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Sublethal effects of metal contamination on marine sponges: responses at different biological levels.

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Sublethal effects of metal contamination on marine sponges: responses at different biological levels

Memòria presentada per Emma Cebrian i Pujol per obtar al títol de Doctor per la Universitat de Barcelona, sota la direcció de la doctora María Jesús Uriz Lespe.

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A la meva mare

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Avui és un dia ventós a Cabrera, no podem sortir, tinc una tesi per acabar i crec que per fi ha arribat el moment d'escriure aquesta part, i és això entre d'altres coses, el què em diu que ja estic al tram final d'aquest llarg, llarg camí.

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Contents

Chapter 1

General introduction

General Introduction

Coastal and marine ecosystems worldwide are under unrelenting stress caused by continued urban development along shorelines, industrial pollution, exposure to trace hazardous substances, extensive fishing and overfishing, habitat destruction, the continued introduction of allocton species, and other threats to oceanic biodiversity (Wells, 1999). The effects of the above-mentioned perturbations are sometimes very evident, leading to the disappearance of some species and the decline of the whole ecosystem. However, in general, transformations of the ecosystems are subtle and known only to a few specialists. In this sense, it is agreed that no current coastal ecosystem is pristine (e.g. Jackson, 2001).

The Mediterranean Sea constitutes a hotspot of marine diversity, with species widespread across a large number of communities (Bianchi and Morri, 2000; Occhipinti-Ambrogi and Savini, 2003; Boudouresque, 2004), but unfortunately, its communities do not escape of the general decline of the whole marine environments. For example, during the last few decades, anthropogenic activities have introduced significant amounts of pollutants into the Mediterranean, threatening community stability and survival of sensitive species. Among them, heavy metals, Polycyclic Aromatic Hydrocarbons (PAH's) and Polychlorinated Biphenils (PCBs) are highly contaminating sediments and waters from both industrialised and agricultural coastal areas (Palanques et al., 1998; Puig et al., 1999). Metals and hydrocarbons are conservative pollutants, which are not subjected to bacterial attack, or if they are, it is on such a long timescale that for practical purposes they are permanent additions to the marine environment. Thus, they represent a real threat for the sustainability and fitness of marine ecosystems (Brown and Ahsnullah, 1971; Berthet et al., 1992; Canesi et al., 1999).

Some examples are reported on the occurrence of acute heavy metal pollution on sessile species that drastically change the composition and spatial distribution of sublittoral communities (i.e. Diaz-Castaneda et al., 1989). However, most often, heavy metals are released to marine coastal ecosystems in low concentrations that produce sublethal effects in organisms with repercussion in the communities at a long-term.

Levels of heavy metals in marine environments are currently measured as their concentrations in waters, sediments and biota. Quantification of heavy metals in the water column is difficult to perform because of methodological constrains for detecting metals at the low concentrations in which they are usually present in seawater. Dissolved metal concentrations, moreover, vary greatly over time, for example with tidal cycle, freshwater run-off, season, and others.

Sediments accumulate heavy metals at high concentrations, what facilitates their measurement. Furthermore, the assessment of heavy metals in sediments allows for some degree of time integration. However, sediment analysis has also some drawbacks since the amount of metals found largely depends on sediment characteristics. In both cases, water and sediment analyses, only the total, not the bioavailable metal, is usually provided because the quantification of the several chemical species (Rainbow, 1995) is difficult and time consuming.

Alternatively, the use of organisms (biomonitoring) has been successfully proposed as an indirect measure of metal abundance in the aquatic environment (Rainbow and Phillips, 1993; Phillips and Rainbow, 1993; Rainbow, 1995). Organisms also provide a time-integrated measure of metal availability (bioavailability), which is responsible for their potential noxious effects (Connell et al., 1999). Thus, organisms are the preferential choice for determining levels of bioavailable heavy metals in marine environments and then are commonly used in biomonitoring studies.

However, several aspects should be taken into account before selecting target species for biomonitoring. Firstly, a suitable biomonitor of heavy metals should reflect the concentrations of the environment; that is, it should accumulate heavy metals as a function of their concentration in the environment. On the other hand, it has to be considered that metal accumulation varies among species even living in the same habitat (Hare, 1992; Phillips and Rainbow, 1993). Moreover, an organism can accumulate differently depending on the tissue considered, on its physiology (reproduction, growth, fitness), season of the year, and the target metal. Temporal variations in heavy metal accumulation in an organism may be due to both, seasonal differences related to the organism ecology/fitness, and variations in the bioavalaible metal. As a result, the significance of a given metal concentration in an organism cannot be interpreted on absolute terms, but depends on all the variables above mentioned.

Thus, an appropriate target organism would be selected for each type of contamination under study. Furthermore, the understanding of the accumulation strategy and the physiological state of the organism would indicate the significance of a measured body metal burden (Rainbow, 1995). Hence, before tackling any biomonitoring study, efforts should be addressed to gain knowledge on all these issues.

Sublethal effects of heavy metals on benthic invertebrates

The literature is replete with examples of metal quantification in an array of marine organisms collected from numerous locations. These surveys are very valuable for determining fluctuations of heavy metals concentrations in biota and for identifying polluted localities. Their limitation is, however, that they do not provide information regarding the ecological/biological effects of a particular metal concentration. In this

sense, several authors underline the need for information on the relationships between bioaccumulation and effects.

Among the effects of heavy metals on marine environments, population-level effects are perhaps the most neglected area in ecotoxicological research (Depledge and Hopkin, 1993). Effects of heavy metals on populations may involve loss of individuals, which are often detectable at a time-scale of days or months. Alternatively, an effective mean to detect changes in populations at shorter-term is by studying their effects at different levels of biological organization, from molecules to populations: e. g. individual growth rates, reduced fecundity and longevity, disturbance of endogenous biological rhythms and molecular and cellular defenses (Gray, 1979; Moriarty, 1983; Depledge, 1984). In contrast, most studies on the effects of heavy metals on invertebrates have addressed a few aspects of the organism biology, such as growth (Tewari et al., 2001; David, 2003; Yang and Wu, 2003) or reproduction (Bhattacharya and Vadya, 1999; Daka and Hawkins, 2002), and rarely attempted to analyse heavy metal effects at different levels of biological organization.

The concentration of a heavy metal in an organism does not indicate fluxes or the extent of their noxious effects. These can only be inferred from experimental approaches *in situ* and in the laboratory. Laboratory studies using target species easy to maintain under laboratory conditions have been the preferential choice (e. g. Brown and Ahsnullah, 1971; Kobayashi, 1980; Rainbow et al., 1980; Rainbow and Wang, 2001). However, it is difficult to predict natural responses from the results obtained under those “artificial” conditions. Moreover, the scarce field studies available tend to be descriptive, usually assessing the contamination effects from observed mortality or changes in community structure. Unfortunately, when changes in the structure of benthic communities are detected, we are restricted to measure the lethal effects of pollution. However, most contaminants enter marine waters at small low-levels, which affect the physiological functions and behaviour of organisms without killing them (e. g. Newton and McKenzie, 1995). On the other hand, experimental studies of deleterious effects of metal pollutants on benthic organisms under field conditions rarely provide conclusive results due to the possible interactions of multiple variables. Field results are to be confirmed through laboratory experiments in which variables can be controlled and their effects quantified.

Thus experimental studies combining both *in situ* and laboratory approaches, and taking into account different levels of biological organization would be crucial for a better understanding of the physiological, biological and ecological effects of the heavy metals.

Sublethal effects on early-stages of development, and cells

Sublethal effects of heavy metals may have drastic repercussions at an ecological level when they alter biological processes of the organisms that indirectly affect to

successive populations. For instance a pollutant may kill a half of the individuals of a species population with little or no ecological significance, whereas a pollutant that does not kill organisms but retards development may have a considerably higher ecological impact (Moriarty, 1983).

Contrasting effects of heavy metals on early life stages and cell behaviour have been reported for invertebrates, depending on the metal concentration and time exposure. High concentrations can be toxic for cells (i.e. Auffret and Oubella, 1997) and larvae (i.e. Wu et al., 1997) and alter larval metamorphosis (Negri and Heyward, 2001). Conversely, the same pollutants, at low concentrations, are reported to positively affect larval settlement in invertebrates (Ng and Keough, 2003). The cellular features underlying settlement enhancement are not fully understood. Nevertheless, available studies indicate that metals can affect the cell calcium homeostasis (Verbost et al., 1989; Viarengo et al., 1994; Marchi et al., 2004), which seems to be involved in the cell rearrangement and aggregation that occur during settlement (Burlando et al., 2000; Pourahmad and O'Brien, 2000).

Consequently, an extensively knowledge on the effects of heavy metals pollutants on adult individuals is not sufficient for a full understanding of its ecological significance, but comprehension of the effects on early-stages and on cellular mechanisms that control them is also necessary.

Biomarkers of heavy metal pollution in marine invertebrates.

A biomarker has been defined as 'the measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response' (Bodin et al., 2004). A more generalized version would include measurements on whole animals expressed in physiological, behavioural or energetic terms' (Ross et al., 2002; Magni et al., 2005).

Ecotoxicologists have realized that biomarkers are powerful tools for investigating contaminant exposure and effects on living organisms (Depledge and Hopkin, 1993). Table 1 shows the hierarchy of biologic markers in relation to specific levels of biological organization, which can be used for the assessment and monitoring of toxic effects on living organisms. The simultaneous use of several biomarkers that reflect toxic exposure and effects at various levels of biological organization will provide information on a number of different consequences of environmental contamination.

Physiological and behavioural markers provide information on the effects (injuries) of pollution at organism and population levels and, as a consequence, at the community level (Table 1.1). Growth, reproduction output, recruitment success, filtration rates, morphological changes, and structural elements, are among others, the

most commonly measured biomarkers that indicate physiological responses at the organism level and may have repercussions on populations and communities.

Level of organization	Type effect	Biological marker	Type of marker	Time scale
Biological molecules	Molecular responses	Genes expression: cell regulation and defense/repair proteins: (e. g. HSPs and metal-binding proteins)	Markers of exposure	Second s
Cell	Tissue and cellular effects	Cell aggregation and motility	Markers of effects	Minutes
Individual	Physiological effects and disease Impaired reproductive capability Death	Reduction in growth rates Changes in reproductive output Respiration, Clearance rates	Markers of ecological change	Hours Days
Population	Decline in reproduction Reduced *sensus* numbers Population decline	Deformities Death Population decline Change of population density		Months
Community	Possible extinction	DNA analyses	Markers of evolutionary change	
Ecosystem	Reduction in biodiversity	Changes in ecological descriptors (e.g. biodiversity, richness, similarity.. indices)		Years

Table 1.1: Some examples of biologic markers employed in ecotoxicological studies (modified from Evenden and Depledge, 1997).

On the other hand, most molecular biomarkers respond faster to toxic exposure than physiological ones and can be used as an early warning system of pollution. Furthermore, they can provide information about the nature of the possible contaminant since some of them use to be pollutant-specific (Depledge and Hopkin, 1993).

Biomarkers are also classed in two categories depending on the information they provide: 1) biomarkers of exposure, when they indicate that the organism has been in contact with a toxicant but it continues to growth and reproduce normally (they should be view as a part of an acclimatation process to altered environmental conditions, rather than a manifestation of the injury, and 2) biomarkers of effect, when they are related with changes in Darwinian fitness parameters (Depledge, 1993) (Table 1.2).

Exposure biomarkers	Effect biomarkers
Accumulation	Scope for growth (SFG)
Metallotioneins	Reproduction
Mixed function oxidase (MFO)	HSPs
Cytochrom P459	Clearance rates

Table 1.2: Some examples of biomarkers of exposure and effect.

Most of the biochemical markers studied are appropriate to signal toxicant exposure rather than deleterious effects in ecological systems (Depledge and Hopkin, 1993). In contrast physiological markers are better biomarkers of effect.

As each type of biomarker has its own pros and cons, an integrated study involving biochemical, physiological and behavioural biomarkers is the best approach in general surveys, as, sources and levels of pollution are commonly uncertain in natural conditions.

Sponges as biomonitors of heavy metals

In the recent years, a considerable number of studies concerning biological monitoring of heavy metal pollution have been conducted. Sessile benthic organisms appear to be particularly suitable tools for local pollution biomonitoring, since they cannot escape from water-borne toxicants released in a given area (Rosenberg et al., 2004; Naranjo et al., 1998; Carballo and Naranjo, 2002). Most of these studies have been carried out on soft bottoms (e. g. Ugolini et al., 2004; Usero et al., 2005), but, despite their ecological significance, rocky bottoms have received far less attention except as for mussels and clams, which have been used in biomonitoring programs (e. g. mussel watch, Claisse, 1989; Jermelov, 1996).

Among benthic organisms inhabiting rocky bottoms, sponges fulfil most of the criteria desirable for biomonitors (Rainbow, 1995): they are sedentary, abundant, widely distributed, long-lived, available for sampling through the year, large enough to

provide sufficient tissue for (individual) analysis, resistant to handling stress, which is required for experimental manipulation (Chapter 3, 4 and 5), and tolerant to environmental variations in physico-chemical parameters (Carballo et al., 1996). Furthermore, sponges are either resistant or susceptible to different heavy metals depending on the metal and the species considered (Perez et al. 2005), and can accumulate toxicants in function of the quantity present in the environment (Olensen and Weeks, 1994; Hansen, et al. 1995).

The suitability of sponges as biomonitors of heavy metal pollution has been indicated by several authors (e. g. Patel et al., 1985; Hansen et al., 1995), but, in contrast, sponges have been underused as biomonitors in global surveys. Perez et al. (2005) reported on the ability of some sponge species to accumulate heavy metal as a function of environmental pollution at a temporal scale. However, there are still many aspects that should be considered before a sponge species is proposed for biomonitoring studies, such species-specific mechanisms of metal uptake, and temporal and spatial variations in metal accumulation, as well as the effects that metals produce on the species selected. .

On the other hand, studies dealing with effects of heavy metal concentrations on sponge populations should also be addressed in order to understand the ecological consequences of a heavy metal burden. Up to now, few studies on the effects of heavy metals on sponges have been performed (e.g. Pérez et al. 2005, Berthet et al., 2005). However, studies dealing with the effects of heavy metals at several levels of sponge organization (from molecules to population), which necessarily involve a broad range of biomarkers, would improve the knowledge on the heavy metals effects on sponge populations.

At the molecular level, the suitability of stress proteins (sometimes referred as heat-shock proteins) as biomarkers of adverse effects by metals has been demonstrated for other invertebrates (Sanders, 1990) but just explored in sponges after exposure to environmental stressors. Their induction by the non-ionic organic fraction from a polluted river, some PCBs, and cadmium has been studied in *Ephydatia fluviatilis* (Müller et al., 1995), *Geodia cydonium* (Wiens et al., 1998), and *Suberites domuncula* (Müller et al., 1998), respectively. However, little is known about heat-shock proteins induction by heavy metal contamination. At the organism level, the metal accumulation in sponge tissues has been analysed (Pérez et al., 2005) but few studies on the ecological effects of heavy metals have been conducted up to now on sponges. These studies should eventually consider physiological variables such as growth, change in morphology, reproduction output, survival and filtration rates, which may alter the species fitness and thus its population dynamics.

By the way, studies directed to attempt the effects of heavy metals on early life stages of sponges are particularly needed since sponge populations, as other invertebrates, mainly rely on their larval settlement stages for maintenance. It is widely recognised that moderate levels of some pollutants that are apparently innocuous for

adult invertebrates can negatively affect the physiology of their juvenile and larval stages (Rinkevich and Loya, 1977; His et al., 1999). Thus, sensitivity of larvae and juveniles to low levels of pollution may largely determine a subtle decadence and even disappearance of sponge populations in heavy metal polluted environments. During larval settlement and metamorphosis of sponges, an extensive reorganization process occurs, which implies movement, self-recognition and aggregation of cells (Weismann et al., 1985). Therefore, modification due to heavy metal pollution in one or several aspects of sponges cell behaviour may cause alterations on sponge settlement.

Thus, although it has been pointed the suitability of sponge species for heavy metal biomonitoring in rocky bottoms, studies on heavy metal accumulation, mechanisms of metals uptake, and temporal and spatial variations in metal accumulation are still needed for the general use of sponges as heavy metal biomonitors. Furthermore, studies on sublethal effects of heavy metals on sponge populations are required to known the further ecological consequences of heavy metal pollution. Here, I have tried to follow the current trend in ecotoxicological studies, which proposes the use of a wide range of approaches involving several methods, each one focusing on a different level of biological organization, to acquire a better understanding of noxious effects of heavy metals on sponge populations. Monitoring, transplantation and laboratory experiments are used in order to improve the assessment of metal pollution in benthic populations of coastal marine environments. This is the first time that a multidisciplinary approach involving the biology, physiology, larval and cell behaviour, and molecule induction/inhibition of sponges has been performed, to tackle the effects of metal pollution in benthic invertebrates. However, some limitations associated to the intensive nature of several chapters and to the field work are to be mentioned: the restriction to a limited number of species (the most adequate ones in our opinion for each particular study) and the selection of the “least bad” zones in the study littoral as control (“clean but not pristin”) and polluted sites for the experiments in the field.

Objectives and structure of the thesis

The general objective of this thesis has been to assess the sponge responses to sublethal concentrations of heavy metals, and to determine the suitability of the sponges as biomonitors. First I aimed to know the extent of metal accumulation by sponges at spatial and temporal scales and then, to analyse the sublethal effects of heavy metals on different levels of biological organization (from molecules to populations) by using a range of biomarkers. I combined field and laboratory experiments in order to better understand the accumulation patterns and the effects according to the species and the metal considered.

The specific objectives were:

- To study heavy metal accumulation by different sponge species, to determine whether they properly reflected the amount of metal in the environment. **Chapter 2**.
- To search for a sponge species able to reflect spatial and temporal variations of the bioavailable heavy metals, which could be proposed as a suitable biomonitor of heavy metals. **Chapter 2.**
- To study the effects of sublethal concentrations of heavy metals on sponge physiology and biology. **Chapter 3 and 4.**
- To assess the induction of HSPs by sublethal concentrations of copper and their potential as a molecular biomarker of copper pollution. **Chapter 5**.
- To examine the effects of low levels of copper pollution on the sponge chemical defences (measured as bioactivity). **Chapter 5**.
- To analyse the effects of moderate concentrations of copper and cadmium on the sponge larval settlement and posterior survival of juveniles. **Chapter 6.**
- To determine the effects of low levels of copper and cadmium on motility and aggregation of sponge cells. **Chapter 7**.
- To analyse the mechanisms by which some heavy metals alter cell motility and aggregation and, as a consequence, sponge settlement. **Chapter 8**.

The study has been divided in three main parts, which dealt with (i) accumulation, and (ii) effects of heavy metals on adults and (iii) on early life stages, respectively:

Part A: Marine sponges as spatial and temporal biomonitors of heavy metals (Chapter 2).

In this part, I studied species-specificity of heavy metal accumulation and determined the ability of sponges to reflect heavy metal concentrations in the environment. I selected four species among the most abundant Mediterranean sponges (*Dysidea avara*, *Phorbas tenacior*, *Crambe crambe* and *Chondrosia reniformis*), which encompass several structural traits that may influence metal bioaccumulation capacities. Then I compared metal concentration in sponges and sediments from three localities with different levels of metal pollution. Afterwards, the most suitable species for monitoring purposes (*Crambe crambe*) among those considered, was selected and used to assess variations at spatial and temporal scales. Spatial variations were analysed by comparing heavy metals accumulation in the target species and in the sediment from 16 localities along the Catalan coast (NE Iberian Peninsula, Mediterranean Sea). Sampling localities were selected in order to embrace different degrees of anthropogenic impact. Temporal variations were assessed by means of a monthly survey of the sponge accumulation in two different localities.

Part B: The ecological effects of heavy metal pollution on two contrasting Mediterranean sponge species (*Crambe crambe* and *Chondrosia reniformis*) (Chapter 3, 4 and 5).

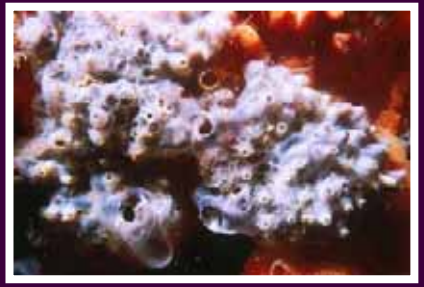
Here, I studied the physiological and biological effects of sublethal concentrations of heavy metals on adults of two contrasting sponge species in terms of bioaccumulation, which also showed different structural organization (mineral vs. collagen skeletons). To achieve this study, I carried out a field experiment where specimens of both species were transplanted from a control area to a polluted site, and the sponge responses at different levels of biological organization (from organisms to molecules) were examined. Organism-level variables, such as changes in morphology, growth and survival rates, collagen content, filtration rates on one hand, and molecular-level responses, such as the production of bioactive substances and the synthesis of heat-shock proteins (HSPs), on the other hand, were assessed. An additional short-term laboratory experiment was run to verify whether copper and not other uncontrolled factors at the polluted site was the variable that affected the HSPs expression and toxicity of sponges in the field experiment. To know whether field variables other than metal pollution may influence the experiment outcomes, I characterised the main environmental variables of the study area (control and polluted sites) along the experiment. Thus, abiotic paramentes such as nutrients, particulate organic matter, sediment rates and quality, water movement, and irradiance, were monitored during the transplant experiment.

Part C: Effects of heavy metals on sponge settlers, juveniles and cells (Chapter 6, 7 and 8).

