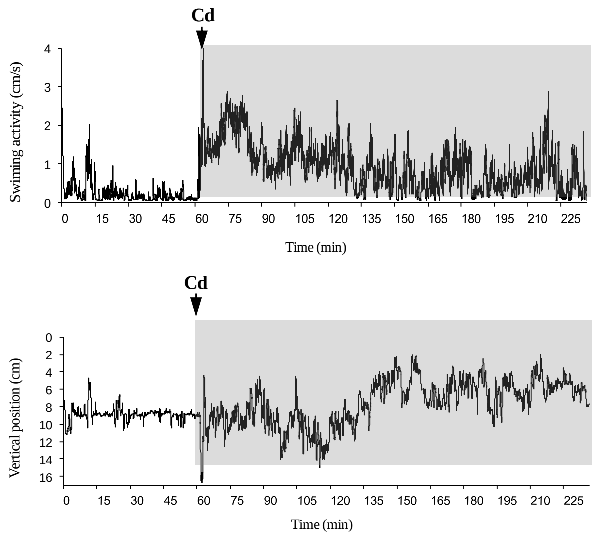

Fig 3. Average locomotor activity (A)and water column position (B) of zebrafish exposed to Cd and filmed at MD (n=7). The black lines represent the mobile average of the original data.

before Cd exposure. However 1 h after Cd was added, zebrafish remained at the bottom of the aquarium (Fig. 2B).At MD, fish activity during the pre-exposure period remained low and stable, between 0.2 and 0.4 cm/s. Once Cd was added to the aquarium there was a significant peak of activity (1.7 cm/s) (paired-samples *t*-test $p<0.05$) but during the following hours gradually decreased and stabilized around 0.8 cm/s (Fig. 3A). However, the average activity levels displayed at MD after Cd exposure were significantly higher than those observed at ML: 0.93 ± 0.01 cm/s against 0.39 ± 0.01 cm/s (t-Student independent samples, $p<0.05$). Regarding fish position, during the hour prior to the exposure, the position of the fish was between 8 and 10 cm depth, but during Cd exposure strong oscillations occurred along the entire water column. After approximately half an hour of exposure, the fish moved closer to the water surface, changing the position between 2 and 8 cm depth (Fig. 3B).

Exp.3. Cd accumulation in zebrafish gills

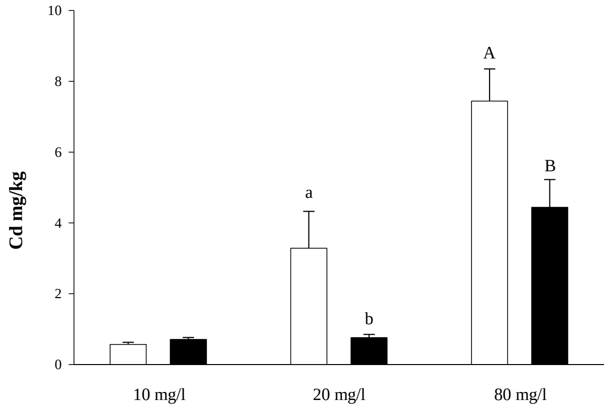

Fig. 4. Cd accumulation in the whole fish at ML (white bars) and MD (black bars) after 3 h of exposure. Superscript lower case letters indicate significant differences between hours of exposition for 20 mg/l Cd concentration (independent samples t-student test, $p < 0.05$). Capital letters indicate significant differences between hours of exposition for 80 mg/l Cd concentration (independent samples t-student test, $p < 0.05$).

The accumulation levels of Cd after exposure to 20mg/l and 80mg/l of $CdCl_2$ were significantly higher at ML than at MD (t-Student, $p<0.05$). Thus, when fish were exposed to 20mg/l, Cd levels in fish gills were 3.28 ± 1.04 mg/kg at ML and 0.76± 0.09 mg/kg at MD, whereas after exposure to 80mg/l Cd levels at ML and MD were 7.44± 0.90mg/kg and 4.44 ± 0.78mg/kg, respectively.In control fish, Cd accumulation was<0.025 mg/kg for both groups (ML and MD). When zebrafish were exposed to 10 mg/l of $CdCl_2$no significant differences were found at ML and MD (0.56 mg/kg and 0.71 mg/kg, respectively)(t-Student independent samples, $p>0.05$).

Exp.4.Daily rhythm of metallothioneins expression (mt1 and mt2)

The expression of *mt1* showed daily variations with a peak located at the end of the light phase. Statistical analysis revealed the existence of significant differences in *mt1* expression between ZT10 and the rest of the sampling points (ANOVA I, $p<0.05$). The expression pattern of *mt1* did not fit a sinusoidal function (Cosinor, $p>0.025$)(Fig. 5). As regards *mt2,*constant expression was observed along the day (ANOVA I, $p>0.05$) with no statistical differences among time-points (data not showed).

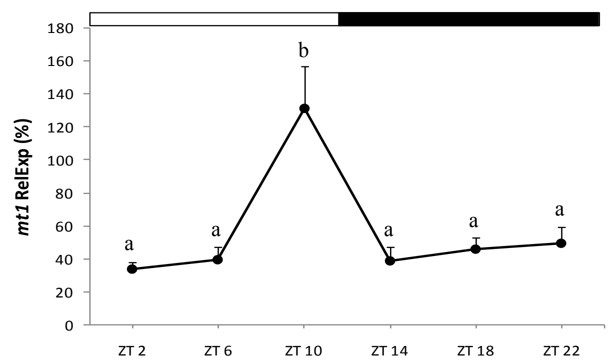

Fig.5. Relative expression of *mt1*gene in zebrafish gills. The white and black bars at the top of the graph indicate the light and dark periods, respectively. Data are shown as the mean ± S.E.M. (n=6). Superscript letters indicate statistically significant differences between sampling points (ANOVA I, $p<0.05$).

Discussion

Our results revealed that both acute lethal toxicity and sublethal effect of Cd in zebrafish differed depending on the time-of-day of exposure. In the toxicity test fish were exposed for 3 h to a Cd concentration of 100 mg/l, the mortality rate 24 h later being significantly higher at ML (77.1%) than at MD (33.3%), suggesting that fish presented more severe damages resulting in a higher mortality rate when they were exposed at ML, corresponding to their active phase. These differential effects are in accordance with recent studies conducted on gilthead seabream and zebrafish, which showed the existence of day/night variations in the toxicity of two anaesthetics (Vera et al., 2010; Sánchez-Vázquez et al., 2011). In these studies, maximum toxicity also occurred during the active phase of fish. Our results showed that a Cd concentration of 40 mg/l did not cause fish mortality but affected their activity patterns. Thus, in both ML and MD, a peak of activity was observed at the beginning of the exposure. This increase of activity corresponds to a typical escape response, where the organism tries to avoid the area impacted by a chemical(Smith E.H., 1988), and might be due to irritation of the fish skin and gills when they get in contact with Cd. Most interestingly, the effect of Cd on locomotor activity during the following hours depended on the time of exposure. In ML fish showed a progressive reduction of activity and after 90 min, swimming was lethargic and fish showed a loss of equilibrium. This behaviour has already been observed in zebrafish exposed to $CdCl_2$ in acute and subacute studies (Karlssonnorrgren et al., 1985) and may reflect the existence of avoidance behaviour, which would reduce the probability of death or metabolic costs invested in maintaining physiological homeostasis(Olla B.L. & Pearson W.H., 1980; Schreck C.B. et al., 1997). On the contrary, in zebrafish exposed to Cd in MD there was an increase of activity which was maintained until 75 minutes of exposure; after this time the activity levels slightly decreased. In addition, fish moved to the water surface, probably due to suffocation caused by the precipitation of mucoprotein coagulators on the gills, which are produced as a defence mechanism but block gas exchange, excretion of waste products and osmoregulation (Tafanelli, R & R.C. Summerfelt, 1975). Many studies have shown that lower concentrations of toxic substances can induce increased swimming activity, while higher concentrations can lead to a decrease in activity(Little et al., 1990). Our behavioural results showed that for the same concentration of $CdCl_2$, the response in ML is similar to that corresponding to exposures with higher concentrations of the contaminant, indicating that zebrafish is more vulnerable to exposure to Cd in ML than in MD, in accordance with the mortality results obtained with lethal doses.

Previous studies using *Gammarus* (a marine amphipod) as a biomarker, also showed that the effect of Cd exposure on locomotor activity was higher at night in this nocturnal animal (Morillo-Velarde et al., 2011). Since zebrafish showed a diurnal activity pattern, Cd absorption through the gills might have also been higher at ML. Thus, for a given concentration, toxicity would be greater during the day since not only swimming activity would be higher but also the respiration and metabolic activities, and as a result, zebrafish would show a greater tolerance to Cd during the night.Whatever the exposure method, Cd accumulates significantly in gills, liver, and kidney (Handy, 1992). Moreover Cd accumulation in tissues of fish depends on factors such as temperature, water hardness, interacting agents(Ay et al., 1999; Saglam et al., 2013), exposure concentration and period(Ay et al., 1999) and according with our results it might be also depending on the time of the day. Cd accumulation in the whole fishincreased with concentration as it was expected and in accordance with many studies showing a linear correlation between metal accumulation and concentration(Arini et al., 2014). However, there are no data related to the daily variations in metal accumulation in fish. Our results showed a highest Cd content in fish exposed at ML for concentrations of 20mg/l and 80mg/l. This fact suggests a higher uptake of Cd from the water during the day and agrees with higher mortality rates and behavioural effects found at ML, supportingthe hypothesis that daily rhythmicity in activity patterns may influence the day/night differences in Cd toxicity.

Metallothioneins (MTs) are enzymes with metal-binding capacity that may also have an effect on the daily variation of Cd accumulation in zebrafish. It is known that biological defense factors showdiurnal variations in the expression levels or their activities. For instance, expression of hepatic drug-metabolizing genes (Zhang et al., 2009) and genes encoding for antioxidant molecules such as glutathion (Xu et al., 2012) present a daily variation in mouse liver. In fact, microarray experiments have provided analysis of 24-h transcriptome regulation in multiple tissues of mammalian species, revealing that approximately 5-10% of gene expression shows daily rhythms, including genes encoding detoxification enzymes (Johnston, 2012). MTs constitute a family or cysteine-rich proteins with important roles such as metal sequestration and detoxification of non-essential metals,such as Cd and Hg (Roesijadi, 1982; van der Oost et al., 2003). Two isoforms for MT genes have been described in zebrafish, *mt1* and *mt2*.Both isoforms are upregulated by metal exposure and *mt1* expression is particularly enhanced by Cd (2015). Gills are the major site of metalsuptake. Therefore,the mechanisms for heavy metals detoxification are present in this tissue (Arini et al., 2015). Our results showed a peak in *mt1*expression at ZT10, just before the light-dark transition. In contrast the expression of *mt2* did not present daily variations. MT1 would bind Cd thereby reducing its toxic effects to zebrafish.This correlation between MTmRNA and MTsproteins in non-exposed fish has been also observed in rainbow trout*Salmogairdneri*and stone loach *Noemacheilus barbatulus* where Norey and collaborators(1990) considered the possibility of a higher resistance of *N.barbatulus*to incoming Cd due to the presence of existing MTs proteins (Norey et al., 1990).Later studies in other fish species have also reported the existence of MTs in individuals non exposed to heavy metals(Kalman et al., 2010;Woo et al., 2006). Furthermore, in rainbow trout gills, the induction of MT gene expression resulted in a gradual increase in MT concentration over the course of a contamination experiment (Norey et al., 1990), indicating acorrelation between gene expression levels and protein availability. In zebrafish, Cd contamination resulted in a strong induction of genes involved in oxidative stress defense, detoxification processes, mitochondrial metabolism, apoptosis and DNA repair (Arini et al., 2014). Heavy metals without redox potential, such as Cd, cause oxidative damage by impairing antioxidant defenses, particularly those antioxidants and enzymes containing thiol groups (Stohs & Bagchi, 1995). Cd can alter reduced glutathione (GSH) levels and affect cell thiol status, inducing the expression of MTs. In addition, Cd can influence antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Sevcikova et al., 2011). Therefore, Cd detoxification is a complex process involving several mechanisms. Furthermore, additionalresearch is required to understand how the circadian clock controls metal kinetics (absorption, distribution, metabolism, and excretion) and dynamics in fish species.

To conclude, in this paper we have found day/night differences in the response of zebrafish to Cd exposure. These differences were reflected by increased mortality and tissue Cd accumulation at ML, as well as a greater reduction in locomotor activity when fish were exposed to a sublethal concentration at this time of the day. Furthermore, the expression of *mt1*showed a peak at the end of the light phase. Altogether, our results indicate that zebrafish is more vulnerable to Cd at ML than at MD, highlighting the importance of considering toxicity rhythm when studying the effects of pollutants.

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CHAPTER III

Behavioural responses of the amphipod *Gammarus chevreuxi* to mercury contaminated sediments

Introduction

In recent years, and following implementation of the European Water Framework Directive, there has been great concern about the environmental quality and ecological condition of European aquatic ecosystems, including coastal and transitional systems such as coastal lagoons. The studies of the impact of priority substances, such as mercury, on marine ecosystems are of extreme importance due to their high toxicity and the possibility of bioaccumulation along trophic chains (Chen et al. 2012; Oken et al. 2012). Traditionally, toxicity assessments have been carried out through the application of bioassays which consider test organism mortality as an end-point (Fay et al. 2002). However, in most cases, potentially toxic substances or elements are present in the environment at sublethal concentrations (with the exception of extremely polluted areas), meaning that sublethal toxicity bioassays (i.e., changes in reproductive output, behavioural changes, etc) might be a more useful tool for impact assessment.

Amphipods are considered key species in coastal food chains, not only due to their abundances in these ecosystems and their role as active herbivores and detritus consumers, but also as a major food source for higher trophic levels (Matthews et al. 1992). Moreover, amphipods are optimal model species for toxicity assessment in laboratory bioassays because of their ubiquitous distribution, sensitivity to disturbance, and suitability for culture and experimentation (Neuparth et al. 2002; Prato and Biandolino 2005). As for other organisms, amphipod behavioural responses are linked to complex biochemical and physiologic changes and act as sensitive indicators of potentially toxic substances' sublethal effects. Although mortality is often the end-point used in bioassays, addressing sublethal toxicity (i.e. behavioural changes) is perhaps more useful, as mortality does not always occur in organisms exposed to potentially toxic substances' in natural settings (Wallace and Estephan 2004). Recent studies on the possible use of behavioural responses as ‘‘early warning signals’’ to contaminant exposure (i.e., presence of elevated concentrations of substances/elements in the environment above the natural background level for the area and for the organism) have demonstrated that the presence of trace metals in seawater affects the swimming behaviour of the amphipods *Gammarus lawrencianus* (Wallace and Estephan 2004), *G. pulex* (Mills et al. 2006; De Lange et al. 2006; Felten et al. 2008) and *G. aequicauda* (Morillo-Velarde et al. 2011). Similar studies carried out on contaminated sediments reported behavioural changes in *Talitrus saltator* (Ugolini et al. 2004, 2012) and *Corophium volutator* (Kirkpatrick et al. 2006).

Contaminants can therefore interfere with amphipod locomotion, which is required by animals to find food, avoid predation, and mate. This interference may decrease the fitness of the affected organisms in their natural environment and have the potential to cause severe impacts on entire populations (Mills et al. 2006).

To date, little attention has been paid to the possible interaction between observed behavioural responses to pollutants and the daily activity rhythms of marine organisms, including amphipods. In a recent study, Morillo-Velarde et al. (2011) demonstrated that the amphipod *G. aequicauda* shows circadian activity rhythms with nocturnal peaks, which implies that similar circadian activity rhythms might occur in other amphipods.These authors also proved that amphipod locomotor responses to toxicants in *G. equicauda* differs depending on the time of the day, as the effects were only measurable during the period of amphipod's maximum activity (Morillo-Velarde et al. 2011).

