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“Pharmaceutical residues in aquatic systems: mode of action and effects on mussel physiology”

Presentata da: Sara Buratti

Coordinatore Dottorato
Prof. Enrico Dinelli

Relatore
Prof.ssa Elena Fabbri

Esame finale anno 2011
Dedicated to my parents,

Emilia and Sauro
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Abstract

Pharmaceutical residues contaminate aquatic ecosystems as a result of their widespread human and veterinary usage. Since continuously released and not efficiently removed, certain pharmaceuticals exhibit pseudo-persistence thus generating concerns for the health of aquatic wildlife.

This work aimed at assessing on mussels *Mytilus galloprovincialis*, under laboratory conditions, the effects of three pharmaceuticals, carbamazepine (antiepileptic), propranolol (β-blocker) and oxytetracycline (antibiotic), to evaluate if the human-based mode of action of these molecules is conserved in invertebrates. Furthermore, in the framework of the European MEECE Programme, mussels were exposed to oxytetracycline and copper at increasing temperatures, simulating variations due to climate changes. The effects of these compounds were assessed evaluating a battery of biomarkers, the expression of HSP70 proteins and changes in cAMP-related parameters. A decrease in lysosomal membrane stability, induction of oxidative stress, alterations of cAMP-dependent pathway and the induction of defense mechanisms were observed indicating the development of a stress syndrome, and a worsening in mussels health status. Data obtained in MEECE Programme confirmed that the toxicity of substances can be enhanced following changes in temperature. The alterations observed were obtained after exposure to pharmaceuticals at concentrations sometimes lower than those detected in the aquatic environment. Hence, further research is advisable regarding subtle effects of pharmaceuticals on non-target organisms.

Furthermore, results obtained during a research stay in the laboratories of Cádiz University (Spain) are presented. The project aimed at measuring possible effects of polluted sediments in Algeciras Bay (Spain) and in Cádiz Bay, by assessing different physiological parameters in caged crabs *Carcinus maenas* and clams *Ruditapes decussatus* exposed in situ for 28 days. The neutral red retention assay was adapted to these species and proved to be a sensitive screening tool for the assessment of sediment quality.
1. Introduction
1.1. Pharmaceuticals as “emerging contaminants”: an issue of concern from different sources.

The presence of pharmaceuticals and personal care products (PPCPs) in the environment has become a recent research topic. The presence of these compounds in the environment is known since decades, but it was only in the mid 90s with advances in analytical techniques that important knowledge on environmental contamination by those compounds grew. Chromatographic-detection techniques improved powerfully, enabling detection limits within the ng/L to µg/L range and allowed researchers to quantify a large number of medicines components (i.e. drugs and excipients) in the environment, thus making the scientific community to reflect on this contamination type as a potential issue meriting concern and further investigation (Santos et al., 2010). The attention to PPCPs originally focused on their occurrence and monitoring, primarily in surface/ground waters and untreated/treated sewage. This research was driven principally by environmental analytical chemists, as new instrument technologies expanded the types of molecules that could be easily identified, as instrument sensitivity increased, and as detection limits of analytical methods were lowered. Approximately during the late 1990s, the interest began to expand to waste treatment and fate/transport. In the last decade, more attention was turned to ecotoxicology, pollution prevention, and environmental stewardship. Likewise, the scope of environmental compartments under investigation has expanded from primarily waters to now include sediments (and suspended particulates), sewage sludge (and biosolids), air (e.g., PPCPs sorbed to suspended particulates), and biota.

Confirming this growing interest, the annual rate of published articles directly relevant to PPCPs has grown exponentially since the middle of 1990s (Cleuvers, 2003; Daughton, 2007) (Fig. 1.1).
Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. These chemicals are designed to have a specific mode of action, and many of them for some persistence in the body. These features among others make pharmaceuticals to be evaluated for potential effects on aquatic flora and fauna (Fent et al., 2006). Some of the chemicals released into the environment may have endocrine-disrupting effects in living organisms, including humans (Nikolaou et al., 2007).

By itself, the word “pharmaceutical” refers to a chemical prepared or dispensed in pharmacies and which treats or prevents or alleviates the symptoms of disease or physiologic function. But in the environment not only these compounds are found. PPCPs include all chemicals used for humans, domestic animals, or agricultural crops that treat disease, alter or improve physiological, cosmetic, or emotional function, appearance, or status, prevent illness (prophylaxis) or maintain health, help in the diagnosis or monitoring of health or disease, or serve to formulate the active ingredient into a commercial product (e.g., excipients and delivery vehicles). These chemicals include human and veterinary prescription and medications and also diagnostic agents (e.g. X-ray contrast media, radiopharmaceuticals), vaccines, “nutraceuticals” (bioactive dietary supplements) and vitamins. Drug consumption originates also from illegal usage; illicit drugs, in particular, comprise an unknown but possibly significant fraction of total drug usage, and consequently contribute to
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individual environmental residues and to the overall environmental loading of PPCPs (Fig. 1.2).

Fig. 1.2. Therapeutic classes detected in the environment, expressed in relative percentage. Data collected from 134 articles published between 1997 and 2009. From Santos et al., 2010.

The universe of PPCPs includes the parent form of the chemicals (the active ingredients or prodrugs, inactive precursors that are converted to the active form by normal metabolic processes), and furthermore their bioactive metabolites and transformation products (including conjugates) (Daughton, 2007).

Pharmaceuticals are conceived primarily to have particular physiological modes of action and frequently to resist to inactivation before exerting their intended therapeutic effect. However, these same properties are paradoxically responsible either for bioaccumulation and toxic effects in aquatic and terrestrial ecosystems (Fent et al., 2006). In a different way from some conventional pollutants (such as pesticides, detergents, fuels, metals and other compounds), medicines are continuously delivered at low levels which might give rise to toxicity even without high persistence rates (Santos et al., 2010).

Pharmaceuticals can be classified according to their purpose and biological activity, like antibiotics, analgesics and anti-neoplastics. Classification according to chemical structure is used mainly for the active pharmaceutical ingredients within sub-groups of medicines, for example within the group of antibiotics. Other
classifications refer to the mode of action; in this case, chemical structures of molecules within the same group can be very different and therefore their environmental fate can be different too (Kümmerer, 2009).

Some drugs are excreted essentially unaltered in their free form, others are metabolized to various extents, still others are converted to more soluble forms by formation of conjugates (Daughton and Ternes, 1999). Many pharmaceuticals indeed undergo a structural change in the body of humans and animals, respectively. The result of such a process is metabolites. After their excretion and introduction into the environment, both parent compounds and metabolites can undergo structural changes by a variety of biotic and non-biotic processes. Pharmaceuticals can be incompletely transformed by organisms such as bacteria and fungi in the environment as well as by light and other abiotic chemical processes. In general, it is supposed that metabolism and transformation of active pharmaceutical ingredients lead to decreased toxicity. In some cases however, metabolism could give origin to more active compounds, for example in the case of prodrugs. This could happen also after photolysis and other oxidizing processes (Kümmerer, 2009) (Fig. 1.3).

Fig. 1.3. Metabolites and transformation products of pharmaceuticals. From Kümmerer, 2009.
The active pharmaceutical ingredients used in medicine as well as the additional compounds in medical formulations may enter the environment by different routes (Fig. 1.4). These include several different non point sources such as manufacturing plants, effluents from sewage treatment plants, waste and landfill runoff.

Fig. 1.4. Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment. From Heberer, 2002.

The consumption of pharmaceuticals is substantial. In the European Union about 3000 different substances are used in human medicine such as analgesics and antiinflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Also a large number of pharmaceuticals are used in veterinary medicine, among them antibiotics and antiinflammatory drugs. Pharmaceuticals are excreted after application in their native form or as metabolites and enter aquatic systems via different ways. The main pathway from humans is ingestion following excretion and disposal via wastewater. Residues of the parent PPCPs (as well as sometimes a complex array of metabolites) are either excreted or are dislodged from skin by sweating, bathing, or swimming (Daughton, 2007). Municipal wastewater is therefore the main route that brings human pharmaceuticals after normal use and disposal of unused medicines into the environment. Hospital wastewater, wastewater from manufacturers and landfill leachates may contain significant concentrations of these drugs. Pharmaceuticals
not readily degraded in the sewage treatment plant are being discharged in treated effluents resulting in the contamination of rivers, lakes, estuaries and rarely, groundwater and drinking water. Where sewage sludge is applied to agricultural fields, contamination of soil, runoff into surface water but also drainage may occur. In addition, veterinary pharmaceuticals may enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture (fish farming) (Fig. 1.5). Investigations conducted in surface waters pointed out that common illicit drugs also contaminate the aquatic environment of populated areas (Zuccato et al., 2008); Zuccato et al. (2005) for example reported that kilograms of cocaine residues travel daily down the River Po (Italy).

Pharmaceuticals have been found in the effluents from medical care units, municipal sewages and the effluents of sewage treatment plants, in surface water, seawater, ground waters and in drinking water (Heberer, 2002; Kümmner, 2009; Murray et al., 2010; Pal et al., 2010). These molecules have also been detected in runoff from landfill sites and even in the arctic environment (Kallenberg et al., 2008). The concentrations of pharmaceuticals in surface water and the effluents
from sewage treatment plants have been shown to lie in the ng/l to µg/l range (Kümmerer, 2009; Murray et al., 2010).

In contrast to the extensive literature describing the occurrence and persistence of pharmaceuticals in freshwater systems, little attention has been paid to their prevalence and quantification in marine ecosystems. In this perspective, Wille et al. (2010) developed and validated a quantitative analytical method for pharmaceuticals in seawater. On the basis of data on the current use in Belgium, 13 environmentally relevant pharmaceuticals were selected from five different therapeutic classes. These included four antibiotics (sulfamethoxazole, ofloxacin, trimethoprim and chloramphenicol), four nonsteroidal anti-inflammatory drugs (NSAIDs) (mefenamic acid, diclofenac, salicylic acid, and ketoprofen), two β-blockers (propranolol and atenolol), two lipid regulators (bezafibrate and clofibrac acid) and one psychiatric drug (carbamazepine).

After administration, some pharmaceuticals are not completely metabolized. The unmetabolized parent drugs and some metabolites are subsequently excreted from the body via urine and faeces. In places with sewage systems, these pharmaceutical residues enter the WWTPs via wastewater (Zhang et al., 2008).

A tangentially related issue regarding sources is the fate of the packaging materials used for PPCPs, such as the materials used for plastic vials, IVs, and syringes, including the drug residues contained therein. Incineration and weathering of these materials are processes perhaps leading to a number of additional unknown products (Daughton, 2009).

The most obvious pathway for environmental contamination of medicines is via the unaltered excretion in urine and faeces although other anthropogenic mechanisms should be assumed, namely:

a) Metabolism post-consumption; since many drugs are metabolised as the organism attempts to convert hydrophobic compounds into more easily excreted polar residues. Their bioconversion into one or more metabolites can occur throughout Phase I and Phase II reactions (Fig. 1.6). Phase I reactions include oxidation, reduction and hydrolysis to modify the original molecule structure by introducing functional groups more receptive to phase II reactions. Phase II reactions (or conjugation reactions) consist of the addition of endogenous groups (like glucuronic acid, sulphate, glutathione, etc.) to receptive functional groups present in the original molecule or in its metabolite derived from phase I.
b) Diagnostic compounds; such as X-ray contrast media are directly discharged in their native forms.

c) Household disposal; either topic formulations or unused medicines (out-of-date or unwanted) are discarded through the sink/toilet or via waste collection, before being taken to landfill sites where they appear as terrestrial ecosystem contaminants. Alternatively, they may possibly leak into surrounding water compartments.

d) Impacts due to anthropogenic activities; as, for instance, sewage treatment plant (STP) sludge, which can carry non-suspected drugs and is frequently used as a fertilizer on agricultural land; veterinary medicines, which are also excreted in urine and faeces by animals before being spread onto land via manure application as fertilisers. Apart from the potential for direct soil contamination, there is also the risk of run-off with heavy rain, thus potentially contaminating both the surrounding surface and groundwater. Other example of an anthropogenic activity is aquaculture, whose pharmaceuticals employed, as well as their metabolites and degradation products, are directly discharged into surface waters. Another important source of environmental contamination by pharmaceuticals is the effluents of pharmaceutical production facilities (Santos et al., 2010).

![Fig. 1.6. Schematic representation of pharmaceutical biotransformation to increase their polarity. Adapted from Daughton and Ternes, 1999.](image)

Wastewater treatment systems are designed for collection and transportation of wastewater and to reduce organic matter that may cause oxygen depletion in the recipient surface water, with the aim of reducing nutrients (nitrogen and phosphorus) that can cause over-fertilisation of recipient lakes, streams and the sea. When pharmaceuticals, or the remains of pharmaceuticals after use, enter the wastewater treatment plants where their fate is governed by the physical, chemical and biological properties of the substance and of the treatment processes in use at the treatment plant. The treatment plants of today are not designed for the reduction or degradation of pharmaceuticals. The fate of the pharmaceuticals in the
plants is therefore determined by the character of the substance itself and of the processes used at particular plants. In some cases the pharmaceuticals can be biologically degraded, if the process conditions are favourable at the plant.

Three main mechanisms have to be taken into account in order to understand the fate of the substances in wastewater treatment systems: evaporation of volatile compounds, compounds ability to adhere to particles and solubility of the pharmaceuticals in water (la Cour Jansen and Ledin, 2009).

What is known today is that very few pharmaceuticals are volatile. This means that evaporation from the plants is not significant. The majority of substances are water soluble and will pass through the plants, unless they are degraded. The major method for handling sludge today is anaerobic digestion, but it was observed that destruction of pharmaceuticals by anaerobic digestion is effective only in a few cases. As a consequence, most of the pharmaceuticals have to be handled in the water treatment part of the STP. Also in this case, biodegradation of these molecules depends on their chemical properties and on the capability of bacteria of degrading them. If pharmaceuticals adhere to particles, physical methods can be used; however, in this case only the result obtained is the separation from the wastewater with no destruction of pharmaceuticals. Besides the removal of the pharmaceuticals all other particles and substances adsorbed to particles are removed. That means in general an improved removal of many other substances from the wastewater, such as particulate organic matter, nitrogen and phosphorus, heavy metals and also pathogens, but it must be kept into consideration that these particles will need further handling. Another way of processing these molecules is the chemical approach. Chemical methods can result in complete or partial destruction of the pharmaceuticals. Partial destruction can lead to the formation of new substances, which are normally less harmful than the original chemical. Examples exist however where newly formed degradation products are more harmful than the parent compound. For example, degradation experiments with carbamazepine have shown the formation of several transformation products including acridine and acrodone; the latter compound is one example of a molecule that have non-wanted biological effects of greater importance then the mother compound since it is known to cause DNA damage and exhibits greater ecotoxicity (la Cour Jansen and Ledin, 2009).
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The evaluation of removal efficiency in STPs (by comparing influent and effluent contents) has been studied in detail, showing removal rates that can differ by up to 99% (Santos et al., 2010). Some studies showed that removal of the parent compound ranged from 7% (e.g. for the antiepileptic drug carbamazepine) to 96% (for the β-blocker propranolol) and that most of the removal efficiencies averaged about 60% (Daughton and Ternes, 1999).

Hence, pharmaceuticals outgo STP barriers and reach water streams where they are detected at concentrations in the range of ng/L to µg/L (Fig. 1.7).

![Graph showing concentration of pharmaceuticals in treated wastewater and surface water](image)

**Fig. 1.7.** Concentration of pharmaceuticals in treated wastewater (a) and surface water (b). From Fent et al., 2006.

Although most pharmaceuticals are designed to target specific metabolic pathways in humans and domestic animals, they can have numerous often unknown effects on metabolic systems of non-target organisms, especially invertebrates. Although many non-target organisms share certain receptors with humans, effects on their physiology are usually unknown. It is important to recognize that for many drugs, their specific modes of action even in the target species are also unknown. For these drugs, it is impossible to predict what effects they might have on non-target organisms. Without knowing the mode of action, coupled with not knowing the
possible receptors, it is impossible to design rational toxicity testing procedures at the organism level.

Moreover, most pharmaceuticals are racemic mixtures. For a specific optically active drug, it is theorized that only one of its optical isomers is responsible for the desired physiologic therapeutic effects; the other isomers are at best inactive, or even worse, responsible for many of the untoward side effects observed (Daughton and Ternes, 1999; Kasprzyk-Hordern, 2010).

The current knowledge indicates that residues of pharmaceuticals at trace quantities are widespread in aquatic systems. Pharmaceuticals in the environment are suggested to pose only a low risk for acute toxicity. For chronic effects, the situation may be different, but there is a considerable lack of information. Investigation of multigenerational life-cycle effects or at various life stages is lacking, although many aquatic organisms are exposed for their entire life. There is a need to focus on long-term exposure assessment regarding specific modes of action of pharmaceuticals to better judge the implications of pharmaceutical residues in aquatic systems. Only after filling these gaps, more reliable environmental risk assessments with much lower uncertainty can be performed (Fent et al., 2006).

1.2. Pharmaceuticals and legislation: what does regulation say?

In spite of the sizeable amounts of human drugs released to the environment, concise regulations for ecological risk assessment are largely missing (Fent et al., 2006). This probably arises because available data is insufficient to quantify a precise contamination profile. Abundant conclusive studies concerning chronic toxicity are also lacking so that it becomes impossible to infer the risks of long-term exposure of pharmaceuticals and their metabolites on fauna and flora (Santos et al., 2010).

For more than 20 years, European legislation has required an environmental risk assessment within the pharmaceutical authorization procedure. Only in the last few years, regulatory agencies have issued detailed guidelines on how pharmaceuticals should be assessed for possible unwanted effects on the environment (Fent et al., 2006). The European Union Directive 92/18/EEC introduced for the first time the
requirement for an environmental risk assessment, as a prerequisite to obtain marketing authorization for veterinary pharmaceuticals. For this purpose, the European Agency for the Evaluation of Medicinal Products (EMEA) published a “Note for Guidance” where guidelines to assess the environmental risk of veterinary medicinal products are established. The European Commission extended its concerns to pharmaceuticals for human use by publishing the Directive 2001/83/EC, which was subsequently amended by the Directive 2004/27/EC. These directives established that marketing authorization for new medical products for human use should be accompanied by an environmental risk assessment, whose guidelines were set out by EMEA. Nevertheless, the environmental impact does not provide sufficient grounds for a refusal (Santos et al., 2010). Environmental risk assessment of both veterinary and human pharmaceuticals is assessed in a step-wise approach, divided into two phases. In phase I drug environmental concentrations are estimated; phase II is an assessment of physical, chemical and ecotoxicological properties. In addition, an exposure threshold value or “action limit” separates the exposure estimation in the first phase from the test requirements in the subsequent second phase. The goals of the risk assessment include protection of the aquatic and terrestrial ecosystems, groundwater, microorganisms in sewage treatment plants and top predators.

The EMEA guidance document for human pharmaceuticals came into force in 2006. In phase I, the drug concentration expected to occur in the aquatic environment is calculated based on the maximum daily dose. If this value is below a defined action limit of 0.01 µg/L it is assumed that this specific medicinal product is unlikely to represent a risk to the environment. In this case the assessment procedure does not continue. If on the other hand the calculated concentration in surface water exceeds the action limit of 0.01 µg/L a phase II environmental fate and effect analysis is required.

When a high risk of toxic effects is supposed (e.g. in the case of potential endocrine disruptors), a phase II assessment should however be carried out irrespective of the predicted environmental concentration. Furthermore, a screening for persistence, bioaccumulation and toxicity is necessary if the octanol-water partition coefficient (log Kow) exceeds 4.5. This identifies a potential risk for bioaccumulation (Fig. 1.8).
Natural substances like vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates, lipids and herbal medicinal products are exempted from environmental assessments.

In the second phase, information on the physical, chemical, and ecotoxicological properties are obtained and assessed in relation to the extent of the environmental exposure. This phase is further split into two tiers, Tier A and Tier B.

In Tier A, possible fate and effects of the pharmaceutical and/or its major metabolites are evaluated; if no risk is identified at the Tier A level, the assessment is considered completed. Conversely, if a risk is recognised at the Tier A level, then an extended ecotoxicity data set is required to be submitted at the subsequent Tier B level. This step focuses on the effects on fauna and flora within environmental compartments that are likely to be affected. However, medicinal products for human use only require Phase II studies if the predicted environmental concentration in surface water is equal to or above 0.01 µg/L (Rechenberg, 2009; Santos et al., 2010).

Authorisation of a human medicinal product, however, can explicitly not be denied if an environmental risk is identified. For veterinary pharmaceuticals the legal situation is different: authorisation may be denied, or may be limited to certain application areas, if evidence for an environmental risk exists (Fent et al., 2006).

In 1996 the EMEA adapted a comprehensive Note for Guidance to assist the applicant in the evaluation of the environmental risk assessment for veterinary medicinal products. A final detailed Technical Guidance Document was drawn up for Europe and came into force in 2007. Similar to the first phase of the environment risk assessment of human pharmaceuticals, phase I identifies veterinary medicinal products that require a more extensive investigation of their
potential environmental effects on non-target organisms. In this step, the potential extent of exposure to the environment from the product, its active substances and other ingredients should be assessed. The evaluation has to take into account the target species, the proposed pattern of use, characteristics of the constituents of the veterinary pharmaceutical and the method of administration. Phase I is further divided into assessments of drugs used in the aquatic and terrestrial systems respectively. With respect to the aquatic branch, any veterinary medicinal product intended for use in open systems are subjected to phase II. The “action limit” settled for pharmaceuticals used in aquatic environment is 1 µg/L, while for terrestrial sector this limit is set up at 100 µg/kg. If these values are exceeded, a phase II assessment is required. As for human pharmaceuticals, phase II is further divided into two tiers (Fig. 1.9) (Rechenberg, 2009; Santos et al., 2010).

![Fig. 1.9. Environmental Risk Assessment of veterinary medical products. From Rechenberg, 2009.](image)

Aiming at estimating potential hazardous compounds, Huggett et al. (2003) proposed the “fish plasma model”. It is based on the comparison of the estimated fish plasma concentration of a given drug to the human therapeutic plasma concentration. The assumption is that the closer the estimated fish plasma concentration and the human therapeutic concentration are, the higher the risk is that the pharmaceutical elicits an effect in fish. Another concept focuses on the homology between human drug targets and potential targets in lower vertebrates; in this case it is assumed that the risk for adverse effects in the environment increases with the degree of homology between human drug target and potential drug targets in lower vertebrates. Moreover, several quantitative structure activity
response (QSAR)-based concepts are developed to aid in the development process of pharmaceuticals (Christen et al., 2010).

In previous studies (Fent et al. 2006; Owen et al., 2007), it was hypothesized that the effects of pharmaceuticals should be better identified by focusing on their mode of actions, or to guide species and toxicity endpoint selection for testing or for development of biomarkers.