In this part, I examine whether sublethal levels of heavy metals (Cu and Cd) affect early-life stages of sponges, which are usually more susceptible to pollutants than adults. Larvae of *Scopalina lophyropoda* and *Crambe crambe* (two representative sponge species of the Mediterranean and the only two which encompass a massive release of larvae in a relatively long time period (1.5-2 months, at the study area) were obtained from ripe sponges. Larvae of both species were submitted to short pulses of Cu (30 $mg \cdot l^{-1}$) and Cd (5 $mg \cdot l^{-1}$) during one week, and settlement and posterior survival of juvelines were monitored. Heavy metals at the concentrations assayed did not affect *C. crambe* settlement and posterior survival, in contrast, short pulses of copper and cadmium enhanced *S. lophyropoda* settlement. As settlement endures intense cell reorganization, I also searched for changes in shape of the sponge cells (as an indication of cell motility) submitted to short pulses of heavy metals, which could explain the settlement responses observed. Cells of *S. lophyropoda* were submitted to Cu (30 $mg \cdot l^{-1}$ and 100 $mg \cdot l^{-1}$) and Cd (5 $mg \cdot l^{-1}$ and 10 $mg \cdot l^{-1}$) for 3 hours. I took advantage of cell changes in shape when crawling and studied the effects of heavy metals on sponge cell aggregation and several shape indices. An additional study was performed in order to known the mechanisms by which copper and cadmium positively affected sponge cells and larvae and whether alteration of calcium homeostasis provoked by heavy metals may be involved in such effects. To achieve this study we incubated *S. lophyropoda* cells and larvae in calcium free seawater under Cu (30 $mg \cdot l^{-1}$) and Cd (5 $mg \cdot l^{-1}$) and monitored the resulting effects. We selected those heavy metal concentrations for laboratory experiments, trying to be realistic because till now, many ecological studies have used unrealistic higher concentrations of heavy metals and therefore they reported on acute effects.

Three chapters of this thesis (Chapter 3, 4 and 5) have been already published. Other three (Chapter 6, 7 and 8) are *in press* and will be released within 2007. The remaining one (Chapter 2) is *submitted*.

Part A



Chapter 2

Sponges as biomonitors of heavy metals in spatial and temporal surveys in northwestern Mediterranean: multispecies comparison.

Chapter 2

Sponges as biomonitors of heavy metals in spatial and temporal surveys in northwestern Mediterranean: multispecies comparison.[1]

Abstract

Contamination by metals has increased drastically in coastal Mediterranean during the last 20 years. A comparative study on metal bioaccumulation by four widespread sponge species has been performed to select the most suitable species for metal monitoring. Copper bioaccumulation fits a net accumulation strategy while lead concentration seems to be regulated in most sponges. *Crambe crambe* was the only studied species that bioaccumulated lead and copper as a function of the bioavailable metal, proving its suitability for monitoring purposes. Then, we examined its effectiveness as a bio-indicator at large spatial and temporal scales, comparing metal accumulation in this species and in sediments. *C. crambe* provided accurate information on the background levels of metals in the area at both spatial and temporal scales, and, furthermore, it reflected seasonal fluctuations of the bioavailable metals, which would be impossible to assess by means of a classical (sediment) survey.

[1] Cebrian E, Uriz MJ, Turon X. Sponges as biomomnitors of heavy metals in spatial and temporal surveys in northwestern Mediterranean: multispecies comparison. *Submitted*.

Introduction

The Mediterranean Sea constitutes a hotspot of marine diversity, with species widespread across a large number of communities (Bianchi and Morri, 2000; Occhipinti-Ambrogi and Savini, 2003; Boudouresque, 2004). During the last few decades, anthropogenic activities have introduced significant amounts of heavy metals into the Mediterranean, threatening community stability and survival of sensitive species.

Heavy metal pollution in the marine realm has rarely been assessed by analysing metal concentration in the water column because of the methodological constraints for detecting metals at the low concentrations in which they are usually present in seawater. In contrast, studies on metal pollution are easier to perform and allow for some degree of time integration in sediments. Sediments accumulate the contaminant metals, and thus metal concentrations can be measured more accurately.

Some studies have reported strong contamination by heavy metals in near-shore sediments from coastal areas of the Mediterranean (e. g. Palanques et al., 1998; Puig et al., 1999). However, sediment analysis has some drawbacks since the amount of metals found largely depends on sediment characteristics, and only the total, not the bioavailable metal, can be considered (Rainbow, 1995). Alternatively, the use of organisms has been successfully proposed as an indirect measure of metal abundance (Rainbow and Phillips, 1993; Phillips and Rainbow, 1993; Rainbow, 1995) as they provide a time-integrated measure of metal availability (bioavailability), which is responsible for the potentially noxious effects of metals on the living components of the ecosystems. Sessile benthic invertebrates appear to be particularly suitable tools for local pollution biomonitoring since they cannot escape from water-borne toxicants released in a given area (Rosenberg et al., 2004; Naranjo et al., 1998; Carballo and Naranjo, 2002; Perez et al., 2005). Among them, sponges are promising biomonitors because they process large amounts of water (Reiswig, 1971; Turon et al., 1997; Ribes et al., 1999), show a wide distribution and year-round availability, are abundant in sublittoral areas (Sarà and Vacelet, 1973), and have a high capacity for accumulating heavy metals (Patel et al., 1985; Olensen and Weeks, 1994; Hansen et al., 1995). Moreover, sponges submitted to metal contamination experience morphological, biological, physiological and biochemical responses, which can be easily monitored (Agell et al., 2001, *Chapter 5*; Cebrian et al., 2003, 2006, *Chapter 3 and 4*). Despite these appropriate features, sponges have been underused in global surveys (except Pérez et al., 2005), probably because most previous studies were just experimental (Hansen et al., 1995), and the few surveys reported up to now embraced small areas (Carballo et al., 1996).

Here, we perform a comparative study on the metal bioaccumulation by four widespread sponge species in order to select the most suitable species for biomonitoring purposes. We also selected one of the species to examine its

effectiveness as bio-indicator at large spatial scales (ca. 250 km), and compare metal accumulation in this species and in sediments. The choice of the contamination sites and target species was based on monitoring criteria that require widely distributed species and study sites with different degrees of pollution. On the other hand, a temporal survey was performed in order to assess whether sponges are able to reflect short-term fluctuations of the bioavailable metals in the environment, which cannot be detected in sediment surveys because bioperturbation quickly homogenises sediment layers.

This study aims to improve the present knowledge of spatial and temporal variations in the concentration of heavy metals along the NE of the Iberian coasts (Mediterranean Sea), and to propose suitable biomonitor species that may help in revealing hotspot polluted zones and assist in decision-making rules for coastal management.

Material and methods

Target species

The target species were four common Mediterranean sponge species: *Crambe crambe* Schmidt 1862, *Chondrosia reniformis* Nardo 1847, *Dysidea avara* Schmidt 1862 and *Phorbas tenacior* Topsent 1925, which encompass several structural traits that may potentially influence metal bioaccumulation capacities. The four species show a wide sublittoral distribution in the western Mediterranean, and they inhabit a wide range of habitats. Further, they are well known from a biological and ecological perspective (Becerro et al., 1994; Turon et al., 1998; Uriz et al., 1995; Wilkinson and Vacelet, 1979; Bavestrello et al., 1998; Garrabou and Zabala, 2001).

Crambe crambe has an encrusting growth habit and is able to live at moderately polluted sites, which makes it suitable for studies of sublethal contamination (Cebrian et al. 2003, *Chapter 3*). It produces large amounts of collagen and has a siliceous skeleton (Galera et al., 2000; Uriz et al., 1995).

Chondrosia reniformis is a thick encrusting widespread species (Lazoski et al., 2001). It can be classed among those called “sensitive organisms” that can hardly survive in polluted habitats (Cebrian et al., 2006, *Chapter 4*). This species does not have mineral or spongin fibre skeleton but contains huge amounts of collagen, with thick and well-developed mesohyl. In contrast, it has a very reduced aquiferous system.

Dysidea avara is an encrusting to massive sponge, with a wide distribution and high filtration and clearance rates (Turon et al., 1997; Ribes et al., 1999). It lacks spicules but has a skeleton of spongin fibres that supports a well-developed system of canals with little intervening mesohyl (Galera et al., 2000).

Phorbas tenacior is an encrusting species sensitive to environmental pollution stress. It was categorised as a stenotipic species, an indicator of non-polluted waters (Carballo et al., 1996). We lack data about its internal organization.

Spatial trends

Three stations with various levels of pollution (La Pilona, Lloret-Castell and St. Feliu de Guíxols) (Fig. 2.1), were selected for a comparative study of metal accumulation by the four target species.

In a second step, 16 sampling stations were selected along the Mediterranean (NE of Spain) sublittoral (Fig. 2.1) in order to embrace heterogeneity of habitats and the different degrees of anthropogenic impact in the area. Stations were selected including moderately polluted areas (Port Balís, and Blanes, St. Feliu de Guíxols and L'Escala harbours), lightly polluted sites such as urbanised beaches (Arenys de Mar, Blanes, Lloret-Castell), and "*a priori*" pristine beaches without significant anthropogenic disturbances (La Fosca, Begur, Illes Medes, Illa Mateua and Port de la Selva, Fig. 2.1).

Temporal trends

The selected sites for the temporal variation study were two "*a priori*" pristine stations (La Fosca and Illa Mateua). This was done in order to test the usefulness of our system to detect even subtle variations, which would be undetected in oversaturated individuals inhabiting highly polluted sites (Cebrian et al., 2003, *Chapter 3*).

Sampling

Sponges were collected randomly among those of uniform size in order to avoid as far as possible variation of metal concentrations due to differences in age. For comparisons of metal content between species, five specimens of *Crambe crambe*, *Chondrosia reniformis*, *Phorbas tenacior* and *Dysidea avara* were collected by scuba diving at the three selected localities in the summer. Sediment samples (N=3) were also collected in the vicinity of the sponge habitat.

For studying the spatial variations in metals, five specimens of *Crambe crambe* per each of the 16 sampling sites were collected in summer. The specimens were collected from vertical rock-faces at 3 and 10 m depth.

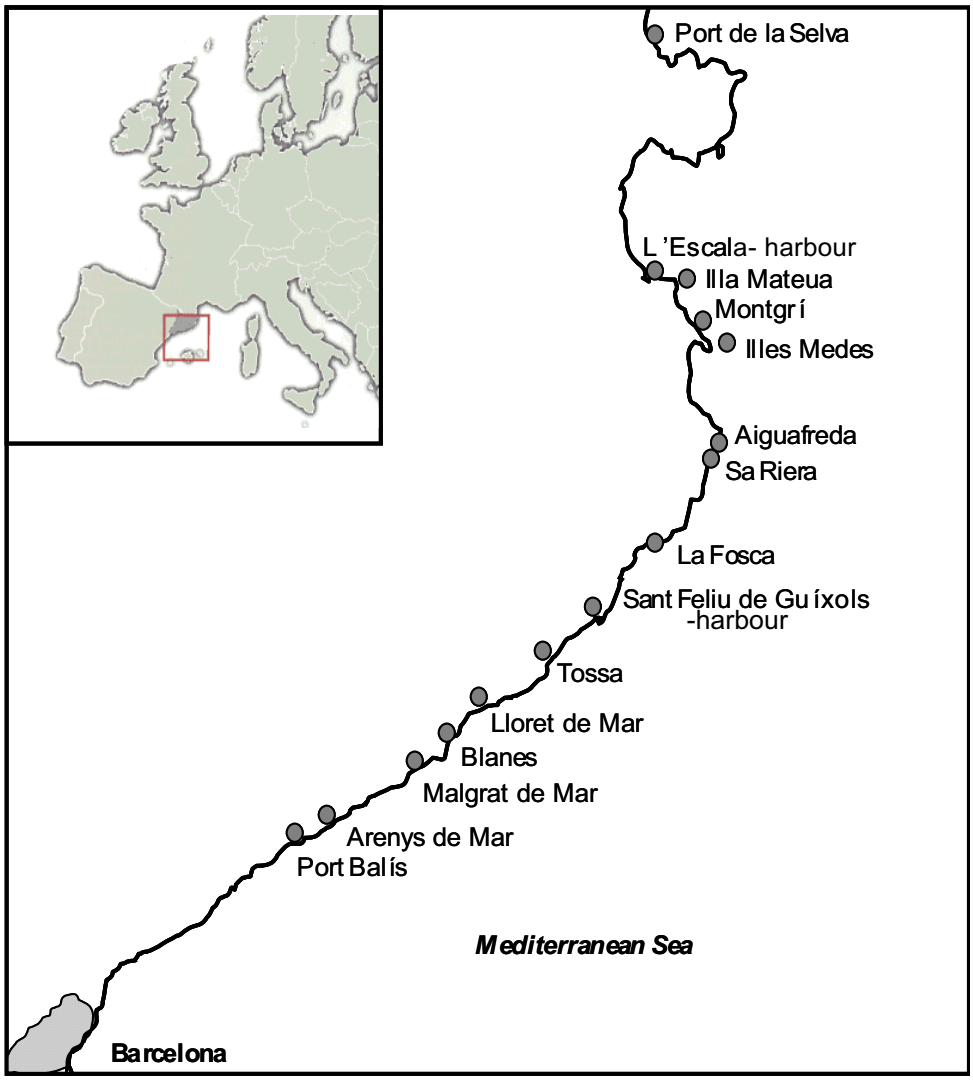


Figure 2.1: Localization of the study sampling sites.

Simultaneously, sediment samples (N=3) were collected in the close vicinity of the sponges using small sediment corers. For the study of temporal variation, five specimens of *Crambe crambe* were collected monthly from January 2003 to February 2004 at La Fosca and Illa Mateua.

Heavy metal analysis

Collected individuals were placed in clean plastic bags and frozen at -20ºC until analysed. Tissues were then removed with plastic instruments and rinsed quickly in distilled water before being placed in acid washed test tubes.

For the analysis of heavy metals (Cu, Pb, Cd, Hg and V), samples (both sponge tissue and sediments) were freeze-dried, carefully cleaned of foreign material (algae, sediment), and ground in a glass mortar. Approximately 0.1 g of sample was subjected to digestion in Teflon reactors to which 3 ml of MerckSuprapur nitric acid at 65% and 1 ml of H_2O_2 were added. The reactors were then placed in an oven at 95º C during 20 h for digestion. Afterwards, the contents were transferred to vials previously weighed. Six ml of mili Q water was added to the reactors and emptied into the corresponding vials, which were weighed again. The attack solutions in the vials were then diluted 1 to 20 with HNO_3 (1%) and 10 ppb of Rh was added as an internal standard. Solutions were measured against a calibration prepared using one blank and four increasing concentrations of commercial standards of Cu, Pb, V, Cd and Hg. Cl was also analysed to estimate possible interference with V. Standards and samples were analysed in an inductively coupled plasma mass spectrometer (Perkin Elmer Elan 6000) under standard conditions. However, when the amount of heavy metals exceeded the optimum range of concentration for ICP-MS, we used an inductively coupled plasma optical emission spectrometer (Thermo Jarrell Ash, ICAP 61E) (Scientific and Technical Services of the University of Barcelona). Results were expressed as μg of metal x g^{-1} (dry weight) of sponge tissue or sediment.

Data analysis

For the two metals accumulated in sponges (copper and lead), a bioaccumulation factor (BF) was calculated from the formula: BF = [metal] sponge tissue / [metal] sediment.

The relationship between metal accumulation in sponge tissues and in sediment was analysed by correlation analysis. Differences in metal accumulation between the 4 sponge species were analysed by two-way ANOVA with site (3 levels) and sponge species (4 levels) as factors. Spatial variation of copper and lead accumulation in *Crambe crambe* and in sediments was analysed separately by one-way ANOVA.

Temporal variation in metal accumulation in *Crambe crambe* was analyzed by two-way ANOVA with locality (2 levels) and month (10 levels) as factors. A second two-way ANOVA test was performed grouping months in two seasons (spring/summer and autumn/winter) with locality (2 levels) and season (2 levels) as factors.

Assumptions of normality and homogeneity of variances were examined using the Kolmogorov-Smirnov and Barlett tests, respectively. When assumptions were not met, we used the rank transformation (Potvin and Roff, 1993), as it was the only one that made the data comply with the requirements of the analyses. The Student-Newman-Keuls (SNK) test was used for post-hoc comparisons. When the interaction factor was significant in the two-way ANOVAs, the levels of each factor were compared within levels of the other factor by the SNK test, using the error MS of the two-way analysis to calculate the standard error of the pairwise comparisons (Underwood, 1981; Day and Quinn, 1989). Statistical analyses were performed with the Sigmastat v 1.0 package.

Results

More than 380 samples of sponges and 80 samples of sediment were analysed for Cu, Pb, Cd, Hg and V. Only copper and lead were present in the samples at reliably detectable concentrations, thus, only these two metals were considered in the study.

Between species comparison

Mean copper and lead concentration measured in both sediment and sponge tissue samples are summarised in Table 2.1. Copper and lead concentrations in sediment differed between localities (ANOVA, $p<0.01$). For copper the three localities had significantly different values (SNK test $p<0.05$), which were highest in St. Feliu-harbour and lowest at Lloret-Castell. Lead concentration in sediments, on the other hand, was only significantly higher ($p<0.05$) in St. Feliu-harbour.

In the 2-way ANOVA between localities and species, both factors, as well as their interaction, were significant for copper (Table 2.2, all $p<0.001$). So the degree of accumulation varied between species in a locality-specific way. The concentration across species and sites ranged between 8.2 and 299.3 $\mu g \cdot g^{-1}$ in *C. reniformis* from La Pilona and *D. avara* from St. Feliu, respectively. Overall, mean values of St. Feliu where higher than in the two other localities. As for species, *D. avara* was the sponge that accumulated the highest copper content in its tissues.

	Locality		
	La Pilona	Lloret-Castell	St. Feliu-harbour
Sediment			
Cu $\mu g \cdot g^{-1}$	5.7 (±0.8)	3.4(±1.2)	16.9(±1.2)
Pb $\mu g \cdot g^{-1}$	13.2 (±1.5)	6.2(±2.8)	32.5(±6.1)
C. reniformis			
Cu $\mu g \cdot g^{-1}$	8.2 (±0.8)	11.3 (±0.8)	11.0 (±0.8)
Pb $\mu g \cdot g^{-1}$	1. 5(±0.4)	2.1(±0.7)	2.1(±1.3)
C. crambe			
Cu $\mu g \cdot g^{-1}$	9.1(±2.1)	9.5(±0.6)	42.2(±14.2)
Pb $\mu g \cdot g^{-1}$	0.3(±0.2)	0.4(±0.2)	1.8(±1.3)
P. tenacior			
Cu $\mu g \cdot g^{-1}$	42.9(±11.3)	34(±10.1)	91(±21.3)
Pb $\mu g \cdot g^{-1}$	0.5(±0.2)	0.6(±0.04)	0.8(±0.7)
D. avara			
Cu $\mu g \cdot g^{-1}$	82.0(±32.3)	47.4(±10.3)	299.3(±82.1)
Pb $\mu g \cdot g^{-1}$	0.7(±0.1)	0.8(±0.2)	0.4(±0.2)

Table 2.1: Mean (± s.d.) copper and lead concentration measured in sediment and sponge tissues ($\mu g \cdot g^{-1}$).

In the presence of a significant interaction term, we ran multiple comparisons between sponges (SNK tests) at each site: *P. tenacior* and *D. avara* accumulated significantly more copper than *C. reniformis* and *C. crambe* at the site with the lowest metal concentration in the sediment (Lloret-Castell) (Fig. 2.2A). In contrast, *D. avara* accumulated more than *P. tenacior* and the latter accumulated more than *C. reniformis* and *C. crambe* at the medium-level polluted site (La Pilona) (Fig. 2.2B). At the most (Fig. 2.2C) polluted site (St Feliu-harbour) differences were significant between the four species. Copper concentration there ranked as *C. reniformis*< *C. crambe*< *P.tenacior* < *D. avara.* Thus, in general, differences among species tended to become more marked as the level of pollution increased.

When we compared sponges across sites *C. reniformis* accumulated significantly less copper (SNK test) at La Pilona than in the other two localities. In contrast, the other three species accumulated significantly more copper in St. Feliu harbour than at the rest of sampling sites. Copper concentration was consistently lower in sediments than in the sponges from the corresponding sites (except for *C. reniformis* at the most polluted site). It ranged from 3.4 $\mu g \cdot g^{-1}$ (Lloret-Castell) to 16.9 $\mu g \cdot g^{-1}$ (St. Feliu harbor) (Table 2.1).