The purpose of this study was to determine if exposure to sediments historically contaminated with mercury alters the daily locomotor activity of *Gammarus chevreuxi* (Sexton, 1913), using the Ria de Aveiro coastal lagoon mercury-contamination gradient. This system was subjected to mercury discharges from a chlor-alkali plant located in the Estarreja industrial complex from the 1950s until 1994, what created an environmental contamination gradient along a 2 Km^2 inner basin (Laranjo) (Pereira et al. 2009). Afterwards, industrial discharges followed EU regulatory levels, although mercury concentrations in the surface sediments of Laranjo Basin are still higher than pre-industrial levels (Pereira et al. 2009).The industrial activity resulted in the accumulation of about 33 t of mercury in the lagoon, from which 82% remained in the Laranjo Basin entirely in the particulate fraction, i.e. sediment-associated(e.g. Pereira et al. 1997). The spatial mercury gradient along Laranjo Basin was reflected in the bioaccumulation patterns of zooplankton communities and benthic organisms (Abreu et al. 2000; Coelho et al. 2005; Gonçalves et al. 2013). Outside the Laranjo Basin, mercury levels are much lower and below the European threshold concentration for fish and seafood consumption (Pereira et al. 2009)

Under controlled laboratory conditions, we exposed *G. chevreuxi* specimens to field sediment samples from the mercury contamination gradient in Ria de Aveiro lagoon. The euryhaline amphipod *G. chevreuxi* was selected as a model-species due to its abundance in the intertidal habitats of costal lagoons and estuaries in European Atlantic coasts (Dexter DM 1992), including Ria de Aveiro (Cunha and Moreira 1995). Since amphipods constitute a vital component in coastal food chains, contaminant-induced behavioural changes in *G. chevreuxi* could have important ecological consequences. By means of video analysis, we followed the movement of individual amphipods and quantified changes in their light/dark dynamics along the day.

Materials and Methods

Sample and animal collection, acclimation and experimental conditions

The present study was conducted with sediment, water and specimens collected in Ria de Aveiro, a temperate shallow coastal lagoon (45 km-length; 10 km-wide) located in the northwestern Atlantic coastof Portugal (40º38'N, 8º 44'W). Within this system, the Laranjo Basin (Fig. 1) is a shallowinner area of 2 km^2 that has been historically contaminated with mercury (Pereira et al. 2009). This inner basin presents a mercury contamination gradient that can be seen as a natural laboratory for mercury exposure studies. Within the Laranjo contamination gradient, three sampling locations were selected, from the most contaminated, Site 1 (3.48km)(Fig. 1), followed by Site 2 (4.74 km), Site 3 (5.80 km) and one reference site located outside the Laranjo Basin at the Mira channel (Fig. 1), The Mira channel has been used as a reference site in previous sediment toxicity studies (Castro et al. 2006).

Three composite sediment samples were collected at each of the four sampling locations. Sediment samples were collected using hand held polycarbonate cores. The top 5 cm of sediment cores collected at each station were stored in 0.5L polyethylene jars, and then transported on ice in the dark to the laboratory. Before setting up the experiment, sediments were homogenized and sieved, removing large pieces of debris and potential predators. All containers used for collecting and storing the sediments were previously washed with Hg-free nitric acid (10%).

Homogenized sediment sub-samples were air-dried and analysed for total mercury content by thermal decomposition atomic absorption spectrometry with gold amalgamation, using a LECO AMA-254 (Advanced Mercury Analyzer). All mercury quantifications were done in triplicate and blank procedures were run in simultaneously. In order to assess the accuracy and precision of the analytical methodology, analyses of certified reference material were carried out (PACS-2 harbor sediment) in parallel with samples and procedure blanks. Certified and measured values were in line with recoveries between 93% and 99%. The organic matter content (SOM) and the proportion of silt-clay in the sediments were also determined. SOM content was determined as the percentage of weight lost on ignition at 550º C for 5 h after drying at 60º C for 24 h. Sediment particle size distribution was determined by mechanical dry sieving (Buchanan 1984). Samples of oven-dried sediments were sieved through a stacked set of graded sieves within the range 2000-62 µm.

Adult *G. chevreuxi* individuals were collected in the Reference site (Mira channel). Amphipods were collected using a 0.5 mm sieve and stored in polyethylene buckets. Food supply consisted of commercial pet fish food (Prodac®). The amphipods were gradually acclimated to test conditions over a period of 72 h. During acclimation, pH (7.6-7.7), salinity (20), and temperature (20ºC) were kept constant. Gravid females and individuals that exhibited evident poor health were excluded from the experiments. All experiments were performed in winter, during January and February 2013. Water used in the tests was also collected at the Reference site and filtered through a GF/F Whatman® (Ø 0.7 µm) filter.

Behavioural tests

To ensure that sublethal concentrations of mercury were been used in the behavioral tests, static 2-day solid-phase sediment toxicity tests were conducted according to methods detailed in the testing manual of the US Environmental Protection Agency (1994). No mortality was observed in any of the samples.

Behavioural tests were designed to record individual amphipod locomotor activity responses along the sediment mercury-contamination gradient. For each of the four treatments (sites), six amphipods were placed into six host aquaria (7 L) containing sediments and water in the proportion 1:4, according to methods detailed in the testing manual of the US Environmental Protection Agency (1994). Individual amphipods swam freely into a prismatic chamber measuring 160 mm x 40 mm x 25 mm within each host aquarium. These prismatic chambers were designed to limit horizontal displacements and were made of polypropylene plastic plates. Each compartment had a 5mm mesh to provide a surface on which amphipods could attach themselves. The back plate was drilled with holes to facilitate water exchange with the aerated water of the 7 L host aquarium. All assays were performed at a constant temperature of 20ºC, with aeration, a salinity of 20 and a photoperiod of 16h/8h (light/dark) (Fig. 2).

Individual amphipod activity was recorded at one frame per second for 24h. To track amphipods' locomotor activity, webcams (Logitech® Webcam C250) were installed in front of each aquarium. Infrared light sources (C-2290, Ø 52mm, Cebek®, 12V) were installed above each aquarium to facilitatenight recording (Fig. 2). The video-recordings were analyzed using the specialized software Fish Tracker®_v1.2. (Sánchez 2010). This software tracked each amphipod and provided their position (X, Y) every second, which generated a file that was exported to Excel for further analysis and graphics creation. Special focus was placed on Y position, which corresponded to vertical swimming activity. It has been demonstrated that vertical swimming is a more sensitive response to contaminants due to the greater energetic costs associated with producing enough thrust to attain the lift required to make a vertical ascent into the water (Wallace and Estephan 2004).

Coordinates were used to compute daily activity patterns and to analyze four behavioural indices: Swimming activity, time in contact with the sediment, total vertical distance covered, and number of surfacings (swimming displacements from the bottom to the water surface). Primatic chambers' dimensions were standardized for the software by considering the total height as the unit. Taking this in to account, 0.2 and 0.8 were deemed as threshold filter positions in the water column to make our calculations (Fig. 2). With this information, the four behavioural indices were calculated from the raw position data provided by the software and integrated by hour: i) swimming activity, defined as the sum of all amphipod movements between two positions separated by at least 0.2 units, constitutes an estimate of the overall amphipod activity and can also be used to evaluate stress or escaping behaviours; ii) time in contact with the sediment, defined as the percentage of time that the amphipod spent between the bottom and the 0.2 threshold in the water column, constitutes an estimate of the animal's relative avoidance of direct contact with the polluted sediment; iii) vertical distance covered, defined as the sum of the total vertical distance covered (difference between two consecutive position records) during amphipod swimming, is another measurement of the overall amphipod swimming performance and can be used to identify differences in activity rhythms and overall amphipod's health status; and iv) number of surfacings (*sensu* Wallace and Estephan 2004), calculated as the number of swimming movements from a position below the 0.2 threshold to a position above the 0.8 threshold in the water column, is related to the general locomotor capacity of amphipods and their ability to perform an effective displacement from the bottom to the surface.

Statistical analysis

Data from locomotor activity tests and physicochemical parameters were analysed for normality and homogeneity of variances with Shapiro-Wilk's test and Hartley's test, respectively. After that, our results were subjected to a parametric analysis of variance (one-way ANOVA, $p< 0.05$), followed by *post hoc* Tukey´s test. Differences between light and dark periods of locomotor activity were analyzed using the t-Student test for comparisons of means. Univariate analyses were carried out with the statistical package SPSS V.19.

Results

Sediment analyses

Measured mercury concentration highlighted the existence of a mercury contamination gradient decreasing with the distance from the industrial effluent, with the highest levels found at site 1 followed by a progressive decreased in site 2 and site 3. The lowest concentrations were found in the Reference site (Table 1). The organic matter content (SOM) ranged from 0.55 to 12.31% with the lowest value registered in Site 3. Silt-clay content was around 20% along the Laranjo de Basin sediments while lower at the Reference site (Table 1).

Behavioural indices

Swimming activity

Swimming activity changed substantially along the mercury-contamination gradient (Fig. 3a). At the Reference site, *G. chevreuxi* showed a clear diurnal activity pattern with a significant higher swimming activity during the light phase (t-test, $p<0.01$). However, this diurnal activity was reduced in Site 3(t-test, $p<0.01$) and was reversed in the most highly-contaminated station (Site 1). There was a significant reduction in Swimming activity in the stations exposed to mercury contaminated sediments compared with Reference site (one-way ANOVA, *post hoc* Tukey´s test, p<0.05). All stations showed similar basal activity during dark conditions (one-way ANOVA, *post hoc* Tukey´s test, p<0.05).

Time in contact with the sediments

This parameter also varied along the mercury-contaminated gradient (Fig. 3b). *G. chevreuxi* spent significantly more time in contact with the sediment at the Reference site and during the dark period. However, the amphipods exposed to mercury significantly reduced the time spent in contact with the contaminated sediments (one-way ANOVA, *post hoc* Tukey´s test, p<0.05).The time spent in sediments (light and dark period) decrease along the contaminated sites respect to the Reference site. It was observed a significant difference in this parameter between light and dark period in Sites 2 and 3 (t-test, p<0.05). This difference was not present in Site 1.

Vertical distance covered

Amphipod total vertical displacement increased during the day in Sites 3 and Reference (Fig. 3c). The daily vertical distancepatterns drastically changed in the most contaminated sediments (Sites 1 and 2) (t-test, p<0.01). Concomitantly, this parameter also differed among sampling sites during the day period. Distance covered decreased in mercury-contaminated sites (1, 2 and 3) during the day period (one-way ANOVA, *post hoc* Tukey´stest, p<0.05). This spatial pattern was less perceptible during the night period. Moreover, it is interesting to note that the day/night movement pattern was inverted in Site 1.

Number of surfacings

The number of surfacing movements were higher during the day period comparatively to the dark period in the Reference site (t-test, p<0.01). This pattern was altered in mercury-contaminated sites, and was even inverted in the most contaminated Site1 (Fig. 3d). Significant differences were found between light/dark periods in sampling sites and the interaction of the light/dark period and sampling sites (one-way ANOVA, p<0.001). The number of surfacing movements decreased along the contaminated sites during the light period (one-way ANOVA, *post hoc* Tukey´s test, p<0.05). Amphipods exposed to the most contaminated sediments (Site 1) presented the least surfacing movements. There were also significant statistical differences between day and night surfacing movements in the most contaminated site (one-way ANOVA, $p<0.05$). There were no significant differences in the number of surfacings during the night period (one-way ANOVA, $p>0.05$).

Discussion

Our results confirmed a clear alteration of *G. chevreuxi* locomotor activity along a spatial mercury contamination gradient in the Ria de Aveiro lagoon. Although non lethal, short term exposure to the mercury contaminated sediments of the study area caused a substantial decrease in *G. chevreuxi* locomotor activity and, therefore, amphipods' ability to find food, escape from predators or undesirable conditions, or mate might be compromise in certain areas of the lagoon, which in turn could have clear deleterious effects on the entire population success. These results are consistent with previous studies in the area which have demonstrated an association between mercury pollution and degraded benthic conditions (e.g. Pereira et al. 2009). The mercury contamination gradient in Laranjo basin (sampling Sites1, 2 and 3) was also associated with reduced total macrofaunal abundance, decreased species diversity and increased dominance of mercury tolerant taxa (Nunes et al. 2008).

The effects of mercury pollution on *G. chevreuxi* locomotion were characterized by an overall decrease in locomotor activity, a decrease in the total vertical distance covered during swimming, and a lowered number of effective surfacing movements. As indicated by Lawrence and Poulter (1998) amphipods may decrease vertical swimming endurance due to a redirection of energy resources in order to cope with expenses of the detoxification of accumulated metal. Similar results were obtained by Wallace and Estephan (2004) when studying the differential susceptibility of horizontal and vertical swimming in *G. lawrencianus*. Our data showed also that amphipods displayed a reduction in the time spent in direct contact with contaminated sediments, which suggests a behavioural pollution avoidance response. The video records showed that amphipods remained attached to aquaria walls rather than on the sediment surface.

A significant interaction between dark–light cycling and mercury contamination was observed in *G. chevreuxi*, which exhibited maximum locomotor activity during the day and a minimum during the night. This suggests that *G. chevreuxi* exhibits daily activity rhythms, as observed in several other amphipod species (e.g. *G. aequicauda* and *C. volutator*) (Harris and Morgan 1984). The lack of activity during the night found in *G. chevreuxi* implies that significant differences in locomotion along the mercury-contamination gradient during dark periods are unlikely. In fact, the main effect observed in Sites1 and 2 was the reduction of the locomotor parameters recorded during the day to the lower basal levels that can be observed during the night. The analysis of changes in locomotor behaviour should be carried out within the context of a previous characterization of daily behaviour patterns of the test species. For example, Morillo-Velarde et al. (2011) recorded a circadian rhythm for *G. aequicauda* with a maximum locomotor activity occurring during the night. Similar results were found in *G. pulex* (Elliott 2002; Mills et al. 2006) and *G. lawrencianus* (Wallace and Estephan 2004). Due to those differences, the results of short-term sublethal bioassays should be interpreted with care, because sensitivity to polluted sediments could differ depending on whether the assays are carried out under dark or light conditions and on the species considered, meaning that there are species-specific responses.

Locomotor activity represents an end-point of particular interest in ecotoxicological studies. The study of swimming behaviour has been used extensively to assess the toxicity of various chemicals to teleost fish and other aquatic crustaceans (Roast et al. 2000, 2001; Sánchez-Vázquez et al. 2011), showing that toxicants can affect swimming performance and compromise vital organism functions (e.g. the ability to escape from predators, feeding activity, migration and reproduction). As for other animals, swimming behavior is a fundamental component of amphipod ecology and appears to be an extremely sensitive metric to a variety of natural factors (e.g Michalec et al. 2012). The overall results for *G. chevreuxi* swimming activity emphasize the deleterious effect of sediments contaminated with mercury, even at concentrations that can be considered non-lethal.

As for other organisms, indirect impact of toxicants on behavioural patterns of amphipods may lead to the mortality of local populations, which has been referred to as 'ecological death' (Roast et al. 2000). Thus an early behavioral assessment could be used to better delineate polluted areas where effects to populations might be occurring. The analysis of behavioural responses may grant a significant contribution to the assessment, interpretation and understanding of the impact of contaminants in marine coastal ecosystem.

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CHAPTER IV

Interacción entre contaminación por metales y cambio climático en el comportamiento locomotor de anfipodos.

1.Introduction

Introducción

El incremento de las concentraciones atmosféricas de dióxido de carbono atmosférico (CO2) debido al cambio climático es una amenaza global para la biodiversidad, así como para la estructura y función de los ecosistemas (McCarthy, 2001). Las previsiones esperan que el aumento de temperatura sea de aproximadamente 1,8 a 4,0 ºC para finales de siglo junto con la probabilidad de escenarios donde las emisiones de gases invernadero conlleven elevadas temperaturas en latitudes altas (Stocker, T.F. et al., 2013). La capacidad de las especies acuáticas para adaptarse a los nuevos regímenes y variaciones de temperatura entre en sus actuales límites de tolerancia térmica(Somero, 2005) será un factor determinante para el éxito de dichas las poblaciones (Pörtner y Knust, 2007). El IPCC prevé que la resiliencia de los ecosistemas se supere en muchas regiones durante este siglo, por una combinación sin precedentes de las perturbaciones del cambio climático y las de muchos otros factores de estrés antropogénicos y naturales (Fischlin et al., 2007). Multitud de actividades humanas liberan una gran variedad de contaminantes ambientales (por ejemplo, metales pesados) a los ecosistemas costeros donde pueden acumularse y reciclarse en los sedimentos y organismos. Los efectos de estas sustancias peligrosas dependerán de la exposición y la susceptibilidad de las poblaciones costeras que pueden ser fuertemente afectados por el cambio climático. Muchos ecosistemas de lagunas costeras también se ven afectados por la liberación de contaminantes de origen terrestre mediante escorrentía y / o deposición atmosférica, que también causan graves efectos sobre las especies acuáticas, así como la salud humana (Gomiero y Viarengo, 2014).