Christen et al. (2010) develop a concept for the identification of highly active compounds that could be used within the risk assessment process for human pharmaceuticals in the framework of the EMEA guideline (2006). The concept was concentrated on human pharmaceuticals, however, it could presumably be applied for veterinary pharmaceuticals in a similar way. As effect concentrations were divided by assessment factors to extrapolate the potential adverse effects of a compound to the ecosystem from laboratory tests on single species to derive predicted no effect concentrations for regulatory purposes, substances with effect concentrations higher than 10 ng/L were also taken into consideration. Even if legislation requires deeper investigations at environmental concentrations higher than 0.01 µg/L, it was demonstrated that there are also compounds which show adverse effects under this value (Tab 1.1).
Tab. 1.1. Summary of pharmaceuticals exerting effects on aquatic organisms at low concentrations. The lowest levels found for lowest effect concentrations (LOEC) are shown. From Christen et al., 2010.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
<th>LOEC</th>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>Femisation</td>
<td>4 ng/L</td>
<td><em>Pimephales promelas</em></td>
<td>Liinge et al. (2001)</td>
</tr>
<tr>
<td>17α-Ethylestradiol EE2 (estrogen)</td>
<td>Complete femisation</td>
<td>4 ng/L</td>
<td><em>Rutilus rutilus</em></td>
<td>Lange et al. (2009)</td>
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<td></td>
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<td>10 ng/L</td>
<td><em>Moxostoma carinatum</em></td>
<td>Schulte-Oehlmann et al. (2004);</td>
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<td></td>
<td>Fecundity, fertility</td>
<td>10 ng/L</td>
<td><em>Oryzias latipes</em></td>
<td>Hutchins et al. (2003)</td>
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<tr>
<td></td>
<td>Femisation</td>
<td>5 ng/L</td>
<td><em>Tilapia</em></td>
<td>Shen et al. (2008)</td>
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<td></td>
<td></td>
<td>4 ng/L</td>
<td><em>Rutilus rutilus</em></td>
<td>Lange et al. (2008)</td>
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<td></td>
<td>Vitellogenesis induction</td>
<td>2 ng/L</td>
<td><em>Danio rerio</em></td>
<td>Xiu et al. (2008)</td>
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<td></td>
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<td>EC50: 3.7 ng/L</td>
<td><em>Solea solea</em></td>
<td>Bjergbaek et al. (2008)</td>
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<td>1 ng/L</td>
<td><em>Pimephales promelas</em></td>
<td>Jobling et al. (2003)</td>
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<tr>
<td>Equilenin (estrogen)</td>
<td>Increase in egg production</td>
<td>1 ng/L</td>
<td><em>Ponometopus antipodarum</em></td>
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<tr>
<td>17β-Dihydroequilenin (estrogen)</td>
<td>Vitellogenesis induction</td>
<td>4.2 ng/L</td>
<td><em>Oncorhyncus mykiss</em></td>
<td>Tyler et al. (2009)</td>
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<td>Trenbolone acetate (androgen)</td>
<td>Sex reversal, all male fish</td>
<td>0.7 ng/L</td>
<td><em>Danio rerio</em></td>
<td>Hoehn et al. (2006)</td>
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<td></td>
<td>Reproductive success</td>
<td>0.8 ng/L</td>
<td><em>Pimephales promelas</em></td>
<td>Zeilinger et al. (2009);</td>
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<td>Effects at 10 – 100 ng/L</td>
<td>Sex reversal</td>
<td>10 ng/L</td>
<td><em>Pimephales promelas</em></td>
<td>Pujiska (2002)</td>
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<tr>
<td>17β-Methyltestosterone (androgen)</td>
<td>Sex-reversal, but temperature dependant</td>
<td>10 ng/L</td>
<td><em>Cynoglossus acusaurus</em></td>
<td>Sebire et al. (2006)</td>
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<td></td>
<td>Fecundity</td>
<td>100 ng/L</td>
<td><em>Oryzias latipes</em></td>
<td>Pujiska (2002)</td>
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<td></td>
<td>Vitellogenesis</td>
<td>100 ng/L</td>
<td><em>Moxostoma carinatum</em></td>
<td>Schulte-Oehlmann et al. (2004);</td>
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<td>Chlortimazole (fungicide)</td>
<td>Inhibition of algal 14α-damathylases</td>
<td>17 ng/L</td>
<td><em>Microcystis aeruginosa</em></td>
<td>Pecunha et al. (2009)</td>
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<td>Effects at concentrations &gt; 100 ng/L</td>
<td>Effects on spermatogenesis</td>
<td>10 μg/L</td>
<td><em>Percula reticulata</em></td>
<td>Rayley et al. (2002)</td>
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<td>Flutamide (antioestrogen)</td>
<td>Courtship behaviour, nest building</td>
<td>100 μg/L</td>
<td><em>Gastrostomus acutus</em></td>
<td>Sebire et al. (2006)</td>
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<td>Reproduction</td>
<td>5 μg/L</td>
<td><em>Ctenopharyngodon idella</em></td>
<td>Henry et al. (2004)</td>
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<tr>
<td>Sertraline (selective serotonin reuptake inhibitor)</td>
<td>Alteration of fecundity</td>
<td>56 μg/L</td>
<td><em>Ctenopharyngodon idella</em></td>
<td>Brooks et al. (2003)</td>
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<td>Fluoxetine (selective serotonin reuptake inhibitor)</td>
<td>Stimulation of reproduction</td>
<td>36 μg/L</td>
<td><em>Ctenopharyngodon idella</em></td>
<td>Brooks et al. (2003)</td>
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pathway for the target (or non-target) species. A flow chart of the present concept is outlined in Fig. 1.10.

![Flow chart](image)

Fig. 1.10. Flow scheme and criteria for the identification of HC among human pharmaceuticals in a stepwise mode of action concept. From Christen et al., 2010.

### 1.3. Pharmaceuticals tested in this study

#### 1.3.1. Carbamazepine

![Structural form of carbamazepine](image)

5H-dibenzo[b,f]azepine-5-carboxamide  
Formula $C_{15}H_{12}N_2O$  
Mol. mass 236.269 g/mol

Fig. 1.11. Structural form of carbamazepine.

In the present study, one of the pharmaceuticals which were taken into consideration was carbamazepine (CBZ) (Fig. 1.11), an anticonvulsant and mood stabilizing drug used primarily in the treatment of epilepsy, bipolar disorder, and
trigeminal neuralgia (Garcia-Morales et al., 2007). CBZ is also used in bipolar depression. This drug is relatively lipophilic, with an octanol/water partition coefficient of 2.2. Reports indicate that CBZ is persistent when released to the environment, and its average removal efficiencies by wastewater treatment plants (WTPs) are below 10% (Zhang et al., 2008). No biodegradation of CBZ at low concentrations in either salt or freshwater was found (Stamatelatou et al., 2003). As a result of its persistence in both municipal wastewater sand surface waters, CBZ is proposed as a suitable anthropogenic marker of urban effluents (Clara et al., 2004). The drug was indeed detected with few exceptions in influents and effluents from WTPs, surface waters, and even seawater. Concentrations as high as 6.3 µg/L have been found in wastewaters; average concentrations up to 2.3 µg/L have been measured in effluent samples; values in the range of 0.1–1 µg/L have been reported for surface waters, with levels up to 1.1 and 0.03 µg/L in ground water and drinking waters, respectively (Fent et al., 2006) (Tab. 1.2).

Tab. 1.2. Examples of concentrations (ng/L) of CBZ measured in different aquatic environments. From Santos et al., 2010.

<table>
<thead>
<tr>
<th>Component</th>
<th>NZO number</th>
<th>Sample</th>
<th>Country</th>
<th>Analytical procedure</th>
<th>USD (ng/L)</th>
<th>Concentration range (ng/L)</th>
<th>Ref.</th>
<th>Taxon</th>
<th>Species</th>
<th>Technological endpoint</th>
<th>Toxicity data</th>
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</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>294-96-6</td>
<td>TEP effluent</td>
<td>Spain</td>
<td>SPE-GC-MS</td>
<td>30</td>
<td>1.00-3.0</td>
<td>[10]</td>
<td>Cenacian</td>
<td>D. magna</td>
<td>IC50 (48h) (growth inhibition)</td>
<td>&gt;1000 ng/L</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>TEP effluent</td>
<td>Spain</td>
<td>SPE-HPLC-MS/MS</td>
<td>1.4</td>
<td>110-230</td>
<td>[10]</td>
<td>Algae</td>
<td>D. subspinosus</td>
<td>IC50 (growth inhibition)</td>
<td>74 ng/L</td>
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<td>Effluent</td>
<td>Spain</td>
<td>SPE-GC-MS</td>
<td>20</td>
<td>2.4-5.0</td>
<td>[10]</td>
<td>Desulfoarcicella</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>25 ng/L</td>
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<tr>
<td>Carbamazepine</td>
<td>Effluent</td>
<td>Spain</td>
<td>SPE-GC-MS</td>
<td>2.8</td>
<td>1.4-4.0</td>
<td>[10]</td>
<td>C. reinhardtii</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>10 ng/L</td>
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<tr>
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<td>SPE-GC-MS</td>
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<td>[10]</td>
<td>C. reinhardtii</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>40 ng/L</td>
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<td>C. reinhardtii</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>40 ng/L</td>
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<td>C. reinhardtii</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>10 ng/L</td>
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<td>Carbamazepine</td>
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<td>0.27</td>
<td>0.1-0.5</td>
<td>[10]</td>
<td>C. reinhardtii</td>
<td>C. reinhardtii</td>
<td>IC50 (7d) (growth inhibition)</td>
<td>76 ng/L</td>
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</tbody>
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From Santos et al., 2010.
Introduction

Approximately 72% of orally administered carbamazepine is absorbed, while 28% is unchanged and subsequently discharged through the faeces. After it is absorbed, carbamazepine is heavily metabolized by the liver: only about 1% of dosage leaves the body in an unaltered form. The metabolites of this drug undergo enterohepatic cycling and finally are excreted with urine. The elimination half-life time of carbamazepine is dose-dependent, but is usually in the range of 25–65 hours post-administration. Its important urine metabolites are 10,11-dihydro-10,11-expoxycarbamazepine (CBZ-epoxide) and trans-10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-diol) (Fig. 1.12). Importantly, the former is pharmaceutically just as active as its parent drug. Furthermore, CBZ-diol is also a metabolite of oxcarbazepine which is a derivative of carbamazepine, with an extra oxygen atom on the dibenzazepine ring (Theisohn and Heimann, 1982). Therefore, one may expect to observe a higher concentration of CBZ-diol in water bodies. Unfortunately, limited studies have been conducted on those metabolites in the aquatic environment to date. Miao and Metcalfe (2003) and Miao et al. (2005) investigated the occurrences of carbamazepine metabolites in a WTP effluent and a surface water. They observed that the concentration of CBZ-diol was approximately three times that of carbamazepine. Therefore, more studies should be conducted on the environmental fate of carbamazepine metabolites (Zhang et al., 2008).

Fig. 1.12. Identified metabolites of carbamazepine and their percentages of oral dosage. Adapted from Zhang et al., 2008.
Clara et al. (2004) studied the degradation and the removal rates of CBZ in several Austrian sewage treatment plants (STPs), with different membrane systems, measuring its concentration in influent and effluent stream flows. They found only in one case a removal rate over 20%, while in all other plants the effluent concentrations were within the same range as those in the influent or even higher. At two STPs effluent concentrations were twice as high as the influent concentrations.

These findings completely agreed with the results that were obtained by the same research group from laboratory experiments.

Moreover, neither in the post-treatment nor during infiltration or in the groundwater a significant removal was detectable. The decrease of CBZ concentrations in the groundwater was only due to dilution processes. The results of the groundwater sampling confirmed that CBZ is a very persistent pharmaceutical active compound, which is not subjected to any degradation or adsorption, neither in waste-water treatment nor during the underground/groundwater passage.

Preuß et al. (2001) obtained comparable results. They studied the behaviour of CBZ during artificial groundwater recharge and reported poor removal during soil passage, too. This behaviour makes CBZ a suitable wastewater marker. In addition, CBZ has the advantage that it is detectable also in higher dilutions.

For all these reasons, carbamazepine showed to be a very persistent substance and seems to be a qualified parameter for detecting wastewater in the aquatic environment. It is neither subjected to degradation nor to adsorption processes during wastewater treatment and as a consequence, these characteristics qualify CBZ as a suitable marker for anthropogenic influences on the aquatic environment. The persistence of this molecule represents also a potential risk factor for drinking water reserves and has already been tracked from wastewater to drinking water. To avoid such contaminations technologies have to be developed that allow their removal.

Antiepileptic drugs act on the central nerve system by decreasing the overall neuronal activity. CBZ achieves it by blocking voltage-dependent sodium channels of excitatory neurons (Fent et al., 2006).

The mechanism of action of carbamazepine and its derivatives is relatively well understood. Voltage-gated sodium channels are the molecular pores that allow
neurons to generate action potentials. After the sodium channels open to start the action potential, they inactivate, essentially closing the channel. CBZ stabilizes the inactivated state of sodium channels, meaning that fewer of these channels are available to subsequently open, making brain cells less excitable.

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<th>Chronic toxicity</th>
<th>PNEC</th>
<th>References</th>
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<tr>
<td></td>
<td>EC_{50}</td>
<td>NOEC</td>
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<tr>
<td>Carbamazepine</td>
<td>&gt;13.8–81 mg L(^{-1})</td>
<td>25–100 mg L(^{-1})</td>
<td>0.42 µg L(^{-1})</td>
<td>Ferrari et al. (2003)</td>
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<td>1–10 mg L(^{-1})</td>
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<td>6.359 µg L(^{-1})</td>
<td>Jones et al. (2002)</td>
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<td>4.5–383.5 mg L(^{-1})</td>
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<td>Lis et al. (2003)</td>
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<td>75.1–502.6 mg L(^{-1})</td>
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<td>Laville et al. (2004)</td>
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</table>

Fig. 1.13. Toxicity data of carbamazepine in the literatures. EC\(_{50}\): concentrations that cause 50% of effect; NOEC: no observed effect concentration; PNEC: predicted no-effect concentrations. Adapted from Zhang et al., 2008.

As previously described, CBZ is widely present in water bodies. Therefore, it is necessary to evaluate its impact on the ecosystems where it is present. Many studies have assessed its ecotoxicology (Fig. 1.13). Ferrari et al. (2003) studied the toxic effects of carbamazepine on bacteria, algae, microcrustaceans, and fish. They observed that it had a relatively limited acute ecotoxicity on the tested organisms. In the worst case of acute toxicity tests, concentration that causes 50% of effect (EC\(_{50}\)) was 13800 µg/L on D. magna over 48 hours. However, chronic tests displayed higher toxicity than acute tests. In this case, the worst no observed effect concentration (NOEC) for CBZ was 25 µg/L on B. calyciflorus over 48 hours. In contrast, Andreozzi et al. (2002) found no toxicity of carbamazepine on algae Ankistrodesmus braunii and also found that the concentration of carbamazepine progressively decreased in the culture of the algae. After 60 days, over 50% of the substance had disappeared from the medium. Furthermore, no significant amounts of CBZ could be detected in A. braunii cells during the course of the experiment. The authors assumed that carbamazepine was taken up by algal cells and entered into biochemical processes. The chronic effects are still not clear. Long term studies are needed for a better characterization of chronic ecological effects. The synergistic toxicity of these drugs with other compounds also needs more studies.
Furthermore, information on the ecological effects of their metabolites and intermediates is also needed (Zhang et al., 2008).

### 1.3.2. Propranolol

![Propranolol Structure](image)

The second pharmaceutical to which mussels were exposed in this work was propranolol (PROP) (Fig. 1.14). Propranolol is available in generic form as propranolol hydrochloride, and it is soluble in water. PROP is a non-selective β-blocker (adrenergic receptor) antagonist, blocking β₁ and β₂-receptors, mainly used in the treatment of hypertension, to treat high blood pressure and other related heart condition illnesses. Due to similarities between human and other vertebrate beta-adrenergic receptors, consequences of β-blocker exposure in vertebrate wildlife, such as fish, are easier to predict. Fish, like other vertebrates, possess β-receptors in the heart, liver and reproductive system so that prolonged exposure to drugs belonging to this therapeutic class may cause deleterious effects (Santos et al., 2010). However, little is known regarding the presence of this receptor in invertebrates although the presence of a β-adrenergic-like receptor in some molluscs has been demonstrated and predicted to have a role in bivalve larval metamorphosis, feeding behaviour in the gastropod Aplysia and bioluminescence regulation in brittlestars. In ascidian larvae (Ciona savignyi), the receptor has been localised and has a role in initiating metamorphosis. Nevertheless, studies conducted in crustacean point to a lack of the β-adrenergic receptor in this group (Solé et al., 2010).

Propranolol and other human and animal pharmaceuticals reach the aquatic environment through a variety of routes. However, the majority of pharmaceuticals are present in the aquatic environment due to incomplete removal
at sewage treatment works (STWs). In Germany it was found that 96% of propranolol was removed from the influent compared to the effluent, yet the 4% remaining is largely why propranolol has been found ubiquitously in rivers and streams in America and Europe at concentrations in the ng/L range, with maximum and median concentrations reaching 590 ng/L and 12 ng/L, respectively (Giltrow et al., 2009).

Although β-blockers act on beta-adrenergic receptors (β-ARs), they can differ greatly in their specificity and lipophilic properties; for example, propranolol has a relatively high log Kow of 3.48, whereas atenolol has a considerably lower log Kow of 0.23 (Owen et al., 2007). Published data show propranolol, out of all the β-blockers investigated, to be the most toxic to aquatic organisms. For example, invertebrate LC$_{50}$ values for metoprolol and propranolol range from 64 to > 100 mg/L and 0.8 to 29.8 mg/L, respectively, showing that propranolol is harmful to invertebrates at much lower concentrations than metoprolol (Cleuvers, 2003; Huggett et al., 2002). In fish studies, the published data on propranolol are limited. However, a report by Huggett et al. (2002) showed propranolol to be relatively toxic with LOEC values of 0.5 µg/L.

Photodegradation of propranolol has been evaluated with half-life of 16.8 days (Cleuvers, 2005).

While propranolol is extensively metabolised in humans, oral ingestion leads to excretion of some of the unabsorbed parent compound, which has consequently been detected in USA and European Sewage Treatment Work (STW) effluents (Owen et al., 2009).

Despite the high rate of removal by sewage treatment plants (about 96% (Fent et al., 2006)), PROP has been detected in STP effluents at concentrations from 30 to 373 ng/L and in surface waters at levels of ng/L. This pharmaceutical has also been found in hospital effluent (Spain) at concentrations that can reach 6.5 µg/L (Tab. 1.3).
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Tab. 1.3. Examples of concentrations (ng/L) of PROP measured in different aquatic environments. From Santos et al., 2010.

<table>
<thead>
<tr>
<th>Propargyl</th>
<th>SD/95-0</th>
<th>STP influent</th>
<th>Seawater</th>
<th>SPE-GC-MS/MS</th>
<th>t1/2</th>
<th>Gram-positive</th>
<th>Gram-negative</th>
<th>RNT</th>
<th>EC50 (mg/L) (inhibition)</th>
<th>7.3 ng·L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>Taihu lake</td>
<td>SPE-GC-MS/MS</td>
<td>0.5</td>
<td>54</td>
<td>[47]</td>
<td>Algae</td>
<td>B. subtilis</td>
<td>EC50 (growth inhibition)</td>
</tr>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>United Kingdom</td>
<td>SPE-GC-MS/MS</td>
<td>10</td>
<td>60-119</td>
<td>[11]</td>
<td>D. summeri</td>
<td>L. minor</td>
<td>IC₅₀ (70%) (growth inhibition)</td>
</tr>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>Spain</td>
<td>SPE-GC-MS/MS</td>
<td>8</td>
<td>200-2000</td>
<td>[73]</td>
<td>Chromatium</td>
<td>T. phaeus</td>
<td>EC₅₀ (48h) (immobilization)</td>
</tr>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>South Korea</td>
<td>SPE-GC-MS/MS</td>
<td>10</td>
<td>40-1.11</td>
<td>[92]</td>
<td>Flab</td>
<td>D. antarcticus</td>
<td>EC₅₀ (48h) (immobilization)</td>
</tr>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>United Kingdom</td>
<td>SPE-GC-MS/MS</td>
<td>10-10</td>
<td>1-10</td>
<td>[94]</td>
<td>Chromatium</td>
<td>M. scim</td>
<td>IC₅₀ (48h) (immobilization)</td>
</tr>
<tr>
<td>Propargyl</td>
<td>SD/95-0</td>
<td>STP influent</td>
<td>Surface water</td>
<td>SPE-GC-MS/MS</td>
<td>+10-27</td>
<td>10-10</td>
<td></td>
<td></td>
<td>NOS (27%)</td>
<td>(immobilization)</td>
</tr>
</tbody>
</table>

1.3.3. Oxytetracycline

![Fig. 1.15. Structural form of oxytetracycline.](image)

Oxytetracycline (OTC) (Fig.1.15) was the second of the broad-spectrum tetracycline group of antibiotics to be discovered.

OTC belongs to the tetracycline class of antibiotics, which includes tetracycline, chlortetracycline, doxycycline, minocycline, and glycyclines. This class of antibiotics represents broad-spectrum agents that act against a range of Gram-positive and Gram-negative bacteria by inhibiting protein synthesis. The tetracyclines have played an important role in human and veterinary medicine, and some have been used as growth promoters for livestock and aquaculture (Li et al., 2010). OTC works by interfering with the ability of bacteria to produce proteins that are essential to them. Without these proteins the bacteria cannot grow, multiply and increase in number. Oxytetracycline therefore stops the spread of the infection and the remaining bacteria are killed by the immune system or eventually die.
Oxytetracycline is a broad spectrum antibiotic that is active against a wide variety of bacteria. However, some strains of bacteria have developed resistance to this antibiotic, which has reduced its effectiveness for treating some types of infection. OTC is still used to treat infections caused by chlamydia (e.g. the chest infection psittacosis, the eye infection trachoma, and the genital infection urethritis) and infections caused by mycoplasma organisms (e.g. pneumonia). It is also used to treat acne, due to its activity against the bacteria on the skin that cause this disease (*Propionebacterium acnes*). It is used to treat flare-ups of chronic bronchitis, due to its activity against the bacteria usually responsible, *Haemophilus influenzae*.

Antibiotics released into the aquatic environment are of concern since: a) they could contaminate raw, treated and recycled water used for drinking, irrigation and recreation; b) they could potentially accelerate widespread bacterial resistance to antibiotics; c) they can have negative effect on important ecosystem bacteria (through death or inhibition). Between 30% and 90% of an administered dose of most antibiotics to humans and animals are excreted in the urine as the active substance (Rang *et al.*, 1999). Antibiotics are predominantly water soluble and enter the aquatic environment through sewage systems following consumption and excretion by humans and via effluent from farms, abattoirs and landfills (Daughton and Ternes, 1999; Costanzo *et al.*, 2005).

Antibiotics are widely used to treat infections in humans and are applied intensively for veterinary purposes. Because they are poorly metabolized in the body and incompletely degraded in wastewater treatment plants, antibiotics are continuously introduced into the environment. Consequently, in spite of their relatively short environmental half-lives, they are ubiquitous in aquatic environments (van der Grinten *et al.*, 2010).

Antibiotics belonging to different classes have been found in different aquatic environments (Tab. 1.4). Oxytetracycline was detected in the Po and Lambro rivers (Italy) at concentrations up to 248.90 and 24.40 ng/L respectively, in combination with tetracycline in American STP influents (47 µg/L) and effluents (4.2 µg/L) and in surface waters (340 ng/L) (Santos *et al.*, 2010).
Tab. 1.4. Examples of concentrations (ng/L) of OTC measured in different aquatic environments. From Santos et al., 2010.