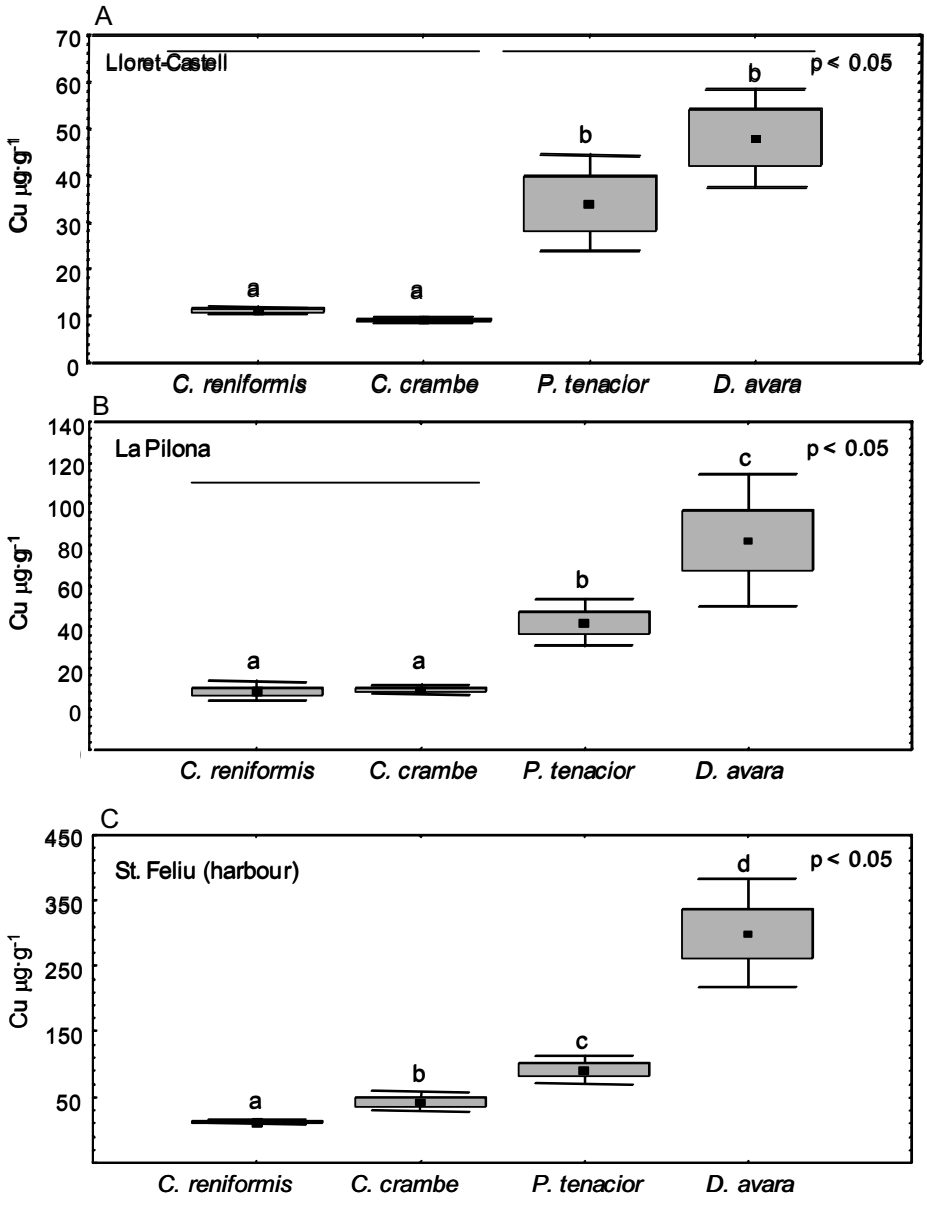


Figure 2.2: Copper concentration in *Chondrosia reniformis*, *Crambe crambe*, *Phorbas tenacior* and *Dysidea avara* tissues ($\mu g \cdot g^{-1}$) at Lloret-Castell, La Pilona and St. Feliu de Guíxols-harbour. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations which proved not significant different in a SNK test were joined by horizontal lines.

Variable	Factor	DF	F	p
Copper ($\mu g \cdot g^{-1}$)	Species	3	93.07	<0.001
	Site	2	42.32	<0.00
	Species & *Site*	6	5.50	<0.002
	Error	48		
Lead ($\mu g \cdot g^{-1}$)	Species	3	31.06	<0.001
	Site	2	3.93	0.026
	Species & *Site*	6	6.03	0.001
	Error	48		

Table 2.2: Two-way ANOVA for species (*Dysidea avara*, *Crambe crambe*, *Phorbas tenacior* and *Chondrosia reniformis*) and site (Lloret-Castell, La Pilona and St. Feliu-harbour) effects on copper and lead concentration in sponges. Variables were rank transformed.

As for lead, its concentration in the sponge tissues was significantly different between localities ($p<0.01$) and species ($p=0.03$) (Table 2.2). The interaction term was also significant ($p<0.01$), indicating different behaviour of these species depending on the locality. Lead concentration in sponge tissues across species and sites ranged between 0.3 and 2.1 $\mu g \cdot g^{-1}$ in *C. crambe* from La Pilona and *C. reniformis* from St. Feliu-harbour, respectively. As in the case of Cu, St. Feliu had the highest overall concentration of Pb, while in this case *C. reniformis* was the species with the highest mean levels of metal accumulation. Lead concentration was consistently higher in sediment than in the sponges of the corresponding sites. Its concentration in sediments ranged from 6.8 $\mu g \cdot g^{-1}$ (Lloret-Castell) to 32.5 $\mu g \cdot g^{-1}$ (St. Feliu-harbor) (Table 2.1)

The within locality comparisons (SNK tests) showed that in the two lesser polluted localities *C. reniformis* accumulated significantly more Pb than the other species, while at St. Feliu *C. reniformis* and *C. crambe* did not differ between themselves but had significantly more Pb than *P. tenacior* and *D. avara* (Fig. 2.3). As for the between localities comparisons, *C. crambe* displayed a significantly higher concentration of lead at the most lead-polluted site (St. Feliu-harbour) than in the other two localities. However, the lead concentration in the remaining three species was not significantly different across sites, which indicates that, except *C. crambe*, no species actively accumulated this metal (Figs. 2.3 A, B, and C).

Mean bioaccumulation factors (BF), for both copper and lead are summarised in Table 2.3. *Dysidea avara* showed the highest bioaccumulation factor for copper whilst *Chondrosia reniformis* showed the highest factor for lead. All the species showed $BF>1$ for copper and $BF<1$ for lead.

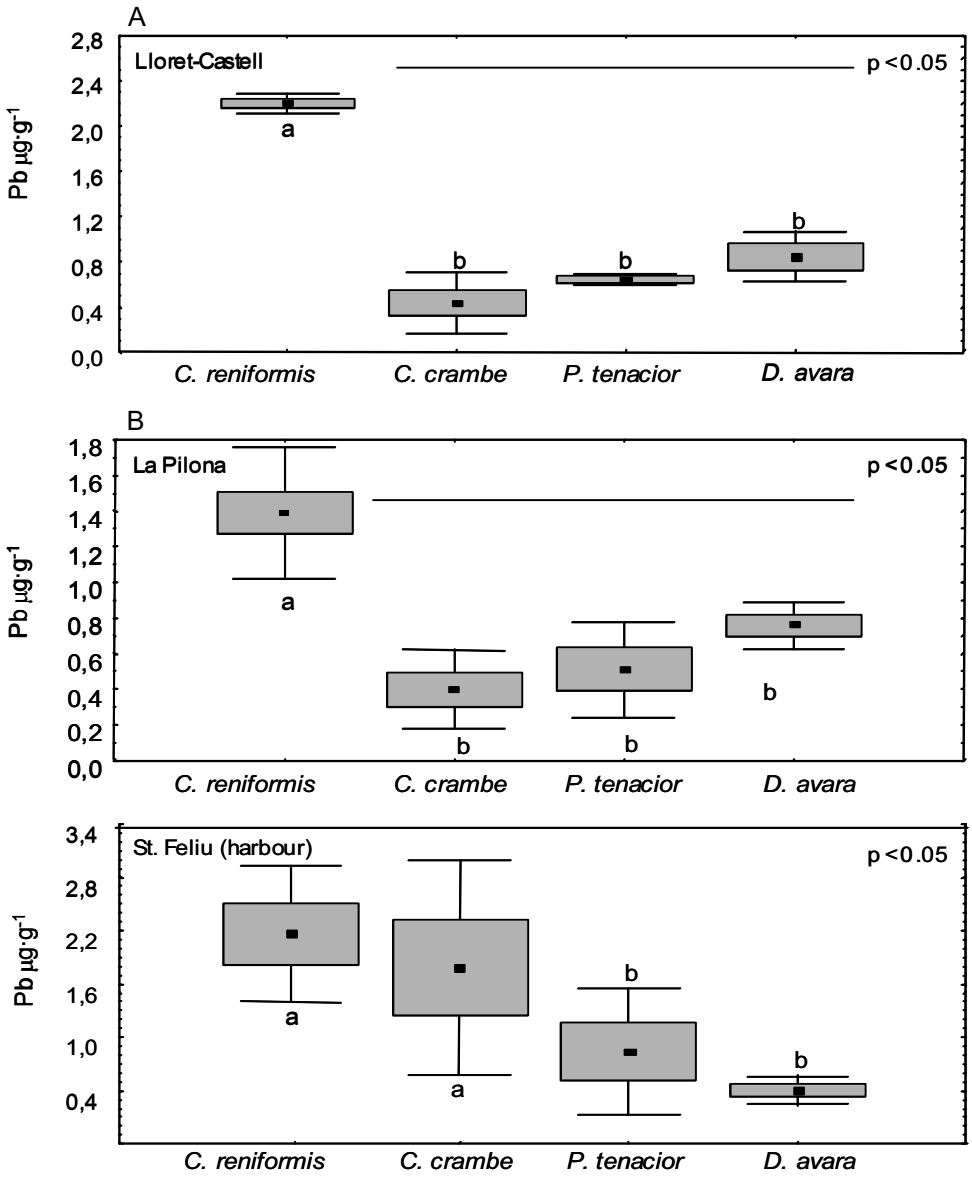


Figure 2.3: Lead concentration in *Chondrosia reniformis*, *Crambe crambe*, *Phorbas tenacior* and *Dysidea avara* tissues ($\mu g \cdot g^{-1}$) at Lloret-Castell, La Pilona and St. Feliu de Guíxols-harbour. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations which proved not significant different in a SNK test were joined by horizontal lines.

	Bioaccumulation Factor	
	Copper	Lead
Chondrosia reniformis	2.01 ± 0.76	0.23± 0.13
Crambe crambe	2.55 ± 0.17	0.05± 0.09
Phorbas tenacior	8.71 ± 1.52	0.07± 0.03
Dysidea avara	17.54 ± 1.84	0.08± 0.05

Table 2.3: Bioaccumulation factors for copper and lead of *C. reniformis*, *C. crambe*, *P. tenacior* and *D. avara* (mean ± s.e).

Spatial and temporal trends

The results obtained in the survey of sponges and sediment at 16 localities are shown in table 2.4. Copper concentration in *C. crambe* tissues was significantly different between localities (ANOVA; $p<0.01$) ranging from 8.51 to 280.31 $\mu g \cdot g^{-1}$ in Sa Riera and l'Escala-harbour respectively (Fig. 2.4). Post-hoc analyses showed that differences between sites were due to the higher values of Port Balís, St. Feliu de Guíxols, Blanes-harbour and l'Escala-harbour, which was in accordance with the higher copper concentration in sediment (Table 2.4). The highest concentration in sponges corresponded to L'Escala-harbour sponges (SNK test, $p<0.01$), followed by Blanes-harbour sponges (SNK test, $p<0.01$), and then Port Balís and St. Feliu de Guíxols, which presented similar copper concentrations (Fig. 2.4A; Table 2.4).

Lead concentration in *C. crambe* tissues was also significantly different between sites (ANOVA; $p<0.01$) ranging from 0.36 to 4.16 $\mu g \cdot g^{-1}$ in Illa Mateua and Blanes-harbour, respectively. Post-hoc analyses (SNK test) showed that differences between sites were due to the higher values of Blanes-harbour (Fig. 2.4B; Table 2.4). A similar trend was observed for lead concentration in sediment.

The possible relationship between metal concentrations in sponge and in sediments was analysed by correlation analysis. Correlation analyses were performed between *Crambe crambe* tissues and sediment samples at all the study localities. We found a significant positive correlation between metal concentration in sponges and sediment, for both copper ($r= 0.987$; $p<0.001$) (fig. 2.6A) and lead ($r= 0.850$; $p<0.001$) (Fig. 2.6B).

Temporal variation of copper and lead in *C. crambe* in specimens collected monthly at La Fosca and Illa Mateua was found. Mean copper concentration in the course of the year ranged from 5.92 to 15.67 $\mu g \cdot g^{-1}$ at l'Escala and from 7.91 to 15.85 $\mu g \cdot g^{-1}$ at La Fosca (Fig. 2.5A). Copper concentration showed significant differences between sites (ANOVA, $p<0.01$) and was always higher at l'Escala. Copper increased in early spring, and then decreased in September. The interaction term was not significant, indicating a similar time course at both localities (Table 2.5, Fig 2.5A). In

addition, significant differences were found between months (ANOVA, $p<0.01$) at both sites. When monthly values were pooled to search for seasonal variations in copper accumulation by *Crambe crambe*, again a significant locality effect was found, and copper concentration was significantly higher during spring/summer, while the interaction term was not significant (Table 2.5).

	Copper ($\mu g \cdot g^{-1}$)		**Lead ($\mu g \cdot g^{-1}$)**		**Sediment ($\mu g \cdot g^{-1}$)**	
	Mean	s.d	Mean	s.d.	Mean Cu	Mean Pb
Port Balís	46.06*	13.41	0.86	0.26	6.66	17.31
Arenys de Mar	13.81	1.99	0.90	0.17	4.17	6.19
La Pilona	9.32	2.13	0.40	0.22	5.71	13.22
Blanes	13.20	4.60	1.43	0.27	6.00	31.00
Blanes-harbour	153.20*	20.12	4.16*	3.09	97.7	69.00
Lloret-Castell	9.09	0.62	0.44	0.27	3.46	6.25
Tossa	12.13	1.67	0.42	0.11	1.30	5.55
Sant Feliu-harbour	42.94*	14.23	1.84	1.34	16.96	32.50
La Fosca	14.63	9.98	0.42	0.12	3.50	5.15
Sa Riera	8.51	1.55	0.66	0.28	3.68	8.96
Aiguafreda	13.00	7.55	0.58	0.74	5.20	6.30
Medes	10.18	3.66	1.69	1.92	5.40	20.80
Montgrí	14.89	4.31	0.49	0.28	12.08	3,53
Illa Mateua	12.97	2.83	0.36	0.06	3.39	6.60
L'Escala-harbour	280.31*	68.91	1.84	0.44	149.32	64.76
Port Selva	10.17	2.13	0.87	0.53	12.88	10.17

Table 2.4: Mean and standard deviations of copper and lead concentrations in *Crambe crambe* tissues and sediments at all localities considered in the survey. *significant differences ($p<0.05$).

Mean lead values ranged from 0.42 to 9.64 $\mu g \cdot g^{-1}$ at L'Escala and 0.38 to 10.78 $\mu g \cdot g^{-1}$ at La Fosca. The temporal trend of bioaccumulation was clearly the opposite of that of copper: lead decreased at both sites in early spring and then increased in September (Fig. 2.6B). Lead concentration was significantly higher at la Fosca (ANOVA, $p=0.04$), and there were significant differences between months ($p<0.01$) at both localities. When months were pooled in two seasons, between-locality differences were no longer significant ($p=0.162$), while lead accumulation by *Crambe crambe* was significantly higher during autumn/winter ($p<0.01$) (Table 2.5). Lead concentration followed the same trend with time at both localities, as there was no significant interaction term between locality and time (Table 2.5).

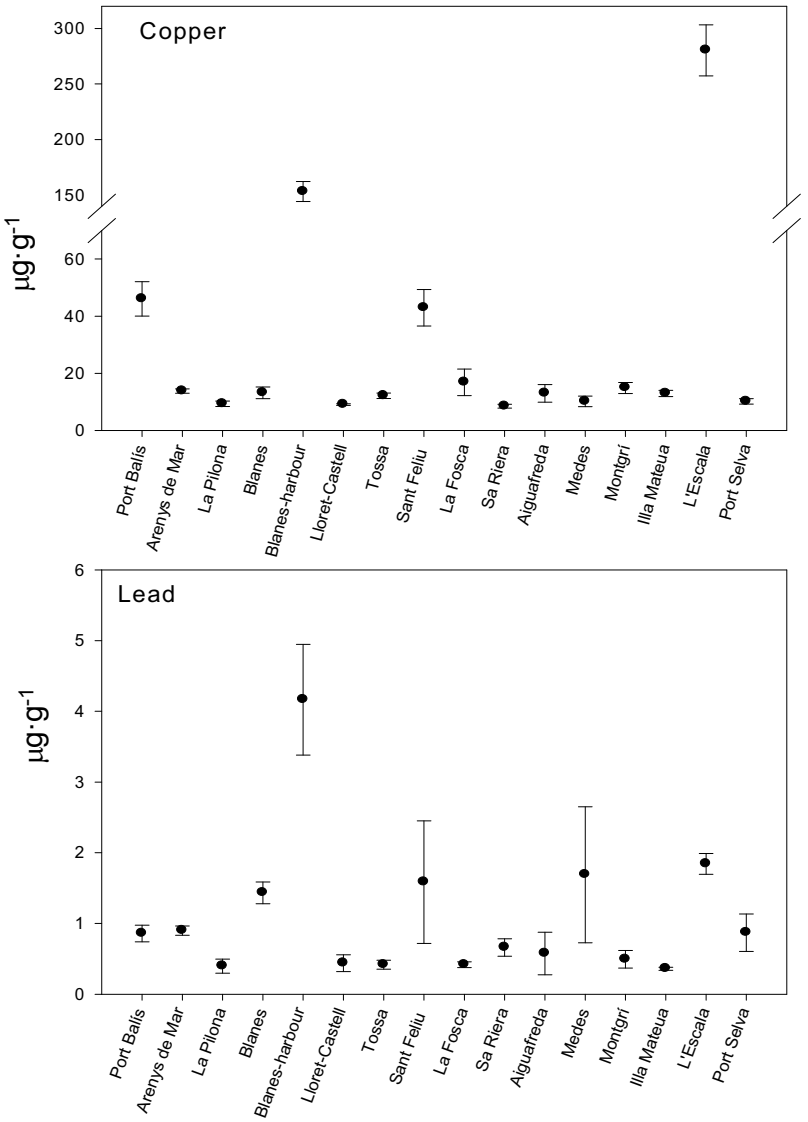


Figure 2.4 : Copper (A) and lead (B) concentrations in *Crambe crambe* tissues at all localities considered in the spatial survey. Vertical bars are standard errors.

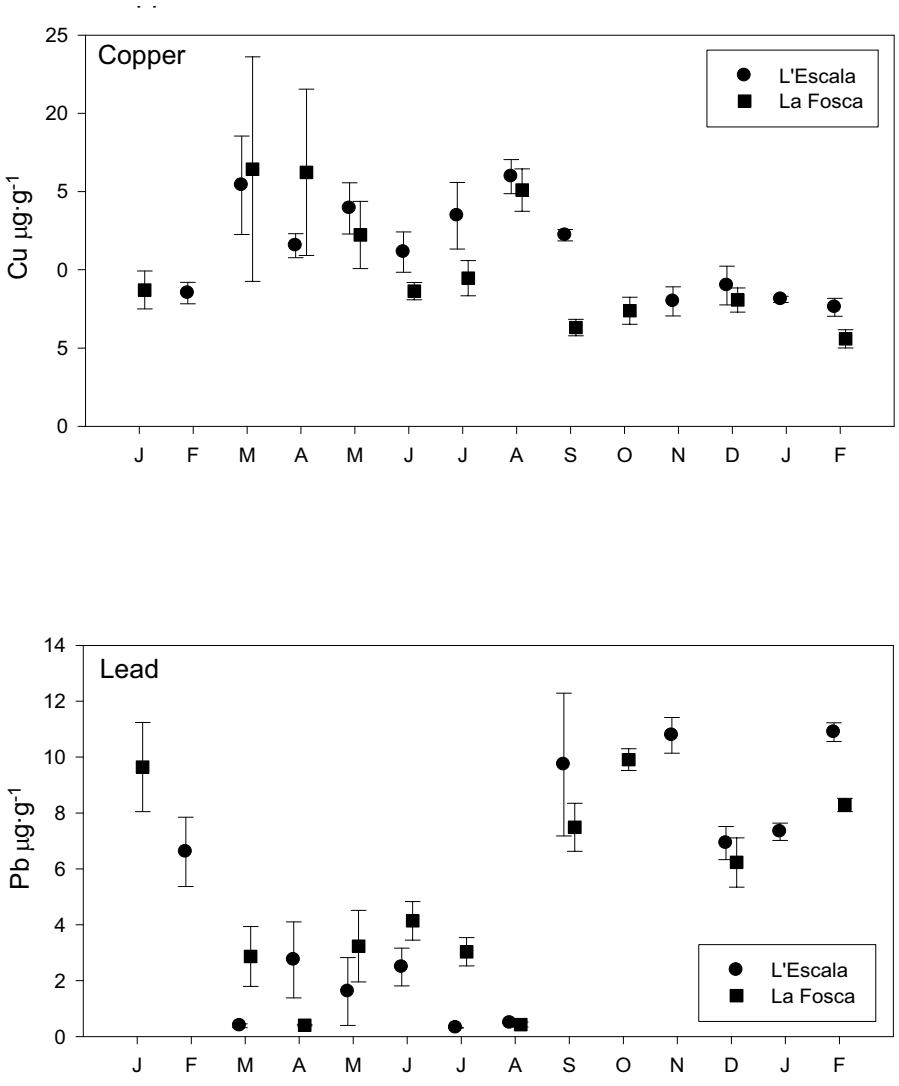


Figure 2.5: Time course of heavy metal concentrations (A: copper; B: lead) in *Crambe crambe* tissues from La Fosca and l'Escala. Vertical bars are standard errors.