El calentamiento climático reciente ya supera la capacidad de algunas especies locales de adaptación, esto puede dar lugar a importantes cambios en la estructura, función y servicios de los ecosistemas (Schiedek et al., 2007). La temperatura y los contaminantes, dos factores estresantes importantes del ecosistema, que a menudo se tratan de

forma independiente, sin embargo su actuación conjunta afectará a procesos fisiológicos y la capacidad de la vida silvestre para mantener la homeostasis (Broomhall, 2004). Para una evaluación eficaz del riesgo global es necesario un enfoque integrado de las interacciones entre los factores de estrés. Además, el impacto del cambio climático será concomitante y en algunos casos agravado por otros factores de estrés de los ecosistemas como las especies invasoras, el exceso de recolección, la destrucción del hábitat y patógenos.

Los metales pesados son un grupo de sustancias químicas ambientales que persisten en el medio ambiente, presentan biomagnificación en la cadena alimentaria y podría causar graves problemas de salud, como anomalías morfológicas, alteraciones neurofisiológicas, carcinogénesis, teratogénesis y mutación (Moore y Ramamoorthy, 1984 ; Rainbow, 1995). Estudios recientes han demostrado que el cambio climático ya está teniendo un impacto significativo en muchos aspectos de la especiación y ciclo de mercurio dentro de los ecosistemas del Ártico (Stern et al, 2012).

Pocos estudios de investigación se centran en la interacción del cambio climático y los contaminantes en los ecosistemas costeros. Los entornos costeros están sometidos a contaminación por metales pesados a través de entradas de las principales fuentes urbanas y depósitos naturales, industriales, atmosféricos que son transportados a través de la descarga del río, los procesos eólicos y deshechos marinos(Prange y Dennison, 2000; Radenac et al., 2001). Las lagunas costeras son cuerpos de agua relativamente cerrados y bajo la influencia tanto del medio ambiente marino como del terrestre. Se encuentran entre los ecosistemas marinos más productivos del mundo, sirviendo como alimentación, rutas migratorias y zonas de cría de muchos organismos (Costanza et al. (1997). Estos ecosistemas reciben limitados intercambios hídricos con las zonas cercanas a la costa y ambientes estuarinos que reciben los subproductos de las actividades humanas procedentes del continente (Yamamuro y Kanai, 2005).

Las condiciones ambientales en las lagunas costeras son muy cambiantes debido a su carácter limitado y su superficialidad. En este sentido, incluso se podría considerar como ambientes estresados de forma natural (Marín-Guirao et al., 2005). En las lagunas costeras, debido su carácter de ecosistemas de transición entre el mar y la tierra, muchas especies viven en el borde de su margen de tolerancia homeostático o fisiológico (Anderson y Peterson, 1969, Gordon, 2003, Heath et al., 1994 y Patra et al. , 2007). Las interacciones complejas entre el cambio climático y los contaminantes pueden ser particularmente problemáticas para estas especies que viven en ecosistemas en transición siendo a menudo los más vulnerables a factores de estrés adicionales, como el cambio climático y la contaminación química (Gordon, 2003, Heath et al., 1994, Heugens et al., 2001 y Patra et al., 2007).

Los ectotermos, tales como crustáceos anfípodos, pueden ser particularmente vulnerables a dichas interacciones. Los anfípodos son considerados especies clave en las cadenas alimentarias costeras, no solo debido a su papel como herbívoros y / o consumidores de detritus, sino también como una fuente de alimento importante para los niveles tróficos superiores (Gosscustard, 1977; Matthews et al., 1992). La mayoría de los anfípodos son pequeños gammaridos bentónicos de vida libre que pueden llegar a densidades tan altas que dominan algunas comunidades (Conradi et al., 1997;. Cunha et al, 2000). Por otra parte, anfípodos son especies modelo óptimas para la evaluación de la toxicidad en bioensayos de laboratorio debido a su distribución ubicua, la sensibilidad a la perturbación y la idoneidad para la cría y experimentación en laboratorio (Neuparth et al., 2002; Prato y Biandolino, 2005). La estrecha interacción entre las variaciones ambientales y el comportamiento de estos anfípodos, han llevado a los investigadores a utilizar sus variaciones de comportamiento como indicador relevante para el monitoreo ecológico de la contaminación ambiental (Scott and Sloman, 2004). Como para otros organismos, las respuestas de comportamiento de los anfípodos están ligadas a cambios bioquímicos y fisiológicos complejos y actúan como indicadores sensibles a concentraciones subletales de tóxicos. Comportamientos como excavación, natación, vigilancia precopulativa, captura de presas han sido utilizados como indicadores de contaminación subletal de metales pesados en crustáceos y otros invertebrados (Roper et al., 1995; Blockwell et al. ., 1998; Wallace et al, 2000)

El uso de la actividad locomotora de anfípodos para monitorear concentraciones subletales de metales en los sedimentos en el medio marino está bien establecida (Morillo-Velarde et al., 2011). Su característica principal es que representa una señal de alerta temprana del deterioro de los ecosistemas al ser sensible a la presencia de concentraciones subletales de contaminantes y sustancias tóxicas.

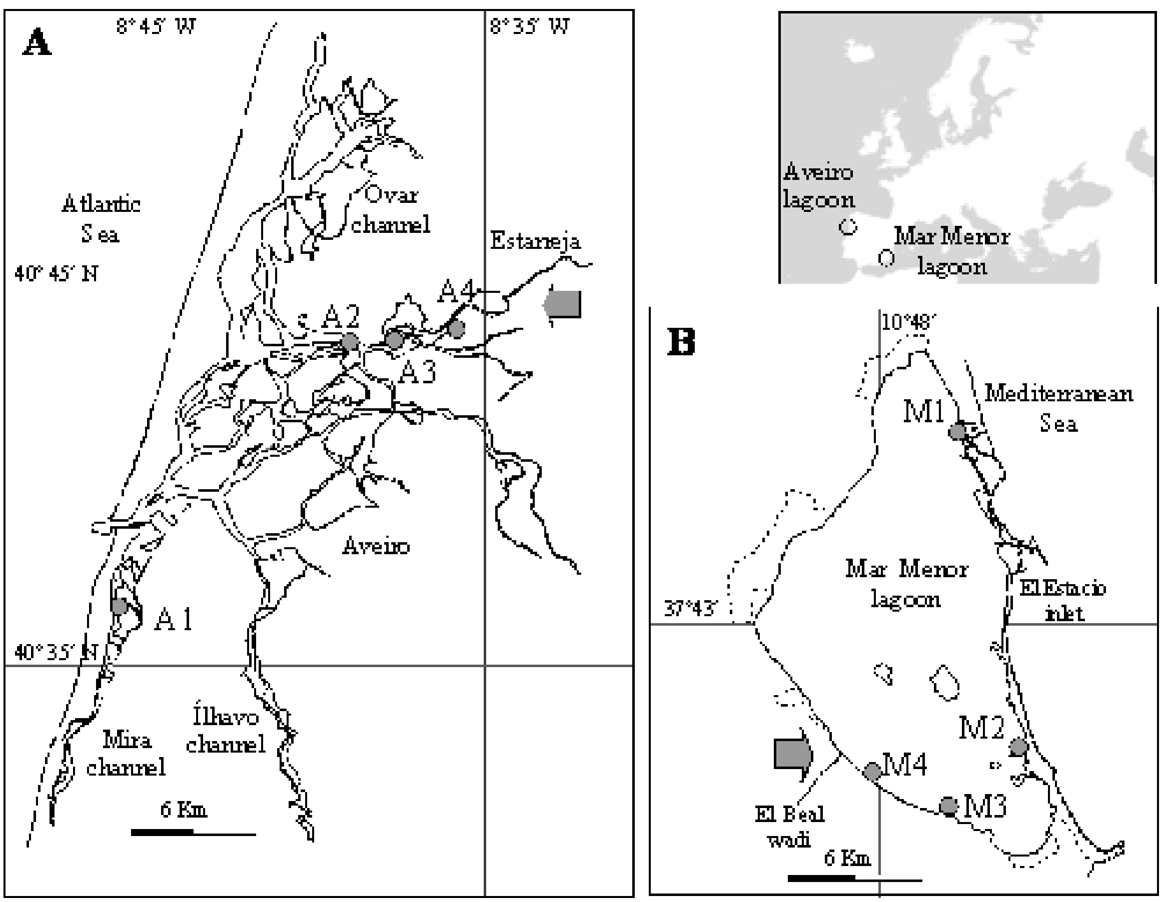
Son escasos los estudios que se centran en la interacción entre el cambio climático y los contaminantes persistentes y aún más los que tienen en cuenta las condiciones ambientales del Océano Atlántico y la costa mediterránea. Las especies con rangos estrechos de tolerancia a condiciones ambientales cambiantes pueden tener dificultades para aclimatarse a un amento de la temperatura debido al Cambio Climático. En este trabajo se comparan las respuestas de comportamiento de los anfípodos del Sur de Europa que se caracterizan por diferentes rangos de tolerancia al calentamiento. El anfípodo Gammarus chevreuxi (Sexton, 1913) ha sido citado en la costa atlántica Europea, mientras que Gammarus aequicauda está ampliamente distribuido a lo largo del Mediterráneo y el Mar Negro, en hábitats caracterizados por una mayor temperatura y salinidad. Estos gammáridos, que viven en el borde de su margen de tolerancia de temperatura, pueden ser menos capaces de hacer frente a los factores estresantes del cambio climático y la exposición de contaminantes. Por lo tanto, consideramos G. aequicauda y G. chevreuxi como especies pertinentes en bioensayos de los sedimentos en el Atlántico y costa Mediterránea. Estos anfípodo se utilizaron en este estudio para investigar si la contaminación por metales en las lagunas costeras dificulta su capacidad para adaptarse a el aumento de temperatura debido al cambio climático.

El objetivo de este trabajo es estudiar si la superposición de factores estresantes como los metales pesados junto con el cambio climático podrían producir alteraciones en el comportamiento de estos anfípodos. Para ello, se han utilizado dos lagunas costeras históricamente contaminados por metales pesados donde se han llevado a cabo una serie de experimentos de mesocosmos manipulados para examinar el posible cambio de comportamiento de la actividad locomotora como marcador de la interacción entre el cambio climático y los contaminantes.

Material y métodos

Área de estudio y sitios de muestreo

El estudio se llevó a cabo en las lagunas costeras de la Ría de Aveiro (Portugal) y Mar Menor (España). Estas dos lagunas se han visto afectados por un incremento de varias actividades antropogénicas (industria química, actividades mineras) que han



contribuido a enriquecer los sedimentos con metales en los últimos años. La Ría de Aveio es una laguna costera templada, poco profunda (45 km de longitud, 10 km de ancho), situada en la costa Atlántica del noroeste de Portugal (40º38'N, 8º 44'O). Fue sometida a los vertidos de mercurio de una planta de cloro-álcali situada en el complejo industrial de Estarreja desde la década de 1950 hasta 1994, creando un gradiente de contaminación ambiental a lo largo de 2 Km^2 de cubeta interior (Laranjo) (Pereira et al., 2009). La actividad industrial dio lugar a la acumulación de aproximadamente 33 t de Hg en Ria de Aveiro, de la cual 82% permaneció enteramente en la cuenca del Laranjo, en fracción de partículas, es decir, sedimento-asociado (Pereira et al., 1997). El gradiente espacial de mercurio a lo largo de la Cuenca Laranjo se reflejó en los patrones de bioacumulación de las comunidades de zooplancton y organismos bentónicos (Abreu et al., 2000; Coelho et al, 2005;. Gonçalves et al, 2013). Fuera de la cuenca Laranjo, los niveles de Hg son mucho más bajos y por debajo del umbral de concentración europeo de pescados y mariscos para el consumo (Pereira et al., 2009).

El Mar Menor es una laguna costera hipersalina (42 a 47) situada en una región semiárida del sureste de España. La laguna ocupa una superficie aproximada de 135 km^2 y un volumen total de 610x103 m^3 (Arévalo, 1988). Tiene una profundidad máxima de 6,5 m y una profundidad media de 3,6 m. Rangos de temperatura? de 10º C en invierno a casi 30º C durante el verano. Las entradas de agua dulce en la laguna se limitan a los cursos de agua efímeros llamados 'wadis' o 'ramblas. Estos barrancos anchos y poco profundas son generalmente inactivos, pero pueden llevar a grandes cantidades de agua y sedimentos durante los episodios de inundación. La laguna del Mar Menor ha sido históricamente afectada por desechos mineros. De hecho, la actividad minera en las montañas que encierran la parte sur de la laguna era una de las más importantes minas de España en los dos últimos siglos. Todas las actividades mineras cesaron en 1991, pero durante los episodios de inundación los metales de los residuos mineros se liberan en las ramblas que vierten en la laguna del Mar

Menor. Se ha observado que se pueden liberar y lixiviar metales durante varios cientos de años después de que la actividad minera haya cesado (Gundersen et al., 2001). Estudios previos llevados a cabo en la zona mostraron que los principales metales residuales de la actividad minera son Zn, Pb y Cd y son liberados directamente en la laguna (Marín-Guirao et al.).

En ambos lugares, se seleccionaron estaciones de muestreo a diferentes distancias de la fuente principal de contaminación por metales en un esfuerzo por abarcar toda la gama de la contaminación por metales causada por la actividad humana. En las estaciones de muestreo Ría de Aveiro fueron seleccionados a diferentes distancias de Estarreja complejo industrial a lo largo del gradiente de contaminación de Hg, RA1 , RA2, RA3 y RA4 de referencia. Las zonas 1, 2 y 3 se localizaron más cercanas a la fuente de la contaminación, mientras que la estación 4 se encuentra en el canal de Mira (Fig. 1). El canal de Mira se ha utilizado anteriormente como un sitio de referencia en estudios de toxicidad de sedimentos (Castro et al. 2006).

En el Mar Menor, los zonas de estudio se encuentran en la cuenca sur de la laguna a lo largo de un gradiente de contaminación a partir de la rambla Beal, donde se establece la corriente más importante drenaje de la zona minera. (Figura 1). Las distancias desde ramblas Beal fueron 0 (MM1), 5 (MM2), 20 (MM3) y 350m (MM4). La estación MM4 se ha utilizado como una estación de referencia en los estudios anteriores, y se ha demostrado ser no tóxico para anfípodo expuesto a 10 días en la internase agua-sedimento (Sanz-Lázaro y Marín, 2009).