<table>
<thead>
<tr>
<th>Component</th>
<th>CVO number</th>
<th>Sample</th>
<th>Country</th>
<th>Analytical</th>
<th>LOD (ng L⁻¹)</th>
<th>Concentration</th>
<th>Ref.</th>
<th>Species</th>
<th>Teratological endpoints</th>
<th>Risk category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daphnia magna</td>
<td>T9-07-2</td>
<td>Surface water</td>
<td>USA</td>
<td>SPE-IL-MS</td>
<td>100</td>
<td>140</td>
<td>[23]</td>
<td>Calanidae</td>
<td>Hepatoceleste</td>
<td>88.4 ng L⁻¹</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>T9-07-2</td>
<td>Surface water</td>
<td>Italy</td>
<td>SPE-HPLC-MS/MS</td>
<td>0.2</td>
<td>ND-112</td>
<td>[24]</td>
<td>Calanidae</td>
<td>Hepatoceleste</td>
<td>30.5 ng L⁻¹</td>
</tr>
<tr>
<td>Daphnia pulex</td>
<td>T0-05-1</td>
<td>Surface water</td>
<td>Tunisia</td>
<td>SPE-HPLC-MS/MS</td>
<td>0.5</td>
<td>2.0</td>
<td>[47]</td>
<td>Calanidae</td>
<td>Hepatoceleste</td>
<td>0.075 ng L⁻¹</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>T0-05-1</td>
<td>Surface water</td>
<td>Luxembourg</td>
<td>SPE-I.C.-MS/MS</td>
<td>0.4</td>
<td>0.5-1</td>
<td>[89]</td>
<td>Calanidae</td>
<td>Hepatoceleste</td>
<td>500 ng L⁻¹</td>
</tr>
</tbody>
</table>

1.3.4. Copper

In the contest of MEECE Programme, mussels were exposed to increasing concentrations of copper.

Copper is an essential micronutrient, it is a cofactor for many enzymes and other proteins implicated in an array of biological processes required for growth, development and maintenance (Trevisan et al., 2011), but it can also have adverse effects on aquatic organisms if bioavailable forms of Cu reach toxic concentrations. Knowing only the total concentration of Cu (i.e., bioavailable and nonbioavailable forms) in the aquatic environment is inadequate to accurately determine the probability of adverse effects. The concentrations of the bioavailable forms of Cu must be determined to better predict potential adverse effects.

Numerous studies in fresh water have demonstrated that complexation of Cu with organic and inorganic ligands as well as competition with dissolved cations (Ca²⁺, Mg²⁺, Na⁺, K⁺, H⁺) for binding and uptake pathways modify Cu bioavailability and
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thus Cu toxicity. Several contemporary studies have similarly demonstrated the
need to quantify the bioavailable fraction to assess potential Cu toxicity and risk in
salt water. Factors that modify Cu bioavailability have been incorporated into a
biotic ligand model (BLM). This model has been modified and adopted by the U.S.
EPA for determining the freshwater Cu quality criteria for the protection of aquatic
organisms and their uses. No such accommodation for factors that modify copper
bioavailability has been adopted for water quality criteria applied to marine waters.
However, it has clearly been shown that dissolved organic matter (DOM) greatly
affects Cu bioavailability in salt water to two sensitive species of mussels (Arnold
et al., 2010).

Increased concerns regarding the use of oceans as a site for the disposal of
anthropogenic wastes and the large scale use of copper and other metals in
antifouling boat paint have prompted evaluation of metal toxicity to marine
species. Today, there is a considerable body of research outlining the toxicity of
various metals to marine species. However, available data often exhibit wide
variation in sensitivity. For instance mussel larvae of the genus Mytilus are widely
accepted to represent one of the most sensitive marine organisms to Cu and
therefore play a critical role in environmental regulations. Yet, the US EPA's
(2003) ambient water quality criteria document reports mean acute values (EC50s
in 48 hours developmental tests) of 21.4 µg/L dissolved Cu (at 20 ppt salinity) for
Mytilus edulis larvae but 6.1 µg/L dissolved Cu (at 28–30 ppt) salinity for Mytilus
spp. respectively. This variation could reflect either the importance of water
chemistry in influencing the bioavailability of the metal, differences in
physiological sensitivity of the organisms at different salinities, or true inter-
species differences. Furthermore, many studies have recently presented evidence
that the toxicity of dissolved copper to Mytilus spp. is a function of the dissolved
organic carbon (DOC) concentration of the test sample (Nadella et al., 2009).

It is known also that high concentrations of Cu may cause oxidative stress by
impairing antioxidant defenses and increasing the generation of ROS leading to
lipid peroxidation and DNA damage (Trevisan et al., 2011).
1.4. Experimental setup: laboratory mussels exposure

In the aquatic environment, organisms are exposed to mixtures of contaminants at different concentrations, that can interact among them and threat animals health. As previously described, among these sources of contamination, also pharmaceuticals are assuming increasing importance and attention.

As to pharmaceuticals exposure, many acute tests and studies were performed, showing effects only at concentrations many times higher than those detected in the environment. Instead, chronic tests are necessary, to understand if these substances constitute a danger for the organisms which are continuously exposed to them even if at very low concentrations.

This kind of survey would be very difficult in natural environment, because of the manifold variables that are involved. To better understand the effects induced by each pharmaceutical, mussels were exposed under laboratory conditions, in order to exclude the interference of other factors.

After acclimatisation, mussels were exposed to the different contaminants in aquaria containing artificial seawater with continuous aeration at 16°C (Fig. 1.16) renewed every day; they were fed once a day and the drug was dispensed at the same hour every day.

Fig. 1.16. Experimental laboratory setup for mussels exposure to pharmaceuticals.
1.5. Why the choice of mussels as sentinel organisms?

Aquatic organisms are particularly important targets for pharmaceuticals, as they are exposed via wastewater residues over their whole life. Standard acute ecotoxicity data have been reported for a number of pharmaceuticals, however, such data alone may not specifically address the question of environmental effects, and subsequently in the hazard and risk assessment (Fent, 2003). The current lack of knowledge holds in particular for chronic effects that have only very rarely been investigated (Fent et al., 2006).

Bivalves are important members of aquatic ecosystems and markedly interact with water and sediment. These sessile and long-lived organisms filter large quantities of surface water for feeding and respiration. They are therefore particularly susceptible to environmental stressors, including point source and diffuse contamination, water-level variations and climatic changes (e.g., temperature fluctuations in shallow water) (Gagné et al., 2006).

Bivalve molluscs, in particular mussels, are useful sentinel organisms to assess chemical pollution in aquatic environment. They are sessile, filter feeding, widely distributed and abundant in coastal and estuarine areas, able to accumulate several classes of pollutants, thus providing a time-integrated picture of their bioavailability. For such characteristics, these organisms are widely used in Mussel Watch monitoring programmes, where chemical analyses are integrated with the use of biomarkers, to evaluate molecular, biochemical and cellular effects induced by pollutants (Bocchetti and Regoli, 2006).

Bivalves have an open vascular system; the haemolymph pervades most organs, favouring direct exposure to the external environment and hence to contaminants. Long-term exposure to contaminants emanating from various sources (urban and industrial wastewaters) could compromise immune function and progressively lead to infectious diseases and cancerous disorders (Gagné et al., 2006). Mussel haemolymph does not coagulate; haemocytes play several functions, in particular they represent the immune defense system, and are important for the accumulation of nutrients, metabolic products and respiratory pigments.

Haemocytes enclose lysosomes, cellular organelles that contain about 40 acid hydrolase enzymes to break up waste materials and cellular debris. Their size varies from 0.1–1.2 μm. The membrane around the lysosome allows the digestive
enzymes to work at the 4.5 pH as they require; this pH differential with respect to cytosol is maintained by pumping protons (H\(^+\) ions) from the cytosol across the membrane via proton pumps and chloride ion channels. The lysosomal membrane protects the cytosol, and therefore the rest of the cell, from the degradative enzymes within the lysosome. The cell is additionally protected from any lysosomal acid hydrolases that leak into the cytosol, as these enzymes are pH-sensitive and do not function well in the alkaline environment of the cytosol. In addition to degrading molecules taken up by endocytosis, lysosomes digest material derived from two other routes: phagocytosis and autophagy. In phagocytosis, specialized cells, such as macrophages, take up and degrade large particles, including bacteria, cell debris, and aged cells that need to be eliminated from the body. Such large particles are taken up in phagocytic vacuoles (phagosomes), which then fuse with lysosomes, resulting in digestion of their contents. The lysosomes formed in this way (phagolysosomes) can be quite large and heterogeneous, since their size and shape is determined by the content of material that is being digested.

Lysosomes are also responsible for autophagy, the gradual turnover of the cell’s own components. The first step of autophagy appears to be the enclosure of an organelle (e.g., a mitochondrion) in membrane derived from the endoplasmic reticulum. The resulting vesicle (an autophagosome) then fuses with a lysosome, and its contents are digested.

The lysosomal-autophagic system appears to be a common target for many environmental pollutants, as lysosomes accumulate many toxic metals and organic xenobiotics, which perturb normal function and damage the lysosomal membrane. In fact, autophagic reactions frequently involving reduced lysosomal membrane integrity or stability appear to be effective generic indicators of cellular well-being in eukaryotes (Moore et al., 2008). Autophagy is often considered to be primarily a survival strategy in multi-cellular organisms, which may be initiated by stressors (e.g., restricted nutrients, hyperthermia, hypoxia and salinity increase), but recent evidence indicates that autophagy is much more than just a survival process and is, in fact, intimately involved in cell physiology. Physiological responses and pathological reactions to environmental stressors in mollusc digestive cells and haemocytes frequently involve destabilizing changes in the lysosomal membrane and the induction of autophagy. Other related lysosomal perturbations can also
occur, such as lysosomal swelling, accumulation of lipid and age pigments or lipofuscins: all of these changes have been described in molluscan hepatopancreatic digestive cells. In eukaryotic cells, the first tier of defense against oxidative damage is provided by xenobiotic transporters, biotransformation enzymes and antioxidant enzymes, such as superoxide dismutase and catalase (Livingstone, 2001; Moore, 2004). Lysosomal autophagy provides a second line of defense, by removing oxidatively damaged proteins, inappropriately folded glycosylated proteins and impaired organelles, and even portions of the nucleus and DNA.

In the field of aquatic toxicology lysosomes have attracted considerable attention in recent years because they a) were shown to be the target for a wide range of contaminants, b) are easy to visualize in blood cells and in reacted tissue cryosections, and c) are present in all nucleated cells and therefore are not species-specific (Viarengo et al., 2007).

Cytochemistry and histochemistry have been used as the main tools in the study of environmentally induced alterations in lysosomes of lower animals for several reasons (Moore, 1990; Moore and Simpson, 1992). Exposure to contaminants gives rise to fairly rapid and characteristic pathological alterations such as enlargement of the digestive cell and hepatocyte lysosomes, which are correlated with increased fragility of the lysosomal membrane, excessive build up of unsaturated neutral lipids (lipidosis) in the lysosomal compartment and accumulation of lipofuscins (lipofuscinosis) (Moore et al., 2008).

1.6. Selected battery of biomarkers

1.6.1. The neutral red retention assay

Neutral red is a lipophilic dye and as such will freely permeate the cell membrane. Within cells the compound becomes trapped by protonisation in the lysosomes and accumulated in these organelles, where it can be visualised microscopically. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump. The
acid environment of lysosomes is maintained by a membrane Mg$^{2+}$-ATPase dependent H$^+$ ion proton pump, the neutral red retention assay reflects on the efflux of the lysosomal contents into the cytosol following damage to the membrane and, possibly, impairment of the H$^+$ ion pump. So any impairment of this latter system will result in a reduction of the dye retention assay. Studies indicate that, similarly to the cytochemical method described above, the neutral red retention assay is sensitive to the main classes of chemical pollutants (UNEP/RAMOGE).

According to this in vitro methodology, the dye is sequestered into the lysosomal compartment when living cells are preloaded with neutral red (NR); if the lysosome membranes are damaged NR leaks out into the cytosol where it can be visualized under the microscope. The time taken for the dye to leak out into the cytosol is related to the degree of membrane damage. In case lysosome membranes are severely damaged (Fig. 1.17a) the dye will leak out within 15 min of incubation, whereas healthy lysosomes (Fig. 1.17b) retain it for up to 180 min (Lowe and Pipe, 1994). The NRR assay has to be considered “a stress on stress test”, in that neutral red is itself toxic for the cells and therefore further damages lysosomal membranes that are potentially already damaged. The additional damage caused by the dye results in a total membrane failure in the case of severely impacted cells, and also in membrane failure and leakage in healthy cells but in this case only after 150–180 min (Viarengo et al., 2007).

Reduced lysosomal membrane stability has to be considered as an indicator of a general physiological stress. Viarengo et al. (2007) proposed the lysosomal membrane stability, assessed either by NRR or by the histochemical technique, as a robust Tier 1 screening biomarker for Environmental Impact Assessments in a 2-Tier approach.
1.6.2. Cytochemical assays: accumulation of lipofuscins (LF) and neutral lipids (NL)

It is very difficult to evaluate the molecular changes affecting the permeability of the lysosomal membrane. These analyses require extensive purified lysosomal membrane preparations and their examination at molecular level. An easier way to assess this parameter is to examine whether its normal physiological function has been altered or disrupted following exposure to pollutants. One tool which links both descriptive morphology and biochemistry to observe such pathological alterations is cytochemistry. Apart from permitting the use of very small samples of tissue, this technique is ideal to detect changes in particular target cells and tissues. Cytochemistry has been successfully applied to assess lysosomal integrity by visualising the hydrolytic enzymes within the lysosomes, and has proved to be a rapid and sensitive investigative tool for evaluating the effects of organic xenobiotics and other injurious agents at very low intracellular concentrations. This generalised response occurs in all cell types ranging from fungi to vertebrates, so that such cytochemical test can be applied on a fairly widespread basis.

Toxic effects of pollutants often depend on their capacity to increase the cellular levels of reactive oxygen species (ROS). When ROS levels production exceeds antioxidant defences, cells experience oxidative stress which causes, among others, membrane lipid peroxidation. The peroxidation end-products are accumulated in lysosomes as insoluble granules containing autofluorescent pigments and are
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usually referred to as lipofuscins (Fig. 1.18). The bulk of lipofuscin granules is constituted by oxidatively modified proteins and lipid degradation products, along with carbohydrates and metals. All lipofuscin pigments are not degradable. The accumulation of these pigments in the lysosome vacuolar system of digestive gland cells of mussels represents an indication of the oxidative stress level in the cells and it is related to the level of membrane lipid peroxidation. During the exposure of mussels to pollutants, this biomarker typically shows a continuously increasing trend, which reaches a maximum level that is determined by the rate of secretion of lipofuscin-rich residual bodies into the external fluids. Lipofuscins represent an end point in the lipid peroxidation process and their accumulation is easily detectable in cells of stressed organisms. Studies carried out by Moore indicated that lipofuscin levels are strongly correlated to lysosomal damage (Viarengo et al., 2007).

The effects of pollutants are often associated with an unbalanced fatty acid metabolism and the accumulation of neutral lipids (Fig. 1.19) in the lysosomal vacuolar system.

The lysosomal storage of neutral lipids in mussel digestive glands has been found to be a useful indicator of a change in the physiology of the cells. As reported by Moore (1988), a build-up of these substances in mussel digestive gland cells may be described as a form of lipidosis induced by toxic chemicals. In fact, cytochemical data clearly indicate an initial stockpiling of neutral lipids in the cytoplasm of cells of pollutant-exposed organisms. Lipids (probably in form of droplets) are then internalised into the lysosomes by autophagic uptake. It is
important to note that such increase in the lysosomal storage of neutral lipids may be related to an increase in the cytosolic lipids content or to a decrease in fatty acid processing (Viarengo et al., 2007).

Fig. 1.19. Accumulation of neutral lipids in lysosomes of mussels *M. galloprovincialis* (magnification 400x).

### 1.6.3. Antioxidant enzymes activities

The assessment of catalase and GST activities, easily carried out by low-cost enzymatic tests, has often been used in laboratory studies and biomonitoring programmes for mussels. These enzymatic tests have proven suitable for monitoring the effects of pollutants on sentinel organisms. The enzyme response to toxic chemicals shows however a bell-shaped trend, with an initial increase due to the activation of enzyme synthesis followed by a decrease in enzymatic activity (due to the enhanced catabolic rate and/or a direct inhibitory action of toxic chemicals on the enzyme molecules). For this reason, enzyme assays should be used in association with other biomarkers such as the lysosomal membrane stability and lipofuscin accumulation assays, which increase or decrease following the development of the pollutant-induced stress syndrome and therefore may help to correctly interpret the “physiological meaning” of changes observed in antioxidative enzymatic activities (Viarengo et al., 2007).

Catalase activity (CAT), although not responding specifically to a group of contaminants, was measured during this work since it has been considered as the primary defense against oxidative damage and it has been studied in bivalve
molluscs around the world. CAT is an enzymatic intracellular antioxidant involved in different defense systems against the radicals generated by the environmental oxidative pollutants. It is a peroxisomal hydroperoxidase that degrades H₂O₂ to H₂O and O₂ (Giarratano et al., 2010). Catalase has one of the highest turnover numbers of all enzymes; one catalase enzyme can convert 40 million molecules of hydrogen peroxide to water and oxygen each second. CAT is considered by many scientists as an important and sensitive biomarker of oxidative stress, revealing biological effects on the redox status of the marine organisms (Vlahogianni et al., 2007).

Chemical compounds, such as heavy metals, are biotransformed to conjugates of reduced glutathione (GSH). Conjugation with GSH is a very important detoxification pathway that is catalyzed by the enzyme GST (Martín-Díaz et al., 2009). Enzymes of the glutathione S-transferase (GST) family are composed of many cytosolic, mitochondrial, and microsomal (now designated as MAPEG) proteins. GSTs are present in eukaryotes and in prokaryotes, where they catalyze a variety of reactions and accept endogenous and xenobiotic substrates. GST contributes to the phase II biotransformation of xenobiotics by conjugating these compounds (often electrophilic and somewhat lipophilic in nature) with reduced glutathione to facilitate dissolution in the aqueous cellular and extracellular media, and, from there, out of the body.

Glutathione S-transferases (GSTs) represent a major group of detoxification isoenzymes whose ‘natural’ substrates range from molecules of foreign origin to by-products of cellular metabolism. GSTs primarily catalyse the conjugation of GSH to various electrophilic compounds, but they can also act as isomerases, or simply as binding proteins sequestering hydrophobic molecules, and can therefore be regarded as playing an antioxidant role (Fernández et al., 2010). Levels of GSTs can be modified by a large range of xenobiotics and also by abiotic factors. Due to their involvement in the detoxification processes of xenobiotics, the GST activity was proposed as a biomarker for several aquatic species such as fishes, crustaceans or molluscs (Bebianno et al., 2007).

Malondialdehyde (MDA) is an intermediate product of lipid peroxidation and prostaglandin biosynthesis that is mutagenic and carcinogenic, and, as reactive toxic metabolite, it is usually rapidly degraded (Viarengo et al., 2007). However, measurements of MDA are still considered a relevant biomarker of lipid

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peroxidation in tissue sample preparations. It is used as a non-enzimatic marker of oxidation of membrane phospholipids through lipid peroxidation and it has been considered as a relevant index of chemical damage induced by toxics in mussels. An increment in MDA level in organisms can be related to degradation of an environmental site by the diminishment of the water quality (Giarratano et al., 2010).

1.7. Cyclic AMP-related cascade reactions

Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3’-5’-cyclic adenosine monophosphate) is a second messenger important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms, conveying the cAMP-dependent pathway.

cAMP is synthesised from ATP by adenylyl cyclase (AC) located on the inner side of the plasma membrane. Adenylyl cyclase is activated by a range of signaling molecules through the activation of adenylyl cyclase stimulatory G (Gs)-protein-coupled receptors and inhibited by agonists of adenylyl cyclase inhibitory G (Gi)-protein-coupled receptors. AC catalyzes cAMP formation from ATP; this nucleotide activates its physiological substrate, the enzyme cAMP-dependent protein kinase A (PKA), which exists as an inactive tetrameric form consisting of two regulatory and two catalytic subunits. By binding to each regulatory subunit, cAMP promotes the dissociation of the holoenzyme in a regulatory dimer and two monomeric and active catalytic subunits. The catalytic subunits in turn phosphorylate substrate proteins in the cytoplasm leading to the regulation of metabolic functions, and/or in the nucleus, modulating gene expression (Fabbri and Capuzzo, 2010) (Fig. 1.20). Until the 1980s, little information was available about cAMP-dependent signalling in bivalves. Further studies indicated that cAMP modulates gonadal functions, muscle contraction, and salt/water homeostasis.
Introduction

Fig. 1.20. Cyclic AMP pathway and putative crosstalk among components in the signal transduction cascade. Dashed lines are used for interactions hypothesized although not demonstrated in bivalves. AC, adenylyl cyclase; CREB, cAMP responsive element binding; DAG, diacylglycerol; GPCR, G protein-coupled receptor; PLC, phospholipase C; IP3, inositol trisphosphate; PDE, phosphodiesterase; PKAi, inactive protein kinase A; PKAα, active protein kinase A; PKC, protein kinase C; TF, transcription factor. From Fabbri and Capuzzo, 2010.

Among several roles ascribed to cAMP in the regulation of mussels physiology, one of the most investigated is the regulation of glycogen breakdown, strictly related to the control of gonad maturation. Glycogen constitutes the main energy reserve in the mantle tissue of molluscs and, as in other tissues, its content depends on the relative activities of the enzymes glycogen synthase and glycogen phosphorylase. A PKA-mediated phosphorylation activity is at the origin of the cascade of reactions that regulate this mechanism.

Furthermore the involvement of cAMP in the regulation of bivalve physiology was recognized relatively to beating of quiescent lateral cilia activation, and induction of spawning. Further on, cAMP was related to cardiac contraction, control of reproduction, mantle and siphon movement regulation, relaxation of the adductor muscle after the “catch” response, and stress response (Fabbri and Capuzzo, 2010).

Several studies about noradrenaline (NA), and adrenaline (A), suggested their interaction with cAMP-modulated processes in bivalves. Adrenergic receptors of α and β type were identified in different tissues of bivalves, showing gross pharmacological properties similar to the mammalian adrenoceptors, although specific studies on subtype classification were not performed in these molluscs.

From various evidence obtained in different bivalve species, α receptors are coupled to the increase of intracellular Ca\(^{2+}\) levels, and β receptors to the enhancement of cAMP concentration. The presence of 5-HT was also detected in several tissues; in bivalves, and more generally in molluscs, the
pharmacological/molecular classification of 5-HT receptors and the correlation with specific physiological functions are mainly unknown, with the exception of a few cases. The serotonergic receptor recognized in molluscs by molecular approaches is of the 5-HT1 subtype (Tierney, 2001) however its pharmacology classifies it as a 5-HT1/5-HT2 mixed receptor. 5-HT1 receptor is coupled to the inhibition of cAMP signalling cascade in vertebrates.

cAMP-dependent signalling is affected by stress factors and environmental or anthropogenic contaminants; for this reason cAMP was proposed as an early and sensitive biomarker (Dailianis et al., 2003).

1.8. Expression of HSP70 proteins

Heat shock protein 70 (HSP70) is a ubiquitous chaperone family preserved in both prokaryotic and eukaryotic cells. HSP70 co-operates with various co-chaperones and therefore serves several cellular functions (Mayer and Bukau, 2005). The most pronounced function, however, is in chaperoning folding and assembly of a vast number of newly synthesised proteins, which in bacteria include 10–20% of total synthesised protein and in eukaryotic cells is predicted to be a larger percentage (Mayer and Bukau, 2005). HSP70 switches between an ATP-bound state, with low substrate affinity, and an ADP-bound state, with high substrate affinity, a process set under the control of co-chaperones belonging to the J-domain protein (JDP) family.

HSP70 proteins can also act to protect cells from thermal and other proteotoxic stress factors. These stresses normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, HSP70 prevents these partially-denatured proteins from aggregating, and allows them to refold.