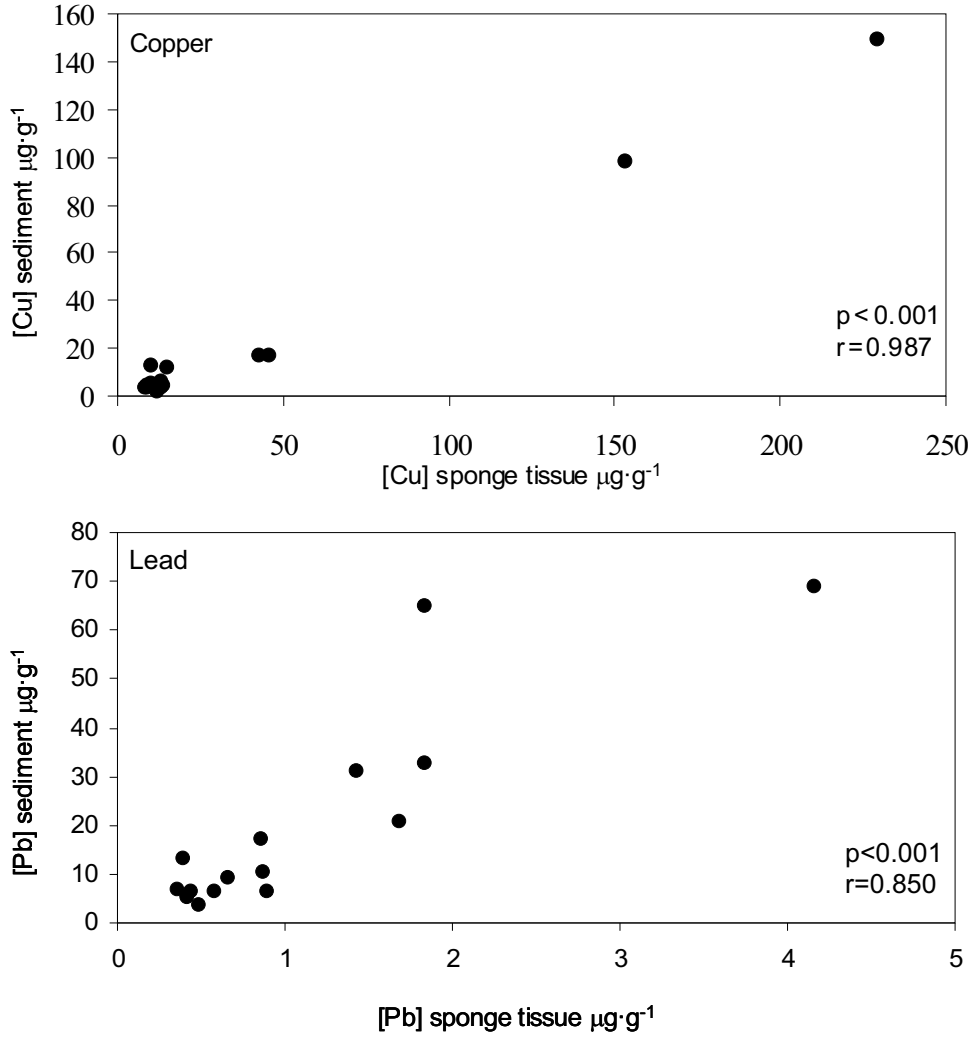


Figure 2.6: Plots of the correlations between heavy metal concentration (A: copper; B: lead) in *Crambe crambe* tissues and sediments.

Variable	Factor	DF	F	p
Copper (μg·g-1) (montly)	Site	1	20.432	0.000
	Time	9	9.595	0.000
	Site & Time	9	1.960	0.054
	Error	83		
Lead (μg·g-1) (montly)	Site	1	4.079	0.046
	Time	9	22.7061	0.000
	Site & Time	9	1.3484	0.225
	Error	83		
Copper (μg·g-1) (seasonal)	Site	1	13.4382	0.003
	Time	1	23.0824	0.000
	Site & Time	1	0.0055	0.940
	Error	99		
Lead (μg·g-1) (seasonal)	Site	1	1.9845	0.162
	Time	1	73.7444	0.000
	Site & Time	1	1.8414	0.177
	Error	99		

Table 2.5: Two-way ANOVA for site (La Fosca and L'Escala) and time (months and two seasons) effects on copper and lead concentration. Variables were rank-transformed.

Discussion

The four sponge species studied always accumulated more copper than sediments did, although copper concentration in the sponge tissues was positively related to copper concentration in sediments. Conversely, lead concentration in sponge tissues never exceeded that in the sediment of the corresponding habitats (except in *C. reniformis* at the most polluted locality), even though lead content in both sponges and sediments was also positively related. These results clearly indicate that sponges accumulate copper more efficiently than lead and that accumulation mechanisms in sponges depend on the metal considered.

Rainbow et al. (1990) suggested that the several mechanisms for metal accumulation of marine invertebrates fit into two main categories: regulation and net accumulation. Regulation is the ability of invertebrates to regulate the body concentration of particular trace metals to an approximately constant level over a wide range of ambient metal availabilities. Net accumulation occurs when invertebrates are

unable to match rates of excretion of trace metals with rates of uptake. A good bioindicator specie should accumulate as a function of the environmental concentration of metal following a linear regresion. The sponges studied, except *C. crambe*, seem to regulate lead concentration in their tissues, since they do not accumulate it above a threshold, irrespective of lead concentration in the environment, while they are accumulators for copper. In any case, further studies concerning subcellular localization and binding of heavy metals are needed to provide essential information on metal bioaccumulation and detoxification mechanisms in sponges.

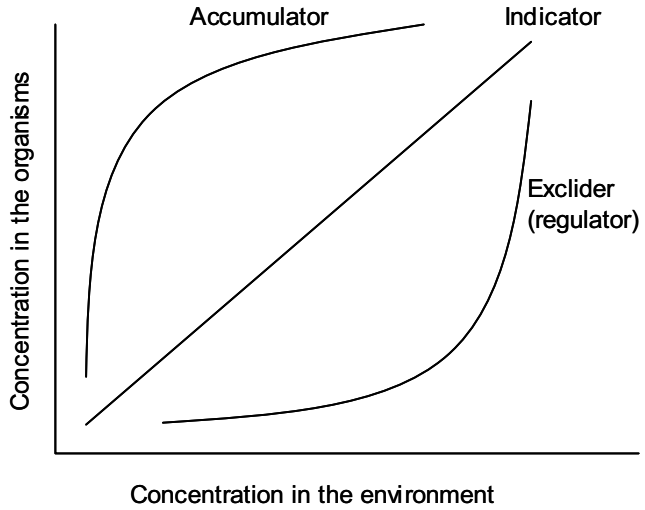


Figure 2.7: Three basic strategies for the uptake of metals by organisms (Adapted from Baker and Walker, 1990).

As for the metal accumulation by the several sponges analysed, *Dysidea avara* displayed the greatest ability to accumulate copper, followed by *P. tenacior, C. crambe* and *C. reniformis*. However, *C. reniformis* did not reflect the high copper concentration present at the most polluted site (St. Feliu-harbour). This behaviour disqualifies *C. reniformis* as a bioindicator of copper pollution, since fluctuations of the bioavailable copper in the environment cannot be inferred from copper concentrations in sponge tissues. If copper uptake by sponges corresponds to an accumulation strategy, as the results found seem to suggest, differences between species might be explained by their respective filtration and clearance rates, which may depend on the relative volume of their aquiferous systems (Turon et al., 1997; Pérez, 2001). This interpretation is supported by the highest copper bioaccumulation by *D. avara,* which also presents the highest volume of choanocyte chambers (Uriz et al. 1995; Galera et al. 2000) and a higher clearance rate than *C. crambe* (Turon et al., 1997). Likewise,

C. reniformis was the species with the smallest volume of choanocyte chambers (authors, current research) and the lowest copper concentration in its tissues.

The higher lead concentration found in *C. reniformis* with respect to the other sponge species is remarkable. Spongin fibres belong to the collagen class (Garrone and Pottu, 1973) and are reported to concentrate some metals to a greater extent than cell tissues (Verdenal et al., 1990). Collagen fibrils might have properties similar to spongin for binding metals, and this might explain why this extremely collagen-rich sponge shows such a high "basal" lead concentration in its tissues.

With respect to lead, *C. reniformis* did not show significant differences among specimens from different localities, despite the differences in lead found in sediments. Similarly, *Phorbas tenacior* did not show differences in lead concentration between localities. Lead concentration was the lowest in *Dysidea avara* inhabiting the most polluted site. *Crambe crambe* was the only species able to accumulate lead at all localities and to reflect the highest proportion of lead availability at the most polluted site. Thus, it is the only sponge species that worked as an indicator for lead. The lack of lead bioaccumulation in *D. avara*, *C. reniformis* and *P. tenacior* tissues suggests the existence of a regulatory mechanism that maintains a fixed level of lead in these sponges.

Copper and lead concentrations in *C. crambe* populations along the coast allowed us to discriminate between polluted and unpolluted sites. These concentrations were positively correlated to those in sediments. Cu concentrations were significantly higher in sponges from all the harbours studied, where copper may be leached from copper-based anti-fouling coatings used on vessel hulls (Schiff et al., 2004).

The source of lead pollution in the littoral studied is more difficult to assess than that of copper. Lead bioaccumulation values in *C. crambe* points to Blanes-harbour as the site with a significantly higher lead concentration. The relatively high lead values in the Blanes surroundings may be due to the influence of the Tordera river, which has been reported to contain high amounts of lead in sediments (up to 5 times higher than in standard coastal sediments; Puig et al., 1999).

As expected, the two localities selected for the study of temporal trends, La Fosca and Illa Mateua, ranged amongst the least polluted for both metals considered. However, a seasonal trend in copper and lead incorporation by *C. crambe* was detected. Both metals showed an opposite seasonal behaviour: while copper accumulated significantly more in summer months, lead concentration was higher in winter samples. These seasonal variations indicate that this sponge is able to reflect not only spatial variations but also seasonal changes in metal concentration of the environment. The higher copper bioaccumulation in summer can be explained by a higher availability of this metal at the two sites because of the copper-based anti-fouling paints extensively used by recreational boats in spring-summer. On the other hand, the higher lead bioaccumulation in winter was unexpected. However, it has

been reported that the main lead input to the marine water comes from the atmosphere (Cossa et al., 1993; Accornero et al., 2004). Thus, any process that promotes seawater/atmosphere exchange may enhance lead deposition in seawater (Migon et al., 2002; Puig pers. com). Sea surge due to climatic conditions may be one of these processes. This hypothesis is supported by data from oceanographic buoys in the vicinity of the two sampling localities. These data showed that months in which lead concentration in *C. crambe* was higher corresponded with periods of increasing sea surge at both localities. Spearman rank correlation between monthly means of wave height and Pb concentration showed a marginally significant positive relationship ($p=0.07$) at both localities.

The efficiency of the target species *C. crambe* in accumulating Cu and Pb, even in an area considered clean *a priori*, has allowed us to detect short-time (monthly) variation in metal availability. These short-time variations would have been impossible to assess by means of a sediment survey since bioperturbation provokes sediment homogenisation, which prevents discrimination of seasonal and even inter-annual variation.

To summarize, metal accumulation in sponges varies between species, levels of pollution and type of metal studied. *Dysidea avara* displayed the greatest capability to accumulate copper, but it did not show any increase in lead concentration when submitted to high lead pollution. Likewise, *Phorbas tenacior* did not bioaccumulate lead, and *Chondrosia reniformis* bioaccumulated neither lead nor copper, as a function of metals concentration in the environment. Among all species studied, *Crambe crambe* offers advantages in the context of biomonitoring as it was the only species capable to bioaccumulate lead and copper as a function of the surrounding bioavailable metal.

The results also suggest that the mode of incorporation varies depending on the trace metal considered. It seems that copper bioaccumulation fits an accumulation strategy in three of the four target species, while lead concentration seems to be regulated in most sponge tissues. The survey of *C. crambe* accumulation along the Catalan sublittoral provided information on the background levels of metals in the area and proved the suitability of this widespread species for monitoring spatial and temporal differences of metal bioavailability in Mediterranean marine environments.

Acknowledgements

The authors thank the Scientific and Technical Services of the University of Barcelona for heavy metal analyses. Ruth Martí and Sònia de Caralt helped us in the fieldwork. The Servei Metereològic de Catalunya provided meteorological data. This research was partially funded by grants from the CE (SPONGES project COOP-CT-205-017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265 /MAR).

Resum

Capítol 2

Les esponges com a biomonitors dels metalls pesants en mostrejos temporals i espaials en el Mediterrani nord-occidental: comparació multiespecífica .

La contaminació per metalls pesants ha augmentat dràsticament durant els últims 20 anys a les costes mediterrànies. En aquest treball, hem dut a terme un estudi comparatiu de l'acumulació de metalls entre quatre espècies d'esponges mediterrànies, a efectes de seleccionar-ne la més apropiada per ser utilitzada en estudis de monitoratge de metalls pesants en el medi marí. Els resultats obtinguts indiquen que la bioacumulació de coure s'ajusta a una estratègia d'acumulació neta, mentre que la concentració de plom sembla estar regulada en la majoria de les esponges estudiades. D'entre totes les espècies estudiades, només *Crambe crambe* bioacumula plom i coure en funció de la disponibilitat d'aquest metalls en l'ambient, el que la fa apropiada pels monitoratges. També s'ha comparat l'acumulació de metalls en *C. crambe* amb la dels sediments, amb l'objectiu d'examinar la seva capacitat com a bioindicador tant a escales espaials com a temporals. Els resultats indiquen que aquesta espècie proporciona informació dels diferents nivells de metalls pesants presents en l'ambient al llarg del temps. Més concretament, *C. crambe* és capaç de mostrar fluctuacions estacionals de la quantitat de metalls disponibles, les quals serien impossibles de detectar mitjançant els mostrejos clàssics (sediments).

Part B

Chapter 3

Sublethal effects of contamination on the Mediterranean sponge *Crambe crambe*: metal accumulation and biological responses

Chapter 3

Sublethal effects of contamination on the Mediterranean sponge *Crambe crambe*: metal accumulation and biological responses. [1]

The effect of low levels of pollution on the growth, reproduction output, morphology and survival of adult sponges and settlers of the sponge *Crambe crambe* were examined. We transplanted sponges from a control area to a contaminated site and measured the main environmental variables (chemical and physical) of both sites during the study period. Except some punctual differences in particulate organic matter, silicates, nitrates, and water motion, most environmental variables in the water were similar at both sites during the study months. Mainly copper, lead and OM concentrations in the sediment, and water motion were significantly higher at the polluted site and may be implicated in the biological effects observed: decrease in the percentage of specimens with embryos, increase in shape irregularity and decrease in growth rate. Individuals naturally occurring at the polluted site and those transplanted there for 4 months accumulated ten times more copper than either untouched or transplant controls. Although lead concentration in water did not differ between sites, native specimens from the contaminated site accumulated this metal more than untouched controls. Vanadium concentration also tended to increase in the sponges living at or transplanted to the contaminated site but differences were not significant. *Crambe crambe* is a reliable indicator of metal contamination since it accumulates copper, lead and vanadium. At the contaminated site, sponge growth, fecundity and survival were inhibited, whereas sponge irregularity ending in sponge fission was promoted. All these effects may compromise the structure and dynamics of the sponge populations in sheltered, metal-contaminated habitats.

[1] Cebrian E, Martí R, Uriz MJ, Turon X (2003). Sublethal effects of contamination on the Mediterranean sponge *Crambe crambe* : metal accumulation and biological responses. Mar. Pollut. Bull. 46 :1273-1284.

Introduction

Many studies on the effects of contaminants on the aquatic environment have been performed in the laboratory using target organisms easy to maintain in laboratory conditions (e. g. Brown and and Ahsnullah, 1971; Kobayaski, 1980; Rainbow et al., 1980; Rainbow and Wang, 2001). Laboratory studies involve the exposure of organisms to well-defined concentrations of one or a few contaminants in controlled environmental conditions. Unfortunately, it is difficult to predict the responses to toxicants in the field from these laboratory experiments, since they do not take into account a variety of complex environmental interactions (Cairns and Pratt, 1989). On the other hand, field studies tend to be descriptive rather than experimental, and contamination impacts are usually assessed from observed mortality or changes in community structure, which are measured by structure descriptors such as diversity indices, species richness, species abundance or presence/absence of indicator species (Carman et al., 1995; Carballo and Naranjo, 2001).

When changes in the structure of benthic communities are detected, we are restricted to measuring the lethal effects of pollution. However, most contaminants enter marine waters as low-level chronic toxicants whose harmful effects are not always obvious and take time to appear (Young et al., 1979). Small doses of pollutants affect the physiological functions and behaviour of organisms without killing them (e. g. Newton and McKenzie, 1995; Agell et al. 2001). Yet, these cryptic effects compromise not only individual fitness but also population success. Most of these studies have been carried out on soft bottom invertebrates (e.g. Ozoh, 1990; Warwick et al., 1990) or plankton species (e.g. Brown and Ahsamullah, 1971; Riisgård, 1979) and scarce data are available on benthic filter feeders other than mussels and clams (Bjerregaard and Depledge, 1994; Abbe et al., 2000). Thus, the sublethal effects of contamination on many invertebrates, which dominate most rocky assemblages, are unknown.

Sponges are among the main constituents of sciaphilous, rocky communities in the Mediterranean benthos (Vacelet, 1979; Uriz et al., 1992a, 1992b), where they play a paramount role in the energy transfer processes (Reiswig, 1971). According to the rare studies on sponge responses to pollution (Alcolado and Herrera 1978; Carballo et al., 1996; Perez, 2001), sponges are either resistant or susceptible to contaminants depending on the contaminant and the species considered.

Here we examine the effect of low levels of pollution on the growth, reproduction output, morphology and survival of adult sponges and settlers. These effects may alter the species fitness and so its population dynamics, which will affect whole benthic assemblages. We transplanted sponges from a control area to a contaminated site and measured the main environmental variables (chemical and physical) of both sites during the study period. Scattered individuals of *C. crambe* inhabited the contaminated site selected, which allows us to state that the levels of contamination of this site were not lethal for the sponge. We subjected the sponges

transplanted to the same environmental conditions affecting the natural populations at both the control and contaminated areas for a four-month period. Since early life stages of benthic invertebrates are usually more susceptible to contamination than adult stages (Kobayashi, 1980), we also studied the survival rates of settlers transplanted to both sites.

Material and methods

Organism and study site

We studied the sponge *Crambe crambe*, a widespread sublittoral sponge in western Mediterranean (Vacelet, 1979; Uriz et al., 1992a), which is well known from a biological and ecological perspective (Becerro et al. 1994; Turon et al., 1998; Uriz et al., 1995). The encrusting growth habit of this sponge allowed us to determine sponge growth and shape by measuring increases in area and the perimeter/area ratio, respectively. Furthermore, *C. crambe* also lives at some polluted sites, what makes it suitable for studies of sublethal contamination.

The study was carried out at the Blanes sublittoral (NE Iberian Peninsula, western Mediterranean) (NE of Spain, 41º 40.4'N, 2º 48.2'E) (Fig. 3.1). The control site was a vertical rocky wall, from 2 to 10 meters deep, facing west. The polluted site, 500 m from the control, was on the inner side of the Blanes harbour breakwater and consisted of a vertical concrete wall from 0 to 5 m deep with similar facing and depth to control site.

From previous studies (Pinedo, 1998) organic matter, most trace metals, TBT, and THC in the sediment at the polluted site were no present at concentrations high enough to be considered pollutants. In contrast, copper was at a mean concentration of 97 $\mu g \cdot g^{-1}$ at the polluted site (versus $6\mu g \cdot g^{-1}$ at the control site) and can be considered a contaminant, according to UNEP policies. Lead was also present in sediments (*Chapter 2*). Contamination in this harbour was expected to be due mainly to cleaning of small ships hulls, and urban sewage.

Environmental variables at the study sites

Chemical parameters

During whole experiment period water was collected weekly with a Niskins sampler from a depth of 3-4 m, at eight sampling points (four at the control site and four at the polluted site).

For the analysis of particulate organic matter (organic carbon and nitrogen), a 2L sample of seawater was screened through a 100 µm pore net to remove large

plankton forms and detritus. Water was then passed through a 0.22 µm diameter, GF/F glass fibre filter, previously exposed to hydrochloric acid vapour for 48 hours in order to eliminate any inorganic material. Filters containing the organic matter were dried and analysed with a C:H:N autoanalyser Eager 200.

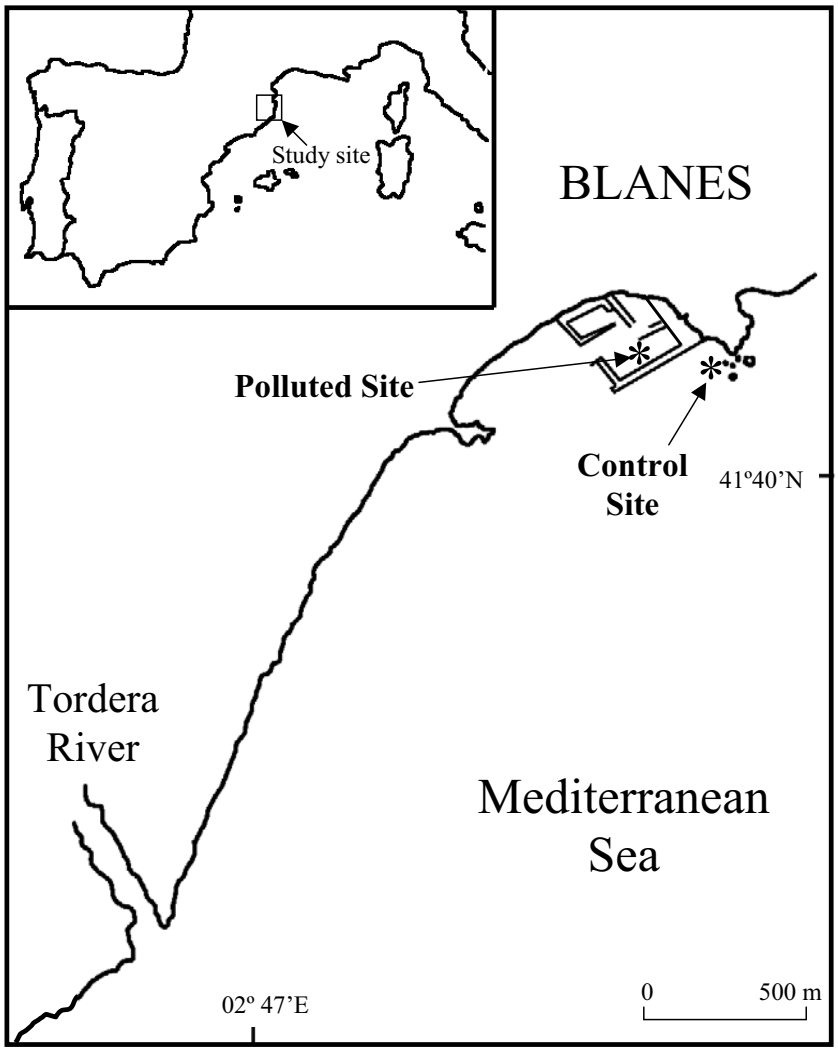


Figure 3.1: Localization of the study site.