Los análisis de sedimentos

Los análisis de sedimentos se llevaron a cabo por triplicado. Las muestras de sedimentos fueron recolectados a través de core de policarbonato. Se recogieron 5 cm de sedimentos por core de cada estación. Se almacenaron en frascos de polietileno de 0.5L, y luego se transportaron refrigerados y en oscuridad al laboratorio. Antes de configurar el experimento, los sedimentos fueron homogeneizadas y tamizadas, se eliminaron grandes piezas de escombros y depredadores potenciales. Todos los recipientes

utilizados para la recogida y almacenamiento de los sedimentos se lavaron previamente con ácido nítrico (10%) libre de Hg. los sedimentos menores de 63 micras fueron digerido con ácido débiles para extraer los metales que estaban vinculados a los sedimentos débilmente, siguiendo las sugerencias de Luoma (1989), en un intento de simular la digestión ácida del intestino del animal. Los recipientes de teflón utilizados para las extracciones se limpiaron en agua caliente y también un triple enjuagado en agua desionizada. Se digirieron alícuotas de aproximadamente 0,3 g de sedimento a temperatura ambiente durante 2 h en HCl 0,6 N. Las extracciones se llevaron a cabo en un agitador orbital plataforma y los sobrenadantes se filtraron inmediatamente a través de membranas de 0,45 micras de nitrato de celulosa y se almacenaron en frascos de HDPE hasta su análisis. Los metales (Zn, Pb, Cu y Cd) se midieron con un espectrómetro de emisión óptica ICP (Optima 2.000 DV- Perkin-Elmer). El control de calidad comprendía una toma al azar en cada serie de análisis de muestras por duplicado incluido, espacios en blanco fueron introducidos al principio y cada 6 muestras, estándares utilizados para la calibración fueron analizados periódicamente, y los métodos analíticos fueron probados con materiales de referencia certificados (1557b NIST y

	Ria de Aveiro				**Mar Menor**			
Estaciones	A1	A2	A3	A4	M1	M2	M3	M4
Hg(µg/g)	74,96±3,67	1.22±0.02	0.13±0.0	0.041±0.0	0.0	0.0	0.0	0.0
Cd(µg/g)	0.1±0.01	0.4±0.02	0.2±0.01	0.066	305 ± 11.2	251±22.4	1496±33.6	7219±11.1
Pb(µg/g)	0.7±0.1	0.3±0.1	0.1±0.01	0.2±0.01	72±4.1	130.76±8.2	471.2±20.7	10006±39.3
Zn(µg/g)	26.7±0.01	68.4±0.01	32.1±0.01	24.6±0.01	0.65 ±0.2	59.45±9.7	75.84±8.8	4120±65.8
OM %	12.31±0.01	3.07±0.01	0.55±0.01	2.21±0.01	1.98±0.19	3.05±0.02	3.18±0.09	8.17 ±0.23
Fines (<63 µm)	2.8	20.8	16.2	2.45	2.1 ±0.06	8.4 ±0.40	4.6 ±0.01	10.5 ±3.76
Salinity	18±4.6	21.3±0.2	20±0.2	30.8±0.2	43±1.2	46.2±0.5	45.5±0.7	46±0.7
pH	8.70±0.0	7.91±0.0	7.91±0.0	7.71±0.0	8.10±0.0	8.18±0.0	8.16±0.0	7.95±0.0

BCR 186), estando la precisión analítica dentro de 15%.

Las sub-muestras de sedimentos homogeneizadas se secaron al aire y se analizaron para el contenido total de mercurio por descomposición térmica espectrometría de absorción atómica con amalgama de oro, usando un LECO AMA-254 (Advanced Mercurio Analyzer). Todas las cuantificaciones de mercurio se realizaron por triplicado y los procedimientos en blanco se realizaron en forma simultánea. Con el fin de evaluar la exactitud y precisión de la metodología analítica, se llevaron a cabo análisis de material de referencia certificado (PACS-2 sedimentos del puerto) en paralelo con las muestras y los blancos de procedimiento. Los valores certificados y medidos estuvieron en línea con las recuperaciones entre 93% y 99%. El contenido de materia orgánica (TOC) y la proporción de limo y arcilla en los sedimentos también se determinaron. Contenido de TOC se determinó como el porcentaje de pérdida de peso por ignición a 550º C durante 5 horas después de secar a 60 ° C durante 24 h. El TOC se determinó con un analizador elemental (Carlo Erba Instruments, EA1108) después de preparada la muestra con HCl 1 N para descomponer los carbonatos (Verdardo et al., 1990). La granulometría de sedimentos fue determinada por tamizado mecánico en seco. Las muestras de sedimentos secadas en el horno se tamizaron a través de un conjunto apilado de tamices graduados dentro del rango de 2000 a 62 micras. El contenido de materia orgánica de sedimentos se determinó como el porcentaje de peso perdido tras la ignición de sedimento seco en un horno de mufla a 450 ° C durante 6 h.

Recolección, aclimatación y mantenimiento de organismos de prueba

Los anfípodos, G. aequicauda y G. chevreuxi, se obtuvieron de los sitios de referencia, donde se llevaron a cabo los experimentos en el Atlántico (estación A4) y Mediterráneo (estación M4), respectivamente. Los anfípodos se recogieron usando un tamiz de 0,5 mm de luz y se almacenaron en frascos de polietileno. Fueron transportados inmediatamente al laboratorio en contenedores de temperatura constante, en el que se mantuvieron en acuarios de vidrio de 20 L que contienen agua de mar natural filtrada (0,45 micras filtro GF / C Whatman) en condiciones controladas para la aclimatación. Se proporcionó aireación y se seleccionó un fotoperíodo de 16: 8h (luz: oscuridad). El suministro de alimentos consistía en comida para peces mascota comercial (Prodac®). Los anfípodos se aclimataron gradualmente para poner a prueba las condiciones durante un período de 72 h, tiempo durante el cual se supervisan la concentración de oxígeno disuelto, pH, salinidad y temperatura. La salinidad se estableció de acuerdo con cada especie: 20 para G. chevreuxi y 41 para G. aequicauda. Las hembras grávidas y los individuos que presentaban evidentes problemas de salud fueron excluidos de los experimentos. Todos los experimentos se realizaron con la cohorte de primavera, que constituyen los individuos adultos durante el verano. Los anfípodos fueron seleccionados al azar, de acuerdo al tamaño (2-4 mm de longitud total) y sólo organismos sanos fueron utilizados los recién nacidos.

La cuantificación, abundancia anual y biomasa del anfípodo G. chevreuxi en el curso superior del Canal de Mira, el brazo sur de la ría de Aveiro fue cuantificada por Subida y su grupo et al (2004). Estos mismos autores indicarón que la población mostró un ciclo de vida semestral, iteróparo, con una vida media de 6 meses. G. aequicauda también tiene un ciclo de vida semestral con una vida útil de 6-9 meses (Prato y Biandolino, 2003). La cohorte de primavera mostró un rápido crecimiento, madurez acelerada y vida útil de unos siete meses, mientras que la cohorte de hibernación mostró una vida útil de alrededor de nueve meses. G. aequicauda muestra una variación estacional de la densidad, con un máximo en primavera y verano y un mínimo en otoño e invierno. Las hembras ovígeras estuvieron presentes durante todo el año en la población y producen 2 generaciones por año: los juveniles nacidos en la primavera, crecieron, maduraron y se convirtieron reproductivamente activas durante los meses de verano, dando lugar a la siguiente entrada de reclutas en otoño (Prato y Biandolino , 2003).

Diseño experimental

Análisis preliminares mostraron una supervivencia del test de comportamiento del 100% en ambos anfípodos. Las pruebas de comportamiento se realizaron para evaluar los efectos subletales de la temperatura del agua y los contaminantes de los sedimentos. Las pruebas de comportamiento fueron diseñados para registrar respuestas de actividad locomotora del anfípodo de forma individual, a lo largo del gradiente de contaminación de los sedimentos en Mar Menor y laguna Ría de Aveiro. Para cada uno de los cuatro tratamientos (un por estación de muestreo), se colocaron seis anfípodos en seis acuarios (7 L) con sedimentos y agua en la proporción 1: 4, de acuerdo con los métodos que se detallan en el manual de pruebas de la Agencia de Protección Ambiental de Estados Unidos (1994). Los anfípodos nadaban libremente en calles individuales dentro de una cámara prismática de 160 mm x 40 mm x 25 mm, alojada en el interior de un acuario mayor tamaño (7 L). Estas cámaras prismáticas de polipropileno fueron diseñadas para limitar los desplazamientos horizontales. Cada compartimiento tenía una malla de 5 mm para proporcionar una superficie de adhesión a los anfípodos. La placa posterior fue perforada con agujeros para facilitar el intercambio de agua y aireación con el acuario de 7 L. Antes de evaluar el efecto de la temperatura sobre los sedimentos contaminados y la influencia de la temperatura sobre la supervivencia de organismos se realizó un ensayo con los sedimentos de referencia (sedimento control negativo). Las temperaturas (15, 20, 25 ºC para G. chevreuxi y 20, 25, 30 ºC para G. aequicauda) fueron seleccionados de acuerdo con el rango de temperaturas registradas en las ubicaciones en las lagunas de la Ría de Aveiro y el Mar Menor. Se realizó una adaptación gradual de los animales a la temperaturas experimentales, variando de 2 ºC /día del valor de la temperatura de campo. La prueba comenzó a las 48 horas después de haber alcanzado la temperatura deseada. Todos los ensayos se realizaron con aireación, una salinidad de 20 (Ría de Aveiro laguna) o 41 (Mar Menor laguna) y un fotoperiodo de 16 h: 8 h (luz / oscuridad) (Fig. 2).

Actividad anfípodo individual fue grabado en un fotograma por segundo durante 24 horas. Para realizar un

seguimiento de la actividad locomotora de los anfípodos ', se utilizaron webcams (Logitech® Webcam C250) instaladas en frente de cada acuario. Las fuentes de luz de infrarrojas (C-2290, 52 mm Ø, Cebek®, 12V) fueron instalados encima de cada acuario para facilitar la grabación en oscuridad (Fig. 2). Las grabaciones de video fueron analizadas utilizando el software especializado Fish Tracker®_v1.2. (Sánchez 2010). Este software registra la posición de cada anfípodo por segundo referenciándolos en los ejes X e Y, lo que genera un archivo que se exporta a Excel para su posterior análisis y creación de gráficos. Se hizo especial hincapié en la posición en el eje "Y" , lo que corresponde a la actividad vertical de la natación. Se ha demostrado que la natación vertical es una respuesta más sensible a los contaminantes debido a los mayores costes energéticos asociados con la producción de empuje suficiente para alcanzar la elevación requerida para hacer un ascenso vertical en el agua (Wallace y Estephan 2.004).

Los movimientos registrados se utilizaron para calcular los patrones de actividad diaria y analizar cuatro índices de comportamiento: la actividad de Natación, número de surfacings (desplazamientos de natación desde el fondo hasta la superficie del agua), porcentaje de tiempo en la columna de agua y la velocidad de natación. Las dimensiones de las cámaras prismáticas fueron estandarizadas para el software teniendo en cuenta la altura total como la unidad. Teniendo esto en la cuenta, 0.2 y 0.8 fueron consideradas como posiciones límites en la columna de agua para clasificar los número de surfacings y el porcentaje de tiempo en la columna de agua (Fig. 2). Con esta información, los cuatro índices de comportamiento se calcularon a partir de los datos de posición proporcionadas por el software e integrados por hora: i) actividad de natación, que se define como la suma de todos los movimientos de anfípodos entre dos posiciones separadas por al menos 0,2 unidades, constituye una estimación de la actividad global anfípodo y también puede ser utilizado para evaluar el estrés o comportamientos de escapatoria; ii) número de surfacings (sensu Wallace y Estephan 2004), calculado como el número de movimientos de natación desde una posición por debajo del umbral de 0,2 a una posición por encima del umbral 0,8 en la columna de agua, se relaciona con la capacidad del aparato locomotor del anfípodos y su capacidad para realizar un desplazamiento eficaz desde el fondo hasta la superficie; y iii) tiempo en contacto con el sedimento, definida como el porcentaje de tiempo que el anfípodo pasó entre la parte inferior y el umbral de 0,2 en la columna de agua, que constituye una estimación de la evitación relativa del animal de contacto directo con el sedimento contaminado.

Durante el período de prueba anfípodos permanecieron en ayunas. Al final del experimento la mortalidad se estimó como porcentaje (± SD) de organismos muertos. Al principio y al final de cada prueba, los parámetros de calidad del agua, incluyendo la temperatura, pH, salinidad y oxígeno disuelto, se midieron (ASTM 1997). El agua utilizada en las pruebas también se recogió en el sitio de referencia y se filtró a través de un / F Whatman® (Ø 0,7 m) de filtro GF.

Para asegurar que se han utilizado las concentraciones subletales de contaminantes en las pruebas de comportamiento, se llevaron a cabo bioensayos de toxicidad con los sedimentos durante 2 días de acuerdo, con los métodos que se detallan en el manual de pruebas de la Agencia de Protección Ambiental de Estados Unidos (1994). No se observó mortalidad en ninguna de las muestras.

Análisis estadístico

Para determinar los efectos principales e interactivos de los tratamientos de nutrientes (con y sin adición) y la exclusión de depredadores (exclusión, trama abierta, control parcial de la jaula). Y para establecer la abundancia de fauna dominante (toda la fauna que comprende al menos el 3% del total de individuos en una combinación estuario / temporada) se utilizó una análisis de varianza de dos vías (ANOVA).

Los datos de los ensayos de toxicidad, expresados como la actividad locomotora y los parámetros físico-químicos fueron analizados para comprobar su normalidad y homogeneidad de varianza con los test de Shapiro Wilk y Hartley respectivamente. Una vez que los datos pasaban las pruebas mencionadas, éstos

eran posteriormente se realizaron análisis para métrico de la varianza (ANOVA de una vía, p <0,05), seguido por la prueba de Tukey post hoc. Los análisis univariado se realizaron con el paquete estadístico SPSS V.19. Este diseño experimental permitió una prueba directa de los principales efectos del tratamiento, así como predecir las interacciones entre las estaciones y las temperaturas.

Resulados y discusión

Los resultados de este estudio mostraron que la contaminación por metales en las lagunas de Ria de Aveiro y Mar Menor modificó el comportamiento locomotor de dos especies de anfípodos (G. chevreuxi y G. aequicauda). Los controles de referencia de ambas especies presentan un perfil similar de comportamiento, con una actividad más alta (actividad de natación, el número de surfacingss) y mayor tiempo en contacto con el sedimento. Este estudio también indica que la influencia de factores de estrés no contaminantes como la temperatura, puede ser particularmente importantes en la respuesta de organismo en los sistemas de estuarios. G. aequicauda y G. chevreuxi fueron más sensibles a sedimentos contaminados por metales pesados a temperaturas más altas. Ambos anfípodos mostraron una disminución de la actividad de la natación, el número de surfacings y menos tiempo en contacto con el sedimento comparado con los sedimentos de referencia a la temperatura más alta. En la laguna del Mar Menor, G. aequicauda tuvo mayor sensibilidad cuando fue expuesto a la temperatura más alta (30º C). Resultados similares fueron encontrados por Prato et al. (2009) en G. aequicauda tras un estudio de laboratorio sobre los efectos de la temperatura y la sensibilidad al cadmio. Estos autores indicaron que G. aequicauda era más sensible a Cd a temperaturas más altas. La actividad locomotora disminuyo en la temperatura más alta tanto en las estaciones de referencia y como las estaciones con sedimentos contaminados. Esto podría ser el resultado de un aumento del metabolismo, aumentando la tasa de respiración (Rathore y Khangarot 2002), y por lo tanto habría un aumento de la

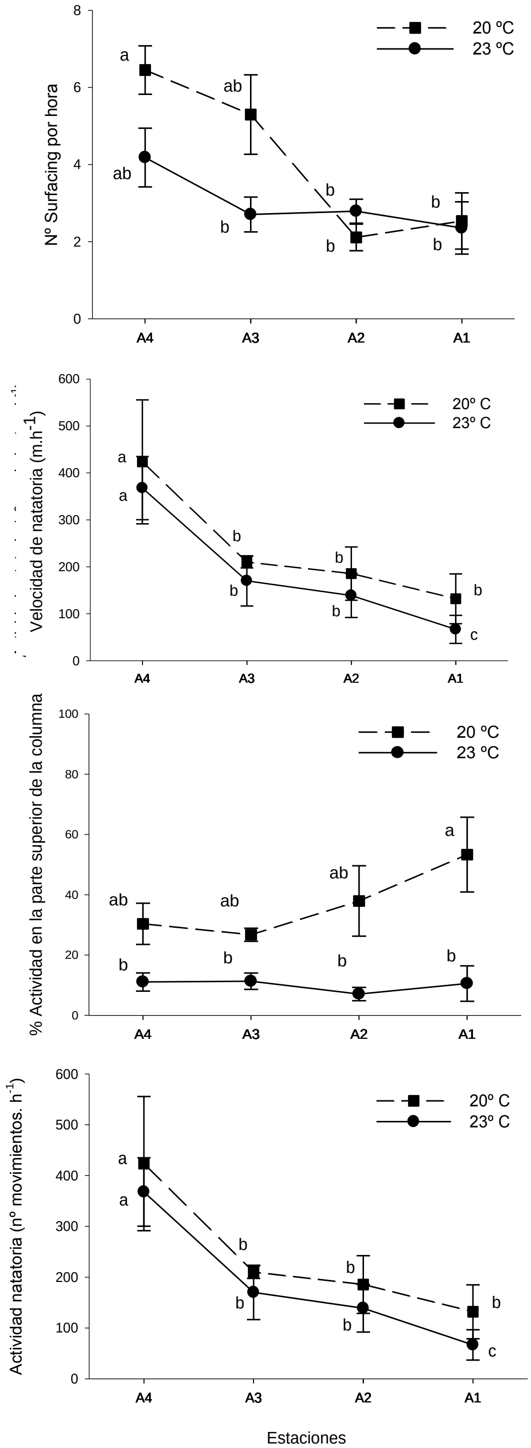


Fig. 1. a,b,c,d. *Gammarus ahevreuxi*. Valores medios, Estaciones con la misma letra no presentan diferencias significativas (p<0.05, ANOVA seguido de la prueba de Tukey HSD).