Cell response to unfavourable conditions includes transient enhancement of the expression of several HSPs. These proteins are indeed classified into families and the best known have a molecular weight of 60, 70, and 90 kDa; amongst them, the HSP70 family appears to be the most evolutionary preserved and distributed in animals (De Maio 1999; Feder and Hofmann 1999). Within the same family, some
HSP isoforms exhibit detectable levels in unstressed cells and, therefore, are addressed as constitutive proteins; others are induced in response to stress exposure and, therefore, are addressed as inducible proteins. For which regards HSP70 proteins, usually a band of molecular weight of at about 77 kDa (constitutive HSP70 isoform) and another one of 72 kDa (inducible HSP70 isoform) are expressed in the gills of mussels in physiological conditions; moreover, a 69-kDa protein is induced following exposure to thermal stress (Piano et al., 2004)

In recent years, interest has grown regarding the cytoprotective and cross-protective role of HSPs in bivalves, often exposed and highly resistant to a variety of environmental stress stimuli (Piano et al., 2004). In fact, a number of HSP inducers other than heat have also been recognized, including hypoxia, heavy metals, oxygen radicals, radiation, osmotic changes, and other factors (Piano et al., 2002) and HSP70 expression was proposed as a biomarker to assess organisms’ health status.

1.9. The MEECE Programme

In the context of global warming, ectothermic animals are supposedly at risk to temperature fluctuations due to their differing thermal dependences to a variety of environments. This is especially true in polluted environments, where increased temperatures could modulate the susceptibility of organisms to contaminants. Bivalves are considered to be particularly at risk to the combined effects of temperature and habitat quality because they are sessile and many of them live in shallow waters where changes in thermal gradients and water levels occur. When organisms are stressed by various physical or chemical agents, there is a metabolic cost for maintaining homeostasis for survival and reproduction. Increased energy to maintain health could be at the expense of growth and gamete development in mussels or resistance to further stressors such as anoxia or temperature (Gagné et al., 2006).
The implementation of the EU’s Marine Strategy Framework Directive (MSFD) requires the application of an ecosystem-based approach to the management of human activities. Priority should be given to achieving or maintaining good environmental status in the Community’s marine environment while enabling sustainable use of marine goods and services. Supporting the delivery of the MSFD is the scientific challenge of investigating and understanding the sensitivities and potential responses of marine ecosystems, to both climatic change and the direct effects of human activity. Without understanding how the ecosystem responds to these multiple drivers, the management of marine ecosystems can result very difficult. Multiple driver assessment may provide the key to managing coastal marine ecosystems, and the goods and services they provide, in a holistic and effective manner.

Led by Plymouth Marine Laboratory, the Marine Ecosystem Evolution in a Changing Environment (MEECE) project brings together 22 European partners to gain a better understanding of the direct and interactive effects of these factors on marine ecosystems. MEECE focuses on the use of predictive models that consider the full range of drivers to elucidate the responses of the marine ecosystem in a comprehensive manner, rather than driver-by-driver as has been done in the past.

MEECE uses predictive models that consider the full range of drivers to explore the responses of the marine ecosystem in a holistic manner, rather than driver by driver as has been done in the past. MEECE will explore the impacts of both climate drivers (acidification, light, circulation and temperature) and anthropogenic drivers (fishing, pollution, invasive species and eutrophication). This approach should help scientists and decision makers to respond to multiple driver impacts with appropriate, knowledge-based, management applications. MEECE also aims at providing methodologies to evaluate these new decision making and management tools.
The project wants to review the impacts of the drivers on the marine ecosystem, to test the impacts of drivers on the structure and functioning of marine ecosystems, to develop indicators of ecosystem status, a coupled model system to predict ecosystem response from plankton to fish and to create a model library of ecosystem modules couplers and decision support tools for management concerning the EC Marine Strategy, EC Maritime Policy and the EC Common Fisheries.

Also the Interdepartmental Centre for Research in Environmental Sciences (C.I.R.S.A.) and the University of Piemonte Orientale were amongst the project partners. For which concerns their contribution, the experimental goal was the assessment of some physiological responses of mussels exposed to increasing concentrations of oxytetracycline and of copper. As previously described, OTC is a widely used antibiotic detected at high concentrations in aquatic environments, included in coastal areas. Cu is an environmental contaminant well studied and largely present in coastal waters. Mussels exposure was carried out at three different temperatures (16, 20 and 24°C), in order to assess if the hypothetical effect of the predicted increase in temperature due to climate changes can alter the effects of the contaminants.

A further step in this perspective will be the combination of the three variables, to assess the interactions of OTC and Cu at increasing temperatures, miming the effects of mixtures really present in natural aquatic environments in which mussels live and to which are continuously exposed.
2. *Aim of the work*
The presence of pharmaceuticals in the environment has become a recent research topic (Santos et al., 2010). The occurrence of pharmaceuticals in different water bodies and the findings of effects on aquatic organisms in ecotoxicity tests have raised concerns about environmental risks of pharmaceuticals in receiving waters (Christensen et al., 2009). As they are continuously added and not efficiently removed by wastewater treatment plants, certain pharmaceuticals exhibit pseudopersistence, giving rise to concerns for the health of aquatic wildlife and a theoretical risk to humans who consume these organisms or drink the water in which they live (Franzellitti et al., 2011).

Residues of human pharmaceuticals have been widely detected in the environment raising concerns about their potential to cause effects in non-target species. Although only present in surface waters at trace concentrations, typically ng-µg/L, some pharmaceuticals cause adverse effects in even lower concentrations (e.g. ≤1 ng/L in some cases). Further, due to the conservative nature of physiological processes, many aquatic species possess similar target molecules to those the drugs are intended to interact with in humans. Thus, given sufficient exposure, pharmacological interactions in non-target species (e.g. mussels) may occur, with potentially adverse effects (Fick et al., 2010).

The aim of this research was the assessment of the physiological effects produced on mussels (Mytilus galloprovincialis) after a sub-chronic exposure under laboratory conditions to three pharmaceuticals belonging to different classes, detected at high concentrations in aquatic environment: an antiepileptic drug (carbamazepine) a β-blocker (propranolol) and an antibiotic (oxytetracycline).

The approach that was followed in this study was based on the concept recently proposed by Christen et al. (2010), the so-called “mode-of-action” based tests, keeping into consideration also inferences from mammalian pharmacokinetic and pharmacodynamic data. This concept is based on three principles: the mode of action of the pharmaceutical, the homology between the human target and the possible targets in the environment, and the importance of the affected pathway for the target species. This was possible for the assessment of the effects due to CBZ and PROP; contrariwise for OTC this approach was not applied since this antibiotic is not supposed to have a direct effect on mussels, but rather on bacteria.
Aim of the work

The results obtained in this work regarding CBZ and PROP, and that will be presented here after, were published on international reviews (Martín-Díaz et al., 2009 for CBZ and Franzellitti et al., 2011 for PROP).
3. Materials and methods


3.1. **Tested substances**

3.1.1. **Carbamazepine**

Carbamazepine was purchased by Sigma (Milan, Italy). Due to its low solubility it was not possible to dissolve CBZ in distilled water; for this reason a stock solution was prepared dissolving CBZ in DMSO (dimethylsulphoxide). This solution was further diluted in order to reach a maximum final DMSO concentration of 0.001% (v/v) once added into the aquaria, in order to avoid a toxic effect due to this solvent. The concentrations chosen for mussels exposure were 0.1 and 10 µg/L.

3.1.2. **Propranolol**

Two experiments were set up to assess the effects of propranolol on mussels. In the first one, mussels were exposed to L-PROP ((S)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol), while in the second one to DL-PROP ((±)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol) hydrochloride (purity ≥ 98%). Both substances were purchased from Sigma Aldrich (Milan, Italy). PROP hydrochloride is readily soluble, so no solvents were necessary for preparation of stock solutions, which were therefore prepared in distilled water. Nominal concentrations were not analytically verified for this study. However, drug degradation during the experiments is unlikely, according to previous studies reporting an average percent recovery for PROP > 80% after longer exposures (Dzialowski et al., 2006; Huggett et al., 2002).

The concentrations chosen for mussels exposure to L-PROP were 300 and 30000 ng/L, while those of DL-PROP were 0.3, 3, 30, 300 and 30000 ng/L. L-PROP is the pure compound commonly used in laboratory experiments. DL-PROP is used as the drug administered in cardiovascular therapies, and therefore corresponds to the compound reported in aquatic systems (Owen et al., 2007).
3.1.3. 

**Oxytetracycline**

Oxytetracycline hydrochloride (purity ≥ 95%) was purchased by Sigma (Milan, Italy).

OTC has a high solubility, so the stock solutions were prepared in distilled water. The concentrations tested on mussels were 0.1, 1, 10, 100 and 1000 µg/L.

3.1.4. 

**Copper**

Copper was added as CuCl₂ (purity ≥ 99%) and was purchased by Sigma (Milan, Italy).

The concentrations to which mussels were exposed were 2.5, 5, 10, 20 and 40 µg/L.

3.2. 

**Experimental animals and holding conditions**

For all the experiments, specimens of Mediterranean mussels (*M. galloprovincialis*) were collected from the north-western Adriatic Sea coast by fishermen of the “Cooperativa Co.pr.al.mo” (Cesenatico, Italy), and rapidly transferred to the laboratory in seawater aerated tanks.

Animals were then acclimated for 3 days in aquaria containing artificial 35 psu seawater at 16°C with continuous aeration and under a natural photoperiod. Each aquarium contained 60 L of water and 30 mussels, which were fed once a day with an algal slurry (Koral filtrator, Xqua, Italy).

15 mussels were sampled after these 3 days of acclimation (zero time) to assess parameters at the onset of each experiment, and to make sure of their good health status.
3.3. Experimental setup and mussel exposure

3.3.1. Exposure to carbamazepine

Thirty mussels per treatment (10 animals per aquaria in 5 L of artificial 35 psu seawater at 16°C with continuous aeration) were exposed for 7 days to CBZ 0.1 and 10 µg/L. Furthermore, 30 unexposed (control) mussels were maintained in parallel to the treated groups. Seawater was renewed every day and the contaminant added as stock solutions properly diluted into DMSO. Mussels were fed once a day.

3.3.2. Exposure to propranolol

3.3.2.1. L-PROP

Thirty mussels per treatment (10 animals per aquaria in 5 L of artificial 35 psu seawater at 16°C with continuous aeration) were exposed for 7 days to L-PROP 300 and 30000 ng/L. Furthermore, 30 unexposed (control) mussels divided into 3 aquaria were maintained in parallel to the treated groups. Seawater was renewed every day and the contaminant added as stock solutions properly diluted into distilled water. Mussels were fed once a day.

3.3.2.2. DL-PROP

Thirty mussels per treatment (10 animals per aquaria in 5 L of artificial 35 psu seawater at 16°C with continuous aeration) were exposed for 7 days to 5 increasing concentrations of DL-PROP. Furthermore, 30 unexposed (control) mussels divided into 3 aquaria were maintained in parallel to the treated groups. Seawater was renewed every day and the contaminant added as stock solutions properly diluted into distilled water. Mussels were fed once a day.
3.3.3. Exposure to oxytetracycline

3.3.3.1. Preliminary test

Forty mussels per treatment (20 animals per aquaria in 10 L of artificial 35 psu seawater at 16°C with continuous aeration) were exposed for 4 days to 5 increasing concentrations of OTC. Furthermore, 40 unexposed (control) mussels were maintained in parallel to the treated groups.

Seawater was renewed every day and the contaminant added as stock solutions properly diluted into distilled water. Mussels were fed once a day.

This experiment was directed to assess if the selected concentrations of the antibiotic could have an effect on mussels physiology.

3.3.3.2. Final test

Unlike all the experiments described above which were performed in the laboratories of the Interdepartmental Centre for Research in Environmental Sciences (C.I.R.S.A.) of Ravenna, this test was carried out in the laboratories of the group of investigation of Prof. Viarengo in Alessandria.

48 mussels per treatment (12 animals per aquaria in 6 L of artificial 35 psu seawater with continuous aeration) were exposed for 4 days to 5 increasing concentrations of OTC, the same tested in the preliminary experiment. Furthermore, 48 unexposed (control) mussels were maintained in parallel to the treated groups.

The possible effects of the antibiotic on mussels were assessed in combination with an increase in water temperature, so the setup described above was maintained to expose mussels to OTC at 16°C, 20°C and 24°C.

Seawater was renewed every day and the antibiotic added as stock solutions properly diluted into distilled water. Mussels were fed once a day.

In order to avoid side effects due only to a sharp thermal stress, mussels were previously adapted to the temperatures of 20 and 24°C by slowly increasing water temperature during 3 days before the exposure to OTC.
This work was (partially) funded by Theme 6 of the EC seventh framework program through the Marine Ecosystem Evolution in a Changing Environment (MEECE No 212085) Collaborative Project.

### 3.3.4. Exposure to copper

Also this test was performed in the laboratories of the group of investigation of Prof. Viarengo in Alessandria.

As described for OTC, 48 mussels per treatment (12 animals per aquaria in 6 L of artificial 35 psu seawater with continuous aeration) were exposed for 4 days to 5 increasing concentrations of Cu. Furthermore, 48 unexposed (control) mussels were maintained in parallel to the treated groups.

Also in this case, the possible effects of the metal on mussels were assessed in combination with an increase in water temperature, so the setup described above was maintained to expose mussels to Cu at 16°C, 20°C and 24°C.

Seawater was renewed every day and the contaminant added as stock solutions properly diluted into distilled water. Mussels were fed once a day.

Likewise it was done for OTC exposure, mussels were previously adapted to the three thermal conditions by slowly increasing water temperature during three days before the beginning of the experiment, in order to avoid side effects due to thermal stress.

This work was (partially) funded by Theme 6 of the EC seventh framework program through the Marine Ecosystem Evolution in a Changing Environment (MEECE No 212085) Collaborative Project.

### 3.4. Tissues preparation

At the end of the exposure period, for all the tested contaminants, a haemolymph sample was withdrawn from the abductor muscle of each mussel using a hypodermic syringe, and immediately processed for the NRR assay.

For which concerns experiments carried out inside the MEECE Programme, the sex of all the mussels was determined by removing part of the gonad and examining it under a light microscope (400x magnification) for the detection of
sperm or eggs (Zouros et al., 1994). Furthermore, haemolymph from mussels exposed to OTC and Cu in Alessandria laboratories was centrifuged and stored at -80°C for proteomic and genomic analysis.

For all the experiments, digestive gland, gills and mantle/gonads were dissected from each individual, immediately frozen in liquid nitrogen and stored at -80°C until the analysis.

3.5. Battery of biomarkers

3.5.1. Stress on stress

This biomarker was performed only on mussels exposed to OTC in the preliminary experiment.

At the end of the exposure period, 10 animals per aquaria were subjected to anoxia by exposure to air at 16°C (Moschino et al. 2010). Survival was assessed daily until 100% mortality was reached.

3.5.2. Neutral red retention assay

LMS was evaluated in mussels haemocytes using the NRR assay described by Lowe et al. (1995) with slight modifications.

Haemolymph was withdrawn from the adductor muscle using a hypodermic syringe containing a physiological solution (20 mM Hepes, 436 mM NaCl, 53 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂; pH 7.3) (1:1 v/v).

A stock solution (s.s.) of neutral red was prepared by dissolving 20 mg of dye in 1 ml of dimethylsulfoxide (DMSO), while a working solution (w.s.) were prepared by diluting this stock solution with the physiological serum: 5 µl of the s.s. with 995 µl of physiological solution.

A volume of 40 µl of haemolymph was placed onto a microscope slide, previously treated with 5 µl of the coating agent poly-L-lysine; the slides were incubated inside a dark humidity chamber for 30 minutes to allow the cells to attach. The excess solution was tipped off by carefully tilting the slides, and 40 µl of the
Materials and methods

neutral red w.s. was added onto the area containing the attached cells. After 15 minutes incubation inside the dark humidity chamber, the excess solution was eliminated, a coverslip was applied and the slides were observed under a light microscope (400x magnification), at 15-minute intervals, to determine the time at which the dye that had been taken up into individual lysosomes (turning them red) had leached out into the cytosol. The test was stopped when dye loss was evident in more than 50% of the haemocytes and the times were recorded; following each inspection, the preparations were returned to the incubation chamber (Lowe et al. 1995).

Data were expressed as destabilization time, representing the time at which more than 50% of the lysosomes released the dye into the cytosol (Martín-Díaz et al. 2009).

For each treatment the mean destabilization time was calculated from 12 different slides prepared from 12 animals.

NRR assay was not performed on mussels exposed to OTC and Cu in the experiments carried out in Alessandria.

3.5.3. Cytochemical assays: accumulation of lipofuscins (LF) and neutral lipids (NL)

Immediately after dissection, digestive glands from 12 mussels per treatment were placed on 3 different aluminium cryostat chucks, frozen in N-hexane pre-cooled at −70°C with liquid nitrogen, and stored at −80°C. The following procedures were carried out according to the UNEP/RAMOGE Manual (1999).

All these tests were performed on 10 µm-thick digestive gland sections, which were obtained using a cryostat (MICROM HM 505 N) at a cabinet temperature of -30°C and transferred onto microscope slides.

The lipofuscin content was determined using the Schmorl reaction. For LF determination triplicate slides of cryostat sections were fixed in calcium formol for 15 min at 4°C, rinsed and immersed in the reaction medium containing an aqueous solution of 1% ferric chloride and 1% potassium ferricyanide in a 3:1 ratio. Sections were stained for 5 min, rinsed in 1% acetic acid and washed in distilled water and mounted with glycerol–gelatine.
Cryosections were examined under a light microscope (Axioskop 40, Carl Zeiss, Milan, Italy) equipped with a 40x objective and a digital camera (AxioCam MRc, Carl Zeiss, Milan, Italy). The volume density of lipofuscins was measured by image analysis. Four images were analysed per each of the 4 sections on the same microscope slides, and 3 slides were analysed for each chuck for each treatment using the Scion Image vers. 4.0.2 image analysis software (Scion Corporation Frederick, MD, USA).

For the assessment of accumulation of neutral lipids, 3 slides per treatment of cryostat sections were fixed in calcium formol for 15 min at 4°C, rinsed and transferred into 60% triethylphosphate for 3 min. Sections were stained in 1% oil red O in 60% triethylphosphate for 15 min in the dark, then rinsed in 60% triethylphosphate for 30 s, washed in distilled water before mounting with glycerol-gelatine.

Tissue sections were quantitatively assessed under a light microscope (Axioskop 40, Carl Zeiss, Milan, Italy) equipped with a 40x objective and a digital camera (AxioCam MRc, Carl Zeiss, Milan, Italy). Four images were analysed per each of the 4 sections on the same microscope slides, and 3 slides were analysed for each chuck for each treatment using the Scion Image vers. 4.0.2 image analysis software (Scion Corporation Frederick, MD, USA).

3.5.4. Antioxidant enzymes activity

For mussels exposed to carbamazepine and to propranolol, for each concentration of exposure, digestive glands, gills, and mantle/gonads tissues from 18 individuals were dissected and rinsed. Tissues were randomly combined into 6 pools and homogenized in 50 mM potassium–phosphate buffer (KPB), pH7.0 containing 0.5 mM Na2EDTA, following the protocol reported by Mimeault et al. (2006).

Total protein content was estimated according to Lowry et al. (1951).

Enzyme activities were measured using a DU 800 Multisample spectrophotometer (Beckman) at 25°C.

Glutathione S-transferase (GST) activity was determined by measuring the increase in absorbance at 340 nm due to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). The reaction was followed for 10 min and the final activity was expressed as nmol/min/mg protein.
Catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm due to the consumption of hydrogen peroxide (55 mM H$_2$O$_2$ in 50 mM KPB pH 7.0). The reaction was followed for 2 min and the final activity was expressed as µmol/min/mg protein.

### 3.5.5. Malondialdehyde (MDA) assay

MDA content was used to assess lipid peroxidation and was carried out following the test described by Banni et al. (2007) with slight modifications. Digestive glands, gills and mantle/gonads tissues were dissected, combined into 6 pools and homogenized in 20 mM Tris–HCl pH 7.4 containing 0.1% β-mercaptoethanol and then centrifuged at 10000 xg for 20 min at 4°C. Total protein content in the supernatant fraction was measured according to Lowry et al. (1951). The supernatants obtained were used for the determination of MDA content: samples were incubated for 40 min in 5 mM methanol/1-methyl-2-phenylindoleinacetonitrile and 6% HCl at 45°C, and subsequently centrifuged at 15000 xg for 10 min at room temperature. Absorbance was read spectrophotometrically at 586 nm against a standard curve of TMOP treated in an identical manner. The final results were expressed as nmol MDA/mg protein. MDA content was not assessed in mussels exposed to OTC or Cu inside the MEECE Programme.

### 3.6. HSP70 determination

The expression of HSP70 proteins was evaluated only in mussels exposed to OTC and Cu in the experiments performed in Alessandria, for all the concentrations of contaminants and all the temperatures tested. For HSP70 detection, gills from 4 individuals for each concentration and for each temperature of exposure were homogenized in an ice-cold lysis buffer and after centrifugation supernatants were diluted 1:3 with Laemmli buffer, boiled for 5 minutes and stored at -20°C. Total protein content was assessed according to Lowry method (Lowry et al., 1951).
20 µg of proteins were loaded onto 30% polyacrylamide gels; electrophoresis was carried out at 28 mA for 150 minutes, after which resolved proteins were transferred onto a nitrocellulose membrane (300 mA, 1 hour at 4°C). Blots were probed with mouse monoclonal anti-HSP70 antibody (1:1000) (Sigma, Milan, Italy) for 1 hour, and then with rabbit anti-mouse IgG polyclonal antibody (1:6000) (Sigma, Milan, Italy) conjugated with horseradish peroxidase for 1 hour. Immunoblots were developed by a chemiluminescence reagent, and a densitometric analysis of the films was performed by Image Master (Amersham-Pharmacia, Milan, Italy) equipped with TotalLab software. All data were normalized on the respective control samples exposed at 16°C.

As control of a correct protein loading, immunoblots were subsequently probed with a monoclonal anti-β-tubulin antibody (1:30000) (Sigma, Milan, Italy) for 1 hour, and then with rabbit anti-mouse IgG polyclonal antibody (1:6000) (Sigma, Milan, Italy) conjugated with horseradish peroxidase for 1 hour. Quantification of proteins expression was carried out as described above for HSP70 detection.

3.7. Cyclic AMP-related cascade reactions

3.7.1. Cyclic AMP content

For mussels exposed to carbamazepine, cAMP content was evaluated following the method described by Fabbri and Capuzzo (2006). About 50 mg of tissues (gills, digestive gland and mantle/gonads) were obtained from 18 individuals; 6 random pools (150 mg each) were homogenized in 5% perchloric acid and then centrifuged at 18000 xg for 20 min at 4°C. The supernatant fraction was neutralized and samples centrifuged again at 18000 xg for 10 min at 4°C. Cyclic AMP contents were evaluated in triplicate in the clear supernatant by radiochemical assay. Results were expressed as pmol cAMP/g fresh tissue.

Cyclic AMP content in mussels exposed to PROP/OTC and Cu was assessed according to Dailianis et al. (2003). Tissues (about 200 mg of gills, digestive gland and mantle/gonads) were homogenized with three volumes of 4 mM EDTA to prevent enzymatic degradation of cAMP. The homogenate was boiled for 5 min
and centrifuged for 5 min at 16000 xg at 4°C. The concentration of cAMP was estimated in the supernatant fraction by the same radiochemical assay described above (Fabbri and Capuzzo, 2006) using \(^3\text{H}\)-cyclic cAMP (Amersham-Pharmacia, Milan, Italy). Results were expressed as pmol cAMP/g fresh tissue.