Filtered water was used to analyse nutrient salts, Polycyclic Aromatic Hydrocarbons (PAHs) and heavy metals. Nutrient salts (nitrites, nitrates, phosphates and silicates) were analysed by colorimetric techniques (autoanalyser Technicon). Total PAHs were determined by gas chromatography coupled to mass spectrometry in the electron impact mode (GC-MS-EI) using a Fison GC8000 series chromatograph interfaced to a Fison MD 800 mass spectrometer (Solé et al., 2000).

For analysis of heavy metals (Cu, Pb, Cd, Hg and V), water samples were subjected to digestion with Merck suprapur nitric acid at 70% within vials previously weighed. The attack solutions in the vials were diluted 1 to 10 with flow injection analysis system and then were measured against a calibration prepared using a blank and increasing concentrations of commercial standards of every element. Standards and samples were analysed in an inductively coupled plasma mass spectrometer (Perkin Elmer Elan 6000) under standard conditions.

Sedimentation

Gross sedimentation rates were assessed by placing sediment traps, placed monthly during the experiment (from February to June) (N = 5), at each study site. Traps remained in the water for three days. Dried sediment was weighed and then combusted at 300ºC for 48 h and the mineral residue was weighed. The organic matter was calculated by subtracting the mineral component of the sediment from the total dry weight. To search for possible differences in granulometry, a constant amount of sediment was suspended in a constant volume of water, and an aliquot (20 µl) of this suspension was placed in a haemocytometer and examined under a light microscope. Microscope fields (N = 3), selected at random, were captured in a digital video camera connected to a computer and the images were used for quantification of particle size-classes with the NIH Image program (public domain). The sediment was classed in very fine (VF) (diameter <12µm), and fine (F) (between 12 and 30 µm in diameter), which can enter the sponge through its inhalant orifices, and medium (M) (between 30 and 100 µm in diameter) and coarse (C) (diameter > 100µm), which cannot enter the sponge via ostia (Galera et al., 2000).

Water motion

Water motion was assessed in various sea conditions (calm; slightly rough; rough; stormy). These measurements were not intended to represent the general trend in water motion of the study sites but were used exclusively for comparative purposes. We submerged $CaSO_4$ balls (N = 5) at the control and polluted sites for three days, and measured their weight losses (Muus, 1968). Results are then expressed in $g \cdot h^{-1}$ of $CaSO_4$.

Irradiance

Incident irradiance was measured by a sensor Licor-SPQA at the same time of the day, on several days, at both the working depth (about 4 m) and the sub-superficial level of both, control and polluted sites.

Transplant experiment

A total of 40 sponge specimens were randomly taken with their substrate from the control site. Twenty of these specimens were transplanted in situ to test possible transplant effects (transplant control, TC). The remaining 20 specimens were placed in separated hermetic bowls underwater and transported to the polluted site, where they were transplanted within one hour of collection (harbour transplant, HT). Twenty more specimens from the control site (control, C) and 20 more from the polluted site (harbour, H) were randomly selected, labelled, and left untouched until the end of experiment. At the end of June, before the sponge larval release (Uriz et al., 1998), we collected the specimens transplanted, and those native to the control and polluted sites, for heavy metal analyses and examination for the presence of embryos.

Sponge descriptors

Metal concentration

In general, accumulation of heavy metals (Cu, Pb, Cd, Hg and V) was quantified using an inductively coupled plasma mass spectrometer (ICP-MS) Perkin Elmer, Elan 6000. When the amount of heavy metals exceeded the optimum range of concentrations for ICP-MS, we used an inductively coupled plasma optical emission spectrometer (Thermo Jarrell Ash, ICAP 61E). Results are expressed in µg.g-1 of metal with respect to sponge tissue (dry weight).

Sponge growth, shape and survival

Sponge growth, shape and survival of transplanted individuals were assessed from monthly photographs from which perimeter and area were calculated with NIH image program for Macintosh.

Growth was estimated from changes in area over time. Since the sponge was thinly incrusting, changes in area are good estimates of changes in biomass (Turon et al., 1998). A monthly growth rate GRm was computed by the formula:

$$GRm = (Am - Am_{-1})/A\ m_{-1},$$

where Am and Am_{-1} are the areas in the month m and in the previous month, respectively.

Sponge shape was approached from the ratio between perimeter and area, which is an estimation of the sponge irregularity, and may increase in stress conditions and precedes sponge fission (Turon et al., 1998).

Sponge fission was recorded monthly for transplanted individuals at both sites.

Reproduction

The percentage of individuals undergoing reproduction at both sites was recorded at the end of the experiment (June), just before larval release. We sampled the natural populations and the transplanted sponges at both sites. The specimens were dissected and examined under a stereomicroscope since embryos were mainly located at the sponge base. We recorded the percentage of sponges that harboured embryos.

Previously (in March), to verify that the sponges transplanted to the contaminated site were undergoing gametogenesis, we took small pieces (3 mm in diameter) and processed them for histological observation. Samples were fixed in 10% formalin/seawater, desilicified in 5% HF, dehydrated and embedded in paraffin. 5µm-thick sponge sections (Autocut 2040 microtome), stained following the Masson's Trichrome technique (Martoja and Martoja, 1970), were examined under the light microscope for the presence of gametes.

Settler survival

Larvae were obtained in the laboratory from ripe sponges by spontaneous release or by shaking the sponges (Uriz et al., 1998). Free larvae settled successfully on Petri dishes (10 larvae per dish), at sea temperature and natural photoperiod. Upon larvae settlement, the Petri dishes were transferred to the control and polluted sites (N =15), and the number of living settlers was subsequently recorded weekly over a month.

Data analysis

Differences in irradiance and medium grain size of the sediment and metal concentration in water between sites were assessed by t-tests.

Differences in particulate organic matter (POM) and soluble nutrients in the water, sedimentation rates, percentage of organic matter in the sediment and water motion between control and polluted sites were analysed by two-way ANOVAs. Metal accumulation within the sponges was analysed by one-way ANOVA (Statistica 4.1 package). The Tukey test was used for post-hoc comparisons. Assumptions of normality and homogeneity of variances were examined using the Kolmogorov-Smirnov and Barlett tests, respectively. Variables were rank-transformed (Conover and Iman, 1981; Potwin et al., 1990) prior to the analysis when assumptions were not fulfilled.

Differences in sponge growth rates, total area and the perimeter/area ratio were analysed by means of the two-level randomisation method based on Manly (1991) and described in Turon et al. (1998) because data did not meet the circularity assumption (Mauchly's sphericity test) required by univariate and multivariate versions of repeated measures analysis of variance (Potvin et al., 1990; Von Ende, 1993). The whole series of data was randomised 4999 times (plus the observed one) to approximate the null hypothesis distribution of the sum of squares for each factor and their interaction, and then we examined how extreme were the observed values in this distribution. An effect was judged significant when the observed sum of squares was exceeded by less than 5% of the corresponding values in the randomisation series.

Differences in the frequency of individuals that incubated embryos between sites were analysed by means of a 2x2 contingency table using the χ^2 statistic.

The percentage of survivors for both settlers and adult sponges at the polluted and control sites were compared using Gehan's Wilcoxon Test.

Results

Environmental parameters

Light

The mean incident irradiance at the control site (371 ± 26.04 µE $m^{-2}\cdot s^{-1}$) (Mean ± SE) did not differ significantly ($p = 0.1123$) from that at the polluted site (439 ± 31.4 µE $m^{-2}\cdot s^{-1}$). These mean values corresponded to a percent surface light of 27% and 32%, respectively.

Organic matter

The time course of the particulate organic carbon (POC), particulate organic nitrogen (PON) in the water, and their ratio at both sites are shown in Fig. 1 and the ANOVA results are listed in Table 3.1. POC concentration ranged from 1300-1500 µg $C\cdot l^{-1}$ in February to 2000-2100 µg $C\cdot l^{-1}$ in April (Fig. 3.2A). POC did not show significant differences between the control and polluted sites during the study period but it varied differently with time (significant interaction term) between sites (Table 3.1). PON ranged from 49-50 µg $N\cdot l^{-1}$ (both sites) to 70 µg $N\cdot l^{-1}$ (contaminated site). It also varied differently with time at both localities, as revealed by a significant interaction between locality and time. The t-test between sites for each time point indicated that PON values were significantly higher at the contaminated site only in May ($p < 0.01$). These differences did not affect the C:N ratio, which followed the same trend and varied significantly with time at both sites (Table 3.1).

Nutrient salts

As for the soluble nutrients, phosphates and nitrites varied with time (Table 3.1). However, while phosphates followed a parallel trend at both sites (no interaction term), nitrites varied differently with time at each site (significant interaction term).

Variable	Factor	DF	F	p
POC ($\mu g.L^{-1}$)	*Site*	1	2.319	0.130
	Time	4	29.110	0.000
	Site&Time	4	4.218	0.003
	Error	134		
PON ($\mu g.L^{-1}$)	*Site*	1	4.068	0.045
	Time	4	4.283	0.002
	Site&Time	4	30.668	0.018
	Error	134		
C:N	*Site*	1	2.391	0.124
	Time	4	10.492	0.000
	Site&Time	4	0.745	0.562
	Error	134		
Phosfates ($\mu mol.L^{-1}$)	*Site*	1	0.013	0.969
	Time	4	8.970	0.000
	Site&Time	4	0.577	0.856
	Error	153		
Nitrites ($\mu mol.L^{-1}$)	*Site*	1	0.664	0.337
	Time	4	88.532	0.000
	Site&Time	4	5.714	0.008
	Error	153		
Silicates ($\mu mol.L^{-1}$)	*Site*	1	7,524	0,002
	Time	4	29,522	0,000
	Site&Time	4	0,728	0,380
	Error	153		
Nitrates ($\mu mol.L^{-1}$)	*Site*	1	4.012	0.013
	Time	4	4.658	0.000
	Site&Time	4	1.027	0.846
	Error	153		

Table 3.1. Two-way ANOVAs for site (control and polluted sites) and time (four months) effects on organic carbon (POC), particulate organic nitrogen (PON), C:N ratio and dissolved nutrients (phosphates, nitrites, silicates and nitrates).

Phosphate concentration followed a similar trend at both sites, except in March when values reached 0.25 µmol.l^{-1} at the control site (Fig. 3.3A). Nitrites peaked in winter months (0.25-0.3 µmol.l^{-1}) and strongly decreased from April to May at both sites, reaching values as low as 0.16-0.18 µmol.l^{-1} in June (Fig. 3.3B). Silicate and nitrate concentrations followed parallel trend but significantly different at both localities and varied with time (Fig. 3.3 C, D) (Table 3.1). Post-hoc tests indicated that the significant differences between sites found in the ANOVA were exclusively due to the higher values of nitrates in April ($p < 0.01$) and silicates in June ($p < 0.05$) at the polluted site.

Hydrocarbons

Neither at the control nor at the polluted site were Polycyclic Aromatic Hydrocarbons (PAHs) detected in water at the depth (4 m) at which the experiment was conducted.

Heavy metals

Water analyses for heavy metal contents only detected copper and lead at both the control and polluted sites. The mean concentration of copper was significantly higher (t-test, $p < 0.05$) at the polluted site (21.8±2.43 µg/L) than at the control site (7.36±0.976 µg/L) (Fig. 4A). Although lead concentrations tended to be higher at the polluted site (3.44±0.64, Mean±SE) than at the control site (2.02±0.29 µg/L) (Fig. 3.4), the t-test failed to detect significant differences between sites due to the higher variance at the polluted site.

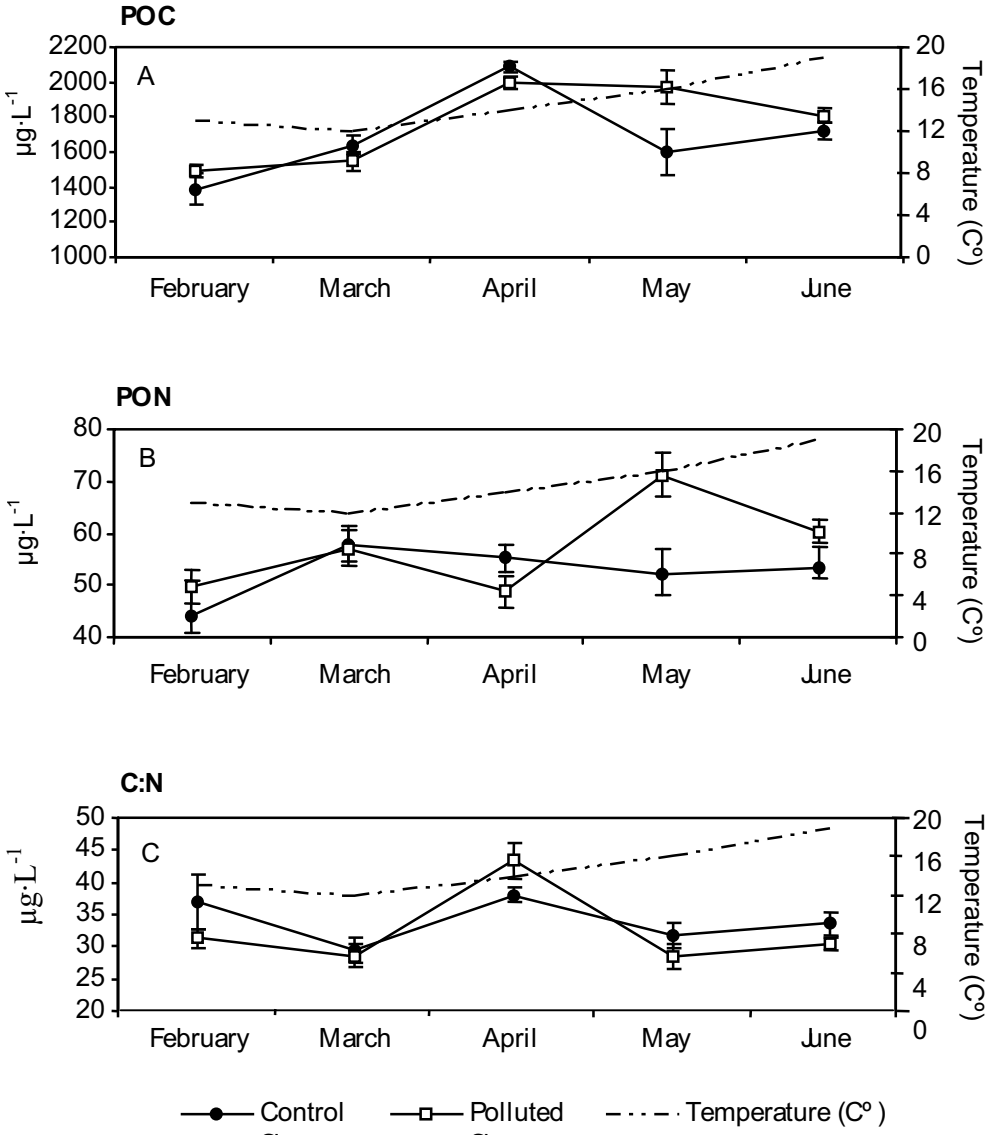


Figure 3.2 : Time course of mean POC (A) and PON (B) concentrations and the C:N ratio (C) during the experiment at the control (solid symbol) and polluted (empty symbol) sites. The time course of the seawater temperature is indicated by a shaded line. Vertical bars are standard errors.

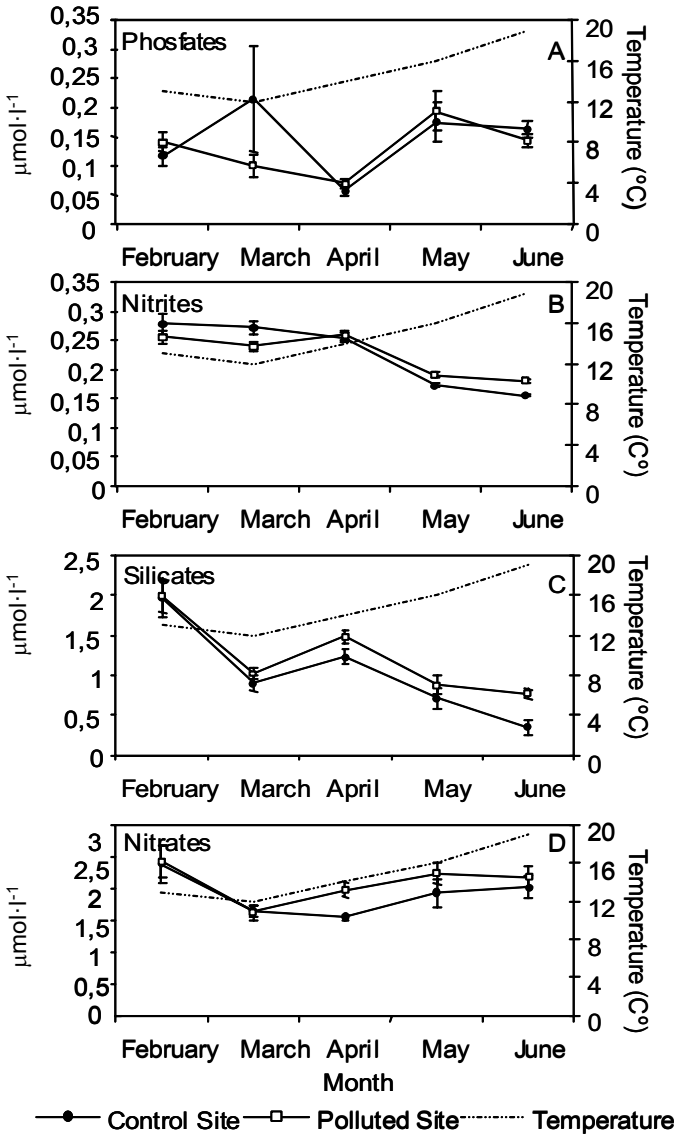


Figure 3.3: Time course of mean concentration for phosphates (A), nitrites (B), silicates (C) and nitrates (D) during the experiment period at the control (solid symbol) and polluted (empty symbol) sites. The time course of the seawater temperature is indicated by a shaded line. Vertical bars are standard errors.

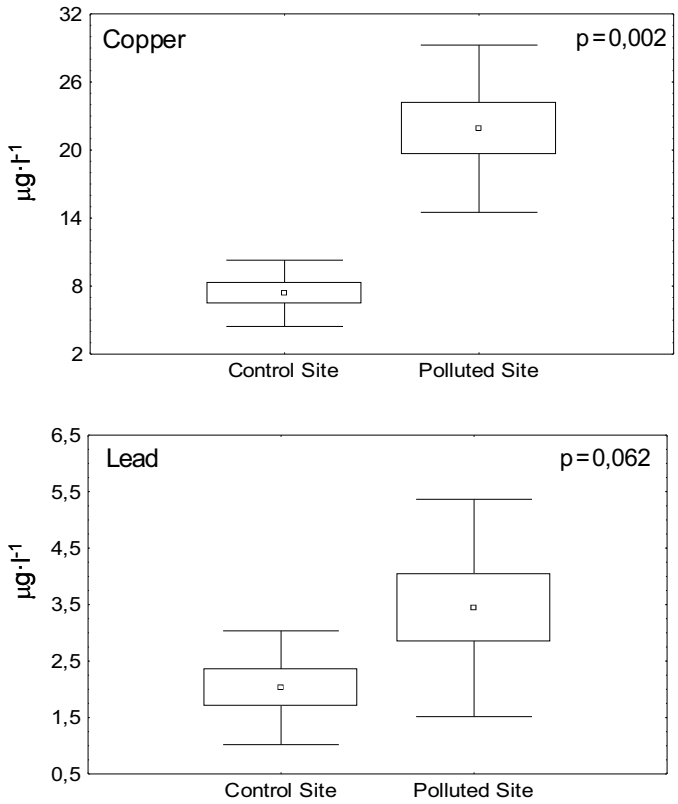


Figure 3.4: Mean copper and Lead concentration in water samples at the control and polluted sites. Boxes represent standard errors; vertical bars are standard deviations.

Sedimentation

Gross sedimentation rates (GSRs), defined as the total amount of sediment per surface and time units recovered in a sediment trap, are presented in Fig. 3.5A. They varied differently with time at both sites (Table 3.2). T-tests between sites at each time point indicated that sedimentation rates were higher at the control site in March and April ($p < 0.01$ in both cases).

Variable	Factor	DF	F	p
GSRs ($g \cdot m^{-2} \cdot d^{-1}$)	*Site*	1	0.257	0.000
	Time	3	24.161	0.855
	Site&Time	3	5.961	0.003
	Error	26		
Organic Matter ($g \cdot m^{-2} \cdot d^{-1}$)	*Site*	1	4.918	0.035
	Time	3	2.453	0.085
	Site&Time	3	4.529	0.011
	Error	26		
Organic Matter (percentage)	*Site*	1	6.397	0.017
	Time	3	2.264	0.104
	Site&Time	3	2.133	0.120
	Error	26		

Table 3.2: Two-way ANOVAs for site and time effects on Gross Sedimentation Rates (GSRs), organic matter, and the percentage of organic matter in the sediment.

The total amount of organic matter (Fig. 3.5B) varied significantly with time at both sites (Table 3.2). T-tests at each time point indicated that the values were higher at the control site in March and April and at the polluted site in May and June ($p < 0.05$).

The time course of the percentage of organic matter in the sediment (Fig. 3.5C) was uniform and followed a parallel trend at both sites but it was significantly higher at the polluted site (Table 3.2). Post-hoc analyses showed that the differences between sites were due to the values of the first two months (Tukey test, $p < 0.05$ and $p < 0.01$, respectively).