acción de iones metálicos en las enzimas celulares y membrana celular, afectando a los mecanismos osmoreguladores e indirectamente el aumento de toxicidad de metales pesados (Yang y Chen 1996;. Serra et al 1999). Un aumento de la temperatura conlleva En generalmente un aumento el consumo de oxígeno, lo que llevaría a un aumento en el gasto de energía. Dingemanse & Wolf, 2010 sugirieron que la exposición a contaminantes puede afectar el comportamiento a través de sus efectos sobre las variables de estado asociados con la adquisición de energía. Tales modificaciones pueden a su vez alterar el costo y beneficios de diferentes comporamientos. La exposición a metales pesados puede tener como resultado una intensificación de los costes metabólicos y un balance energético negativo (Massarin et al, 2010;. Massarin et al., 2011). Este hecho podría explicar por qué ambos anfípodos disminuyeron su actividad locomotora a temperaturas más altas, que podrían ser una adaptación bioenergética a esta situación. Además, la presencia de los metales pesados también podría afectar el comportamiento del aparato locomotor,

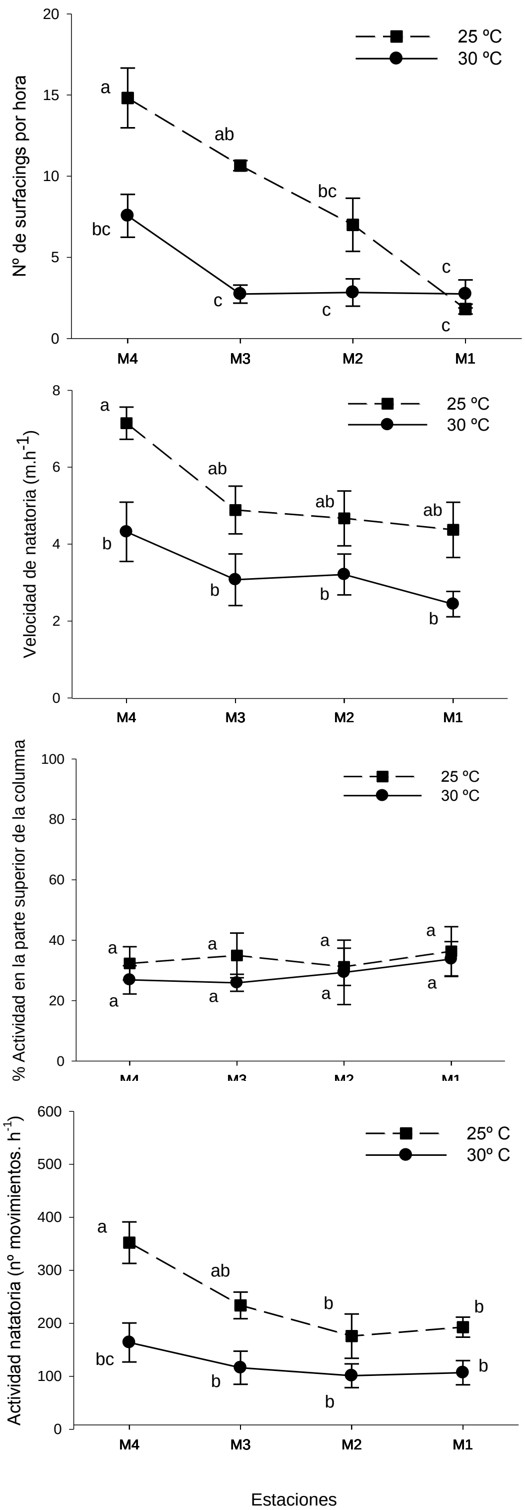


Fig2 a,b,c,d. *Gammrus chevreuxi*. Valores medios, Estaciones con la misma letra no presentan diferencias significativas (p<0.05, ANOVA seguido de la prueba de Tukey HSD).

al determinar la energía que los anfípodos asignan a varias funciones como el crecimiento, la reproducción y el mantenimiento del cuerpo (Montiglio, royauté, 2014). Se especuló que la disminución de la actividad de natación de G. duebeni tras la exposición a Cu se debida a una posible reorientación de los recursos energéticos con el fin de hacer frente a la desintoxicación de metales (Lawrence y Poulter, 1998). Sin embargo, la influencia de la temperatura sobre la toxicidad de metales se puede atribuir a los cambios relativos en la tasa de absorción del metal, la eliminación, la difusión y biotransformación de los organismos. Aunque las razones específicos tanto bioquímicas como fisiológicas que pueden alterar la actividad de la natación en estos anfípodos son dudosas, este trabajo hace hincapié en la interacción entre el calentamiento climático y la contaminación a lo largo de los gradientes de contaminación de las dos lagunas.

El tiempo en la columna de agua está relacionado no solo con la actividad de natación, sino también con la capacidad de agarre de los amphipodos a sustratos verticales. Dicho comportamiento disminuyó por la temperatura y la contaminación con metales, en ambos anfípodos, lo que revela efectos subletales sobre su comportamiento. Al investigar el tiempo en la columna de agua de anfípodos del Atlántico y el Mediterráneo, se observó un patrón entre las dos lagunas costeras, en el que disminuía el tiempo en la columna de agua conforme aumentaba distancia de las zonas con sedimentos más contaminados por metal .

Sin embargo, nuestros resultados mostraron también un fuerte efecto de la temperatura sobre el tiempo en la columna de agua. En sedimentos contaminados, los anfípodos estuvieron más expuestos a la contaminación por metales debido a que pasaron más tiempo en contacto directo con el sustrato. Estos comportamiento inadecuados a estímulos externos, pueden ser debido a los efectos nocivos de los contaminantes, pudiendo tener consecuencias graves para la supervivencia a nivel individual y poblacional (Webber y Haines, 2003).

El presente documento pone de relieve la importancia del número de surfacings como indicador valioso del estado perturbación del ecosistema.

Este comportamiento es el que exige un mayor gasto energético, siendo por tanto un indicador de fitness. En un estudio de actividad de natación horizontal y vertical de Gammarus lawrencianus, Wallace y Estephan (2004) especularon que de los dos comportamientos, la actividad vertical de la piscina es más sensible a la exposición Cd porque conlleva una mayor coste energético ara alcanzar el elevación necesaria para hacer un ascenso vertical en el agua. Este ascenso vertical completo en la columna de agua requiere de un golpeo constante de los pleopodos para superar la gravedad, por lo que es probable que sea más costoso desde el punto de vista energético que los desplazamientos cortos (Boudrias, 1991; Wallace y Estefan, 2004).

Se observaron un menor número de surfacings tras la exposición a sedimentos de estaciones con concentraciones relativamente altas de metales. Esto sugiere que en estas estaciones ya se ha producido una incapacidad fisiológica seguida de una pérdida del control de movimiento. En

un trabajo anterior, Morillo-Velarde et al. (2011) indicaron que la concentración requerida para estudiar una disminución significativa en la actividad de natación de G. aequicauda era 0,24 mg l^{-1} Cd en condiciones de oscuridad, siendo mucho más baja que la CL50 (48 h) encontrada para esta especie (1,71 mg Cd l^{-1}). Los hallazgos de este estudio sugieren que en el Mar Menor, existe una difusión de metal de sedimentos a la columna de agua con un efecto de menor toxicidad que la CL50 (48 h) de Cd. Además, no se produjo mortalidad en nuestros ensayos de actividad locomotora, lo que indica que las características de comportamiento de anfípodos pueden ser indicadores útiles a exposición subletal de sedimentos contaminados por metales en Mar Menor

Actividad Natación y número de surfacings son parámetros ecológicamente relevantes en anfípodos, porque sus actividades vitales como la alimentación, evitación de los depredadores y la reproducción dependen de él (Scott y Sloman, 2004). La reducción de la actividad locomotora probablemente tiene un impacto negativo en la evitación de depredadores, la busqueda de alimento o la reproducción sexual. Esto puede tener consecuencias ecológicas importantes como la disminución de la densidad de población. Los estudios de Weis et al. (2001) en ciprinidos indicaron que la reducción de densidad de la población una zona industrial de Nueva Jersey,Que contaminante? se debía a una reducción del crecimiento y longevidad y a una mayor vulnerabilidad a la depredación debido a que la actividad natatoria de los peces era menor lo que dificultaba la captura de alimento. Esto concuerda con los hallazgos de Nunes et al. (2008) en la Ría de Aveiro, que también mostró que el aumento de la contaminación por mercurio se asoció con una reducida abundancia total (incluyendo G. chevreuxi) y menor diversidad de especies de comunidades bentónicas. La distribución espacial de mercurio en los sedimentos que se encuentran en este trabajo está de acuerdo con estudios previos en la zona (Pereira et al., 1998; Ramalhosa et al, 2006;. Nunes et al (2008) Nunes et al (2008). Estos autores encontraron que la concentración de mercurio en el sedimento fue factor determinante más fuerte que influyó en la composición de la comunidad. Se observó que la densidad de crustáceos (incluyendo G. chevreuxi) disminuía a lo largo del gradiente de contaminación por mercurio. Del mismo modo, la concentración de mercurio orgánico en macroalgas disminuía con la distancia a la fuente (Coelho et al., 2005). De la misma forma Marin-Guirao et al. (2005) también encontró un cambio en la estructura de las comunidades de invertebrados asociados a los sedimentos contaminados del Mar Menor. No hubo diferencias significativas entre la estación de muestreo debido a la baja densidad de los anfípodos Microdeutopus sabatieri y G. aequicauda en sedimentos contaminados. En conjunto, estos hallazgos y los resultados de este trabajo de apoyo la hipótesis de que la inhibición de la actividad locomotora por sedimentos contaminados tienen consecuencias negativas sobre las poblaciones naturales.

El fuerte efecto de la temperatura sobre G. aequicauda y G. chevreuxi sugiere que las poblaciones de anfípodos de la Europa del sur podrían ser amenazados si la temperatura aumenta por encima de 23 ºC en la Ría de Aveiro y 30ºC en el Mar Menor. El modelado hidrodinámico sigue siendo una herramienta importante para predecir futuros cambios en los ecosistemas lagunares. La simulación de cambio climático para el Mar Menor muestra un aumento de la temperatura media anual de 3,28 ºC y una disminución del valor de la salinidad de 1,53 para el escenario A2 del IPCC en 2100 (De Pascalis et al., 2012). G. aequicauda puede sobrevivir y crecer en un amplio rango de salinidades entre 2 y 40 (Delgado et al. 2011) y temperaturas (Prato et al 2012), lo que sugiere una mayor capacidad de resistencia al cambio climático. Esto sugiere que el factor limitante de G. aequicauda en la laguna Mar Menor será la temperatura y la salinidad no de acuerdo con esta predicción. En el impacto del cambio climático Ría de Aveiro en la salinidad y la temperatura también se predijo (proyecto Lagunas?) Especialmente en las piscinas intermareales. Gammrus chevreuxi, con una tolerancia térmica relativamente baja, es incapaz de tolerar temperaturas

altas sostenidos de 25 ° C y baja salinidad. Por lo tanto, temperaturas elevadas sostenida, especialmente durante el bajo nivel de las mareas, es probable que limiten la distribución de Gammrus chevreuxi en las costas atlánticas del sur de Europa. Subida et al (2004) en un estudio llevado a cabo en la Ría de Aveiro indicó que la abundancia y biomasa de G. chevreuxi no mostraron patrones estacionales claros pero si se asociaba con la variación en la salinidad, oxígeno disuelto y concentración de clorofila a. La abundancia y biomasa máxima se produjeron durante la primavera con una temperatura relativamente baja y una alta disponibilidad de alimentos, condiciones que probablemente aumenten las tasas de supervivencia y / o reproducción (Subida et al 2004).

A pesar de que el aumento de la temperatura afecta a las dos especies de anfípodos, el impacto del cambio climático podría ser más fuerte en las poblaciones naturales debido a la influencia de otros factores ambiental covariantes durante el período de verano (por ejemplo, los regímenes de oxígeno, salinidad). Las poblaciones naturales de anfípodos expuestos a sedimentos contaminados son sometidas a mayor estrés debido a los retos fisiológicos de parámetros ambientales variables. El grupo de Prato et al. 2009 indicó que G aequicauda presenta una mayor sensibilidad al cadmio durante el verano. Estos autores sugirieron que esto es presumiblemente debido a la entrada de la materia orgánica más alto en la cuenca del Mar Piccolo, que causa crisis anoxia en la parte inferior, haciendo que los organismos más estresados. Esto es particularmente importante en los sistemas de estuarios donde los factores ambientales pueden ser más variables.

Eriksson y Weeks (1994) expusieron a Corophium volutator a concentraciones de oxigeno diferentes, en condiciones control y exposición a concentraciones de cobre de 50 y 100 µg. En este experimento, las concentraciones de cobre en el cuerpo fueron las mismas para todas las condiciones, pero se observó un aumento de la mortalidad y una alteración en el comportamiento cuando C.volutator se expuso a niveles de oxígeno reducidos. Estos hallazgos sugieren que la interacción de los contaminantes metálicos y el aumento de temperatura podría acoplarse con otros co-factores de estrés indirectos asociados al cambio climatico. Además, la biodisponibilidad de los metales pesados en las lagunas costeras podría variar debido a fenómenos meteorológicos extremos asociados al cambio climático. El transporte y la transformación de mercurio se ve afectado por una variedad de factores ambientales (por ejemplo, la concentración de oxígeno, la cantidad de materia orgánica, la concentración de sulfato y pH) que controlan la biodisponibilidad de mercurio y a las bacterias metilantes. La mayoría de estos factores están relacionados con los cambios de los cuerpos de aguas superficiales causadas por la modificación del clima (PONER CITAS). La salinidad en las lagunas costeras es el factor de control para el reparto de contaminantes entre los sedimentos suprayacentes y aguas intersticiales. La salinidad es también el factor natural más importante en el control de la distribución de los organismos estuarinos (CHAPMAN * y FEIYUE WANG, 2001). En este estudio consideramos este factor como constante, aunque podría ser un factor variable debido al cambio climático pudiendo aumentar o disminuir las entradas de agua dulce. La exposición a corto plazo a los tóxicos como Cd y Hg podría ocurrir en el Mar Menor y la Ría de Aveiro, respectivamente, en eventos como escorrentía de aguas pluviales pesado después de una fuerte lluvia.

Estos resultados sugieren que poblaciones de anfípodos del sur de Europa podría estar amenazadas si el aumento de temperatura alcanza los 25ºC a 30ºC en las lagunas costeras del Océano Atlántico y Mediterráneo. Respecto a la sensibilidad a los sedimentos de referencia, G. aequicauda y G. chevreuxi se han visto influenciadas por las temperaturas de experimentación, particularment, los organismos expuestos a altas temperatura (23ºC y 30ºC respectivamente) mostraron una elevada sensibilidad en todas las estaciones de muestreo.