### 3.7.2. PKA activity

PKA activity was assayed in gills, digestive glands and mantle/gonad tissues in the mussels exposed to CBZ and PROP.

Tissues were homogenized in cold PKA extraction buffer (25 mM Tris–HCl, pH 7.4 containing 0.5 mM EDTA, 0.5 mM EGTA, 10mM β-mercaptoethanol and 50-fold diluted proteinase inhibitor cocktail). Total protein content was determined according to Lowry et al. (1951). Supernatants were assayed for PKA activity using the non-radioactive PepTag PKA assay kit (Promega, Milan, Italy) with dye-labeled Kemptide as a substrate according to manufacturer’s protocol. Results were expressed as nmol/min/mg total protein.

### 3.8. Statistical analysis

Data relative to the exposure to CBZ were analysed by SPSS/PC+ statistical package. Significant differences between exposed and control samples were determined using a one-way ANOVA followed by the multiple comparison Dunnett’s test. Pair-wise correlations were obtained through the Spearman’s rank order correlation test. Statistical difference was accepted when p < 0.05.

All the other data were analysed using the SigmaStat statistical pakage. As for data about CBZ, significant differences between exposed and control samples were determined using a one-way ANOVA followed by the multiple comparison Dunnett’s test. Pair-wise correlations were using the Person’s correlation test. Statistical difference was accepted when p < 0.05.

Data about OTC and Cu were analysed using a two-way ANOVA followed by the multiple comparison Bonferroni test. Statistical difference was accepted when p < 0.05.
4. Results
4.1. CARBAMAZEPINE

4.1.1. Lysosomal membrane stability (NRR assay)

Fig. 4.1. Lysosomal membrane stability assessed in haemocytes from mussels exposed to CBZ by NRR assay. Asterisks indicate values significantly different from control: ** p < 0.01.

The effect of CBZ on mussel haemocyte LMS is illustrated in Fig. 4.1. NRRT was about 90 min in haemocytes from control animals; it was significantly reduced to about 35 min (~60%) and 18 min (~80%) after a 7-day exposure to 0.1 and 10 µg/L CBZ, respectively.

4.1.2. Cytochemical assays

4.1.2.1. Accumulation of lipofuscins

Fig. 4.2. Accumulation of lipofuscins in mussels exposed to CBZ. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.
Results

The content of LF in mussels exposed for 7 days to 0.1 and 10 µg/L CBZ (Fig. 4.2) was slightly but significantly increased with respect to the control animals at both concentrations tested.

4.1.2.2. Accumulation of neutral lipids

![Graph showing accumulation of neutral lipids](#)

Fig. 4.3. Accumulation of neutral lipids in mussels exposed to CBZ. Asterisks indicate values significantly different from control: ** p < 0.01.

The content of NL (Fig. 4.3) in mussels exposed to CBZ was significantly increased with respect to the control animals, even if of a low percentage.

4.1.3. Antioxidant enzymes activity

4.1.3.1. Catalase

![Graph showing catalase activity](#)

Fig. 4.4. Catalase activity in digestive gland, gills and mantle/gonad tissues of mussels exposed to CBZ. Asterisks indicate values significantly different from control: ** p < 0.01.
Results

CAT activity (Fig. 4.4) was significantly increased in digestive gland after exposure to 0.1 and 10 µg/L CBZ. No effect was observed in gills, while CAT activity was significantly increased in mantle/gonads but only at 10 µg/L CBZ. Independent of the treatment, enzyme activity was significantly higher in digestive gland compared with gills and mantle/gonads.

4.1.3.2. Glutatione S-transferase

![Graph showing GST activity](image)

Fig. 4.5. GST activity in digestive gland, gills and mantle/gonad tissues of mussels exposed to CBZ. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.

GST activity (Fig. 4.5) was significantly increased in digestive gland after exposure to 0.1 µg/L CBZ, while it was not significantly different from controls at 10 µg/L CBZ. No effect was observed in gills, while GST activity was significantly increased in mantle/gonads at both CBZ concentrations. Independent of the treatment, enzyme activity was significantly higher in gills compared with digestive gland and mantle/gonads.
4.1.4. MDA assay

Fig. 4.6. MDA content in digestive gland, gills and mantle/gonad tissues of mussels exposed to CBZ. Asterisks indicate values significantly different from control: ** p < 0.01.

Lipid peroxidation assessed as MDA content (Fig. 4.6) showed no effect of CBZ in digestive gland, while a significant increase in MDA content was observed in gills of mussels exposed to 0.1 and 10 µg/L and in mantle/gonads after exposure to 10 µg/L CBZ.

4.1.5. Cyclic AMP-related cascade reactions

4.1.5.1. cAMP content

Fig. 4.7. cAMP content in digestive gland, gills and mantle/gonad tissues of mussels exposed to CBZ. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.

Content of cAMP in different tissues from mussels of control and mussels exposed to CBZ is shown in Fig. 4.7. cAMP from control mussels was about 190, 160, and 120 pmol/g fresh tissue in digestive gland, gills, and mantle/gonads, respectively.
Results

Significant reductions were observed in cAMP content in digestive gland and gills at 10 µg/L CBZ, and in mantle/gonads at 0.1 and 10 µg/L CBZ.

4.1.5.2. PKA activity

Fig. 4.8. PKA activity in digestive gland, gills and mantle/gonad tissues of mussels exposed to CBZ. Asterisks indicate values significantly different from control: ** p < 0.01.

The activity of PKA was assessed in the same tissues analysed for cAMP content, and similar trends were observed (Fig. 4.8). PKA activities from control mussels were about 2.0, 1.7, and 3.0 nmol/min/mg protein in digestive gland, gills, and mantle/gonads, respectively. Significant reductions in PKA activity were observed in digestive gland and gills at 10 µg/L CBZ, and in mantle/gonads at 0.1 and 10 µg/L CBZ.

4.1.6. Sensitivity of biomarkers and cAMP-related parameters to CBZ exposure

A pair-wise correlation analysis was performed considering CBZ concentrations and the biological endpoints analysed in digestive gland, gills, and mantle/gonads of mussels. LMS was included although analysed on haemocytes, since this parameter is highly related to the mussel general health status. LMS values were negatively correlated with increasing CBZ concentrations (Tables 4.1–4.3). Moreover, they were positively correlated with the reduction of cAMP levels and PKA activity in all tissues. For which regards the other biomarkers, NL content in digestive gland was negatively correlated with the reduction of LM destabilization times and positively correlated with the increase of CBZ concentrations. CAT
activities in digestive gland and mantle/gonads increased with increasing CBZ concentrations and GST activities. Moreover, CAT activities in digestive gland were negatively correlated with the reduction of LMS values. Increase of GST activities in mantle/gonads was positively correlated with CBZ concentrations and negatively correlated with LMS values.

Levels of cAMP in digestive gland and gills and PKA activity were negatively correlated with increasing CBZ concentrations; in all tissues analysed decrease of cAMP contents was strongly correlated with the reduction of PKA activities.

Tab. 4.1. Correlation coefficients amongst the biological endpoints measured in digestive gland of mussels exposed to CBZ.

<table>
<thead>
<tr>
<th>[CBZ]</th>
<th>LMS</th>
<th>LF</th>
<th>NL</th>
<th>MDA</th>
<th>CAT</th>
<th>GST</th>
<th>cAMP</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMS</td>
<td>-0.896**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>0.474</td>
<td>-0.517</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL</td>
<td>0.873**</td>
<td>-0.678*</td>
<td>0.142</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>0.264</td>
<td>-0.333</td>
<td>0.383</td>
<td>0.167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>0.843**</td>
<td>-0.700*</td>
<td>0.467</td>
<td>0.828**</td>
<td>0.500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>0.580</td>
<td>-0.600</td>
<td>0.300</td>
<td>0.002</td>
<td>0.617</td>
<td>0.650*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>-0.738*</td>
<td>0.617*</td>
<td>-0.117</td>
<td>-0.603*</td>
<td>0.333</td>
<td>-0.333</td>
<td>-0.450</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>-0.896**</td>
<td>0.733*</td>
<td>-0.150</td>
<td>-0.762</td>
<td>-0.400</td>
<td>-0.783**</td>
<td>-0.500</td>
<td>0.992**</td>
</tr>
</tbody>
</table>

[CBZ], carbamazepine concentration; LMS, lysosomal membrane stability; LF, lipofuscin; NL, neutral lipids; MDA, malondialdehyde; CAT, catalase; GST, glutathione S-transferase; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase.

* p < 0.05 according to the Spearman’s test.

** p < 0.01 according to the Spearman’s test.

Tab. 4.2. Correlation coefficients amongst the biological endpoints measured in gills of mussels exposed to CBZ.

<table>
<thead>
<tr>
<th>[CBZ]</th>
<th>LMS</th>
<th>MDA</th>
<th>CAT</th>
<th>GST</th>
<th>cAMP</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMS</td>
<td>-0.896**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>0.185</td>
<td>-0.067</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>0.791**</td>
<td>-0.600</td>
<td>0.310</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>0.949**</td>
<td>-0.733**</td>
<td>0.243</td>
<td>0.850**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>-0.580</td>
<td>0.990**</td>
<td>-0.109</td>
<td>-0.383</td>
<td>-0.567</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>-0.632</td>
<td>0.946**</td>
<td>-0.109</td>
<td>-0.267</td>
<td>-0.517</td>
<td>0.983**</td>
</tr>
</tbody>
</table>

[CBZ], carbamazepine concentration; LMS, lysosomal membrane stability; MDA, malondialdehyde; CAT, catalase; GST, glutathione S-transferase; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase.

** p < 0.01 according to the Spearman’s test.
Tab. 4.3. Correlation coefficients amongst the biological endpoints measured in mantle/gonads of mussels exposed to CBZ.

<table>
<thead>
<tr>
<th></th>
<th>[CBZ]</th>
<th>LMS</th>
<th>MDA</th>
<th>CAT</th>
<th>GST</th>
<th>cAMP</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMS</td>
<td>-0.896**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>0.474</td>
<td>-0.483</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>0.316</td>
<td>-0.033</td>
<td>-0.067</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>0.105</td>
<td>-0.067</td>
<td>-0.150</td>
<td>0.450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>-0.978*</td>
<td>0.617</td>
<td>-0.083</td>
<td>-0.367</td>
<td>-0.200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>-0.988**</td>
<td>0.683*</td>
<td>-0.417</td>
<td>-0.350</td>
<td>0.183</td>
<td>0.935**</td>
<td></td>
</tr>
</tbody>
</table>

[CBZ], carbamazepine concentration; LMS, lysosomal membrane stability; MDA, malondialdehyde; CAT, catalase; GST, glutathione S-transferase; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase.

* p < 0.05 according to the Spearman’s test.
** p < 0.01 according to the Spearman’s test.

4.2. L-PROPRANOLOL and DL-PROPRANOLOL

As previously described, two experiments were set up to assess the effects of propranolol on mussels. The first one aimed at investigating the effects of two concentrations of L-PROP (300 and 30000 ng/L) mainly on cAMP-related parameters in the digestive gland, gills and mantle/gonads. In the second experiment, five concentrations of DL-PROP were tested on the same parameters assessed for L-PROP as well as on a suite of stress-response related parameters; only digestive gland and mantle/gonads were assessed as these tissues showed the highest sensitivity to L-PROP in the first experiment carried out.

4.2.1. Lysosomal membrane stability

![Figure 4.9](image)

Fig. 4.9. Lysosomal membrane stability assessed in haemocytes from mussels exposed to L-PROP (a) and DL-PROP (b) by NRR assay. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.
Results

Significant reductions in LMS values were detected both in mussels exposed to L-PROP (Fig. 4.9 a) and to DL-PROP (Fig. 4.9 b), at all the concentrations tested.

4.2.2. Cytochemical assays

These tests were performed only in the second experiment, in which mussels were exposed to DL-PROP.

4.2.2.1. Accumulation of lipofuscins

Statistical analysis did not find significant differences in lipofuscins accumulation between mussels exposed to DL-PROP and control animals (Fig. 4.10).

4.2.2.2. Accumulation of neutral lipids

Statistical analysis did not find significant differences were found between exposed mussels and controls.
No significant differences in lipofusins accumulation were found by statistical analysis between mussels exposed to DL-PROP and control animals (Fig. 4.11).

4.2.3. Antioxidant enzymes activity

4.2.3.1. Catalase

Fig. 4.12. Catalase activity in digestive gland (a) and mantle/gonad (b) of mussels exposed to DL-PROP. Asterisks indicate values significantly different from control: * p < 0.05.

Catalase activity was assessed only on mussels exposed to DL-PROP.

CAT activity significantly increased in the digestive gland of mussels exposed to 3 ng/L DL-PROP, and decreased below control levels at higher concentrations (Fig. 4.12 a). In mantle/gonads CAT activities significantly decreased at all DL-PROP concentrations tested (Fig. 4.12 b).
4.2.3.2. Glutathione S-transferase

Fig. 4.13. GST activity in digestive gland (a), mantle/gonad (b) and gills (c) of mussels exposed to L-PROP. No significant differences were found between exposed mussels and controls.

Fig. 4.14. GST activity in digestive gland (a) and mantle/gonad (b) of mussels exposed to DL-PROP. Asterisks indicate values significantly different from control: * p < 0.05.

No effects were observed in GST activity in the tissues of mussels exposed to L-PROP (Fig. 4.13 a, b and c).
Contrariwise GST activity significantly increased in the digestive gland of mussels exposed to DL-PROP (Fig. 4.14 a), whereas activities were significantly reduced in mantle/gonads (Fig. 4.14 b).
4.2.4. Cyclic AMP-related cascade reactions

4.2.4.1. cAMP content

Mussels exposed to L-PROP had significantly decreased cAMP levels in the digestive gland at both concentrations tested. No effect was observed in gills, while cAMP levels were significantly increased in mantle/gonads at 30000 ng/L L-PROP (Fig. 4.15).

Cyclic AMP levels in mussels exposed to DL-PROP were significantly reduced in the digestive gland (Fig. 4.16 a) and significantly increased in mantle/gonads (Fig. 4.16 b) even at the lowest concentration used (0.3 ng/L).
4.2.4.2. PKA activity

Fig. 4.17. PKA activity in digestive gland, gills and mantle/gonad tissues of mussels exposed to L-PROP. Asterisks indicate values significantly different from control: * p < 0.05.

Fig. 4.18. PKA activity in digestive gland (a) and mantle/gonad tissues (b) of mussels exposed to DL-PROP. Asterisks indicate values significantly different from control: * p < 0.05.

PKA activity was decreased in the digestive gland of mussels exposed to 30000 ng/L of L-PROP; no significant differences were observed in gills while a significant increase occurred in mantle/gonads at the highest L-PROP concentration (Fig. 4.17).

In mussels exposed to DL-PROP, PKA activities were significantly decreased in the digestive gland of animals exposed to 0.3, 300 and 30000 ng/L DL-PROP (Fig. 4.18 a).

In mantle/gonads, PKA activities increased after exposure to DL-PROP concentration between 3 and 300 ng/L; activities were similar to control levels at 30000 ng/L (Fig. 4.18 b).
4.2.4. Sensitivity of biomarkers and cAMP-related parameters to PROP exposure

A pair-wise correlation analysis was performed to compare L- and DL-PROP concentrations and the biological endpoints analysed in the different tissues (Tab. 4.4-4.5).

Taking into consideration organisms exposed to L-PROP, statistical analysis showed a negative correlation between PKA activity in the digestive gland and a positive correlation in mantle/gonads. cAMP levels were positively correlated with L-PROP concentrations in gills and mantle/gonads.

For which concerns data relative to exposure to DL-PROP, statistical analysis was performed on endpoints analysed in the digestive gland and mantle/gonads of mussels. LMS was included although analysed on haemocytes, since this parameter is highly related to the mussel general health status. LMS values were significantly negatively correlated with increasing DL-PROP concentrations. Moreover, LM destabilization times were positively correlated with reduced cAMP levels and PKA activities in digestive gland, while negatively correlated with the same parameters in mantle/gonads.

Tab. 4.4. Correlation coefficients amongst the biological endpoints measured in digestive gland, gills and mantle/gonads of mussels exposed to L-PROP.

<table>
<thead>
<tr>
<th></th>
<th>Digestive gland</th>
<th>Gills</th>
<th>Mantle/gonads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>PKA</td>
<td></td>
</tr>
<tr>
<td>[L-PROP]</td>
<td>-0.361</td>
<td>-0.971**</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>-0.361</td>
<td>0.128</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[L-PROP]</td>
<td>0.979*</td>
<td>0.096</td>
<td>0.857*</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.979*</td>
<td>0.096</td>
<td>0.857*</td>
</tr>
<tr>
<td>PKA</td>
<td>0.096</td>
<td>0.991**</td>
<td>0.917**</td>
</tr>
</tbody>
</table>

[L-PROP], L-Propranolol concentration; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase.
* p < 0.05 according to the Pearson’s test.
** p < 0.01 according to the Pearson’s test.
Results

Tab. 4.5. Correlation coefficients amongst the biological endpoints measured in digestive gland and mantle/gonads of mussels exposed to DL-PROP.

<table>
<thead>
<tr>
<th></th>
<th>cAMP</th>
<th>PKA</th>
<th>LMS</th>
<th>CAT</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digestive gland</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[DL-PROP]</td>
<td>0.291</td>
<td>-0.646**</td>
<td>-0.839**</td>
<td>-0.312</td>
<td>0.294</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.621</td>
<td>0.591</td>
<td>-0.088</td>
<td>-0.646**</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td></td>
<td>0.818**</td>
<td>0.293</td>
<td>-0.736**</td>
<td></td>
</tr>
<tr>
<td>LMS</td>
<td></td>
<td></td>
<td>0.404*</td>
<td>-0.728**</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
<td>-0.088</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mantle/gonads</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[DL-PROP]</td>
<td>0.396</td>
<td>-0.107</td>
<td>-0.839**</td>
<td>-0.332</td>
<td>-0.551*</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.137</td>
<td>-0.627**</td>
<td>-0.480*</td>
<td>-0.511*</td>
<td>-0.256</td>
</tr>
<tr>
<td>PKA</td>
<td></td>
<td>-0.346</td>
<td>-0.511*</td>
<td>-0.236</td>
<td></td>
</tr>
<tr>
<td>LMS</td>
<td></td>
<td></td>
<td>0.665**</td>
<td>0.751**</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
<td>0.746**</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[DL-PROP], DL-Propranolol concentration; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; LMS, lysosomal membrane stability; CAT, catalase; GST, glutathione S-transferase.

* p < 0.05 according to the Pearson’s correlation test.

** p < 0.01 according to the Pearson’s correlation test.

4.3. OXYTETRACYCLINE

4.3.1. Preliminary experiment

4.3.1.1 Lysosomal membrane stability (NRR assay)

![Graph showing lysosomal membrane stability](image)

Fig. 4.19. Lysosomal membrane stability assessed in haemocytes from mussels exposed to OTC by NRR assay. Asterisks indicate values significantly different from control: ** p < 0.01.

Significant reductions in LMS values were registered in mussels exposed to all concentrations of OTC (Fig. 4.19). At the concentration of 1000 µg/L it was
possible to observe a slight increase in lysosomal membrane destabilization time, but the values were in any case significantly lower than control ones.

### 4.3.1.2. Stress on stress

Mussels subjected to anoxia by exposure to air after 4 days of treatment with increasing OTC concentrations survived on average 6 days (Fig. 4.20 a and b). No differences with respect to control in mussels exposed to OTC 0.1, 1 and 10 µg/L were observed, while it was possible to observe a significant increase of survival in organisms exposed to the two highest concentrations of the antibiotic (Fig. 4.20 a). All the mussels used in this experiment were subjected to sex determination, so it was possible to compare survival of male organisms with females (4.21 a and b). No significant differences were found between the two genders at all the concentrations tested (Fig. 4.22).

---

**Fig. 4.20.** Average time (a) and percentage (b) of survival in air of mussels exposed to OTC. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.
Results

Fig. 4.21. Percentage of survival in air of female (a) and male (b) mussels exposed to OTC.

Fig. 4.22. Average time of survival in air of female and male mussels exposed to OTC. No differences were observed between the two genders.

4.3.1.3. Cytochemical assay: accumulation of lipofuscins

Fig. 4.23. Accumulation of lipofuscins in mussels exposed to OTC. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.
The content of lipofuscins (Fig. 4.23) in mussels exposed for 4 days to increasing concentrations of OTC was slightly but significantly increased with respect to control animals.

**4.3.2. Final experiment**

**4.3.2.1. HSP70 determination**

In the contest of the European MEECE Programme, mussels were exposed to OTC 0.1, 1, 10, 100 and 1000 µg/L at 16, 20 and 24°C, in order to assess the effects due to the combination of the antibiotic and the increase in temperature. The concentrations of the antibiotic were the same employed in the preliminary experiment.

The expression of HSP70 proteins was assessed for each concentration and at all the temperatures of exposure.

Normally, two bands of molecular weight estimated at about 77 kDa (constitutive HSP70 isoform) and 72 kDa (inducible HSP70 isoform) are shown in the gills of mussels in physiological conditions; moreover, a 69-kDa protein is induced after exposure to thermal stress (35°C for 1 hour, plus 24 hours of recovery at 16°C) (Fig. 4.24).

Samples from mussels exposed in this experiment to OTC at the three different temperatures never showed the expression of the 69-kDa protein, but only of the 77 and 72 kDa proteins.

![Western blotting to detect proteins of the HSP70 family in gills of *M. galloprovincialis*. Heat shocked animals (stressed) were exposed to 35°C for 1 hour and allowed to recover in aquaria at 16°C for 24 hours before collecting samples.](image)

Fig. 4.24. Western blotting to detect proteins of the HSP70 family in gills of *M. galloprovincialis*. Heat shocked animals (stressed) were exposed to 35°C for 1 hour and allowed to recover in aquaria at 16°C for 24 hours before collecting samples. a = constitutive isoform (77 kDa); b = inducible isoform (72 kDa); c = strictly inducible isoform (69 kDa).
It was possible to exclude the risk that the differences observed in HSP70 expression were due to unequal protein loading, as blots were probed with a monoclonal anti-β-tubulin antibody, showing no differences in the expression of this protein, which keeps constantly expressed even in mussels exposed to stress factors (Fig. 4.25).

Fig. 4.25. Western blotting to assess β-tubulin protein expression in gills of *M. galloprovincialis* exposed to increasing OTC concentrations at 20°C. A representative immunoblot out of at least 3 separate blots run in duplicate is reported.

### 4.3.2.1. Exposure to OTC at 16°C

Fig. 4.26. Western blotting to assess HSP70 protein expression in gills of *M. galloprovincialis* exposed to increasing OTC concentrations at 16°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.
Mussels exposed at 16°C for 4 days to 5 concentrations of OTC (from 0.1 to 1000 μg/L) showed an increase in the inducible HSP70 protein expression, with a maximum effect of about 185% with respect to control values reached at 1 μg/L OTC and slightly decreasing thereafter (Fig. 4.27 b). The expression of the constitutive isoform was not changed after mussels exposure to OTC (Fig. 4.27 a).