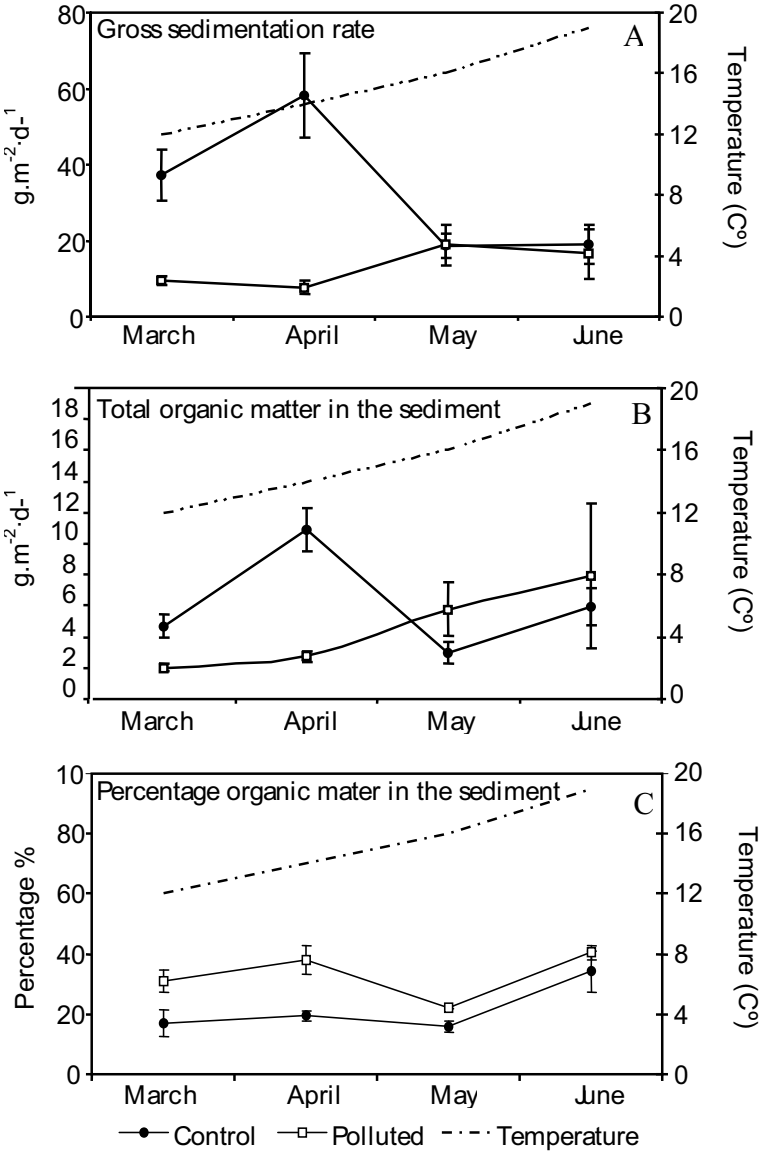


Figure 3.5: Time course of gross sedimentation rates (A), total organic matter (B) and percentage of organic matter (C) in the sediment during the experiment at the control (solid symbol) and polluted (empty symbol) sites. The time course of the seawater temperature is indicated by a shaded line. Vertical bars are standard errors.

The medium grain size of the sediment was significantly larger (t-test, $p < 0.05$) at the control site (Fig. 3.6). Coarse sediment over 100µm in diameter was only present in the control traps and never exceeded 5% of the total (Fig. 3.7). The medium-sized fraction (M) (30-100 µm in diameter) was about 20-30% in all the traps except in June at the contaminated site. The fine (F) fraction (grain size 12-30 µm) was the main component of the sediment at both sites in all the sampled months except in June at the polluted site, when the fraction smaller than 12µm (VF) accounted for most of the sediment (Fig. 3.7).

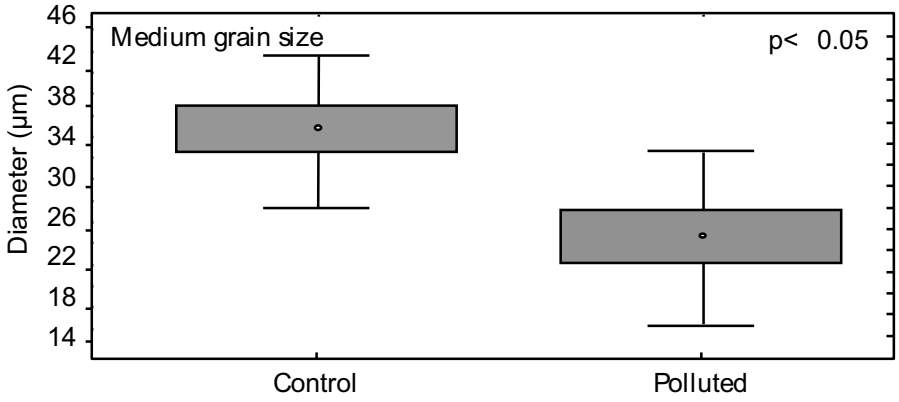


Figure 3.6. Mean medium grain-size (µm) at the control and polluted sites. Boxes represent standard errors; vertical bars are standard deviations.

Water motion

Water motion level was significantly higher at the control site (Fig. 3.8) and varied similarly at both sites (no interaction between site and time) depending on the sea condition (Table 3.3). Nevertheless, no differences were found (Tukey-test, $p > 0.05$) between sites in very calm and stormy sea conditions (Fig. 3.8).

Variable	**Factor**	**DF**	**F**	**p**
Water motion ($g \cdot h^{-1}$)	*Site*	1	41.556	0.000
	Sea conditions	3	17.412	0.000
	Site & Sea Con.	3	0.929	0.436
	Error	37		

Table 3.3: Two-way ANOVA for site (control and polluted sites) and sea condition (calm, slightly rough, rough and stormy) effects on water motion.

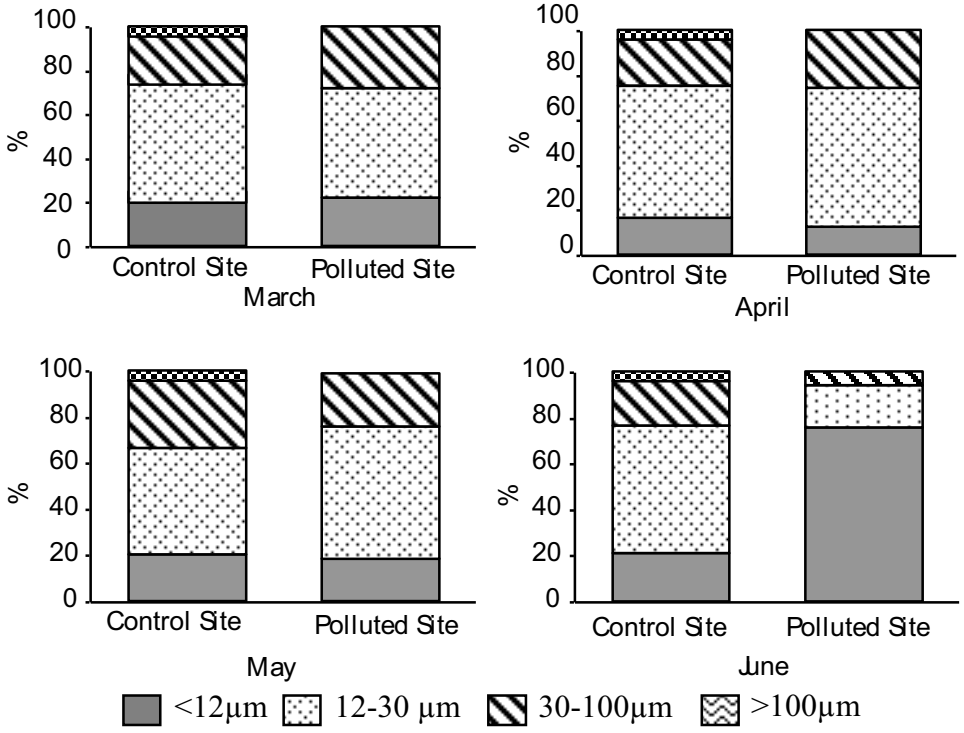


Figure 3.7: Granulometric sorting of the sediment at the control and polluted sites for different experiment months. Very fine sediment (VF<12µm), fine sediment (12µm<F> 30µm), medium sediment (30µm<F>100µm) and coarse sediment (C <100µm).

Sponge descriptors

Metal accumulation

Copper, lead and vanadium were the only heavy metals detected in the sponge tissues (Fig. 3.9). Copper accumulation was significantly higher (Tukey test, $p < 0.05$) in the specimens naturally occurring at the polluted site (H) and in those transplanted there for 4 months (HT) than in both the specimens from the control site (untouched control, C) and those transplanted *in situ* (transplant control, TC) (Table 3.4) (Fig. 3.9 A). Lead concentration in sponges tissues featured in the ANOVA in an ambiguous manner. They were not significantly different between sponges naturally occurring at the control site, and those transplanted at the both the control and the polluted sites (Fig.3.9B). And neither between sponges transplanted at the polluted site and those naturally living there (Fig. 3.9 B). Tukey-test ($p<0.05$) only indicated

significant differences between specimens naturally occurring at the polluted site and those at the control site. Although not statistically significant (Table 3.4), vanadium concentration also tended to be higher in the sponges native or transplanted to the contaminated site (Fig. 3.9C).

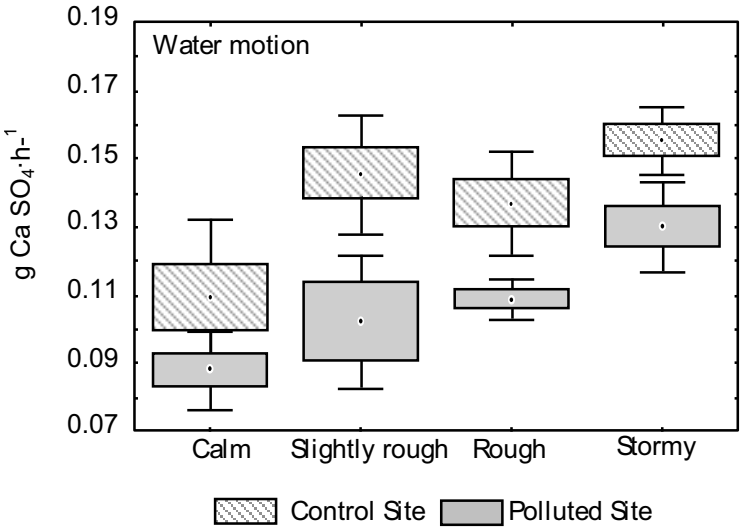


Figure 3.8: Water motion as measured by CaSO4 losses at the control site and polluted sites for all the sea conditions studied.

Variable	Factor	DF	F	p
Copper (µg.g^{-1})	*Treatment* *Error*	3 12	34.231	<0.000
Lead (µg.g^{-1})	*Treatment* *Error*	3 12	3.901	0.037
Vanadium (µg.g^{-1})	*Treatment* *Error*	3 12	2.460	0.112

Table 3.4: One-way ANOVAs for sponge treatment effect (untouched control, transplant control, native at the polluted site and transplanted to the polluted site) on metal accumulation.

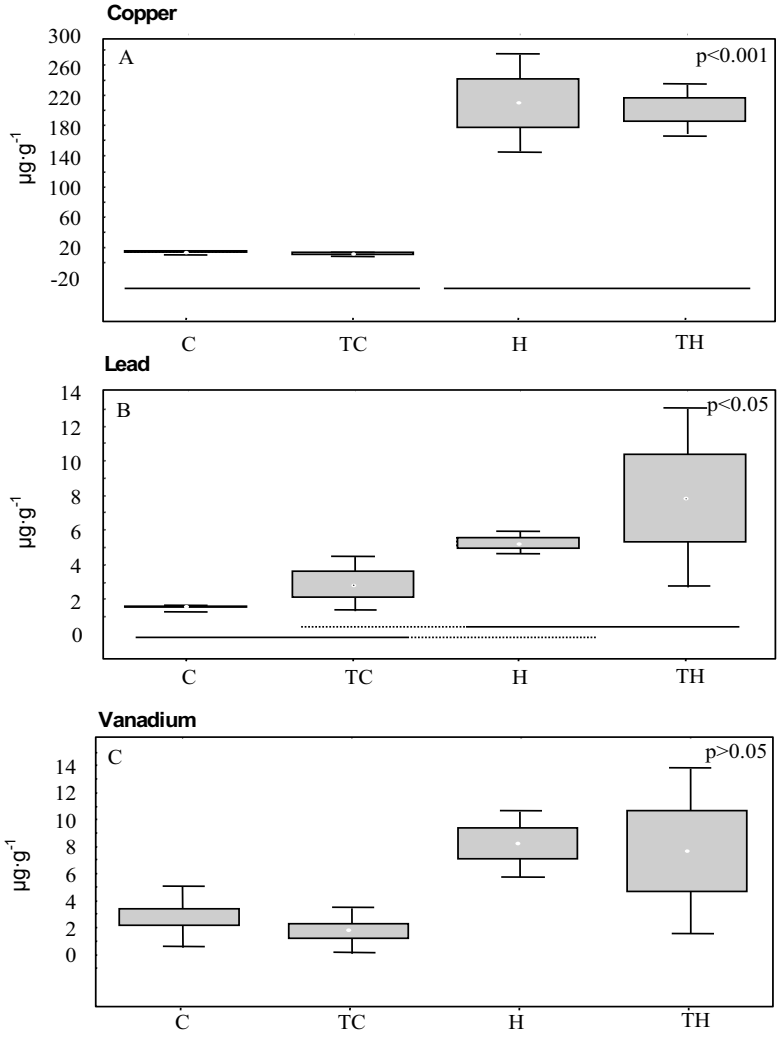


Figure 3.9: Mean concentration of copper (A), lead (B), and vanadium (C) present within the sponges tissues. C, untouched control; CT, transplant control; H, harbour native; HT, harbour transplant. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations, which proved not significantly different in a Tukey test were joined by horizontal lines.

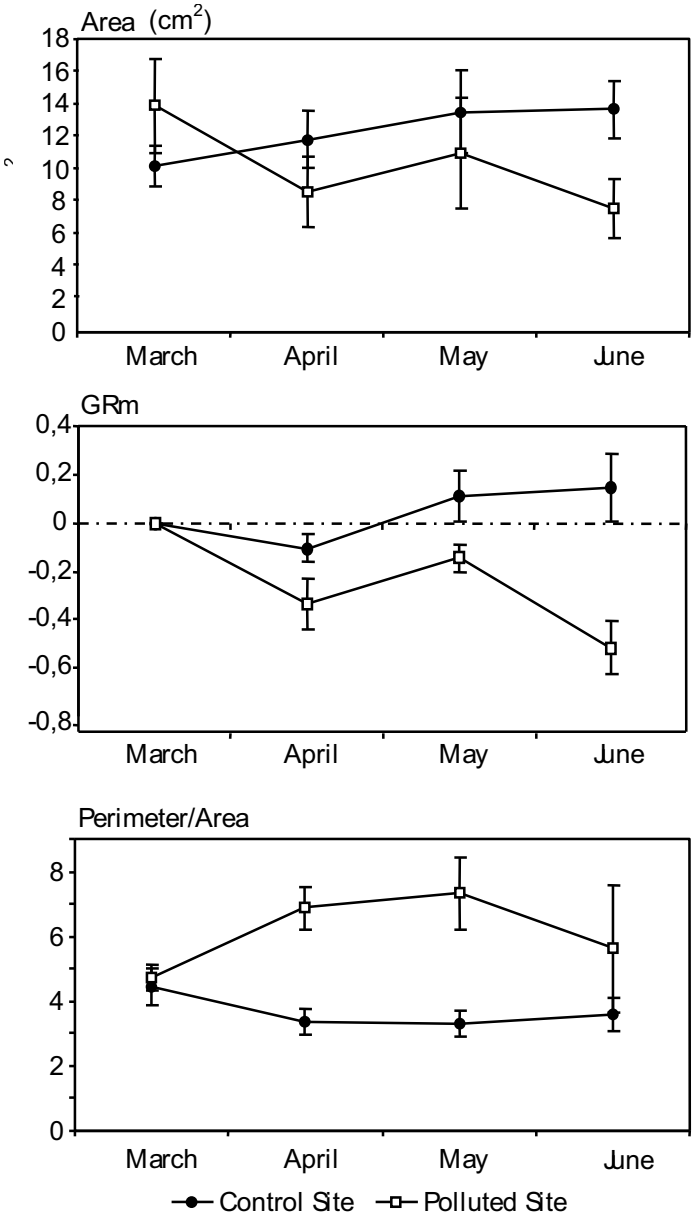


Figure 3.10. Time course of the mean sponge area (A), monthly growth rate (B) and perimeter/area ratio (C) of the sponges transplanted to the control site (solid symbol) and to the polluted site (empty symbol). Vertical bars are standard errors.

Growth

At the beginning of the experiment, the sponge mean area was nearly the same at both sites. Thereafter, the area of the specimens transplanted to the control site (TC) increased slightly, while that of the sponges transplanted to the contaminated site (HT) decreased meaningfully (Fig.3.10A, Table 3.5). The site (polluted *vs.* non polluted site) had a significant effect on the sponge monthly growth rate (GRm): while growth rates of the individuals transplanted to the control site (TC) were close to zero, growth rates of the specimens transplanted to the polluted site were negative throughout experiment (Table 3.5, Fig.3.10B).

	Percentage of randomisation SS that exceeds the observed sum of squares		
Source of variation	**Area**	**GR_m**	**Perimeter/Area**
Site	0.134	0.000	0.000
Time	0.279	0.180	0.256
Site&Time	0.382	0.027	0.985

SS: Sums of squares

Table 3.5. Significance levels obtained by randomisation for the repeated measures analyses of the area, monthly growth rate and perimeter/area ratio.

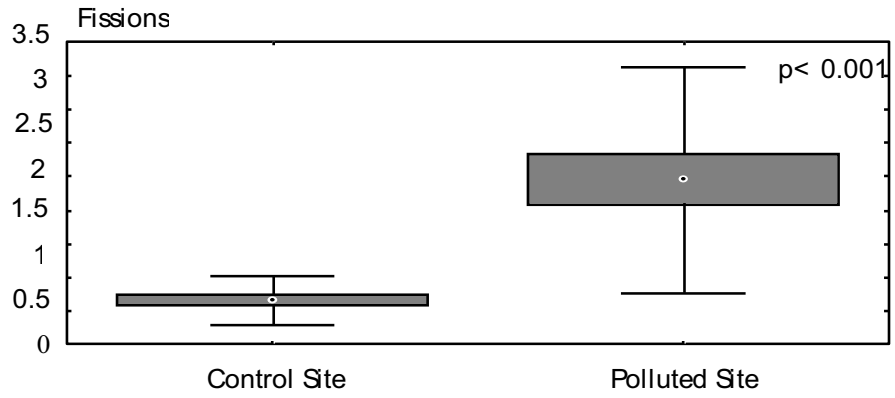


Figure 3.11: Mean number of fissions at both sites. Boxes represent standard errors; vertical bars are standard deviations.

Shape

The site also had a significant effect on sponge irregularity, as measured by the perimeter/area ratio. This ratio was similar for individuals from both sites at the beginning of the experiment but increased significantly with time among the individuals transplanted to the polluted site (Table 3.5). Conversely, individuals transplanted to the control site maintained that ratio more or less constant. Transplantation *per se* did not enhance sponge irregularity (Fig. 3.10C).

Sponge fission was significantly enhanced (t-test, $p < 0.001$) in individuals transplanted to the polluted site (1.94 ± 0.38) compared to the control site (0.15 ± 0.08; Mean ± SE) (Fig. 3.11).

Adults survival

Survival in transplanted individuals was 90% (polluted site) and 85 % (control) during the first month of the experiment (Fig. 3.12). No decrease in survival was detected from March to May at the control site, while some of the sponges transplanted to the polluted site died (Fig. 3.12). Nevertheless, the differences in survival between sites were not significant (Gehan's Wilcoxon Test, $p = 0.086$).

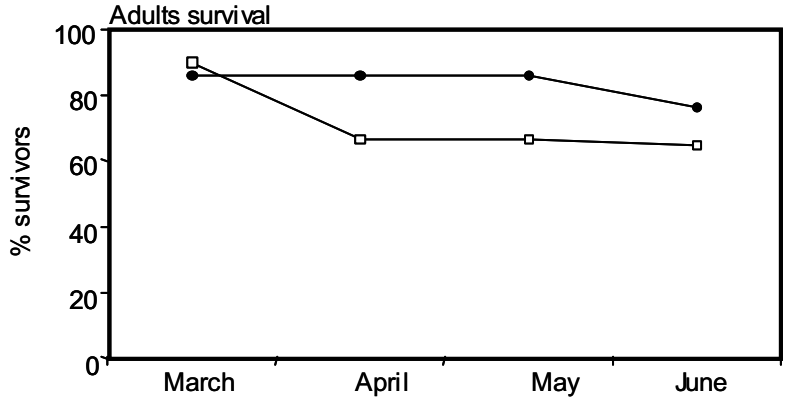


Figure 3.12: Percentage of survivors among sponges transplanted to the control (solid symbol) and polluted (empty symbol) sites.

Reproduction

The percentage of sponges harbouring embryos in June was lower among individuals transplanted (25%) and naturally occurring (11,7%) to the polluted site than in those untouched (82%) and transplanted (50%) to the control site (Fig. 3.13; $p < 0.01$). Light microscope examination of paraffin sections of the transplanted sponges indicated that all of them underwent gametogenesis in March. Thus, the low number of sponges harbouring embryos among those transplanted to the polluted site is due to causes other than the absence of initial reproduction.