Conclusión

Nuestros resultados sugieren que la actividad locomotora y el número de surfacings se presentan cono una señal temprana de la interacción de los factores co-estresantes temperatura y sedimentos contaminados. Los cambios en la actividad locomotora, surfacings, porcentaje de tiempo en la columna de agua y velocidad de natación tienen una conexión clara con la supervivencia de los organismos pueden tener una relevancia ecológica.

la actividad locomotora de G. aequicauda y G. chevreuxi se vio influida negativamente por el aumento de temperatra del agua. Un incremento de la temperatura del agua del mar de 23ºC a 25ºC respectivamente mostró efectos remarcable en la actividad locomotora de los anfípodos. Se encontró además un efecto aditivo causado por el aumento de temperatura y la contaminación de los sedimentos por metal en ambos anfípodos. Estos hechos resaltan las posibles interaccíónes complejas entre el cambio climático y los gradientes de contaminación en las lagunas costeras. A este respectos, uno de los objetivos del la directiva marco del agua (WFD, 2000/60/EC; European Commission, 2000) es obtener un buen estatus químico de todas los cuerpos de agua en Europa para el 2015. La WFD

La evaluación de la DMA del estado químico de una masa de agua se basa, junto con los niveles de fondo utilizadas como condiciones de referencia, el cumplimiento de normas de calidad ambiental (NCA) que, de cumplirse, permiten que el estado químico de la masa de agua que se describe como bueno. En este trabajo se indica que, incluso con los mismos niveles de fondo, las NCA de los sedimentos contaminados disminuirá en lagunas costeras debido a la vinculación ecológica del climático cambio. Ésta conexión ecológica y la sensibilidad encontrada en este estudio para el aumento de la temperatura y los contaminantes indican que la actividad locomotora se debe considerar en el evaluación de los efectos ecológicamente relevantes del cambio climático en estas especies y potencialmente en otros organismos acuáticos en ecosistemas costeros contaminados.

Las respuestas comportamentales están conectadas con complejos cambios bioquímicos y fisiológicos y deben actuar como indicadores sensibles de efectos subletales a contaminantes (Blockwell et al. 1998; Morillo-Velarde et al. 2011) o factores estresantes naturales (por ejemplo la temperatura). Los habitat costeros alterados debido al cambio climático podrían empujar a las especies de anfípodos a ambientes sub-ópitmos de regiones del sur de Europa donde pueden experimentar una reducción global de fitness y disminuir la tolerancia a la exposición a tóxicos. Este trabajo muestra la habilidad de los anfípodos G. aequicauda y G. chevreuxi a tolerar elevadas temperaturas puede ser perjudicada con la exposición conjunta a tóxicos, afectando de ese modo al comportamientos como actividad locomotora e indirectamete al mantenimiento de poblaciones naturales en el sur de Europa. Aún así, son varios los autores que son precabidos a la hora de extrapolar datos del laboratorio al medio natural (Ann-Kristin Eriksson Wiklund, Brita Sundelin Dag Broman 2005), ya que generalmente en los experimentos controlados son mayores las cantidades de contaminantes que en el medio natural.

General Discussion

4. General discussion

Hoy en día, la cronotoxicidad es unadisciplinaemergente dentro del campo de la toxicología y son excasos los trabajos centrados en metales pesados por la dificultad que conlleva elestudio de sus efectos sobre los ritmos circadianos. Por ello, ha sido un reto profundizar en un campo tan novedoso y más concretamente en el estudio de éste en aguas de transición. Por eso, los capítulos de esta tesis han ido recorriendo un camino de exploración y conocimiento progresivo a través del laboratorio (capítulo 1 y 2) donde se comenzó por el uso de técnicas tradicionales para el estudio de las variaciones diarias en la toxicidad de xenobióticos en una especie modelo,el pez cebra.Para elloelegimos una sustancia bien conocida e interesante por sus implicaciones biomédicas como es el etanol y posteriormente el Cd, como metal pesado de interés tanto para salud pública como para el medio ambiente. En ambos casos combinamos el el uso de varias técnicas actuales tales como el “video-tracking” para el estudio del comportamiento animal y los análisis transcriptómicos para el estudio de la expresión de genesimplicados en los procesos de detoxificación.

Sin querernos detener aquí, nos propusimos un planteamiento ascendente en el estudio de la toxicidad y ya más concretamente de los metales pesados. Por ello, en los capítulos 4 y 5 extendemos el uso de las técnicas de “video-tracking” al estudio de los efectos de los metales pesados y sus posibles variaciones diarias para otra especie muy importante de las aguas en transición comoson los gammáridos. Para ello utilizamos sedimentos de zonas de estudio con contaminación por metales pesados debido a actividades antropogénicas desarrolladas en el pasado, y planteamos un contexto medioambiental más realista en el que además intervinieran también factores externos cómo temperatura.

Estudios e cronotoxicidad en laboratorio con pez cebra (*Danio rerio*) como modelo de vertebrados.

En el primer bloque de ensayos escogimos zebrafish por ser un excelente modelo de vertebrados ya que comparte muchas características moleculares, bioquímicas, celulares y fisiológicas con los vertebrados superiores (usar los números 1 y 2 de referencias del

Existe un amplio abanico de estudios realizados con pez cebra y tóxicos a diferentes niveles fisiológicos (añadir más referencias, otros toxicos(Arenzana et al., 2006; Hallare et al., 2006). Sin embargo, son escasos los estudios basados en los efectos de los xenobióticos y metales pesados en función de la hora del día en peces y los resultados de los que se disponen han sido extraídos de ensayos donde la hora de la experimentación es escogida arbitrariamente. En nuestros trabajos se han mostrado por primera vez evidencias de diferencias diarias en la mortalidad y efectos en el comportamiento en peces cebra.

Los estudios clásicos de toxicidadaguda que realizamostuvieron resultados robustos en las diferencias de mortalidad de los animales en función de la hora del día para los tóxicos estudiados. Para el etanol dichas diferencias se observaron ya en larvas (5dpf) donde se obtuvouna mayor tasa de mortalidad en las horas de luz en comparación con la mortalidad a misma concentración por la noche. Similares resultados se obtuvieron al estudiar el efecto de un metal pesado, el cadmio, en peces cebras adultos, donde la tasa de mortalidad fue también significativamente mayor cuando la exposición se realizaba en la mitad de la fase de luz. Análogos resultados se habían observado anteriormente tras la exposición a anestésicos como el MS-222 y eugenol (comúnmente utilizados en acuicultura)(Sánchez-Vázquez et al., 2011) Del mismo modo, otras especies como la dorada (*Sparus aurata*) también mostraron mayor toxicidad durante el día para MS-222 (Vera et al., 2010).

En cuanto a los efectos de concentraciones subletales de etanol y cadmio, lo estudios de comportamiento mediante "video trackinig" nos proporcionaron datos de la actividad y posición de los peces cada segundo tanto con luz como en oscuridad. Los análisis de estos test señalaron que la exposición a los tóxicos de estudio durante el día tiene un mayor efecto en los peces, que mostraron una reducción significativa de sus niveles de actividad. Sin embargo, durante la noche mostraron menor o ningún efecto en su actividad natatoria. La posición de los peces en la columna de agua también se ve afectada durante las exposiciones para ambos tóxicos. Los cambios de comportamiento durante exposiciones subletales han sido estudiados por muchos investigadores (Tran & Gerlai, 2013; Pannia et al., 2014), Karlossonnorrgren y colaboradores (1985) ya observaron una reducción progresiva de la actividad con concentraciones subagudas de $CdCl_{2,}$ estoscambios podrían corresponderse con un comportamiento de evitación, que buscaría reducir la posibilidad de muerte al reducir el gasto metabólico en mantener la homeostasis fisiológica fisiológica (Olla B.L. & Pearson W.H., 1980; Schreck C.B. et al., 1997). Similar comportamiento natatorio fue observado por Gerlay y su grupo de investigación tras exposiciones a Etanol y lo atribuyeron a un efecto sedativo de éste, ya que afecta al sistema nervioso central (Gerlai et al., 2000). Todos estos estudios realizaban las exposiciones dentro del horario laboral de los centros de invesetigación sin tener en cuenta los ritmos diarios de los peces. Sin embargo, recientes estudios de la variación diaria del comportamiento de dorada a concentraciones subletales de anestésicos, obtuvieron respuestas comportamentales diferentes en función de la hora del día, (Vera et al., 2010; Sánchez-Vázquez et al., 2011) como las obtenidas en nuestros estudios.

Siendo dos sustancias tóxicas muy diferentes, losresultados de comportamiento muestran que para la misma concentración de tóxico (etanol o cadmio), la respuesta durante la fase de luz es similar a la que corresponde a exposiciones de altas concentraciones de contaminantes, indicando que el pez es más vulnerable a la exposición durante la fase más activa de éste,que en caso de el pez cebra es durante el día(Lopez-Olmeda, JF, 2010).Por eso, para una misma concentración, la toxicidad es mayor durante el día ya que no solo su actividad natatoria sino también la actividad respiratoria y la tasa metabólica son mayores.Estudios previos con *Gammarus aequicauda*, utilizado como biomarcador, también mostraron que la exposición a cadmio producía una mayor efecto durante la noche, correspondiento en este caso con la fase activa de este animal (nocturno)(Morillo-Velarde et al., 2011) al igual que en roedores (Spanagel et al., 2005)

El hecho de que el pez cebra presente estas diferencias diarias sugiere una relación entre los ritmos diarios de actividad y los de toxicidad.

Dicha ritmicidad diaria que se presenta ante tóxicos debe ser, en parte, el resultado de una ritmicidad diaria a nivel fisiológico en los procesos metabólicos de absorción y distribución.Es sabido que los factores de defensa biológica muestran variaciones diarias en sus niveles de expresión y actividad. Por ejemplo, la expresión de genes que metabolizan drogas (Zhang et al., 2009) y genes que codifican moléculas antioxidantes como glutatión(Xu et al., 2012) presentan una variación diaria en hígado de ratón. En el caso del etanol, recientes estudios de Trany colaboradores (2015) indican que las respuestas del comportamiento del pez cebra frente a etanol tienen que estar relacionadas con cambios en la actividad enzimática y en la expresión de los genes que las codifican en los órganos implicados en la detoxificación. Esto nos llevo a profundizar a nivel molecular, donde buscamos diferencias en la expresión de genes en órganos que codifican los enzimas detoxificantes. En nuestro estudio hemos observado diferenciasdía/noche en la expresión del gen que codifica para la Aldehido deshidrogenasa (*aldh2*) en hígado, aunque no hemos encontrado diferencias en el gen que codifica la Alcohol deshidrogenasa (*adh8a*). Este hecho podría estar relacionado con la disposición temporal de la enzima en la oxidación catalítica. Esto explicaría que la exposición a etanol durante el día tenga un mayor efecto en el pez cebra, tal y como se observó en el test de mortalidad y comportamiento. Para el estudio de genes detoxificantes de cadmio nos centramos en dos isoformas de los genes de metalotioneína han sido descritas en branquias de pez cebra, *mt1* y *mt2*. Nuestros resultados mostraron un pico en la expresión de *mt1*, justo antes de la transición día-noche, aumentando la disponibilidad de la proteina MT1 durante la noche, en contraste con la expresión de *mt2* que no presenta variación diaria. La mayor disponibilidad de MT1 durante la noche podría hacer que éste se una al Cd reduciendo por tanto sus efectos tóxicos.Esta correlación entre el ARNm de MT y la proteína en peces no expuestos también ha sido observada en la trucha arcoíris (*Salmogairdneri)*y en la barbatula (*Noemacheilus barbatulus)* donde Noey y colaboradores (1990) consideraron la posibilidad de una mayor resistencia al cadmio debido a la preexistencia de MTs en los tejidos de estos peces. Los metales pesados sin potencial redox, como el cadmio, causan daño oxidativo al afectar a las defensas antioxidantes, particularmente aquellos antioxidantes y enzimas con grupos tiol (Stohs & Bagchi, 1995)induciendo la expresión de MTs. Además, el cadmio puede influenciar enzimas antioxidantes, como la superóxido dismutasa (SOD) y la catalasa (CAT)(Sevcikova et al., 2011). Por lo tanto, la detoxificación de cadmio es un proceso complejo que envuelve varios mecanismos, así que se presenta como una emocionante linea de futuros trabajos para ampliar la investigación del reloj circadiano para un mejor entendimiento de la cinética de metales (absorción, distribución, metabolismo y excreción) y la dinámica en especies de peces.

Tras encontrar diferencias diarias en la toxicidad en ambos ensayos y la posible relación de estos ritmos en su detoxificación, decidimos estudiar también las posibles diferencias en la acumulación en el caso del cadmio, como metal pesado. Es sabido que el cadmio se acumula significativamente en branquias, hígado y riñones (Handy, 1992). La acumulación de este metal en tejidos de peces depende de factores como temperatura, dureza del agua, interacción de agentes, concentración y periodo de exposición (Ay et al., 1999; Saglam et al., 2013) y también incrementa con la concentración de exposición, como era de esperar (Arini et al., 2014). Sin embargo, nuestros resultados mostraron además un mayor contenido de cadmio en peces expuestos en mitad de la fase de luz. Este hecho sugiere que hay una mayor captación de cadmio en el agua durante el día y coincide con los resultados de variaciones diarias en la tasa de mortalidad y efectos en el comportamiento, apoyando la hipótesis de la toxicidad del cadmio es mayor durante la fase de luz. Estas diferencias pueden deberse en parte a la existencia de un ritmo diario de actividad motora y como sugieren nuestros estudios de expresión de metalotioneina, también a una mayor presencia de MT1 durante la fase de oscuridad.

Estudios e toxicidad en laboratorio con anfípodos como modelo de invertebrados.

En este punto ya sabemos que el momento de día en el que se estudian los animales es importante para extraer conclusiones sobre los modelos más cercanas a la realidad y así poder avanzar en el estudio del los effectos de los tóxicos.

A continuación estudiaremos los effectos de sedimentos contaminados con metales pesados que se encuentran en lagunas costeras, aguas en transición con antiguas actividades de extracción e industria y ver como afecta en el comportamiento de otro animal muy utilizado como bioindicador de ecositemas acuáticos, los gammaridios.

En la ria de Aveiro (Portugal) nuestros resultados confirman una clara alteración de la actividad de *Gammarus chevreuxi* a lo largo del gradiente de contaminación en la Ria de Aveiro. Aunque las concentraciones no son letales, exposiciones cortas a sedimentos contaminados con mercurio del área de estudio causaron un decrecimiento substancial en la actividad locomotora y por lo tanto los comportamientos de búsqueda de comida, escape de depredadores o de condiciones peligrosas, o la búsqueda de pareja reproductora puede estar comprometidas en ciertas áreas de la Ria. Estos resultados son apoyados por estudios previos en el área que demuestran una asociación entre la polución por mercurio y la degradación de las condiciones bentónicas (Pereira et al., 2009). El gradiente de contaminación por mercurio en el Laranjo de Basin fue asociado también con la reducción de la abundancia total de macrofauna, decrecimiento de especies y un aumento del dominio de los taxones de especies tolerantes al mercurio(Nunes et al., 2008).

Los efectos en la locomoción en *G. chevreuxi* por polución de mercurio fueron caracterizados sobretodo en la actividad locomotora, en decrecimiento total de la distancia vertical recorrida, una disminución del número de sufacings efectivos. Como ya indicaban Lawrence y Poulter (1998) los anfípodos pueden disminuir su resistencia a los movimientos verticales debido al redireccionamiento de sus recursos energéticos con el fin de detoxificar la acumulación de metal. Resultados similares fueron obtenidos por Wallace y Estephan (2004) cuando estudiaron las diferencias en la susceptibilidad de los movimientos verticales y horizontales en *Gammaruslawrencianus*. Nuestros datos muestran que los anfípodos muestran una reducción en el tiempo de contacto con los sedimentos contaminados, lo que sugiere una respuesta de evitación a los sedimentos contaminados.

Las grabaciones de video muestran que los anfípodos permanecen agarrados a las mallas del acuario en vez de en la superficie del sedimento.

Una interacción significativa entre el ciclo día y noche y la contaminación por mercurio fue observada en *G. chevreuxi*, éste mostró una actividad elevada durante el día y mínima durante la noche. Esto sugiere que *G. chevreuxi* posee un ritmo diario, cómo ha sido observado en otras especies de anfípodos (*G. aequicauda* y *C. volutator*) (Harris & Morgan, 1984)la falta de actividad durante la noche en *G. chevreuxi* implica una improbable actividaddurante la noche. De hecho, en efecto observado fue la reducción de los parámetros registrados durante el día a los niveles basales alcanzados durante la noche. El análisis de los cambios en el comportamiento de la actividad locomotora debenllevarse a cabo dentro del contexto de una caracterización previa del patrón de comportamiento diario de la especie de estudio. Por ejemplo, Morillo-Velarde et al. (2001) registro el ritmo circadiano de *G. aequicauda* con la máxima actividad locomotora durante la noche. Resultados similares fueron encontrados en *G. pulex* y (Elliott, 2002; Mills et al., 2006) y *G. lawrencianus* (Wallace & Estephan, 2004). Debido a estas diferencias, el resultado de los experimentos subletales deben ser interpretado con cuidado, porque la sensibilidad a sedimentos contaminados puede ser diferente dependiendo de la forma de realizar los ensayos en condiciones de luz u oscuridad y las especies a considerar, lo que significa que hay repuestas diferentes dependiendo de la especie.