4.3.2.1.2. Exposure to OTC at 20°C

Fig. 4.28. Western blotting to assess HSP70 protein expression in gills of M. galloprovincialis exposed to increasing OTC concentrations at 20°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.
The exposure of mussels at 20°C to OTC caused an increase in the inducible HSP70 protein expression compared to control samples at 16°C, with a maximum effect of about 185% with respect to control values at 100 μg/L OTC, followed by a strong decrease at 1000 μg/L OTC until reaching control values (Fig. 4.29 b). The expression of the constitutive isoform did not change at any OTC concentration at 20°C (Fig. 4.29 a).

4.3.2.1.3 Exposure to OTC at 24°C

Fig. 4.30. Western blotting to assess HSP70 protein expression in gills of *M. galloprovincialis* exposed to increasing OTC concentrations at 24°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.
Mussels exposed at 24°C for the same period and to the same range of OTC concentrations showed significant increases in inducible HSP70 expression with respect to controls at 16°C at all concentrations tested excluding 0.1, 1 and 1000 µg/L OTC (Fig. 4.31 b). The expression of the constitutive isoform after OTC exposure at 24°C was significantly higher only at 100 µg/L OTC (Fig. 4.31 a).

### 4.3.3. Statistical analysis

A two-way ANOVA was performed to assess if there were significant interactions between the factors (temperature, OTC concentrations and variations in HSP70 protein expression).

Regarding HSP70 constitutive isoform, only a significant interaction between increase in temperature and HSC70 expression was found (p < 0.01). No interactions were found among the other factors.

For which concerns HSP70 inducible isoform, there is no statistically significant difference with increase in temperature, while contrariwise there is significant difference (p < 0.01) amongst the different OTC concentrations.

Statistical analysis showed a significant interaction (p < 0.01) between increase in temperature and OTC concentrations, which affect variations in HSP70 inducible isoform expression.
### 4.4. COPPER

#### 4.4.1. HSP70 determination

In the framework of the European MEECE Programme, mussels were exposed to five increasing concentrations of Cu (2.5, 5, 10, 20 and 40 µg/L) at 16, 20 and 24°C, in order to assess the effects due to the combination of this metal and the increase in temperature.

As to OTC exposure, the expression of HSP70 proteins was assessed for each concentration and at all temperatures of exposure.

Western blotting analysis carried out in gills from mussels exposed to Cu at the three different temperatures never showed the expression of the 69-kDa protein (Fig. 4.24), but only of the constitutive and of the inducible ones, as it was described also above for mussels exposed to OTC.

As for OTC exposure, it was possible to exclude the risk that the differences observed in HSP70 expression were due to unequal protein loading, as blots were probed with a monoclonal anti-β-tubulin antibody, showing no differences in the expression of this protein, which keeps constantly expressed even in mussels exposed to stress factors (Fig. 4.32).

#### 4.4.1.1. Exposure to Cu at 16°C

![Fig. 4.32. Western blotting to assess HSP70 protein expression in gills of *M. galloprovincialis* exposed to increasing Cu concentrations at 16°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa); β-TUB = β-tubulin (55 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.](image-url)
Results

Fig. 4.33. Constitutive (a) and inducible (b) HSP70 protein expression in mussels exposed to Cu at 16°C. Data are expressed as % vs control samples. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.

Mussels exposed at 16°C for 4 days to 5 concentrations of Cu (from 2.5 to 40 μg/L) showed an increase in the inducible HSP70 protein expression yet at the lowest concentration tested, with a maximum effect of about 185% with respect to control values reached at 5 and 20 μg/L Cu and slightly decreasing thereafter (Fig. 4.33 b). The expression of the constitutive isoform was significantly increased in mussels only after exposure to 5 and 20 μg/L Cu (Fig. 4.33 a).

4.4.1.2. Exposure to Cu at 20°C

Fig. 4.34. Western blotting to assess HSP70 protein expression in gills of *M. galloprovincialis* exposed to increasing Cu concentrations at 20°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.
Results

Fig. 4.35. Constitutive (a) and inducible (b) HSP70 protein expression in mussels exposed to Cu at 20°C. Data are expressed as % vs control samples at 16°C. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.

The exposure of mussels to Cu at 20°C caused an increase in the constitutive HSP70 protein expression at all the concentrations tested with respect to control samples at 16°C (Fig. 4.35 a).

The expression of the inducible HSP70 proteins, was induced at 2.5 μg/L Cu, increasing to reach a maximum effect at 20 μg/L Cu (about 250% with respect to control values at 16°C), followed by a strong decrease at the highest copper concentration (Fig. 4.35 b).

4.4.1.3. Exposure to Cu at 24°C

Fig. 4.36. Western blotting to assess HSP70 protein expression in gills of M. galloprovincialis exposed to increasing Cu concentrations at 24°C. HSC70 = constitutive isoform (77 kDa); HSP70 = inducible isoform (72 kDa). A representative immunoblot out of at least 3 separate blots run in duplicate is reported.
Results

![Graph showing constitutive and inducible HSP70 protein expression in mussels exposed to Cu at 24°C.](image)

Fig. 4.37. Constitutive (a) and inducible (b) HSP70 protein expression in mussels exposed to Cu at 24°C. Data are expressed as % vs control samples at 16°C. Asterisks indicate values significantly different from control: * p < 0.05; ** p < 0.01.

Mussels exposed at 24°C for the same period of time and to the same range of Cu concentrations showed significant increases in inducible HSP70 expression with respect to controls at 16°C at all concentrations tested excluding 5 µg/L Cu (Fig. 4.37 b). The expression of the constitutive isoform after Cu exposure at 24°C was not modified compared to control samples at 16°C (Fig. 4.37 a).

4.4.2. Statistical analysis

As for OTC, a two-way ANOVA was performed to assess significant interactions between the factors (temperature, Cu concentrations and variations in HSP70 protein expression).

Regarding HSP70 constitutive isoform, only a significant interaction between increase in temperature and HSC70 expression was found (p < 0.01). No interactions were found among the other factors.

As regards the HSP70 inducible isoform, there is statistically significant difference with increase in temperature (p < 0.01), and also significant difference (p < 0.05) amongst the different Cu concentrations.

Statistical analysis excluded a significant interaction between increase in temperature and Cu concentrations.
5. Discussion
5.1. CARBAMAZEPINE

The exposure of mussels to the anticonvulsant drug CBZ aimed at evaluating the chronic toxicity of environmentally relevant CBZ concentrations and at investigating whether the drug interacts in these organisms with the same cellular targets through which it exerts its therapeutic effects in human patients.

Carbamazepine is an anticonvulsant and mood stabilizing drug used mainly in the treatment of epilepsy, bipolar disorder, and trigeminal neuralgia (Garcia-Morales et al., 2007). As previously described, CBZ is persistent when released to the environment, it is poorly removed by wastewater treatment plants (Zhang et al., 2008) and not biodegradated (Stamatelatou et al., 2003). For these reasons this drug was detected with few exceptions in influents and effluents from WWTPs, surface waters, and even seawater. Concentrations as high as 6.3 µg/L have been found in waste waters; average concentrations up to 2.3 µg/L have been measured in effluent samples; values in the range of 0.1–1 µg/L have been reported for surface waters, with levels up to 1.1 and 0.03 µg/L in groundwater and drinking waters, respectively (Fent et al., 2006).

The range of CBZ concentrations tested in the present work (0, 0.1 and 10 µg/L) was chosen as representative of those detected in the aquatic environment. The possible chronic toxicity of CBZ was evaluated through the assessment of biomarkers of stress and exposure. To understand whether environmental CBZ concentrations produce the same effects on non-target organisms as those documented in humans, we also evaluated the effects on cAMP-dependent pathway.

CBZ is widely used in human therapies, and it is known that it produces side effects after prolonged administration, including enhancement of human erythrocyte glutathione and glutathione peroxidase, and cytotoxicity at the cell membrane level, neurite swelling, and hepatotoxicity (e.g. Suwalsky et al., 2006), oxidative stress, and alteration of membrane phospholipids (Santos et al., 2008).

The battery of biomarkers analysed in this study included several parameters mainly related to mussel health status (LMS) and oxidative stress (CAT, GST, LN, LF, MDA). The lysosomal system in digestive cells of mussels is involved in pathological changes occurring in individuals exposed to environmental pollutants,
including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Moore, 1988). The effects of pollutants are often associated with an unbalanced fatty acid metabolism and with the accumulation of NL and LF in the lysosomal vacuolar system.

LMS was assessed in mussels haemocytes using the NRR assay. This test measures the lysosome membrane integrity by the capability of these organelles to retain the acidophilic vital dye neutral red (Canesi et al., 2007). The results obtained in this study showed a significant reduction in lysosome membrane stability after exposure to 0.1 and 10 µg/L CBZ. Viarengo et al. (2007) proposed LMS as a robust Tier1 screening biomarker for Environmental Impact Assessments based on the 2-tier approach. Following the data obtained in this experiment, we conclude that a stress syndrome is induced in mussels after a 7-day exposure to environmental concentrations of the drug.

The lysosomal storage of NL in mussel digestive glands was confirmed a useful indicator of alteration of cell physiology (Koehler, 2004), and accumulation of LF represents an indication of the oxidative stress level in the cell, related to the level of membrane lipid peroxidation (Viarengo et al., 2007). In the present study, small increases in NL and LF contents were detected in digestive glands from mussels exposed to CBZ. Since it was demonstrated that both lipofuscins and neutral lipids accumulated over time in organisms exposed to CBZ, it is possible to hypothesise that the results obtained in this study after a 7-day exposure to environmental concentrations of CBZ represent an early phase of mussels’ response to the pharmaceutical.

MDA is a marker of oxidative stress. It is the product of the decomposition of unsaturated fatty acid peroxides, and as an intermediate product it is usually rapidly degraded. However, in this study MDA content was measured in mussel tissues assuming the hypothesis of observing the early phases of the oxidative stress response. MDA content was significantly increased in gills at both CBZ concentrations tested, while only the highest concentration enhanced the MDA content in mantle/gonads. No effect was observed in digestive glands. Considering these results together with the slight increase in LN and LF accumulation in digestive gland, it is possible to affirm that the process is weakly developed in digestive glands, although lipid peroxidation was observed in gills and mantle/gonads after 7 days of exposure. The induction of MDA content is in
accordance with the results described by Gagné et al. (2006) after exposure of rainbow trout hepatocytes to CBZ.

Second phase detoxification enzymes catalyze the synthetic conjugation reactions of the xenobiotics and their metabolites. Gagné et al. (2004) showed a significant increase of GST activity in the mussel *Elliptio complanata* after exposure to primary-treated municipal effluent plumes containing pharmaceuticals and personal care products. In accordance with these studies, the data obtained in the present study indicated that GST activity was significantly enhanced in mussel digestive glands and mantle/gonads after a 7-day exposure to 0.1 and 10 µg/L CBZ.

Another enhancement in defence systems aimed at inhibiting oxyradical formation was observed through the activity of antioxidant enzymes. In the experimental conditions set up in this study, CBZ exposure significantly increased CAT activity in mussel digestive gland and mantle/gonads. Interestingly, CBZ did not affect CAT and GST activities in gills; contrariwise, in this tissue, the highest MDA content was measured.

Altogether, the biomarkers analysed indicate that a 7-day exposure to CBZ at environmentally relevant concentrations reduced the health status of mussels and induced oxidative stress.

Li et al. (2010) assessed antioxidant activity in juvenile rainbow trout after a 96-hour exposure to 5, 10, 15, 20, 25 and 30 mg/L CBZ. A significantly higher activity of catalase in liver, muscle and intestine of CBZ exposed organisms was observed when compared to the control animals. In contrast, CAT activity in gills and brain of CBZ exposed group was significantly lower than in control group.

The same scientists demonstrated variations in oxidative stress indices and antioxidant defence system in brain homogenates of rainbow trout *in vitro* treated with CBZ, at concentrations similar to those used in the present study (1 µg/L CBZ) observing a higher sensitivity of this methodology of exposure (Li et al., 2010).

Oxidative metabolism was indeed induced by CBZ also in the cnidarian *Hydra attenuata* (Quinn et al., 2004).

LMS demonstrated to be an early warning indicator of CBZ contamination, as its reduction was strongly correlated with increasing CBZ concentrations. Other biomarkers showed variable responses, mainly depending on the degree of tissue
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vulnerability to CBZ toxicity, even though side effects due to non-specific interactions with cellular constituents can not be excluded.

The pharmacological mechanisms by which CBZ exerts its therapeutic effects are not completely clear, although its molecular basis of action was ascribed to voltage-dependent inhibition of Na$^+$ and Ca$^{2+}$ channel currents. However CBZ induces at least part of its effects by interacting with the adenylyl cyclase system and lowering the intracellular levels of cAMP in mammalian brain (Chen et al., 1996; Montezinho et al., 2007). It is well known that cyclic AMP regulates several functions in molluscs (Valbonesi et al., 2004), and amongst others the nucleotide modulates the availability of mantle glycogen as a fuel for gonadal maturation (Díaz-Enrich and Ibarguren, 2003).

The effects of CBZ we observed on the cAMP-related cascade parameters indicated that the specific molecular target of the drug found in mammals is conserved also in mussels. Cyclic AMP content was in fact significantly reduced in all tissues of mussels exposed to CBZ. PKA activity was also reduced after exposure to the antiepileptic drug. In particular, PKA activities in mantle/gonads were inhibited at 0.1 and 10 µg/L CBZ, while only the highest concentration was effective in digestive gland and gills. It was also possible to observe that PKA activities were the highest in mantle/gonads, both in control and treated animals.

The mantle/gonads complex showed the highest sensitivity to the drug with respect to gills and digestive gland, and thus it appears the most suitable tissue to suppose CBZ effects on mussels. The important role expressed by the cAMP-related system in gonadal maturation could explain the effects observed in mantle/gonads complex of mussels exposed to CBZ. Indeed, a great availability of its specific molecular target could lead to a more relevant action of the drug in this tissue.

Rao et al. (2007) assessed CBZ effects on cAMP-related system in rats, administering it intraperitoneally during 30 days. Likewise the results described above, they observed a decrease in cAMP levels and a consequent decrease in PKA activity.

The attention on the effects due to environmental stressors on cell signalling has been neglected for sometime, while nowadays this system is a question of growing interest, and researchers have demonstrated that the cAMP pathway in mussels is affected in animals living in polluted waters (Dailianis et al., 2003; Dailianis and Kaloyianni, 2007), or by heavy metals (Fabbri and Capuzzo, 2006). Gagné et al.
(2004) showed a modification of serotonin and dopamine levels in *E. complanata* exposed to municipal effluents; these data are very interesting since both substances modulate cAMP-dependent pathways. Considering the physiological roles ascribed to the cAMP/PKA pathway, continuous release of CBZ from wastewaters may pose a serious risk to the health status and fitness of local mussel populations.

In this sense, it is noteworthy the strong correlation observed between the negative modulation of the cAMP-related pathway and the reduction of LMS, a parameter which points out the development of a stress syndrome.

### 5.2. PROPRANOLOL

PROP is a typical β-AR antagonist used in human therapies in the treatment of cardiovascular pathologies (Weir, 2009). It is effective on different organs and tissues at low concentrations both in mammals and in lower vertebrates; we reported a Ki(PROP) in fish liver membranes of 0.01 nM consistent with reports in mammalian liver (Fabbri *et al.*, 1992). In addition PROP is an effective serotonin (5-hydroxytryptamine; 5-HT) receptor antagonist and serotonin affects invertebrate physiology at many levels (Tierney, 2001).

PROP is widely detected in aquatic environments. The maximal PROP concentrations reported in estuaries are 56 ng/L (Thomas and Hilton, 2004), 590 ng/L in rivers (Ashton *et al.*, 2004), 1900 ng/L in wastewater effluents (Huggett *et al.*, 2002), and 6500 ng/L in hospital effluents (Santos *et al.*, 2010). The drug was recently reported to bioconcentrate up to about 360 µg/g w.w. in mussel tissues (Ericson *et al.*, 2010), and to reach mg/L levels in fish blood (Giltrow *et al.*, 2009).

The present study investigated whether PROP, a human β-blocker that is found in the aquatic environment, interferes with physiological processes in Mediterranean mussels, *M. galloprovincialis*.

Two experiments were set up to assess propranolol effects on mussels. The first measured the effects of L-PROP on mussel cAMP pathway in three different tissues, digestive gland, gills and mantle/gonads. In the perspective of obtaining the clearest evidence for an effect, the two chosen concentrations were in the upper
range (300 ng/L) or exceeded (30000 ng/L) the environmental levels (Huggett et al., 2002; Santos et al., 2010). In the second experiment, 5 concentrations of DL-PROP in a range between 0.3 and 30000 ng/L were chosen for mussels exposure. The lowest concentration tested (0.3 ng/L) was about 200-times lower than the highest PROP concentration found in estuaries (Thomas and Hilton, 2004). As only digestive gland and mantle/gonads had shown changes in parameters of the cAMP signalling pathway after exposure to L-PROP, these tissues were chosen to assess effects due to DL-PROP. The two isomers were chosen for mussels exposure since L-PROP is the pure compound commonly used in laboratory experiments, while DL-PROP is used as the drug administered in cardiovascular therapies, and therefore corresponds to the compound reported in aquatic systems (Owen et al., 2007). Besides the effects on LMS and cAMP pathway (cAMP levels and PKA activity), CAT and GST activities and accumulation of lipofuscins and neutral lipids in lysosomes in digestive gland sections were assessed to better understand the effects of this β-blocker on mussel physiology. A subchronic 7-day exposure was chosen since preliminary experiments showed that this exposure period was appropriate to reach significant and consistent cAMP changes in mussels exposed to modulators of the signalling pathway (Martín-Díaz et al., 2009). The consistency of the time of exposure also allowed comparison with previous data.

The effects of DL-PROP on the cAMP-dependent pathway confirmed the results obtained in mussels exposed to L-PROP. In particular, a 7-day exposure significantly lowered cAMP levels and PKA activities in digestive gland while increased levels in mantle/gonads.

The crucial role of cAMP in the modulation of bivalve physiology was recently reviewed (Fabbri and Capuzzo, 2010), and it is known that cAMP signalling is triggered by the interaction of a diverse group of extracellular ligands, including noradrenaline, adrenaline, and serotonin, with a large family of G-protein coupled receptors. In contrast to statements by Santos et al. (2010) who excluded the presence of ARs in invertebrates, ARs of the α- and β-type are reported in all tissues of bivalves studied to date (Fabbri and Capuzzo, 2010), and evidence obtained in different bivalve species find that β-AR occupancy is coupled to increased tissue cAMP concentrations (Dailianis et al., 2005; Koutsogiannaki et al., 2006; Lacoste et al., 2001; Shpakov et al., 2005).
Although considered a prototypical β-blocker, PROP acts also as a 5-HT1 receptor antagonist. Unlike any described vertebrate receptor, a 5-HT receptor structurally homologous to the mammalian 5-HT1 but showing a mixed 5-HT1/5-HT2 pharmacology is shared by a number of bivalves (Tierney, 2001). The most recent information on this 5-HT receptor reported that the partially sequenced 5-HT receptor from the gonads of the marine mussel *Mytilus edulis* was homologous to the mammalian 5-HT1 sub-group (Cubero-Leon et al., 2010). This 5-HT1 receptor subtype is coupled to the cAMP-mediated signalling but in an inhibitory manner (Tierney, 2001).

Confirming the role of PROP as a β-AR blocker and inhibitor of the cAMP-signalling pathway, cAMP levels and PKA activity decreased in digestive gland of mussels exposed to L-PROP or DL-PROP, possibly by antagonizing the effects of physiological adrenergic activators. Viceversa, the observed increases in cAMP levels and PKA activities in mantle/gonads support an indirect stimulation of PROP by blocking 5-HT1 receptors. In agreement with our hypothesis, Garnerot *et al.* (2006) showed that serotoninergic fibers are apparently absent or scarce in bivalve digestive gland, whereas a high concentration of these fibers is detected in gonads. The scarce knowledge regarding receptor distribution in bivalves does not allow any explanation regarding the lack of effects observed in gills. We hypothesize, however, that the absence of PROP effects in gills may result from the presence of receptor types that have roles on cAMP signalling such as 5-HT1 and β-receptors.

Although the effects of PROP on cAMP-related parameters are quite clear, correlations with exposure concentrations were in some cases inconsistent. It could be hypothesized that these discrepancies are due to different tissue receptor populations which indeed produce differential effects. Further information on receptor distribution and pharmacology in mussels is needed to support the above hypothesis.

It might be expected that the alterations of the cAMP-dependent pathway described above would interfere with neuro-endocrinological modulation of important functions (Fabbri and Capuzzo, 2010). However, the results obtained in this work demonstrate no overt detrimental effects to the mussels. Nevertheless, recently PROP was reported to lower the scope for growth, byssus strength and abundance (Ericson *et al.*, 2010), and feeding (Solé *et al.*, 2010) of mussels. In this direction,
in the present study the LMS was assessed in haemocytes of mussels exposed to both L-PROP and DL-PROP, as a sensitive biomarker of animal health status that could predict consequences at the organism and population level (Moore et al., 2006; Viarengo et al., 2007). A significant concentration-dependent reduction in the LMS was observed following mussel exposure to L-PROP 30 and 300 ng/L and to DL-PROP for 7 days in the range of 0.3–30 ng/L, pointing out the development of a stress response. In the experiment reported above about exposure of mussels to carbamazepine, it was demonstrated that this antiepileptic drug significantly and in a concentration-dependent manner decreased LMS in exposed mussels (Martín-Díaz et al., 2009). LMS is therefore a suitable biomarker of mussel exposure to pharmaceuticals of diverse chemical classes and therapeutic effects. The effects on LMS were highly correlated with the action of both pharmaceuticals on the cAMP-signalling pathway. Lysosomes undergo oxidative damage, leading to lipid peroxidation, loss of membrane fluidity and thus lowering membrane stability (Terman et al., 2006). Probably, oxidative stress is a common factor through which PROP and other pharmaceuticals lower LMS. The oxidative effects of carbamazepine are indeed a side effect demonstrated in human patients that have been observed also in mussels, as previously described (Martín-Díaz et al., 2009). Differently, antioxidative effects on human cells have been ascribed to PROP at the mg/L range, possibly improving its therapeutic use (Gomes et al., 2006). Solé et al. (2010) reported that mussels exposed to PROP at 11 µg/L did not show variations in CAT activities but their GST activity was reduced in digestive gland. In the experiments carried out in this study, CAT activities were significantly increased in the digestive gland of mussels exposed to DL-PROP between 0.3 and 30 ng/L but decreased at higher DL-PROP concentrations, in agreement with the bell-shaped response pattern often reported for this enzyme (Viarengo et al., 2007). Thus mussels showed an activation of antioxidant responses to PROP in concentration ranges that are below those reported in the environment. In the same tissue, DL-PROP exposure led to a significantly increased GST activity. A significant reduction in both CAT and GST activities was conversely observed in mantle/gonads after DL-PROP exposure. On the other hand, mussels exposed to L-PROP registered no variations in GST activities in none of the tissues analysed. In agreement with previous studies (Solé et al., 2010), the different responses in
digestive gland and mantle/gonads tissues may relate to their different antioxidant capacities. For what concerns lipid peroxidation, no effects were observed either in lysosomal lipofuscin nor in neutral lipids accumulation in digestive gland of mussels exposed to DL-PROP. Maybe a 7-day exposure of mussels to the β-blocker is not enough to induce a significant alteration of these parameters.