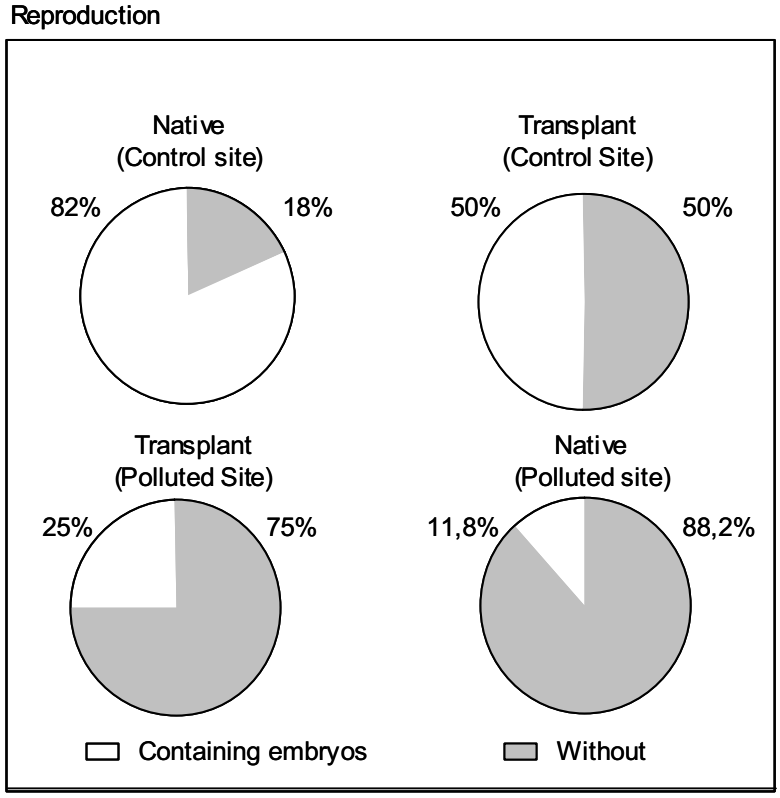


Figure 3.13: Percentage of sponges harbouring embryos in the various treatments.

Settlers survival

A similar pattern of settler mortality was observed at the control and polluted sites (Gehan's Wilcoxon Test, p = 0.47). Most of the settlers disappeared during the first week of the experiment at both sites (Fig. 3.14). Thereafter, the number of settlers remained more or less constant until the end of the experiment (4 weeks).

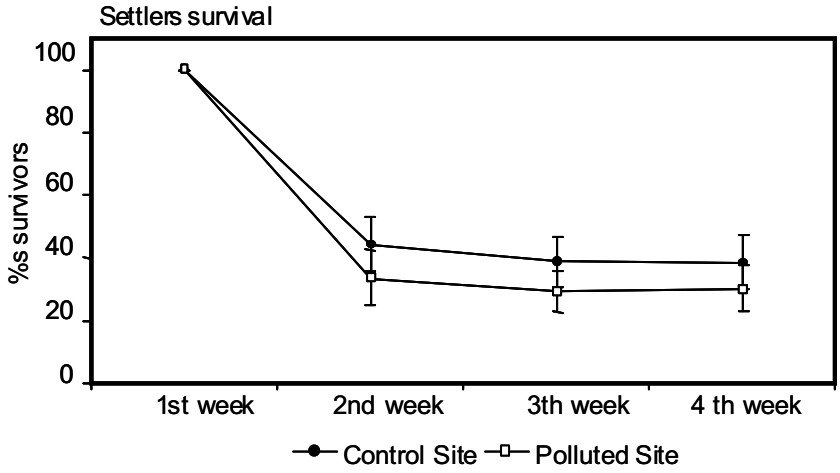


Figure 3.14. Settler survival (%) at the control (solid symbol) and polluted (empty symbol) sites. Vertical bars are standard errors.

Discussion

We aimed to experimentally address the sublethal effects of metal contamination on the widespread Mediterranean sponge *Crambe crambe* by means of a transplant experiment. However, since environmental parameters other than metals could also vary between the control and the polluted sites selected, we also analysed the most relevant water and sediment variables at both sites. Given the small size of the harbour selected as the contaminated site, the presence of organometallics (e.g. TBT) was discarded because the use of these products is prohibited in antifouling paints for small ships.

The mean values of POM (carbon and nitrogen) and dissolved nutrient salts at both sites were within the range for coastal Mediterranean waters (Mann, 1982; Ballesteros 1992) and the differences found were due to very punctual values, and thus, they do not appear to be relevant enough to exert significant effects on the biology of the sponge. It seems that only organic matter in the sediment and water movement can affect the sponge biology along with, of course, the higher concentration of some metals at the polluted site.

The lower water motion detected at the polluted site is inherent to harbours and is usually associated with high sediment rates, which can affect negatively sponges (Verdenal, 1986). However, in our case, the gross sediment rates (GSRs) were higher at the control site in winter months, probably owing to the re-suspension of coarse particles under storms (Grémare et al., 1998). The sedimentation rates recorded at both sites were within the range reported for other Mediterranean areas (Buscail, 1991; Grémare et al., 1998) and therefore do not suggest particular adverse effects for the sponge. Only during the last month of the experiment (June), the fine fraction was conspicuously higher at the polluted site. Thus, the fine sediment, although it may be deleterious for the sponge in summer by clogging its inhalant orifices, did not affect the sponge variables measured during the previous months.

Sheltered conditions, copper concentration in the water, together with organic matter in the sediment when the temperature increases above 16ºC (i.e. late May and June), appear to be the most significant environmental variables that differed between the control and the polluted sites.

Sponges filter a large volume of water and accumulate heavy metals (e.g. Patel et al., 1985, Verdenal et al., 1990; Perez, 2001; Hansen et al., 1995). However, accumulation seems to depend on the metal and the species considered (Perez, 2001). Our results indicate that the sponge *Crambe crambe* efficiently concentrates heavy metals from seawater. Specimens from the control and contaminated sites accumulate copper, lead and vanadium, although the two last metals were accumulated at lower concentrations. Differences in copper were particularly relevant between specimens transplanted to the polluted and to the control sites. Moreover, the higher organic contents of the sediment at the contaminated site during the two last months of the experiment may have promoted copper and lead accumulation by metal binding, which would favour further ingestion by the sponge cells. There were no significant differences in copper contents between sponges inhabiting the contaminated site for four months (transplants) and those living there for more than three years (native sponges). This suggests the existence of an intracellular control of metal concentration in the sponge (Philp, 1999).

As for the biological variables, negative effects were observed in the sponges transplanted to the polluted site. The sponge growth rate was negative in these sponges, while it was close to 0 in the transplant control during the study, as expected, since the sponge does not grow in winter (Turon et al., 1998).

The sponge shape was more irregular in the individuals transplanted to the contaminated site than in transplant controls, which may be a result of sponge stress (Agell et al., 2001). As reported for other invertebrates (Turon and Becerro, 1992), changes in the perimeter of *C. crambe* also appear to respond to environmental pressures (Becerro et al., 1994).

The percentage of individuals containing embryos was notably lower in the sponges at the polluted site than in transplant and untouched controls. Although this

suggests that contamination hinders embryo production, and copper has been proved to be responsible for inhibition of embryo development in other invertebrates (Bellas, 2001), a lower density of individuals at the contaminated site may also shrink fertilisation success and contribute to the lower fecundity observed. Manipulation during transplantation also seems to affect sponge reproduction since the number of transplanted individuals that incubated embryos was 20% lower than that of the untouched individuals at the control site. Stress-induced gamete re-absorption may be responsible.

Sponge survival was relatively high until June. Mortality was higher at the control site than at the harbour during the first month of the experiment, probably owing to the exposed conditions of the control site. Mortality rates similar to those recorded for transplanted specimens control site have been reported for natural populations of *C. crambe* in the same area (Turon et al., 1998).

Survival of the three-weeks old settlers was similar at both sites, which indicates that they endured the conditions of the polluted site and explains the presence of a natural population of the sponge in this site. However, there was a noticeable mortality during the first week of the experiment (70%). This high mortality is similar to that observed for newly settlers in the field (Uriz et al., 1998) and to that reported for early post-metamorphic stages of several invertebrates (Gosselin and Qian, 1997).

To summarize, this study confirms that the sponge *Crambe crambe* can be a suitable indicator of metal contamination thanks to its notable capacity to accumulate copper and, to a lower extend, lead and vanadium in its tissues, and because it experiences degrees of behavioral and physiological responses such as changes in shape, growth rates, reproduction, which can be easily monitored (Phillips and Rainbow, 1994). We can attribute the sublethal effects on the biology of the sponge mainly to water movement, metal content (mainly copper) and organic matter in the sediment. They inhibited sponge growth, increased sponge irregularity and fission and decreased fecundity. All these effects may compromise the structure and dynamics of the sponge populations in moderately metal-contaminated habitats.

Acknowledgements
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Resum

Capítol 3

Efectes subletals dels metalls en l'esponja mediterrània *Crambe crambe*: acumulació dels metalls i respostes biològiques.

S'han examinat els efectes de baixes concentracions de metalls pesants en el creixement, esforç reproductiu, morfologia i supervivència dels adults i en l'assentament larvari i supervivència post-assentament de l'esponja *Crambe crambe*. Esponges d'una zona control, "*a priori* considerada neta", es van trasplantar tant en la mateixa zona (control del efecte transplantament) com a una zona contaminada propera. Després de 4 mesos, es van mesurar les variables mencionades a les esponges intactes de la zona neta, a les transplantades a mateixa zona, a les transplantades a la zona contaminada, i a les esponges que vivien de forma natural a la zona contaminada. També es van mesurar les principals variables ambientals (químiques i físiques) durant tot l'experiment a ambdues zones. A excepció de diferències puntuals en la quantitat de matèria orgànica particulada, silicats, nitrats i hidrodinamisme, la majoria de variables ambientals es van comportar de forma similar als dos llocs. En canvi, el coure a l'aigua, i la concentració de matèria orgànica en el sediment van ser significativament més altes a la zona contaminada. Aquest dos factors podrien estar implicats en els efectes observats: disminució del percentatge d'especímens amb embrions, morfologies més irregulars, i disminució de les taxes de creixement, en els individus trasplantats a la zona contaminada. Els individus que vivien a la zona contaminada i els trasplantats a aquesta zona durant 4 mesos, van bioacumular per terme mig 10 vegades més coure, 2,5 vegades més plom, i 3,2 vegades més vanadi que els de la zona control. Les diferències van ser fortament significatives en el cas del coure ($p<0.001$),lleugerament significatives ($p<0.05$) en el cas del plom (segurament degut a l'alta variabilitat entre els especímens) i no significatives en el cas del vanadi ($p>0.05$). Per tant, podríem dir que *C. crambe* és un bon indicador de la contaminació per metalls ja que acumula metalls en altes concentracions. D' altra banda, les esponges del lloc contaminat van presentar un creixement menor, fecunditat i supervivència més baixes i un augment de la irregularitat en la forma que en alguns casos acabava en fissions. Per tant, els hàbitats contaminats per metalls pesants poden comprometre a mig/llarg termini, l'estructura i dinàmica de les poblacions de l'esponja.

Chapter 4

Response of the Mediterranean sponge *Chondrosia reniformis* Nardo to copper pollution.

Chapter 4

Response of the Mediterranean sponge *Chondrosia reniformis* Nardo to copper pollution. [1]

Abstract

Here we examined the effects of exposure to copper pollution on the sponge *Chondrosia reniformis*. We transplanted sponges from an unpolluted control area to a harbour with copper contamination. No effect of this habitat was observed on sponge growth, shape, heat-shock protein expression or metal accumulation. However, a decrease in the clearance rate, an increase in the collagen/cell rate and a lower survival rate after 4 months of the sponges transplanted to the harbour confirmed the strong effect of pollution on this organism. Our results indicate that environmental conditions in the harbour are responsible for the drastic decrease in pumping rates observed, which indirectly provoke trophic depletion and a consequent decrease in cellular elements, which results in an increased collagen rate. Here, we suggest that metal pollution, mainly by copper, may alter the sponge physiology, by reducing pumping capacity, which may ultimately lead to sponge death.

[1] Cebrian E, Martí R, Agell G, Uriz MJ (2006). Response of the Mediterranean sponge *Chondrosia reniformis* Nardo to heavy metal pollution. Environ. Poll. 141 : 452-458.

Introduction

Marine environments and, in particular, coastal waters, are subjected to increasing contamination by heavy metals (e.g. Wells, 1999). Copper, is one of the most abundant heavy metals in the Mediterranean littoral (Tankere and Statham, 1996), and has been reported to produce harmful effects on benthic invertebrates (e.g. Gnassia-Barelli et al., 1995; Webster, 2001).

The healthy state of marine ecosystems is often indirectly monitored by means of periodic measurements of physical and chemical characteristics of water and sediment. However, the results are hardly comparable between areas because of the distinct sensitivity of instruments and performance of laboratories. Furthermore, chemical analyses often include compounds that are not available to biological systems (AbdAllah and Moustafa, 2002). Conversely, metal concentrations in biota, and biological indicators automatically take into account metal bioavailability and integrate pollution during a specified period of time (Connell et al., 1999). Consequently, organisms are useful tools to detect small doses of pollutants that affect their physiological functions and behaviour before dying and may therefore indicate sublethal effects of pollution.

Benthic organisms are useful indicators of pollution in marine environments because of their reduced motility, high diversity and filter-feeding or detritus-feeding habits. Most studies on pollution have been carried out on soft-bottom invertebrates, or on mussels and clams (Bryan et al., 1985; Duquesne and Riddle, 2001). Thus, despite the increasing number of studies in recent years (e.g. Pérez, 2001; Agell et al., 2001; De Caralt et al., 2002; Bellas et al., 2001, 2003, Cebrian et al., 2003), the sublethal effects of contamination by heavy metals on invertebrates such sponges and ascidians, which dominate most rocky assemblages, are unknown. Sponges have been proposed as biomonitors for heavy metals because they accumulate metals, and are sensitive to these pollutants in a short time (Olesen and Weeks, 1994; Hansen et al., 1995; Agell et al., 2001; Cebrian et al., 2003, *Chapter 2*). Moreover, sponges are, in general, long-lived organisms and in contrast to other seasonal macro-invertebrates (Carballo and Naranjo, 2002), they are not greatly affected by seasonal or transient environmental changes.

Here we used experimental studies *in situ* and in the laboratory to examine the responses, at different levels of biological organization (from molecules to organisms), of a widespread littoral sponge to copper contamination. Organism-level variables, such as changes in morphology, growth and survival rates, collagen content, and the synthesis of heat-shock proteins (HSP's), were assessed. The accumulation of copper and lead were also analysed. Additional short-term laboratory

experiments were conducted to try to differentiate the effects of a range of copper concentrations on the production of HSP's from other environmental variables associated with copper pollution in the field.

The aim of our work was to examine the responses of a widespread sublittoral sponge to copper contamination, at different levels of biological organization (from molecules to organisms). At organisms level, changes in morphology, growth and survival rates and collagen content were assessed, while at molecular level the synthesis of heat-shock proteins (HSP's) was studied. Moreover, a additional laboratory experiment was conduced in order to study responses of *Chondrosia reniformis* to short-term copper exposure. Short-term responses were studied by means of HSP's quantification since the other variables did not respond fast enough to produce measurable changes in five days.

Materials and Methods

Material and study site

The sponge *Chondrosia reniformis* Nardo, 1847 is a widely distributed species (Lazoski et al., 2001) that commonly inhabits shaded walls of littoral zones (0-50m) (Wilkinson and Vacelet, 1979). As a result of its ubiquity and abundance in sublittoral rocky assemblages, several studies have addressed various aspects of its biology and ecology (e.g. Wilkinson and Vacelet, 1979; Bavestrello et al., 1998; Garrabou and Zabala, 2001; Nickel and Brümmer, 2003). The study site was located along the Blanes littoral (western Mediterranean). The control site was a vertical west-facing rocky wall from 4 to 10 m depth (Santa Anna Point). The polluted site selected was the inner side of the Blanes harbour, which was reported to be copper contaminated (mean copper concentration in the sediment 97 $\mu g \cdot g^{-1}$ at the polluted site vs. 6 $\mu g \cdot g^{-1}$ at the control site) (Pinedo, 1998). The harbour breakwater was placed 150 m from the control zone, and consisted of a vertical concrete wall with similar facing and depth to the control site. Both walls received similar incident light, as measured by LiCor sensor (Lincoln, NE, USA). Other physical and chemical variables, such as sedimentation rates, nutrients, particulate organic matter, PAHs, and trace metals in the water at both sites, were monitored (Cebrian et al., 2003, *Chapter 3*). Most variables behaved similarly in both zones, except for copper concentration and water movement, which were significantly higher and lower, respectively at the contaminated site (*Chapter 3*). Differences in water movement, which was lower at the polluted site, did not involve parallel differences in quality and quantity of sedimentation, which has been reported to affect sponges negatively in confined environments (Verdenal, 1986). These results are described in detail in Cebrian et al. (2003), *Chapter 3*.

Field experiment

The field experiment involved transplanting sponges from an "a priori" clean (control) site to a clearly polluted site. A total of 40 specimens were randomly taken with their substrate from the control site by Scuba diving. Twenty of these were transplanted *in situ* to test possible transplant effects (transplant control, TC). The remaining 20 specimens were transplanted to the polluted site, within one hour of collection (harbour transplant, HT). Twenty more specimens from the control site (control, C) were randomly selected, labelled, and left untouched until the end of the experiment to monitor stress caused by handling. All transplanted specimens were taken with their substrate and placed in separate bowls to be glued in the new site using a two-component epoxy resin (IVEGOR ®) within one hour. Transplants and in situ organisms were monitored monthly, from February to June. Then all the individuals still alive were collected, and stored at –80ºC for further analysis.

Sponge descriptors

Sponge growth, shape and survival

The growth, shape and survival of transplanted and untouched specimens were assessed monthly. Sponges were outlined on acetate paper underwater and then drawings were digitised with a Nikon LS-2000 Scanner to analyse perimeter and area by means of the NIH image program for Macintosh.

Growth was estimated from changes in area over time and a monthly growth rate GR_m was computed as:

$$GR_m = (A_m - A_{m-1})/A_{m-1},$$

Where A_m and A_{m-1} are the areas in the month m and in the previous month, respectively.

Sponge shape was calculated from the ratio between perimeter and area. As reported for other modular encrusting invertebrates (Turon and Becerro, 1992), changes in the perimeter of *Chondrosia reniformis* were expected to respond to environmental pressures (Wilkinson and Vacelet, 1979). The perimeter/area ratio is an estimation of sponge irregularity, which may increase under stress and precedes sponge fission (Turon et al., 1998). Sponge survival (number of living specimens) was recorded monthly.

Collagen content

Given that the main structural material of *Chondrosia reniformis* is a dense collagen-rich mesohyl, we quantified collagen fibrils content in all treatments at the end of the experiment. A sponge fragment of approximately 1 cm^3, containing ectosome and

choanosome, was taken from each individual, it was then frozen, lyophilised and weighed. All fragments were then submerged in H_2O_2 for 24 h to digest the cellular matrix but not the collagen fibrils (Olivella, 1977; Uriz; 1986; Cristobo et al., 1992). Then, fragments were frozen, lyophilised and weighed again. The proportion of collagen content was calculated as:

Cr = (Wt-Wc)/Wt

Where Cr is the collagen rate, Wt is the total weight of the fragment and Wc is the fragment collagen weight.

Metal concentration

Freeze-dried fragments of the sponges were rinsed in ultrapure deionised water and ground in a glass mortar. Approximately 0.1g of tissue was acid digested (in a 3:1, HNO_3/H_2O_2 mixture) in an oven at 95º C during 20 h and analysed with inductively coupled plasma mass spectrometer (Perkin Elmer Elan 6000). The samples were analysed in batches with reagents blancs and certified reference material. When the concentration of copper exceeded the optimum range for ICP-MS, we used an inductively coupled plasma optical emission spectrometer (Thermo Jarrell Ash, ICAP 61E).

Clearance experiments

To assess the effect of contamination (mainly cooper) on sponge filtration rates, we incubated sponges at the control site and to the polluted site for 1h and studied its filtration capacity. Whole sponge specimens were removed from their substrate, cleaned of macroepibionts and attached to floor tiles. Tiles with attached sponges were kept at both, the control and polluted sites for adaptation for 5 days. Incubations were conduced in 4 l hemispherical transparent bags (Ribes et al., 1999). At each study site, an experimental chamber was then placed on each specimen attached to a floor tile (N =3; treatment chambers) and 3 chambers more covered tiles without specimens (control chambers). Water samples of 50 ml (N =3) were collected from each chamber (treatment and control) at the beginning of the experiment (initial water samples). After 1 hour, 3 more water samples were collected from each chamber (final water samples). Grazing (clearance rate) was calculated from decreases in prey concentration in the experimental relative to the control chambers (Ribes et al., 1999).

Particle assessment protocol: pico- and nano-plankton provide a stable baseline of food for some sponges reaching 85% of the ingested carbon (Reiswig 1971, Pile et al., 1996; Ribes et al., 1999). To study these potential food sources, we analysed heterotrophic and autotrophic bacteria (*Synechococcus* sp.), and autotrophic pico and nanoeukaryotes, using flow cytometry. Water samples (2 ml) were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), frozen in liquid

nitrogen and stored at -80ºC or in dry ice. To determine cell abundance, we used a BandD FACScalibur bench machine as described in Gasol and Morán (1999).

HSP analysis

Samples were hand homogenized in an ice bath in 1: 2 (w/v) of a calcium- and magnesium-free solution (pH 7.3) containing 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl supplemented with 1 mM dithiothreitol (DTT), phenylmethylsulfonide (PMFS), 1 mM Tripsin Inhibitor and 1 % Igepal Ca-630 (Sigma). Homogenates were gently stirred and smashed and then sonicated for 90 seconds. Centrifugation was performed at 15 000 x *g* at 4ºC for 1 hour, and the supernatants were stored at –80 ºC. Total protein content was determined by the method described by Lowry et al. (1951) with BSA as a standard. Frozen supernatants were slowly thawed in an ice bath. They were then boiled for 5 minutes after the addition of SDS-PAGE sample buffer (Laemli et al., 1970).