La actividad locomotora representa un criterio de particular interés en los estudios ecotoxicológicos. Los estudios del comportamiento natatorio has sido extensamente utilizados para evaluar la toxicidad de varias sustancias químicas en pecesteleósteos y otros crustáceos marinos (Roast et al., 2000, 2001; Sánchez-Vázquez et al., 2011). Mostrado que los tóxicos pueden afectar a la actividad natatoria y comprometer las funciones vitales de los organismo (ej. la habilidad para escapar de los depredadores, la actividad para búsqueda de alimento, migración , reproducción) . Igual que para otros animales, para los anfípodos la actividad natatoria es un componente fundamental de su ecología y parece ser un elemento de medida extremadamente sensible a las variaciones de los factores naturales (Michalec et al., 2012).

Y para los otros organismos, el impacto indirecto en el comportamiento de los patrones de anfípodos puede conducir a la muerte de las poblaciones locales, lo que se conoce como "muerte ecológica" (Roast et al., 2000). De este modo una evaluación temprana podría usarse como una mejor medida para trazar las áreas contaminadas donde los pueden producirse efectos en las poblaciones. El análisis de las respuestas de comportamiento podrá conceder una contribución significativa en su evaluación, interpretación y comprensión del impacto de los contaminantes en los ecosistemas marinos.

Los resultados de este estudio mostraron que la contaminación por metales en las lagunas de Ria de Aveiro y Mar Menor modificó el comportamiento locomotor de dos especies de anfípodos (G. chevreuxi y G. aequicauda). Los controles de referencia de ambas especies presentan un perfil similar de comportamiento, con una actividad más alta (actividad de natación, el número de surfacingss) y mayor tiempo en contacto con el sedimento. Este estudio también indica que la influencia de factores de estrés no contaminantes como la temperatura, puede ser particularmente importantes en la respuesta de organismo en los sistemas de estuarios. G. aequicauda y G. chevreuxi fueron más sensibles a sedimentos contaminados por metales pesados a temperaturas más altas. Ambos anfípodos mostraron una disminución de la actividad de la natación, el número de surfacings y menos tiempo en contacto con el sedimento comparado con los sedimentos de referencia a la temperatura más alta. En la laguna del Mar Menor, G. aequicauda tuvo mayor sensibilidad cuando fue expuesto a la temperatura más alta (30º C). Resultados similares fueron encontrados por Prato et al. (2009) en G. aequicauda tras un estudio de laboratorio sobre los efectos de la temperatura y la sensibilidad al cadmio. Estos autores indicaron que G. aequicauda era más sensible a Cd a temperaturas más altas. La actividad locomotora disminuyo en la temperatura más alta tanto en las estaciones de referencia y como las estaciones con sedimentos contaminados. Esto podría ser el resultado de un aumento del metabolismo, aumentando la tasa de respiración (Rathore y Khangarot 2002), y por lo tanto habría un aumento de la acción de iones metálicos en las enzimas celulares y membrana celular, afectando a los mecanismos osmoreguladores e indirectamente el aumento de toxicidad de metales pesados (Yang y Chen 1996;. Serra et al 1999). Un aumento de la temperatura conlleva En generalmente un aumento el consumo de oxígeno, lo que llevaría a un aumento en el gasto de energía. Dingemanse & Wolf, 2010 sugirieron que la exposición a contaminantes puede afectar el comportamiento a través de sus efectos sobre las variables de estado asociados con la adquisición de energía. Tales modificaciones pueden a su vez alterar el costo y beneficios de diferentes comporamientos. La exposición a metales pesados puede tener como resultado una intensificación de los costes metabólicos y un balance energético negativo (Massarin et al, 2010;. Massarin et al., 2011). Este hecho podría explicar por qué ambos anfípodos disminuyeron su actividad locomotora a temperaturas más altas, que podrían ser una adaptación bioenergética a esta situación. Además, la presencia de los metales pesados también podría afectar el comportamiento del aparato locomotor, al determinar la energía que los anfípodos asignan a varias funciones como el crecimiento, la reproducción y el mantenimiento del cuerpo (Montiglio, royauté, 2014). Se especuló que la disminución de la actividad de natación de G. duebeni tras la exposición a Cu se debida a una posible reorientación de los recursos energéticos con el fin de hacer frente a la desintoxicación de metales (Lawrence y Poulter, 1998). Sin embargo, la influencia de la temperatura sobre la toxicidad de metales se puede atribuir a los cambios relativos en la tasa de absorción del metal, la eliminación, la difusión y biotransformación de los organismos. Aunque las razones específicos tanto bioquímicas como fisiológicas que pueden alterar la actividad de la natación en estos anfípodos son dudosas, este trabajo hace hincapié en la interacción entre el calentamiento climático y la contaminación a lo largo de los gradientes de contaminación de las dos lagunas.

El tiempo en la columna de agua está relacionado no solo con la actividad de natación, sino también con la capacidad de agarre de los amphipodos a sustratos verticales. Dicho comportamiento disminuyó por la temperatura y la contaminación con metales, en ambos anfípodos, lo que revela efectos subletales sobre su comportamiento. Al investigar el tiempo en la columna de agua de anfípodos del Atlántico y el Mediterráneo, se observó un patrón entre las dos lagunas costeras, en el que disminuía el tiempo en la columna de agua conforme aumentaba distancia de las zonas con sedimentos más contaminados por metal .

Sin embargo, nuestros resultados mostraron también un fuerte efecto de la temperatura sobre el tiempo en la columna de agua. En sedimentos contaminados, los anfípodos estuvieron más expuestos a la contaminación por metales debido a que pasaron más tiempo en contacto directo con el sustrato. Estos comportamiento inadecuados a estímulos externos, pueden ser debido a los efectos nocivos de los contaminantes, pudiendo tener consecuencias graves para la supervivencia a nivel individual y poblacional (Webber y Haines, 2003).

El presente documento pone de relieve la importancia del número de surfacings como indicador valioso del estado perturbación del ecosistema.

Este comportamiento es el que exige un mayor gasto energético, siendo por tanto un indicador de fitness. En un estudio de actividad de natación horizontal y vertical de Gammarus lawrencianus, Wallace y Estephan (2004) especularon que de los dos comportamientos, la actividad vertical de la piscina es más sensible a la exposición Cd porque conlleva una mayor coste energético ara alcanzar el elevación necesaria para hacer un ascenso vertical en el agua. Este ascenso vertical completo en la columna de agua requiere de un golpeo constante de los pleopodos para superar la gravedad, por lo que es probable que sea más costoso desde el punto de vista energético que los desplazamientos cortos (Boudrias, 1991; Wallace y Estefan, 2004).

Se observaron un menor número de surfacings tras la exposición a sedimentos de estaciones con concentraciones relativamente altas de metales. Esto sugiere que en estas estaciones ya se ha producido una incapacidad fisiológica seguida de una pérdida del control de movimiento. En un trabajo anterior, Morillo-Velarde et al. (2011) indicaron que la concentración requerida para estudiar una disminución significativa en la actividad de natación de G. aequicauda era 0,24 mg l^{-1} Cd en condiciones de oscuridad, siendo mucho más baja que la CL50 (48 h) encontrada para esta especie (1,71 mg Cd l^{-1}). Los hallazgos de este estudio sugieren que en el Mar Menor, existe una difusión de metal de sedimentos a la columna de agua con un efecto de menor toxicidad que la CL50 (48 h) de Cd. Además, no se produjo mortalidad en nuestros ensayos de actividad locomotora, lo que indica que las características de comportamiento de anfípodos pueden ser indicadores útiles a exposición subletal de sedimentos contaminados por metales en Mar Menor

Actividad Natación y número de surfacings son parámetros ecológicamente relevantes en anfípodos, porque sus actividades vitales como la alimentación, evitación de los depredadores y la reproducción dependen de él (Scott y Sloman, 2004). La reducción de la actividad locomotora probablemente tiene un impacto negativo en la evitación de depredadores, la busqueda de alimento o la reproducción sexual. Esto puede tener consecuencias ecológicas importantes como la disminución de la densidad de población. Los estudios de Weis et al. (2001) en ciprinidos indicaron que la reducción de densidad de la población una zona industrial de Nueva Jersey,Que contaminante? se debía a una reducción del crecimiento y longevidad y a una mayor vulnerabilidad a la depredación debido a que la actividad natatoria de los peces era menor lo que dificultaba la captura de alimento. Esto concuerda con los hallazgos de Nunes et al. (2008) en la Ría de Aveiro, que también mostró que el aumento de la contaminación por mercurio se asoció con una reducida abundancia total (incluyendo G. chevreuxi) y menor diversidad de especies de comunidades bentónicas. La distribución espacial de mercurio en los sedimentos que se encuentran en este trabajo está de acuerdo con estudios previos en la zona (Pereira et al., 1998; Ramalhosa et al, 2006;. Nunes et al (2008) Nunes et al (2008). Estos autores encontraron que la concentración de mercurio en el sedimento fue factor determinante más fuerte que influyó en la composición de la comunidad. Se observó que la densidad de crustáceos (incluyendo G. chevreuxi) disminuía a lo largo del gradiente de contaminación por mercurio. Del mismo modo, la concentración de mercurio orgánico en macroalgas disminuía con la distancia a la fuente (Coelho et al., 2005). De la misma forma Marin-Guirao et al. (2005) también encontró un cambio en la estructura de las comunidades de invertebrados asociados a los sedimentos contaminados del Mar

Menor. No hubo diferencias significativas entre la estación de muestreo debido a la baja densidad de los anfípodos Microdeutopus sabatieri y G. aequicauda en sedimentos contaminados. En conjunto, estos hallazgos y los resultados de este trabajo de apoyo la hipótesis de que la inhibición de la actividad locomotora por sedimentos contaminados tienen consecuencias negativas sobre las poblaciones naturales.

El fuerte efecto de la temperatura sobre G. aequicauda y G. chevreuxi sugiere que las poblaciones de anfípodos de la Europa del sur podrían ser amenazados si la temperatura aumenta por encima de 23 ºC en la Ría de Aveiro y 30ºC en el Mar Menor. El modelado hidrodinámico sigue siendo una herramienta importante para predecir futuros cambios en los ecosistemas lagunares. La simulación de cambio climático para el Mar Menor muestra un aumento de la temperatura media anual de 3,28 ºC y una disminución del valor de la salinidad de 1,53 para el escenario A2 del IPCC en 2100 (De Pascalis et al., 2012). G. aequicauda puede sobrevivir y crecer en un amplio rango de salinidades entre 2 y 40 (Delgado et al. 2011) y temperaturas (Prato et al 2012), lo que sugiere una mayor capacidad de resistencia al cambio climático. Esto sugiere que el factor limitante de G. aequicauda en la laguna Mar Menor será la temperatura y la salinidad no de acuerdo con esta predicción. En el impacto del cambio climático Ría de Aveiro en la salinidad y la temperatura también se predijo (proyecto Lagunas?) Especialmente en las piscinas intermareales. Gammrus chevreuxi, con una tolerancia térmica relativamente baja, es incapaz de tolerar temperaturas altas sostenidos de 25 ° C y baja salinidad. Por lo tanto, temperaturas elevadas sostenida, especialmente durante el bajo nivel de las mareas, es probable que limiten la distribución de Gammrus chevreuxi en las costas atlánticas del sur de Europa. Subida et al (2004) en un estudio llevado a cabo en la Ría de Aveiro indicó que la abundancia y biomasa de G. chevreuxi no mostraron patrones estacionales claros pero si se asociaba con la variación en la salinidad, oxígeno disuelto y concentración de clorofila a. La abundancia y biomasa máxima se produjeron durante la primavera con una temperatura relativamente baja y una alta disponibilidad de alimentos, condiciones que probablemente aumenten las tasas de supervivencia y / o reproducción (Subida et al 2004).

A pesar de que el aumento de la temperatura afecta a las dos especies de anfípodos, el impacto del cambio climático podría ser más fuerte en las poblaciones naturales debido a la influencia de otros factores ambiental covariantes durante el período de verano (por ejemplo, los regímenes de oxígeno, salinidad). Las poblaciones naturales de anfípodos expuestos a sedimentos contaminados son sometidas a mayor estrés debido a los retos fisiológicos de parámetros ambientales variables. El grupo de Prato et al. 2009 indicó que G aequicauda presenta una mayor sensibilidad al cadmio durante el verano. Estos autores sugirieron que esto es presumiblemente debido a la entrada de la materia orgánica más alto en la cuenca del Mar Piccolo, que causa crisis anoxia en la parte inferior, haciendo que los organismos más estresados. Esto es particularmente importante en los sistemas de estuarios donde los factores ambientales pueden ser más variables.

Eriksson y Weeks (1994) expusieron a Corophium volutator a concentraciones de oxigeno diferentes, en condiciones control y exposición a concentraciones de cobre de 50 y 100 µg. En este experimento, las concentraciones de cobre en el cuerpo fueron las mismas para todas las condiciones, pero se observó un aumento de la mortalidad y una alteración en el comportamiento cuando C.volutator se expuso a niveles de oxígeno reducidos. Estos hallazgos sugieren que la interacción de los contaminantes metálicos y el aumento de temperatura podría acoplarse con otros co-factores de estrés indirectos asociados al cambio climatico. Además, la biodisponibilidad de los metales pesados en las lagunas costeras podría variar debido a fenómenos meteorológicos extremos asociados al cambio climático. El transporte y la transformación de mercurio se ve afectado por una variedad de factores ambientales (por ejemplo, la concentración de oxígeno, la cantidad de materia orgánica, la concentración de sulfato y pH) que controlan la biodisponibilidad de mercurio y a las bacterias metilantes. La mayoría de estos factores están relacionados con los cambios de los cuerpos de aguas superficiales causadas por la modificación del clima (PONER CITAS). La salinidad en las lagunas costeras es el factor de control para el reparto de contaminantes entre los sedimentos suprayacentes y aguas intersticiales. La salinidad es también el factor natural más importante en el control de la distribución de los organismos estuarinos (CHAPMAN * y

FEIYUE WANG, 2001). En este estudio consideramos este factor como constante, aunque podría ser un factor variable debido al cambio climático pudiendo aumentar o disminuir las entradas de agua dulce. La exposición a corto plazo a los tóxicos como Cd y Hg podría ocurrir en el Mar Menor y la Ría de Aveiro, respectivamente, en eventos como escorrentía de aguas pluviales pesado después de una fuerte lluvia.

Estos resultados sugieren que poblaciones de anfípodos del sur de Europa podría estar amenazadas si el aumento de temperatura alcanza los 25ºC a 30ºC en las lagunas costeras del Océano Atlántico y Mediterráneo. Respecto a la sensibilidad a los sedimentos de referencia, G. aequicauda y G. chevreuxi se han visto influenciadas por las temperaturas de experimentación, particularment, los organismos expuestos a altas temperatura (23ºC y 30ºC respectivamente) mostraron una elevada sensibilidad en todas las estaciones de muestreo.

Observaciones finales y aplicaciones:

Las variaciones fisiológicas juntos con sus implicaciones ecológicas forman un campo saludable y dinámico de la investigación actual a través de la incorporación de enfoques que van desde la genómica funcional a la macrofisiología (Chown 2008). Resumiendo, los resultados obtenidos en esta investigación acentúan la importancia de el estudio de los ritmos de toxicidad así como las condiciones de experimentación (luz, oscuridad, temperatura) como factores cruciales para la obtención de resultados los más rigurosos y reproducibles en el estudio de el efecto de los tóxicos.

Para futura investigación básica y salud y seguridad ocupacional.