In agreement with previous reports on other contaminants (Contardo-Jara et al., 2010; Martín-Díaz et al., 2009), the data obtained in this study indicated that digestive gland is a suitable tissue for detecting PROP effects. Mantle/gonads showed a good sensitivity to PROP, however, since cAMP levels are physiologically modulated during gonad development, attention must be paid to the reproductive stage of the experimental animals. Haemocytes appear highly sensitive targets to PROP, and may be collected without animal sacrifice to follow up the stress syndrome development through LMS evaluation. It was proven that they possess receptors affected by PROP (Fabbri and Capuzzo, 2010), thus it is not possible to exclude that the effect observed is related to receptor-mediated specific pathways.

In summary, exposure to L- or DL-PROP at environmentally relevant concentrations affected the cAMP-mediated transduction pathway in mussel tissues. PROP indirectly lowered cAMP levels in digestive gland, possibly by blocking β-ARs thus reducing the effects of physiological adrenergic stimulators. In a contrary way, PROP increased cAMP levels in the mantle/gonads possibly by blocking 5-HT1 receptors thus reducing the effects of physiological inhibitors of the cAMP-pathway, such as serotonin.

According to the criteria proposed by Christen et al. (2010) for the identification of highly active human pharmaceuticals in aquatic ecosystems, PROP fulfils the requirements to be included on the list. It affects specific and evolutionally conserved biochemical pathways for which it was designed. The mode of action of PROP in mussels is related with its therapeutic effects, and a significant homology with the human targets is suggested. The novelty of the present study is that these conclusions were reached through the study of a marine invertebrate. Investigations addressing toxicity of pharmaceuticals in marine species are under-represented in the literature, despite the fact that certain pharmaceuticals may exhibit higher toxicity in the relatively high pH of the marine compared with the freshwater environment (Valenti et al., 2009). Lipophilicity as defined by logK\textsubscript{ow}
values of PROP increases linearly over the 6–9 pH range starting from 0.92 to 5.7 (Owen et al., 2009) which may explain the effects triggered by PROP at very low concentrations to marine mussels; unlike these results, PROP was suggested instead not to pose a risk to freshwater fish at the environmental concentrations (Huggett et al., 2002; Owen et al., 2009).

The concentrations used in this study are in the range of those found in water bodies, mostly in the lower range. The three lowest concentrations (0.3, 3 and 30 ng/L) are 10–100 times lower than the concentrations measured in estuarine waters (Thomas and Hilton, 2004), and effects were found at PROP concentrations (ng/L) far below those used in ecotoxicological approaches. The studies published in the literatures show that the PROP effective concentrations are in the μg/L and mg/L or mM range, and that endpoints assessed were of high ecological relevance, while the specific PROP targets were never analysed. In principle, specifically acting compounds interfering with physiological processes are more prone to act at low concentrations than those acting via unspecific mechanisms. Moreover, molecular responses are induced earlier than those at the individual or population levels (Franzellitti et al., 2010). Therefore, it is intelligible that in the present study PROP effects were observed at very low concentrations, since specific responses in animals possessing the same targets for which the human drug was designed were analysed.

The data obtained in this work do not demonstrate that PROP effects are detrimental to mussels; nevertheless they must be considered as indications of animal vulnerability. This is particularly relevant when related to potential synergistic effects of pharmaceuticals and other environmental stressors. The cAMP pathway significantly affected by PROP is involved in a variety of mussel physiological functions. Amongst these, it modulates the energy availability for gonad development being therefore crucial for mussel reproduction (Fabbri and Capuzzo, 2010). PROP also reduced LMS, hence affecting the health status of the animals and having the potential to jeopardize the fitness of individuals within a natural population (Moore et al., 2006).

As a concluding remark, we may observe that studying the possible alterations of each single physiological pathway by environmental pharmaceuticals represents a demanding and virtually never-ending process. It may be an important approach when dealing with precise targets in aquatic organisms, as in the case of PROP.
Considering the plethora of pharmaceutical residues found in aquatic environments, and the urgent need to assess the risk posed by their occurrence, a holistic approach through –omic methodologies may provide wide-spectrum indications and direct further more specific investigations.

5.3. OXYTETRACYCLINE

OTC belongs to a class of broad-spectrum antibiotics that is active against a wide variety of bacteria and works by interfering with their ability to produce proteins that are essential to them. Antibiotics are extensively used worldwide and their global annual consumption has been estimated to be between 100000 and 200000 tons (Tambosi et al., 2010). As previously described, between 30% and 90% of an administered dose of most antibiotics to humans and animals are excreted in the urine as the active substance (Rang et al., 1999); antibiotics are predominantly water soluble and are continuously introduced into the aquatic environment through sewage systems. As a result, in spite of their relatively short environmental half-lives, they are ubiquitous in aquatic environments; for these reasons, aquatic organisms are constantly exposed to these substances (van der Grinten et al., 2010).

The preliminary experiment in which mussels were exposed to oxytetracycline was aimed at assessing if this antibiotic can alter organisms physiology at a range of concentrations detected in aquatic environments.

The range of OTC tested (0.1, 1, 10, 100 and 1000 µg/L) was chosen as representative of those detected in aquatic environment. This first exposure of mussels to the antibiotic was performed at a temperature of 16°C.

The parameters assessed in this preliminary work aimed at evaluating mussel health status (LMS and “stress on stress” response) and oxidative stress (LF accumulation).

LMS assessed through NRR assay showed a significant reduction in lysosome membrane stability at all the concentrations tested. As NRR assay was indicated as a valid screening tool for Environmental Impact Assessment in a two-tier approach (Viarengo et al., 2007), these results demonstrated the development of a stress
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syndrome in mussels after a 4-day exposure to the antibiotic tested. The data relative to LMS tested through NRR assay obtained in this study, together with those achieved after CBZ and PROP exposure, allow to recommend this test as a reliable tool to assess the effects due to pharmaceuticals exposure.

Mussels subjected to anoxia by exposure to air after OTC treatment survived on average 6 days (both males and females without differences between the two genders), showing no differences amongst the lowest concentrations. These data are in accordance with the results obtained in previous studies (Viarengo et al., 1995; Viarengo et al., 2007; Moschino et al., 2010). Nevertheless an increase in survival times (up to 8 days) was observed in mussels exposed to the two highest concentrations of the antibiotic (100 and 1000 µg/L OTC); this could be due to a positive effect of the antibiotic, which acts against bacteria, removing therefore an external source of stress and slightly increasing mussel health status. This beneficial effect could be noted also in LMS, where a slight increase in destabilization times at the highest concentration (1000 µg/L OTC) was observed; nevertheless, it must be underlined that NRR values were significantly lower than in control organisms, showing in any case a negative effect of the pharmaceutical in the general health status of the mussels exposed.

The lysosomal storage of lipofuscins is representative of the oxidative stress level related to lipid peroxidation of the membranes (Viarengo et al., 2007). After the 4-day exposure of mussels to OTC carried out in this study a slight but significant increase in LF accumulation in digestive gland at all the concentrations tested (with the exception of 1 µg/L OTC) was observed. Likewise after CBZ exposure, this increase in LF storage in lysosomes allows to hypothesise that the results obtained represent an early phase of mussels response to an oxidative stress caused by the antibiotic.

After the preliminary experiment, in the contest of a European Programme (Marine Ecosystem Evolution in a Changing Environment, MEECE), it was decided to test the effects of OTC on mussels in combination with an increase in temperature, in order to simulate the effects due to climate changes.

The concentrations of the antibiotic that were tested were the same chosen in the preliminary experiment: 0.1, 1, 10, 100 and 1000 µg/L OTC. The setup of the experiment was maintained constant at the three temperatures of exposure (16, 20 and 24°C).
Discussion

In the present study, only data relative to HSP70 protein expression in gills of mussels are presented and discussed.

It is known that cellular exposure to stress factors induces a number of anomalies in cellular function, including a general inhibition of protein synthesis, alterations in protein structure and function, cytoskeleton rearrangements, shifts in metabolism, alterations in cell membrane dynamics and fluidity. These anomalies trigger major changes in gene transcription and protein synthesis, known as the cell response to stress mediated by HSP, metallothioneins, antioxidant enzymes and other defense mechanisms (Fabbri et al., 2008). Production of high levels of heat shock proteins can be triggered by exposure to different kinds of environmental stress conditions, such as thermal stress, infection, inflammation, exercise, exposure of the cell to toxins, starvation, hypoxia, or water deprivation. Consequently, the heat shock proteins are also referred to as stress proteins and their upregulation is sometimes described more generally as part of the stress response.

In physiological conditions, two western blotting bands are detectable in the gills of mussels, a constitutive isoform, with molecular weight estimated at about 77 kDa, and an inducible one, with molecular weight of about 72 kDa. Moreover following a thermal stress it is possible to observe a further band with molecular weight of about 69 kDa representing a strictly inducible isoform of HSP proteins (Piano et al., 2004). In none of the samples analysed it was observed the expression of the 69-kDa protein; this result makes possible to hypothesise that the temperatures tested in this study are not high enough to induce the expression of this isoform, in accordance with previous reports by Hofmann and Somero (1995).

After a 4-day exposure to OTC at 16°C, no differences were observed in the expression of the constitutive HSP70 isoform. Contrariwise, the expression of the inducible isoform showed a bell-shaped trend, with a maximum effect of about 185% with respect to control at 1 µg/L OTC. Hence, it is possible to hypothesise that the presence of the antibiotic induces a stress syndrome in mussels, which try to counteract it by activating defense mechanisms, including the induction of HSP70 proteins expression.

Mussels exposed to the same concentrations of OTC but at the temperature of 20°C showed a similar response to the antibiotic: no differences in constitutive isoform of HSP70 proteins was observed, while an increase in the inducible form
expression was registered in correspondence of the increase in OTC concentration, with a maximum effect of about 185% with respect to control at 16°C at 100 µg/L OTC, followed by a sharp decrease at 1000 µg/L. Also in this case we would ascribe the induction of HSP70 expression to the antibiotic and not to the temperature, since the constitutive isoform was not modified and moreover no differences were observed between HSP70 expression in control mussels at 16 and 20°C.

For which regards mussels exposure to OTC at 24°C, HSC70 proteins did not show significant differences with respect to control at 16°C, except for mussels exposed to 100 µg/L OTC. And induction of a stress response was hypothesised given the increase of the HSP70 inducible form: yet control mussels at 24°C showed an increase in protein expression, which was maintained also at the highest concentrations of OTC. In this case it is more difficult to ascribe such increase in HSP70 expression to the antibiotic, but it must be taken into consideration an effect due also to the increase in temperature.

At the end of this work, a two-way ANOVA was performed, in order to understand which was the factor (between temperature and OTC concentration) that determined the changes in HSP70 expression and if there was an interaction between these factors. For HSC70, only temperature seemed to be the driving factor that induced variations in protein expression. Contrariwise, the alterations in the inducible isoform can be ascribed to the increase in OTC concentrations; this would demonstrate that the antibiotic has an effect on mussel health status at environmental concentrations, inducing the activation of stress responses, such as heat shock proteins expression. Statistical analysis highlighted also an interaction between temperature and OTC concentration. This is very important in the contest of climate changes and a consequent expected increase in seawater temperature (Noyes et al., 2009); also pharmaceuticals (in this case an antibiotic) showed to modify their toxic effect on organisms depending on the temperature, as it was previously demonstrated for other classes of environmental contaminants (Noyes et al., 2009; Borgå et al., 2010; Pandolfo et al., 2010). An increase in toxicity of pharmaceuticals in parallel to the increase in water temperature was observed also by Kim et al. (2010) after exposure of *Daphnia magna* to 7 classes of pharmaceuticals. To our knowledge, the studies focused on sub-chronic or chronic
toxic effects of pharmaceuticals on marine organisms are scarce, so further investigation is needed to confirm the results obtained in this work.

5.4. COPPER

Copper is an essential micronutrient, important in many physiological functions, but it can also have adverse effects on aquatic organisms if bioavailable forms of Cu reach toxic concentrations.

Cu is a metal that is often detected at high concentrations in aquatic environment, including coastal areas. For this reason, the studies regarding this contaminant and its effects on aquatic organisms are numerous and very important to understand the ecological impact of this metal. The attention is focused on bioavailability of Cu, on its bioaccumulation in organisms’ tissues and on alterations of physiological parameters of the organisms tested (Le Bris and Poulíquen, 2004; Ait Fdíl et al., 2006; Julshamn et al., 2001; Giarratano et al., 2010). Amongst these parameters, also HSP70 proteins expression is often assessed as indicator of induction of defense mechanisms.

Although the term HSP specifically refers to heat shock, the accumulation of these proteins is not increased only by heat. A large body of evidence indicates that there is a variety of stimuli that result in an increase in their concentrations. The question arises of how different harmful stimuli can provoke such a similar effect (Fabbri et al., 2008). Some data are available on the HSP response after bivalve exposure to essential metals, namely copper and zinc. It is well known that prolonged exposure to Cu or Zn produces toxic effects and decreases animal fecundity, hatchability and reproduction, although these metals have a physiological role (Fabbri et al., 2008).

Sanders et al. (1994) found that M. edulis exposed for 7 days to increasing Cu concentrations (30-100 μg/L) showed an increased expression of Hsp60 and Hsp70 in the mantle, and to a greater extent in the gills.

The induction of HSP proteins by environmental contaminants is widely documented and HSP have been often considered as potential biomarkers to be included in biomonitoring programmes. One of the advantages of this choice would be that they give information about general conditions of health and provide
Discussion

early warnings of intoxication, before complex functions are compromised (Fabbri et al., 2008). The results obtained in this study confirm the important role of HSP70 expression in the detection of a stress syndrome development after contaminant exposure.

In the contest of the MEECE Programme, mussels were exposed for 4 days to increasing concentrations of copper (2.5, 5, 10, 20 and 40 µg/L) at three different temperatures (16, 20 and 24°C).

As previously described for OTC exposure, in the present study, only data relative to HSP70 protein expression in gills of mussels exposed to Cu are presented and discussed.

Exposure of mussels to Cu at 16°C induced a slight but significant increase in HSC70 expression at the concentrations of 5 and 20 µg/L. The inducible isoform showed an increase in its expression already at the lowest concentrations tested, underlining a toxic effect of the metal and the induction of defense mechanisms by mussels.

A 4-day exposure to the 5 concentrations of Cu at 20°C showed an increase in HSP70 constitutive isoform with respect to mussels exposed at 16°C. As no differences were observed among the different concentrations of Cu at 20°C, this increase in protein expression must be ascribed only to the raise in temperature and not to the metal. In the inducible isoform protein expression, it was observed a bell-shaped trend, that reached the maximum induction of about 250% with respect to control at 16°C at the concentration of 20 µg/L Cu, followed by a strong decrease at the highest concentration. This low expression of the inducible isoform observed in the gills of mussels exposed to 40 µg/L Cu lets us hypothesize that the high concentration of the metal constitutes a factor of stress too strong for the organism up to partially compromising the biochemical machinery at the base of the heat shock response; an activation of the heat chock response could be ongoing at the molecular level, but transcription could be not synchronized with this process. Similar effects were observed by Piano et al. (2004) following a severe increase in temperature.

In mussels exposed to Cu at 20°C, it is conceivable that the alteration of protein expression was due to the presence of the metal, but it must not be excluded a contribution due to the increased temperature.
Mussels exposed at 24°C for the same period and to the same range of Cu concentrations did not register any modification in HSC70 proteins expression in comparison with control at 16°C nor with control samples at 24°C. Significant increases in inducible HSP70 expression with respect to controls at 16°C were observed at all concentrations tested excluding 5 μg/L Cu. As no differences were detected amongst the different copper concentrations, these alterations in protein expression could be ascribed to the increase in temperature, but it can not be excluded an interaction of this factor with the effects due to the presence of the metal.

Pandolfo et al. (2010) assessed the toxic effects of Cu on the early life stages of freshwater mussels, which were indicated as more sensitive to Cu than most aquatic organisms. They observed that increase in temperature reduced the survival of juvenile mussels exposed to different concentrations of copper, showing interactive effects between these two factors. In this study, statistical analysis showed that the differences observed in the expression of the HSP70 constitutive isoform were due to the increase in temperature and not to the presence of Cu, and hypothetically no interaction between temperature and metal concentration was evidenced. For which regards inducible HSP70 isoform, both temperature and Cu concentration seemed to alter protein expression in mussel gills. Nevertheless, the two-way ANOVA excluded the possibility of an interaction between these two factors.
6. Conclusions
This work aimed at assessing the effects of three different pharmaceuticals detected in aquatic environment on mussels physiology: carbamazepine (an antiepileptic), propranolol (a β-blocker) and oxytetracycline (an antibiotic). The data obtained do not demonstrate that these molecules pose a risk to biodiversity. However they must be regarded as indications of animal vulnerability according to the concept developed by Dagnino et al. (2008) (Fig. 6.1). This is particularly relevant when related to potential synergistic effects of pharmaceuticals and other environmental stressors. The decrease in LMS observed in all the experiments is an indication of the induction of a stress syndrome in mussels, and a worsening in their health status after the exposure to these substances, that could have the potential to jeopardize the fitness of individuals within a natural population.

Fig. 6.1. Different biological toxic effects induced by environmental contamination and revealed by biomarkers, bioassays, and ecological surveys. From Dagnino et al., 2008.

Alterations of several physiological parameters were observed, as the induction of oxidative stress, the alteration of cAMP-dependent pathway and the induction of defense mechanisms (such as HSP70 proteins expression). One important consideration is that these effects were observed in mussels after exposure at very low concentrations of pharmaceuticals, sometimes lower than those detected in aquatic environment. These same substances after acute tests showed to be dangerous for organisms only at very high concentrations. In this sense, more attention to the chronic effects of pharmaceutical residues is required to better define risk assessment policies. Further research is advisable regarding subtle effects of pharmaceuticals on non-target organisms.
Conclusions

The data obtained in the contest of the MEECE Programme, in the perspective of climate changes, suggest to pay attention to variations in temperature, as the toxicity of the substances can be enhanced following changes of natural environmental factors.

Moreover it must be taken into consideration that in natural environment, the organisms are not exposed to a single pharmaceutical, but to mixtures of substances that could interact and amplify or anyhow modify their effects on individuals’ physiology. For this reason, studies on mixtures of pharmaceuticals should be carried out at least under laboratory condition in order to understand the effects due to these compounds and possibly distinguish them from the interaction of all the other factors involved in the natural environment.
7. Activity of research at Cádiz University
7.1. Introduction

Thanks to a grant conferred by the Marco Polo Programme, I had the opportunity to spend a 3-month research stay in the laboratories of the Department of Physical Chemistry of the Faculty of Marine and Environmental Sciences of Cádiz University (south-west Spain).

The research project aimed at measuring the possible effects of polluted sediments in Algeciras Bay (Gibraltar Strait, south Spain) and in Cádiz Bay assessing different physiological parameters of caged marine invertebrates exposed in situ.

The three sentinel species chosen for this study were the crab *Carcinus maenas*, the clam *Ruditapes decussatus* and the marine polychaete *Arenicola marina*. Here below, only data obtained from crabs and clams are presented.

The areas of investigation chosen were located in estuaries or ports; sediment quality in these zones is a crucial topic, because they are often subjected to dredging activities that involve the excavation and the removal of large volumes of sediment and rocks from the bottom of rivers, lagoons, channels, and other water bodies in order to maintain an adequate depth and width for ship navigation through these fairways (Torres et al., 2009). Dredging can also be used for the environmental restoration of impacted sites, or can be conducted also to provide material for beach nourishment projects, to create or expand wetlands, or other beneficial uses (Munns et al., 2002).

During these activities, sediments can enhance their capacity to mobilize contaminants; for this reason, they could represent an environmental impact that should be taken into account (Del Valls et al., 2004). Special concern arises on the disposal of dredged material; the selection of the best management option mostly depends on the quality of this material, and hence it is necessary to apply a reliable assessment of the sediments in order to assure that the disposal of such material will be environmentally harmless (as well as cost-effective) (Choueri et al., 2009).

In this sense, sediments assessment and management play an important role in dredging activities.

Sediments are an important component of the aquatic environment. A large variety of contaminants (e.g. synthetic organic chemicals, polycyclic aromatic hydrocarbons, trace metals, pharmaceuticals, etc.) from industrial, agricultural,
urban and maritime activities can accumulate in this matrix concentrations frequently higher than those detected in the water column (Hartwell et al., 2010; den Besten et al., 2003). In the sediment-water interface, these compounds may be resuspended, transported and redeposited far from their original source, producing negative effects to the benthic biota and to the organisms that feed on the benthos or on the sediment, leading also to threaten human health (Cesar et al., 2007; Hartwell et al., 2010).

From an ecological perspective, the assessment of sediments’ contamination and their appropriate management become extremely critical whenever remediation, enlargement or deepening of water basins, through dredging activities, are needed (Leotsidinis et al., 2008).

In this direction, each country has developed particular guidelines in order to manage dredged material, usually based on a chemical approach. In Spain, two action levels and three concentrations categories were proposed, defined on the basis of the concentration of a list of contaminants detected in the sediments (DelValls et al., 2004). Chemical characterization of the sediment alone is not enough to understand if there is a concrete risk for the ecosystem and the organisms that live in contact with this matrix. This characterization does not provide information about the bioavailability of contaminants bound to sediment, and the possible adverse effects that these contaminants may cause to the ecosystem. With this regard, the assessment of sediment quality using an integrated approach which takes into account different lines of evidence (LOEs), that may be physical, chemical, ecotoxicological and biological (Torres et al., 2009) could lead to a better knowledge of the environmental risk of contaminants bound to sediments and a proper sediment management.

The presence of contaminants in sediment is not directly correlated to their toxicity, which is influenced by numerous factors. Despite it is indispensable to know exactly what kind of contaminants are present in the sediments and at which concentrations, it is also necessary to assess if they are bioavailable and potentially toxic for the organisms.

Different tests can be applied to assess sediment contamination through various pathways of exposure, using whole sediment or elutriate, and exposing different species with distinctive feeding habits. Nevertheless, these toxicity assays are focused in the determination of acute and chronic responses under laboratory
conditions.

Biological data obtained in laboratory studies could overestimate or underestimate the effective contamination of the sediments, and could not allow to predict potential negative effects in the field; on the other hand, assessing sediment toxicity in situ, using animals sampled from the site under investigation, could provide misleading results, because these organisms could have developed adaptation and defense mechanisms to the medium in which they are living. The transplantation of caged animals to assess sediment quality is a useful tool that was introduced in Environmental Risk Assessment (ERA) programmes in the last years; this methodology allows to integrate field information with laboratory analysis (Martín-Díaz et al., 2008b,c; Ramos-Gómez et al., 2008; Morales-Caselles et al., 2008c; Viarengo et al., 2007).

These approaches have induced researchers to find early-warning signals, or biomarkers, which can rapidly and preventively reflect the adverse biological responses of organisms towards anthropogenic environmental contaminants as well as chronic toxicity. Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host reaction. These responses are sensitive indicators which are able to demonstrate that toxicants have entered the organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (van der Oost et al., 2003).

In this perspective, the application of biomarkers can be very helpful: a) their responses may indicate the presence of biologically available contaminants in the environment; b) they can perceive intermittent pollution events even when they are no longer chemically detectable; c) they have the advantage of being easier to perform and less expensive than a wide range of chemical analysis and; d) it allows to evaluate the impact of environmental pollution in its developing phase (Handy et al., 2003; Dagnino et al., 2007).

Incorporation of biological assays in marine ecosystems monitoring programmes is widely recommended by international organizations, although they have not been included in legislative technical procedures and in the activities of environmental agencies yet. In this context, the application of biomarkers has become increasingly important in the last years, and, among them, the lysosomal membrane stability (LMS) test has been proposed as an indicator of state or marine
pollution index (MPI) for inclusion in various national monitoring programmes (Martínez-Gómez et al., 2008).

International expert organizations proposed two techniques for the assessment of the LMS in molluscs: a cytochemical assay on unfixed frozen cryostat sections of the digestive gland and the neutral red retention (NRR) assay in living haemocytes. The lysosomal compartment is an ideal target to investigate cellular responses in organisms, especially in mussel. It has been demonstrated that lysosomes can accumulate a diverse range of contaminants, but this causes an intensification in toxicity and cell injury through lysosomal membrane damage and alterations, causing the leakage of hydrolytic degradative enzymes into cells cytosol, possibly leading to a more severe damage and to cell death (Lowe and Pipe 1994; Viarengo et al., 2007). NRR assay allows to evaluate the health status of the organisms and to understand the entity of the stress conditions they are exposed to.

Monitoring studies as well as in situ studies using caged organisms have validated the applicability of the NRR assay in wild mussel populations from different localities of the Iberian peninsula (Martinez-Gómez et al., 2008) and in the Mediterranean Sea respectively (Gorbi et al., 2008; Dagnino et al., 2007; Viarengo et al., 2007). For these reasons, NRR assay has been proposed as a first screening sensitive tool in a “two-tier approach”, that would permit to decide if the level of stress is too high so that further investigation have to be performed, for example applying a wider battery of biomarkers (Viarengo et al., 2007).

Most biomarkers have been validated mainly on bivalve molluscs (especially mussels), but during the last years some studies have been conducted in order to extend these analysis to other species with different feeding habits and different way of exposure to contaminated sediments (Brown et al., 2004). LMS through NRR assay has been assessed in clams, both under laboratory conditions (Matozzo et al., 2001; Brown et al., 2004), and on animals collected from polluted sites (Martins et al., 2005). This technique was adapted also to crab haemocytes, on individuals collected from the sites of investigation (Wedderburn et al., 1998; Brown et al., 2004). Nevertheless, to authors’ knowledge, little is known about the application and validation of NRR assay in sediment bioindicator species and under field conditions using caged transplanted animals.

Hence, the aim of this work was to set up NRR assay in clams *Ruditapes decussatus* and crabs *Carcinus maenas* transplanted in cages into potentially
contaminated sites over a 28-day exposure period, in order to assess if this technique could be a sensitive screening tool for the assessment of sediment quality in Spanish ports.

7.2. Materials and methods

7.2.1. Study site

Three sampling stations were chosen to assess sediment quality in Algeciras Bay (SW Spain): one in the Saladillo Port and two sites in the Estuaries of the Rivers Palmones and Guadarranque. Another site was placed in the Port of Cádiz (Cabezuela), which is characterized by the absence of concrete sources of contamination (Riba et al., 2004; Casado-Martínez et al., 2006; Martín-Díaz et al., 2007) (Fig 7.1). Moreover nearby areas have been used as reference site in other studies (Jiménez-Tenorio et al., 2008; Ramos-Gómez et al., 2008).

Algeciras Bay presents high levels of contaminants, due to the large number of ships that transit the strait, to the presence of a widely developed industrial area, with important chemical factories, oil refineries, thermal power plants, iron works, paper mills and ship yards (Guerra-García et al., 2009). This area has suffered a chronic impact lasting several decades, and presents pollution due to hydrocarbons, not only as a result of accidents, sinking and oil spills, but also as a consequence of frequent tank cleaning operations that take place in the Bay and deliberate discharges from commercial shipping (Morales-Caselles et al., 2008b).
7.2.2. Sediment characterization

Superficial sediment samples were collected at each sampling station using a 0.025 cm² Van Veen grab, transported to the laboratory in plastic dark refrigerated containers, and subsampled for physical-chemical and toxicological characterization. Macrofauna and larger particles were removed by sieving with a 1 mm mesh, and sediment samples were kept at 4°C in the dark until the analysis. Analysis of sediments were performed following Spanish recommendations for dredged material (CEDEX 1994). The following parameters were measured: grain size, organic carbon, metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) and polycyclic aromatic hydrocarbons (PAHs) (naphthalene, acenaphtalene, acenaphthene, fluorene, fenanthrene, anthracene, fluorantene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno, dibenzo(a)anthracene, benzo(g,h,i)perylene, perylene, benzo(e)pyrene).

Metals were determined in microwave acid-digested samples (HNO₃ and aqua regia in a proportion 1:3) in Teflon vessels and adjusted to volume with boric acid 5.6%. For Hg, the cold vapour technique was used and for As hydride generation, and both quantified using atomic absorption spectrometry. The concentrations of Cd, Pb, Cu, Zn and Cr were determined using flame or furnace atomic absorption spectrometry, depending on the metal content. PAHs were quantified after extraction with cyclohexane and dichloromethane by means of ultrasound treatment and concentration and clean-up with column chromatography.
Concentration of acenaphtylene, acenaphtene, anthracene, benzo(a)anthracene, benzo(a)pyrene, chrysene, dibenzo(a,h)anthracene, phenanthrene, fluoranthene, fluorene, naphthalene and pyrene were determined with HPLC with fluorescence detection (EPA 8310) (Casado-Martínez et al., 2006).

The PAHs analysis were carried out according to US EPA SW-846 Method.

### 7.2.3. In situ study: selection of organisms and animals handling

As described above, specimens of clams *Ruditapes decussatus* and of crabs *Carcinus maenas* were chosen as bioindicator species for this research work. These organisms have different feeding habits (filter feeder and burrowing respectively), a different way of exposure to contaminated sediments and different physiological characteristics. Both species have been deeply studied and used in biomonitoring surveys in contaminated sites (Wedderburn et al., 1998; Brown et al., 2004; Martín-Díaz et al., 2007; Banni et al., 2009; Bebianno and Barreira 2009).

In this work, individuals of intermoult female *C. maenas* (50–55 mm carapace width) and specimens of *R. decussatus* of the same size (35–45 mm shell width) were purchased from an aquaculture farm and acclimatized in the laboratory (Martín-Díaz et al., 2007) before the transplantation to the field.

20 clams and 20 crabs were placed into cages (100 x 100 x 25 cm) made of plastic net; clams and crabs were kept separated in order to avoid that the clams could be eaten by the crabs (Fig. 7.2).

Two cages were utilized per sampling point, to provide duplicate assays; cages were anchored to the sediment by a scuba-diver, and were exposed to the sediments for 28 days (Martín-Díaz et al., 2009a).
Specimens of *C. maenas* and *R. decussatus* (n = 4) were collected from the cages at each sampling station on days 0, 7, 14, 21 and 28, and they were rapidly transferred to the laboratory for further analysis. After 24 hours depuration period in the laboratory, haemolymph samples were withdrawn in order to perform NRR assay.

In clams, haemolymph was withdrawn from the adductor muscle using a hypodermic syringe containing a physiological solution (20 mM Hepes, 436 mM NaCl, 53 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂; pH 7.3) (1:1 v/v). Regarding crabs, haemolymph was obtained inserting a needle into the haemocoel through the arthodial membrane at the base of the third walking leg (Brown *et al.*, 2004). Before the extraction of the haemolymph it was necessary to add to the physiological solution inside the syringe (10 mM Hepes, 466.5 mM NaCl, 10.9 mM KCl, 12.6 mM CaCl₂•2H₂O, 19.5 mM MgCl₂•6H₂O; pH 7.4) an anticoagulant solution (113.8 mM glucose, 2.7 mM citrate, 7.2 mM NaCl (Liu *et al.*, 2006)). Samples were diluted 2:1:1 haemolymph/saline solution/anticoagulant solution.

### 7.2.4. Lysosomal membrane stability (LMS): the neutral red retention (NRR) assay

LMS was evaluated in clam and crab haemocytes using the NRR assay described by Lowe *et al.*, (1995) and adapted to the species used in this study. A stock solution (s.s.) of neutral red was prepared by dissolving 20 mg of dye in 1 ml of dimethylsulfoxide (DMSO), while two different working solutions (w.s.) were prepared by diluting this stock solution with the respective physiological
solutions: for clams, 5 µl of the s.s. with 995 µl of physiological solution; for crabs 25 µl of s.s. with 975 µl of physiological solution.

A volume of 40 µl of haemolymph was placed onto a microscope slide, previously treated with 5 µl of the coating agent poly-L-lysine; the slides were incubated inside a dark humidity chamber for 30 minutes to allow the cells to attach. The excess solution was tipped off by carefully tilting the slides, and 40 µl of the neutral red w.s. was added onto the area containing the attached cells. After 15 minutes incubation inside the dark humidity chamber, the excess solution was eliminated, a coverslip was applied and the slides were observed under a light microscope (400x magnification), at 15-minute intervals, to determine the time at which the dye that had been taken up into individual lysosomes (turning them red (Fig. 7.3 a and b)) had leached out into the cytosol. The test was stopped when dye loss was evident in more than 50% of the haemocytes and the times were recorded (Fig. 7.4 a and b); following each inspection, the preparations were returned to the incubation chamber (Lowe et al., 1995).

Data were expressed as destabilization time, representing the time at which more than 50% of the lysosomes released the dye into the cytosol (Martín-Díaz et al., 2009b). For each sampling station the mean destabilization time was calculated from four different slides (both of clams and crabs).

Fig. 7.3. Haemocytes from crabs C. maenas (a) and clams R. decussatus (b) retaining the neutral red inside the lysosomes.
7.2.5. Statistical analysis

Biomarkers results were analyzed using SPSS/PC+ statistical software. Significant differences between organisms exposed to control sediments and organisms exposed to contaminated areas were assessed using a one-way ANOVA followed by a multiple comparison of Dunnett’s tests; the significance level was set at p < 0.05.

Significant correlations between chemical concentrations in sediments and biomarker responses were examined using a Spearman’s correlation analysis. The level of significance was set at p < 0.05.

7.3. Results

7.3.1. Chemical characterization of sediments

Concentrations of metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) and PAHs were measured in the sediments from each sampling site.

All the results of this chemical characterization of sediments are shown in Table 7.1 and Table 7.2.

It has been observed that sediments from Cabezuela were mainly contaminated by Zn and PAHs. Sediments from Saladillo Port were highly contaminated by both metals (especially Zn and Cu) and PAHs, that reached a total concentration of 2189.83 µg/kg. In Guadarranque and Palmones Estuaries PAHs concentrations were under the limit of detection, while it was possible to detect a moderate contamination by metals, mostly by Zn, Cu and Cu.
Taking into consideration the Spanish Guidelines for Dredged Material Characterization (CEDEX 1994) based on the chemical-physical characterization of sediments, the potential biological effect was determined and showed on Table 7.3.

In this table, data are expressed as ratios (RAL1 and RAL2) between the concentration determined in the samples and Action Levels (AL) applied in Spanish guidelines for dredged material management as described in a previous work by Martín-Díaz et al., (2008d). These indexes provide information about the potential biological effect of the compounds detected in the sediments.
Risk regarding each concentration according to Action Level 1 is calculated as:

\[ RAL1(X)_y = \frac{Cm - AL1}{AL1} \times 100 \]

This index represents the risk associated to compound X measured at the concentration Cm in the sampling site y.

A similar index of risk is calculated in the same way according to Action Level 2:

\[ RAL2(X)_y = \frac{Cm - AL2}{AL2} \times 100 \]

RAL1 and RAL2 values under 0 are considered not to have associated risks, while values equal or higher than 0 could represent a potential biological risk for the organisms that live in that sediment.

Chemical analysis of sediments collected in the four sampling stations, showed that the concentrations of all the metals were lower than the limits described by CEDEX recommendations for dredged material management. Only Cu exceeded AL1 in Saladillo sediments (116.5 µg/g) and RAL1 showed indeed a positive value (16.45), which represents a potential biological risk.

**Table 7.3.** Potential biological effects based on chemical characterization for each sampling station.

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<th>PLM</th>
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Positive RALs are bold characters

7.3.2. Ecotoxicological responses

A percentage of survival of a 100% was observed in organisms exposed to sediments from Cabezuela from the beginning to the end of the exposure period. Contrariwise, clams mortality analyzed in Saladillo, Palmones and Guadarranque reached a percentage of 100 after 14 days of exposure while crabs mortality reached a 100% after 21 days of exposure.

Summarized lysosomal membrane stability determined through NRR assay in the haemolymph of the clams *R. decussatus* and of the crabs *C. maenas* over time are shown in Fig. 7.5 and Fig. 7.6.

![Fig. 7.5](image1)

![Fig. 7.6](image2)

Fig. 7.5. Lysosomal membrane stability assessed through NRR assay in haemocytes from clams exposed to sediments from Cabezuela (a), Saladillo Port (b), Palmones Estuary (c) and Guadarranque Estuary (d). Significance is indicated by * for p < 0.05 and by ** for p < 0.01 as compared to control.
NRR times determined in *R. decussatus* used as bioindicator species exposed to sediments in the Cabezuela ranged between 49 and 79 minutes over time. In clams exposed to sediments from Saladillo Port, NRR times ranged from 49 to 60 minutes, while in those exposed to sediments from the two Estuaries NRR times ranged from 30 to 60 minutes.

No significant differences were observed over time since the beginning of the exposure until the end in *R. decussatus* from Cabezuela.

Regarding LMS of clams in contaminated sites, a significant decrease of 50% in NRR times was observed in organisms exposed to Palmones (p < 0.05) and Guadarranque (p < 0.01) sediments after 7 days of exposure.

Regarding responses observed in the crab *C. maenas*, NRR assay applied to haemolymph of exposed crabs from Cabezuela did not show significant differences during the exposure period in NRR times, where NRR times ranged between 49 and 79 minutes, as it was observed in *R. decussatus*.

NRR times of haemocytes of crabs exposed to sediments from Algeciras Bay ranged from 27 to 75 minutes in crabs from Saladillo Port, from 23 to 75 minutes in those from Palmones Estuary and from 34 to 75 minutes in specimens of *C. maenas* exposed to sediments from Guadarranque.

After 7 days of exposure of crabs to contaminated sites in the Bay of Algeciras, it was observed a significant decrease in NRR times: in Saladillo destabilization...
times decreased of 65% compared to those determined on day 0 (T0) (p < 0.01), in Palmones of 70% (p < 0.01) and in Guadarranque Estuary of 55% (p < 0.05).

After 14 days of exposure, a recuperation in crabs health status was registered, showing NRR times similar to those registered at day 0, with the exception of organisms exposed to Saladillo sediments, where NRR times were still 35% lower than T0 (p < 0.05).

Regarding LMS measured on day 21 in crabs, no significant differences in NRR times compared to day 0 were observed in Saladillo site, while there was a significant decrease in destabilization times in crabs exposed to sediments from the two Estuaries (50% lower than T0 in Palmones, p < 0.01, and 45% lower than day 0 in Guadarranque, p < 0.05).

Spearman’s correlation analysis showed a significant correlation between NRR assay response in clams and metals concentration (with Cr, Ni, Zn, Cd, Cu (p < 0.01) and with As and Pb (p < 0.05)) (Table 7.4), and between the decrease in NRR times and the total concentration of PAHs (p < 0.01) (Table 7.5).

Also Spearman’s analysis performed on data regarding crabs showed a significant correlation between NRR assay and metals concentrations (with Zn, Pb and Cu (p < 0.01) and with Cr, Ni, As and Cd (p < 0.05)) (Table 7.6), and between NRR times reduction and total PAHs concentration (p < 0.01) (Table 7.7).

Table 7.4. Spearman’s correlation coefficient between NRR times and metal concentrations in clams

<table>
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* p < 0.05
** p < 0.01
Table 7.5. Spearman’s correlation coefficiente between NRR times and PAHs concentrations in clams

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<td>ΣPAHs</td>
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* p < 0.05  
** p < 0.01

Table 7.6. Spearman’s correlation coefficiente between NRR times and metal concentrations in crabs

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* p < 0.05  
** p < 0.01
Table 7.7 Spearman’s correlation coefficient between NRR times and PAHs concentrations in crabs

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<td>ΣPAHs</td>
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* p < 0.05  
** p < 0.01

7.4. Discussion and conclusion

The present study aimed at evaluating whether lysosomal membrane stability performed through the neutral red retention assay on transplanted caged clams *R. decussatus* and crabs *C. maenas* exposed to potentially contaminated sediments is a sensitive and useful screening tool for the assessment of dredged material quality together with the chemical characterization procedure described by CEDEX (1994).

Several studies used caged crabs or clams to assess sediment quality (Martín-Díaz *et al.*, 2007; Nigro *et al.*, 2006; Morales-Caselles *et al.*, 2008a,b,c), but to our knowledge, no studies have been performed using NRR assay on transplanted *C. maenas* or on *R. decussatus* to determine sediment quality.

The results obtained in this study showed the limitations of the mere chemical analysis and the higher sensitivity of biological assays in the assessment of
dredged material, confirming the high functionality of this biomarker in sediment quality assessment.

Chemical characterization of sediments showed the presence of metals and PAHs in all the sampling stations, at very low concentrations in Cabezuela, intermediate in Palmones and Guadarranque Estuaries, and high levels in Saladillo Port. Following Spanish recommendations for dredged material, only sediments from Saladillo would be considered category II, indicating moderate contamination, mainly due to the presence of Cu, whose concentration exceeded AL1. This material would need further study before an eventual disposal could be allowed.

Only considering chemical assessment, no further analysis would be necessary neither in Cabezuela nor in Guadarranque and Palmones Estuaries, and no biological risks would be expected in these sites.

Biological assays performed in this study on the contrary demonstrated that even lower concentration of heavy metals and PAHs (which are not taken into consideration in CEDEX guidelines) could cause adverse effects to organisms’ health.

Statistical analysis showed a significant correlation between NRR assay and sediment contamination. The increase in pollutants concentration was attended by a decrease in NRR times, indicating a worsening of organisms’ health (p < 0.05).

Although metals and some PAHs were detected in Cádiz sediments, neither crabs nor clams exposed to this site seemed to be affected. NRR times did not vary during the exposure and the organisms survived until the end of the study. Other authors have described the relationship between grain size and bioavailability of metals, showing a higher availability of metals bound to sediment when increasing the percentage of fines (Langston et al., 2010). In fact in Cabezuela the fine fraction of sediment was only a 26%, while in the other sampling stations ranged between 83 and 95%.

Crabs showed a significant reduction (p < 0.05) in LMS after one week of exposure to sediments in the sampling stations of Saladillo, Palmones and Guadarranque, revealing a toxic effect of these polluted matrices. The increase after 14 days of NRR times in all these sites could be due to osmoregulation ability and the effort of the organisms to adapt themselves to the contaminated sites and to the defense mechanisms that they start up to survive (Wedderburn et al., 1998). After 21 days of exposure, LMS was then significantly lower than T0 (p < 0.05).
only in haemolymph of crabs from Palmones and Guadarranque Estuaries, revealing the presence of sources of stress in the environment. This reduction in crabs has been correlated with the presence of metals (with Zn, Pb and Cu (p < 0.01) and with Cr, Ni, As and Cd (p < 0.05)) and PAHs (p < 0.01) bound to sediments.

Clams *R. decussatus* showed a similar behaviour, but revealed a lower sensitivity to sediment contamination. Indeed, NRR times did not suffer any alteration in Saladillo Port, the station in which contaminants concentrations were the highest detected. A significant reduction (p < 0.05) in LMS was registered only after 7 days in Palmones and Guadarranque Estuaries, but the organisms rapidly recovered their health status, showing no differences in NRR times compared to T0 on day 14.

Many studies used transplanted clams to assess sediment quality, but NRR assay was performed only using other species of clams (e.g. *R. philippinarum* (Nigro et al., 2006; Coughlan et al., 2009), *Anomalocardia brasiliana* (Martins et al., 2005)). On the contrary, NRR assay was previously used in field trials with *C. maenas* to demonstrate differences between polluted and non-polluted sites (Wedderburn et al., 1998; Brown et al., 2004). The baseline NRR times registered in this study were similar to those reported previously, and the assay demonstrated to be enough sensitive to disclaim between the different levels of contamination detected in the sediments.

Nevertheless the results that were obtained in this study showed similarities with other research works that were performed by other research groups at comparable conditions (Wedderburn et al., 1998; Petrović et al., 2001; Pisoni et al., 2004; Nigro et al., 2006; Fang et al., 2010), and are therefore encouraging for the application of NRR assay as a screening tool in dredged material assessment.

LMS is considered the most reliable of the recommended biomarkers in water quality assessment, also in field studies (Pisoni et al., 2004). Lysosomal damage is diagnostic for environmental stress, including contaminants, and LMS is recommended as biomarker of subcellular toxicity and stress, that could provide “early-warning” distress signals about deleterious changes in the environment, before the later detection of biological harms at the organism and population level (Petrović et al., 2001).
Also in this research study, statistical analysis showed significant correlations between NRR assay response and contamination by metals and organic compounds. Both clams and crabs pointed out a worsening in health condition, expressed through a decrease in haemocyte lysosomal membrane stability, in presence of high concentrations of metals and PAHs in the sediments they were exposed to.

Similar results were found in in situ studies performed in sites with high levels of contaminations from PAHs and PCBs. Petrović et al., (2001) registered a reduction in LMS up to 70% compared to control organisms in mussels collected from Rijeka Harbour, one of the heaviest polluted areas due to municipal, industrial and commercial harbour waste-waters.

Pisoni et al., (2004) observed a significant reduction in LMS in mussels from Mar Piccolo and Mar Grande sites with high concentrations of metals and PAHs, in particular in areas with considerable industrial and naval activity and urban sources.

All these results are in accordance with the reduction in NRR times that were registered in Saladillo and in the two estuaries in this study, where the sources of contamination are similar to those reported in the research works cited above.

Aarab et al., (2008) assessed the effects of PAHs contamination on caged mussels *M. edulis* in two Norwegian fjords. In this field study the destabilization periods were significantly shorter in mussels from the PAHs contaminated site compared to samples from the reference one, and the organisms exposed to the contaminated sediments showed moreover strong histological and histochemical alterations. On the other hand, Fang et al., (2010) assessed haemocytic lysosomal integrity in the mussel *Perna viridis* through NRR assay to evaluate the presence of PAHs and PCBs both in laboratory and in field studies, and demonstrated the independence of this biomarker from seasonal variations of seawater temperature and salinity. For this reason, it is possible to infer that changes in LMS detected in the present study could be ascribed to sediment contamination and not to other physical factors.

To conclude, NRR assay applied to the species *C. maenas* and *R. decussatus* has demonstrated to be a useful tool to assess sediment quality when helping to assess bioavailability and toxicity of contaminants in sediments and how bioavailability and toxicity affect organisms’ health status.

LMS assayed through NRR technique demonstrated to be a powerful and sensitive
biomarker of stress to be applied in sediment quality evaluation, since it provided information about the possible adverse effects of bioavailable contaminants bound to the sediments. The biomarker tested allowed to assess pollution of sediments due to contaminants not taken into consideration by CEDEX guidelines, as PAHs, and those present in the sediments at concentrations lower than AL1 and AL2 proposed for Spanish legislation.

Nevertheless improvements and modifications are needed in order to obtain a still more functional and accurate screening tool and to augment its capability as a low-coast, quick, reliable and sensitive biomarker for future studies (Coughlan et al., 2009).

The methodology could be refined by reducing interval times between slides observation at the microscope from 15 to 10 minutes, in order to be able to better appreciate differences from stressed and healthy organisms. Furthermore, the application of NRR assay to transplanted caged organisms could be integrated by the collection and a parallel analysis of wild native organisms of the same species in the areas under investigation, in order to be able to assess very long-term effects caused by chronical contaminant exposure and discriminate between adaptation responses, or accumulation of chemicals in the tissues, and stress effects induced in control organisms (Nigro et al., 2006).

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References

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