Ritmos de toxicidad son importantes:

- A la hora de determinar la dosis-respuesta a la hora de calcular LD 50 y otros índices de mortalidad en esta especie.
- En el diseño de estudio de neuro-comportamiento, claves en el establecimiento de criterios de estudio de los efectos deletéreos del etanol.

Para estudios ecotoxicológicos: es sabido que los cambios de temperatura en el agua afecta a numerosos parámetros de los individuos y poblaciones, por lo que en los futuros estudios de los efectos de los contaminantes se deberían tener en cuenta los regímenes térmicos de exposición, de esa forma podríamos abrir un camino a la medición de las consecuencias del cambio climático.

Futuros retos:

1. Hoy en día existen un mejor conocimiento de los ritmos biológicos en plantas, animales incluido el hombre. El estudio de la cronobiología en la salud y enfermedades tiene un impacto incluso en las terapias medicas(Smolensky & Peppas, 2007). De ahí la importancia de incluir chronotoxicología para estudiar diferencias de susceptibilidad y tolerancia de drogas y agentes tóxicos. Solo la combinación de la investigación básica y ambiental permitirá alcanzar este objetivo.

2. Desde el punto de vista medioambiental, la investigación en tóxicos y sus impactos han experimentado un gran progreso. Sin embargo, todavía existe la necesidad de entender el impacto que tienen la presencia de combinados de metales en el medio.

Conclusions

5. Conclusions

1. Exposure of zebrafish using ethanol bath at different times of day shows a rate of toxicity reflecting a higher rate of mortality in larvae during the day compared to the effects overnight. Also effects in adult zebrafish are observed in their swim and activity position in the water column to be significantly more severe during the light phase.

2. Exposure zebrafish using cadmium bath during different times of day reflected an increase in mortality and cadmium accumulation in tissues during half of the light phase and a further reduction of locomotor activity when the fish is exposed to sublethal concentrations.Expression of *mt*1 shows a peak before the end of the light phase. All these results indicate that zebrafish are more vulnerable to cadmium during the light in the dark phase.

3. The exposure of *Gammarus chevreuxi* by mercury-contaminated sediments showed a deleterious effect on the swimming activity even considered non-lethal concentrations. These effects were most pronounced during the light phase, which corresponds to the most active animal.

4. Our results suggest that the locomotor activity and the number of surfacings an early sign of the interaction of co-temperature stressors and contaminated sediments occur cone. The behavioral parameters studied in *Gammarus. aequicauda* and *Gammarus. chevreuxi* have a clear connection to the survival of organisms and can have an environmental relevance.

General Bibliography

6. General Bibliography

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Summary in Spanish

Esta tesis tiene como objetivo investigar los efectos del etanol y metales pesados (cadmio y mercurio) en los patrones diarios de dos especies acuáticasclave: pez cebra (*Darío rerio*) y anfípodo gammarus (*G. aequicauda* y *G. chevreuxi*). La investigación se realizó en dos niveles organizativos: comportamentaly molecular. Con este fin, hemos establecido cuatro objetivos específicos:

1. Investigar los efectosal etanolen el pez cebra en función en su ritmo circadiano.

2. Determinar la existencia de un ritmo diario de la toxicidad del cadmio en el pez cebra.

3. Caracterizar en *Gammarus chevreuxi*los efectos enla actividad locomotora debido a sedimentos contaminados con mercurio.

4. Evaluar la calidad ambiental de los sedimentos de la cuenca sur del Mar Menor influenciados por históricasactividades mineras.

Sobre la base de lo expuesto esta tesis, es el objetivo ha sido combinar los estudios tradicionales de toxicología con nuevos enfoques en el estudio del comportamiento y técnicas moleculares para explorar algunos de los principales efectos de los xenobióticos y metales pesadospueden tener en animales utilizados como modelos experimentales.

Por lo tanto, estos nuevos datos tiene la intención de señalar la importancia de aplicar un enfoque multidisciplinario para estudios toxicológicos, así como elevar la importancia de la elección del momentoadecuado para realizar el ensayo de toxicidad.

Capítulo 1. Diferencias en la toxicidad del etanol dependiendo del momento del día: estudio cronobiológico en pez cebra (*Danio rerio*)

El Etanol es la droga cuyo abuso está más extendido, también se utiliza como solvente de sustancias químicas lipofílicas en bioensayos de toxicidad. Sin embargo, es escasa la información disponible sobre sus efectos nocivos dependiendo de la hora del día de su aplicación o consumo o la existencia de ritmos de detoxificación. En este capítulo investigamos los ritmos diarios en: la mortalidad de larvas de zebrafish (5 días después de la fertilización) expuestas a concentraciones subletales de etanol (1% v/v de etanol, durante 15 minutos). Además, estudiamos la expresión relativa de dos genes que codifican enzimas encargados de detoxificación del etanol (la alcohol deshidrogenasa, *adh8a*y el aldehído deshidrogenasa, *aldh2*), localizados en el hígado de pez cebra adulto. Los resultados muestran que larvas mantenidas bajo un ciclo 12h:12h luz- oscuridad presentan una mayor tasa de mortalidad (80%) al comienzo de la fase de luz, mientras que no se registró mortalidad al final de la fase de oscuridad. En cuanto a los efectos subletales, la exposición a etanol redujo en gran medida la actividad locomotora durante la mitad de la fase de luz, hecho que no se observó durante la mitad de la fase de oscuridad. Los resultados coincidieron con los obtenidos en la expresión de *aldh2* en hígado. Enzima encargado de la degradación del producto del *adh8a*, cuyo pico de expresión se sitúa justo antes de que comience la fase de oscuridad.

En resumen, en nuestro trabajo se ha observado por primera vez evidencias de diferencias diarias en la mortalidad y comportamiento de peces zebra expuestos a Etanol. Además, tras el estudio de la expresión de los genes implicados en la detoxificación del etanol hallamos variaciones diarias en la expresión relativa de la aldehido deshidrogenasa. Estos hallazgos proporcionan la primera evidencia de un ritmo diario en los mecanismos de detoxificación del pez cebra. Resaltando la importancia de considerar la hora del día a la que se realizan las exposiciones a etanol durante ensayos toxicológicos.

Capítulo 2. Cronotoxicidad del cadmio en pez zebra*(Danio rerio)*: patrones de mortalidad, comportamiento, acumulación y detoxificación en función de la hora del día.

Los peces poseen osciladores circadianos endógenos de ahí que su fisiología y respuesta a xenobióticos sea rítmica. Sin embargo, en estos organismos el estudio de ritmos de cronotixicidad y detoxificación está por desarrollar. El objetivo de este trabajo fue evaluar las respuestas del pez cebra expueto a concentraciones de cadmio letales y subletales en mitad de la fase de luz (ML) y en mitad de la fase de oscuridad (MD). Además, también se estudió las diferencias en la acumulación de cadmio en branquias dependiendo de la hora del día y la expresión de los genes implicados en la detoxificación del metal pesado. Los resultados venticuatro horas después de la exposición aguda de 100mg/l de cadmio, se observo una diferencia significativa en tasa de mortalidad en el pez cebra siendo mayor en ML que en MD (77% respecto a 33%, respectivamente). Además, se observó un cambio en los patrones de comportamiento tras 3 horas de exposición a 40mg/l, siendo su efecto mayor en ML reduciendo significativamente la actividad locomotora después de una hora de exposición mientras que en MD los niveles de actividad no se redujeron incluso después de 3 horas de exposición. Estos resultados coinciden con una mayor acumulación de cadmio en branquias durante ML, indicando que la captación de este por agua puede ser mayor durante el día, cuando el pez cebra presenta su mayor actividad. Finalmente, el patrón diario de la expresión de los genes de metalotioneina (*mt1* y *mt2*) fue medido en branquias del pez cebra para estudiar la correlación temporal entre las variaciones de efecto del cadmio y los mecanismos de detoxificación. Nuestros resultados muestran que mt1 tiene un pico al final del día, lo que sugiere que habra mayor concentración de proteína MT presente durante la noche y como consecuencia se produciría una mayor detoxificación de Cd aumentando su tolerancia. Curiosamente la expresión de *mt2* fue constante durante todo el día. Teniendo en cuenta nuestros resultados la susceptibilidad y tolerancia del pez cebra a los metales pesados como el cadmio muestra diferencias diarias reguladas por su sistema circadiano. Por lo tanto, estos resultados deben ser tenidos en cuenta en el diseño experimental de estudios ecotoxicológicos donde se utilice el pez cebra como modelo.

Capítulo 3. Respuestas comportamentales del anfípodo *Gammarus chevreuxi* a sedimentos contaminados por mercurio

El propósito de este trabajo fue determinar si la exposición a sedimentos contaminados históricamente por mercurio puede alterar la respuesta del gammarido; *Gammarus chevreuxi.* La respuesta del comportamiento locomotor fue medida bajo condiciones de laboratorio controladas utilizando un gradiente de sedimentos contaminados con mercurio procedentes de la zona de estudio, cuenca de Laranjo (en la laguna costera de Aveiro, Portugal). Para estudiar los cambios en la actividad locomotora del anfípodo se analizarosmúltiples parámetros durante 24 horas siguiendo su patrón natural de luz/oscuridad. Los anfípodos expuestos a los sedimentos contaminados experimentaron una reducción de la actividad natatoria, evitaron el contacto directo con los sedimentos, cubrieron una menor distancia vertical en sus desplazamientos y realizaron un menor número de surfacings (desplazamientos en un movimiento único desde el fondo hasta la superficie). Este comportamiento estuvo condicionado por patrones circadianos de comportamiento de la especie de estudio, que exhibió una mayor actividad locomotora durante el día. Los resultados de los experimentos apoyan el uso de la actividad de organismos autóctonos como una herramienta adecuada para la evaluación de sedimentos contaminados por mercurio, y por lo tanto los test de actividad locomotora pueden ser utilizados para relacionar los niveles de contaminación con los efectos sobre las poblaciones autóctonas y los cambios potenciales en los estados de los ecosistemas.

Capítulo 4. Efectos de los metales pesados y cambio climático en moralidad y comportamiento de *Gammarus aequicauda* y *Gammarus chevreuxi*

La peninsula Iberica cuenta con dos de las mayores lagunas costeras del sur este de Europa, Mar Menor en el SE de España y Ria de Aveiro en el NE de Portugal. Actividades mineras e industriales desarrolladas históricamente en ambas lagunas han causado la contaminación por metales de sus sedimentos, siendo Cd y Hg los metales más abundantes, respectivamente. La temperatura y su capacidad de alterar las propiedades químicas de muchos contaminantes, es otro factor importante a estudiar. Fenómenos derivados del cambio climático como son el aumento de la temperatura media es otros de los agentes a los que se exponen las comunidades bentónicas de ambas lagunas. El efecto de la contaminación de los sedimentos por metales combinado con los incrementos de temperatura previstos en las zonas de estudio se determinaron por medio de bioensayos de toxicidad. Se emplearon dos especies de anfípodos, cada una característica de su laguna. Dichos efectos se valoraron a través de análisis de comportamiento de los anfípodos. Los resultados mostraron que la exposición metales causó una disminución de la actividad locomotora en ambos anfípodos, como se evidencia por una disminución marcada y dosis dependiente en la actividad de natación, número de surfacings y los valores de la velocidad de natación. Como conclusión, los bioensayos de actividad locomotora con ambos gammáridos parecen ser un indicador sensible de la contaminación por metales pesados.

Conclusiones

1. La exposición de pez cebra mediante baño a etanol a diferentes horas del día muestra un ritmo de toxicidad que refleja una mayor tasa de mortalidad en larvas durante el día comparado con los efectos durante la noche. Así mismo en adultos de pez cebra se observan efectos en su actividad natatoria y posición en la columna de agua siendo significativamente más severos durante la fase de luz.

2. La exposición de pez cebra mediante baño a cadmio durante diferentes horas del día reflejó un incremento de la mortalidad y acumulación del cadmio en tejidos durante la mitad de la fase de luz, así como una mayor reducción de la actividad locomotora cuando el pez es expuesto a concentraciones subletales, además la expresión de *mt1* muestra un pico antes al final de la fase de luz. Todos estos resultados indican que el pez cebra es más vulnerable al cadmio durante la fase de luz que en la fase de oscuridad.

3. La exposición de *Gammarus chevreuxi* mediante sedimentos contaminados con mercurio mostró un efecto deletéreo en la actividad natatoria incluso en concentraciones consideradas no letales. Dichos efectos fueron más acusados durante el la fase de luz, que corresponde con la más activa del animal.

4. Nuestros resultados sugieren que la actividad locomotora y el número de surfacings se presentan cono una señal temprana de la interacción de los factores co-estresantes temperatura y sedimentos contaminados. Los parámetros comportamentales estudiados en *Gammarus*. *aequicauda* y *Gammarus*. *chevreuxi*tienen una conexión clara con la supervivencia de los organismos y pueden tener una relevancia ecológica.

Annex

7. Annex

Annex I: Scientific production resulting from the experiments on the present PhD thesis:

<u>Scientific papers</u>

C. Bello, T. Monero, J. Lloret, F.J Sánchez-Vázquez, A.M.V.M. Soares, A. I. Lillebø[2],A. Marín. The effect of historical mercury contamination in sediments on amphipod locomotor activity.

Bello, C.; Sánchez-Vázquez*, F.J; Vera, L.M. Ethanol toxicity differs depending on the time of day: a chronobiological study in zebrafish (*Danio rerio*).

Bello, C.; Sánchez-Vázquez*, F.J; Vera, L.M. Cadmium chronotoxicity in zebrafish *(Danio rerio)*: mortality and behavioral responses

<u>Collaborations</u>

Paper focus group y ecosistemsa.Lloret J.

Bioindicadores. Marin A. , Lloret J., Velasco F., Bello C. 2015

Transboundary water management across borders and interfaces: present and future challenges (2013). Susan Baggett, Geoffrey D Gooch, Sarah Hendry, Małgorzata Bielecka, Olena Katerusha, **Carolina Bello** Marin, Lisa P Sousa.

Using participatory methods for coastal lagoon management and climate change. Susan Baggett, Geoffrey D Gooch, Sarah Hendry, Małgorzata Bielecka, Olena Katerusha, **Carolina Bello Marin**, Lisa P Sousa (2013) In: Roebeling, P.C. & Rocha, J. (Eds), 2013. Proceedings of the TWAM2013 International Conference & Workshops (CD-ROM).CESAM – Department of Environment & Planning, University of Aveiro, Portugal. 93pp. ISBN: 978-972-789-377-5. Aveiro, March 2013.

Congress contributions:

LAGOONS International Conference "Between the River and the Sea", Dundee, 16-18 September 2014. Oral presentation

CBF, 11th International Congress on the Biology of Fish. 3-7 August, 2014, Heriot-Watt University, Edinburgh. Poster

SETAC. 9º Congreso Ibérico y 6º Iberoamericano de Contaminación y Toxicología Ambiental.(Valencia , Spain 1-4 July of 2013) :

Poster: Susceptibility of locomotor activity to polluted sediments from Ria de Aveiro (Portugal) in a gammaridean amphipod (*Gammarus chevreuxi* Sexton, 1913). C. Bello, T. Monero, J. Lloret, A. Marín, A.M.V.M. Soares, I. Lopes, A. I. Lillebø

Comunicación oral :Effects of mining wastes on locomotor activity rhythms of the amphipod*Gammarus* aequicauda (Martynov, 1931). C.Bello, J. Lloret, A. Marín, F.J. Sánchez-Vázquez

XIII Congress of the European Biological Rhythms Society. *Oxford, UK. 20-26 August 2011*

Poster: Cadmium chronotoxicity in zebrafish *(Danio rerio)*: mortality and behavioral responses

Book chapters

The Physio-geographical Story of Mar Menor.A Marín, J Lloret, J Velasco and **C Bello**.

The Management Story of Mar Menor. J Lloret, A Marín, J Velasco and **C Bello**.

Lagoons response using key bio-indicators & and implications onecological status (WFD). A Marín, J Lloret, J Velasco and **C Bello**

The DPSIR framework applied to the society vision for tourism in 2030 in European coastal lagoons. M Dolbeth, AI Lillebø, P Stålnacke P, GD Gooch, L Sousa, FL Alves, J Soares, **C Bello**, A Marín, V Khokhlov, Y Tuchkovenko, M Bielecka, G Różyński, A Reda and B Chubarenko.

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