1-D electrophoresis: Samples of equal amounts of protein (100 μg) were loaded onto 10 % polyacrylamide running gels with 4 % stacking gels. Western blot process was carried out following Agell et al. (2001) using a dilution of 1:2500 of secondary antibody. To visualize the bound sites of the antibody, the substrates p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used. One replicate of each control and treatment was assayed in the same Western blot for comparison. Western blots were scanned with Bio-Rad Fluor-S^{TM} Multilmager equipment, and band density was measured using the software Quantity One (Bio-Rad). Thus, semi-quantitative data on protein concentrations are provided since it was not possible to use pure proteins as an internal standard.

Laboratory experiment

In order to know whether short-term exposure of copper induced HSP expression in sponges at different concentrations, we performed a laboratory experiment. Fifteen randomly selected individuals of *C. reniformis* were taken from the control site. The experiment (17 ºC, natural light/dark photoperiod) lasted for five days. Two treatments consisting of copper 30 μg/L and 100 μg/L, and a seawater control were set up. We used individual polystyrene tanks (N=5) with 1 L of 0.7μm filtered and aerated seawater. The copper solution ($CuCl_2$/seawater) was freshly made and changed daily to maintain nominal copper concentrations. Seawater in the controls was also changed daily. At the end of the experiment, samples were frozen at –80ºC until analysis.

Data analysis

Metal accumulation within the sponges (HT, TC and C) was analysed by one-way ANOVA (Statistica 4.1 package). Assumptions of normality and homogeneity of

variances were examined using the Kolmogorov-Smirnov and Barlett tests, respectively.

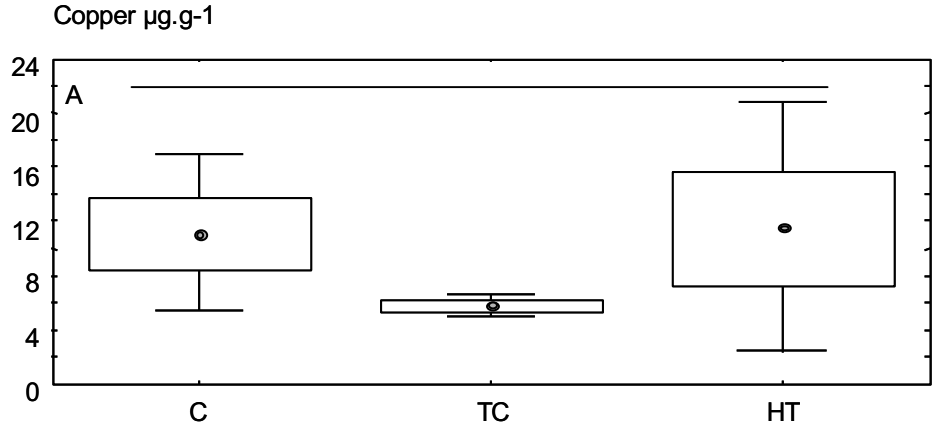
Differences in sponge growth rates, total area and the perimeter/area ratio were analysed by the two-level randomisation method based on Manly (1991) and described in Turon et al. (1998). The whole data series was randomised 4999 times (plus the observed one) to approximate the null hypothesis distribution of the sum of squares for each factor and their interaction, and then we examined the extremeness of the values observed in this distribution. An effect was judged significant when the sum of squares observed was exceeded by less than 5% of the corresponding values in the randomisation series.

Differences in collagen content and clearance rate were assessed by t-tests.

HSP data from the field and laboratory experiment that met the assumptions of parametric procedures were analysed by a randomised blocking design taking blots as the blocking factor.

Results

Metal concentration



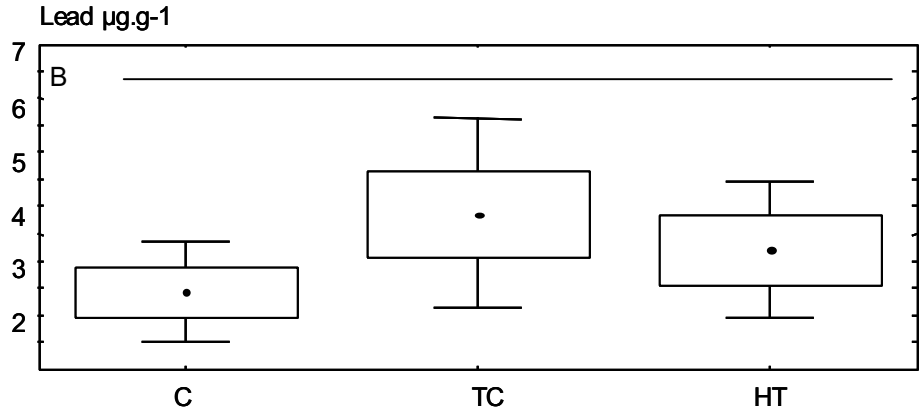


Figure 4.1. Mean concentration of copper (A) and lead (B) in sponge tissues. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Copper concentration did not differ significantly between sponges living in the "clean" site and those transplanted to the polluted site ($p>0.05$). The levels of copper ranged from 7.6 to 21 µg·g in C sponges, from 5 to 9 $\mu g \cdot g^{-1}$ in TC sponges, and from 6 to 27 $\mu g \cdot g^{-1}$ in HT sponges (Fig. 4.1 A). Similarly, lead concentration in sponge tissues did not differ among treatments ($p> 0.05$) (Fig. 4.1 B).

Sponge growth, shape and survival

Sponge mean area did not change during the experiment ($p>0.05$) in any treatment (Table 4.1; Fig. 4.2A). The mean area of untouched sponges was significantly larger than that of transplanted individuals throughout the experiment (5 months) (Tukey-test; $p<0.05$).

The relationship between perimeter and area was significantly lower for the untouched specimens (C). It followed a parallel trend in all treatments (no interaction term) except at the end of the experiment for the HT group, which showed an increased rate (Table 4.1; Fig. 4.2B).

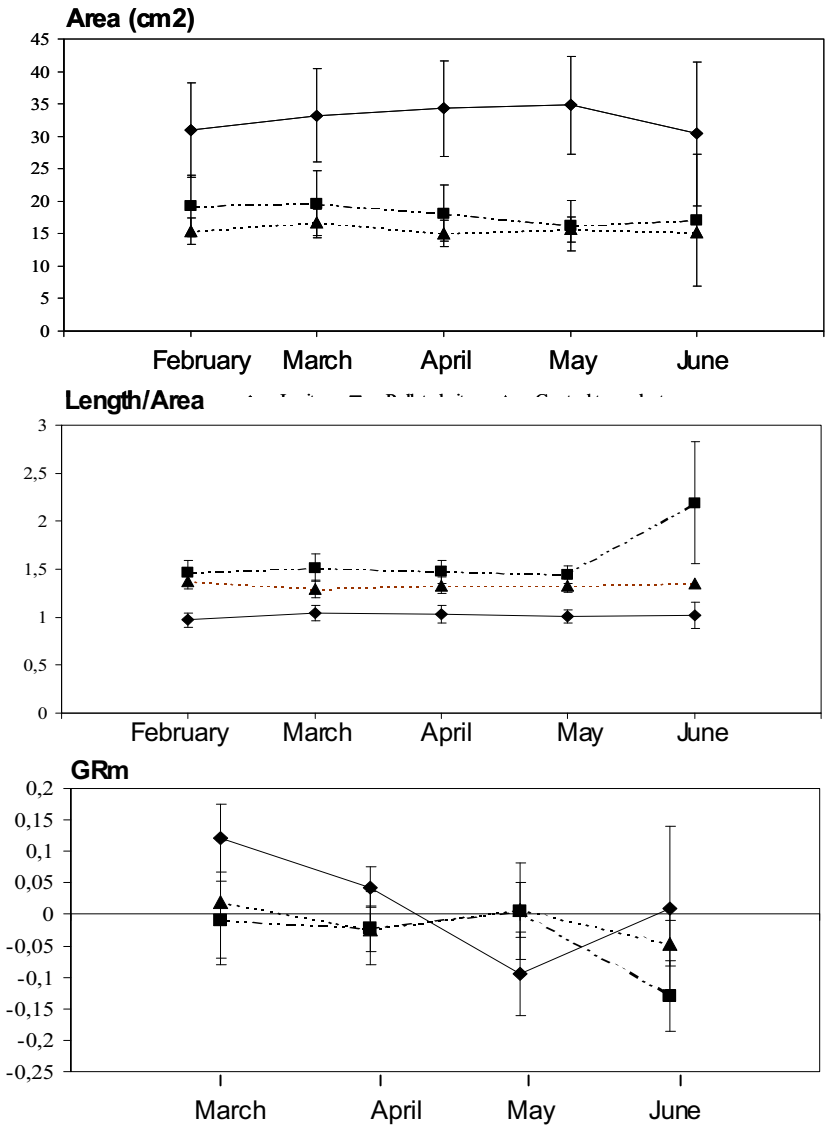


Figure 4.2. Time course of the mean sponge area (A), perimeter/area ratio (B) and monthly growth rate (C) of the specimens maintained in situ (rhombus) transplanted to the control site (triangles) or to the polluted site (quadrates). Vertical bars are standard errors.

Percentage of randomisation SS that exceeds the observed sum of squares			
Source of variation	**Area**	**GR_m**	**Perimeter/Area**
Site	0.0152	0.1936	0.0066
Time	0.1916	0.4782	0.5354
Site&Time	0.9586	0.4520	0.9992

SS: Sums of squares

Table 4.1: Significance levels obtained by randomisation for the repeated measures analyses of the area, monthly growth rate and perimeter/area ratio.

No significant differences in the monthly growth rate GR_m were found between sponges in C, and those in the TC and HT treatments (Fig. 4.2C). However, although not statistically significant, the monthly growth rate of specimens in the HT group was lower than for those in C and TC in June.

Survival

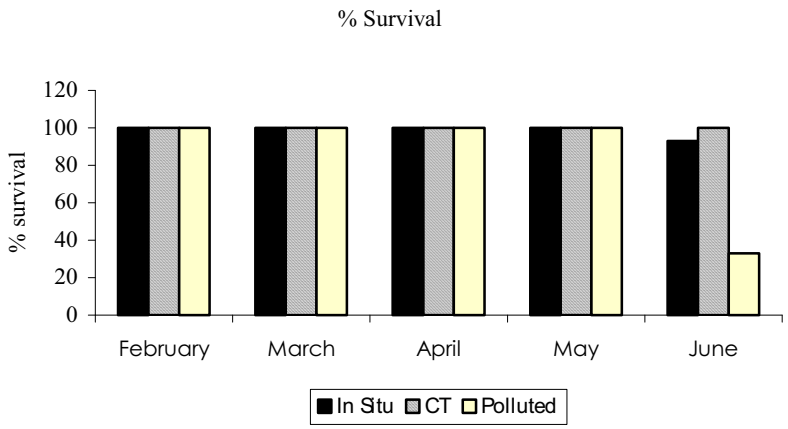


Figure 4.3. Percentage of survivors among sponges maintained *in situ* and those transplanted to the control and polluted sites.

Survival was 100% in all cases from February to May (Fig. 4.3). In June, this variable was significantly lower for the HT specimens (33%) than for C (93%) or TC (100%) (Tukey-test, p=0.0002).

Collagen content

The proportion of collagen was significantly higher (ANOVA, p< 0.05) in the HT group (Fig. 4.4) than in C or TC sponges (Tukey-test; p>0.05), the two latter groups with similar amounts of collagen.

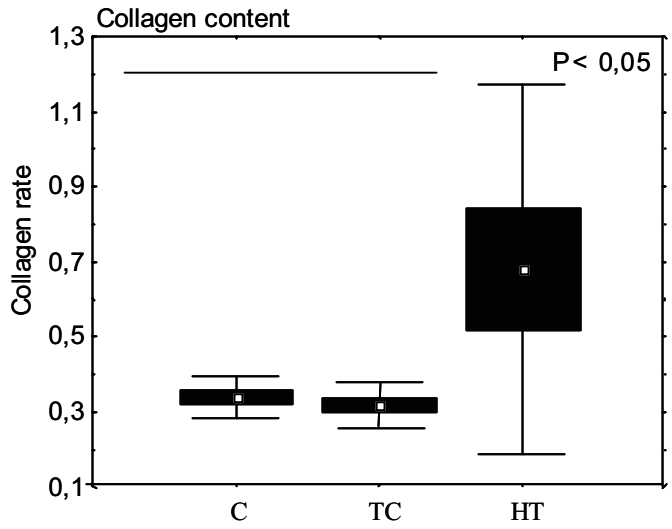


Figure 4.4. Mean collagen content for specimens maintained in situ and those transplanted to the control and polluted sites. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Clearance rate

Clearance rates of the specimens incubated varied depending on the prey group considered. Although not statistically different from each other (Tukey t-test; p>0.05) clearance rate for picoeukaryotes and *Synechococcus* sp. was higher than that on heterotrophyc bacteria and nanoeukaryotes. Differences in clearance rates for picoeukaryotes and *Synechococcus* sp. (the most efficiently retained) were significantly higher in the TC group than in HT sponges (t-test; p<0.05) (Fig. 4.5).

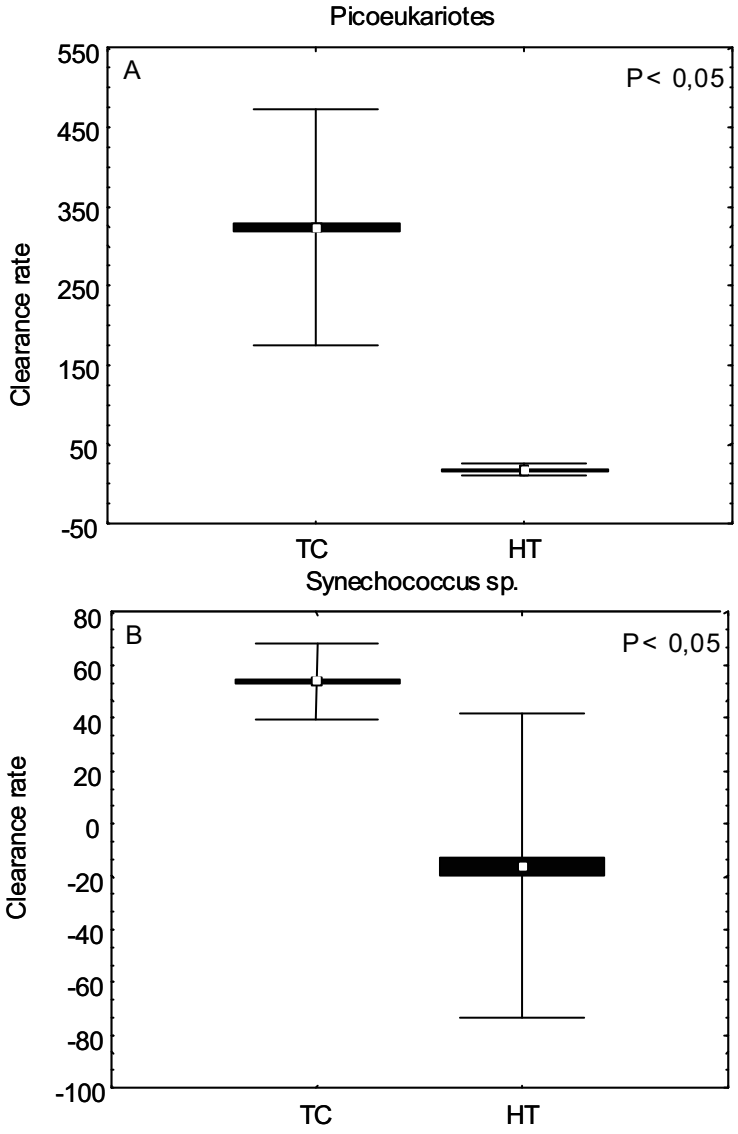


Figure 4.5. Mean clearance rate for Picoeukariotes (A) and Synechococcus (B) of specimens incubated at the control site and at the polluted site.

HSPs

The monoclonal antibody against bovine HSP70 used in this study cross-reacted in *Chondrosia reniformis,* showing one band of 60 kDa. In the field experiment, no significant differences in HSP expression were observed between treatments (C, TC, HT) (Randomised blocking design, F= 0.087, p>0.05) (Fig.4.6).

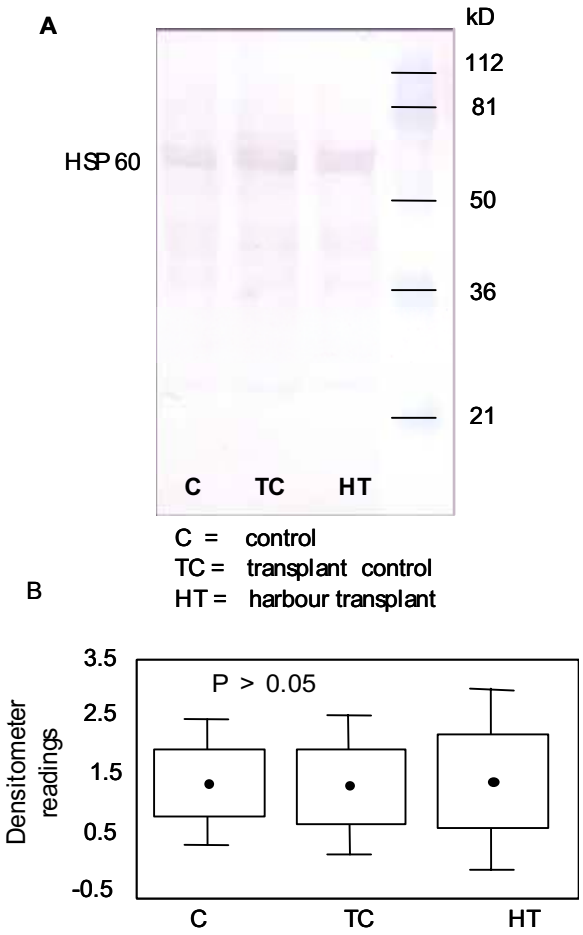


Figure 4.6. (A) Western blot of *Chondrosia reniformis* heat-shok proteins (HSP) from the field experiment (N=20). Bands in the right lane are molecular-weight markers, which were run in parallel. The bands corresponding to Hsp60 are indicated on the left. (B) Densitometer readings of Hsp60 bands in sponges from the field experiment.

Similarly, no significant increase in HSP production was observed in the laboratory experiment between copper treatments and controls (Randomised blocking design, F= 1.944, p>0.05) (Fig. 4.7).

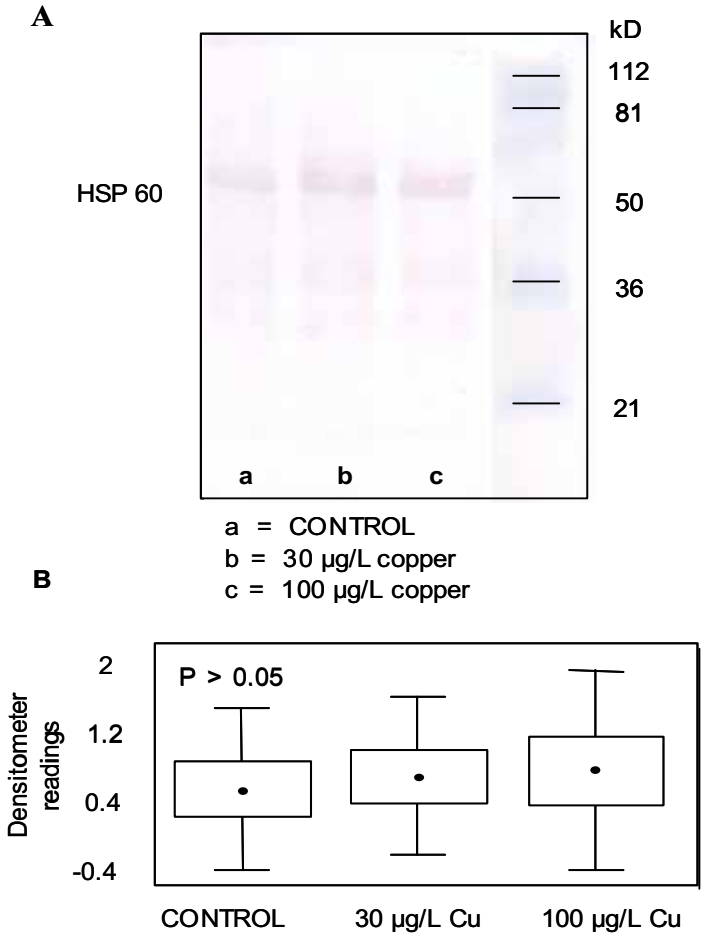


Figure 4.7. (A) Western blot of *Chondrosia reniformis* heat shock proteins (HSP) from the laboratory experiment (N=15). Bands on the right lane are molecular-weight markers, which were run in parallel. The bands corresponding to Hsp60 are indicated on the left. (B) Densitometer readings of HspP60 bands in sponges from the laboratory experiment.

Discussion

Several sponge species resistant to pollution have been proposed as indicators of water quality (Carballo and Naranjo, 2002). In contrast, we selected for the study a species typical of clean waters in order to ascertain its response to metal-contaminated environments.

In contrast to that reported for the sponge *Crambe crambe*, which inhabited moderately polluted environments (Cebrian et al., 2003; *Chapter 3*), no effect was observed in growth and shape, Hsp expression and metal accumulation in the sponge tissues of *C. reniformis*. However, although no changes were manifested externally, a lower survival rate of the sponges transplanted into the harbour and a decrease of their clearance rates on picoeukariotes and Synechococcus suggests a decline in the sponge physiological conditions. Moreover, a collapse of its molecular repair mechanisms (no changes in HSP), indicate a strong negative effect of the polluted environment.

Growth rates close to 0 are typical of many Mediterranean sponges in winter (Wilkinson and Vacelet, 1979; Turon et al., 1998). If metal pollution affects sponge growth, we would expect negative GRm for the sponges transplanted to the polluted site. Conversely, no significant differences in GRm were found between treatments. Similarly, no changes in external shape were found. Thus, no effects on these biological variables due to heavy metal contamination (mainly copper) were observed, although the thick collagen cortex of this species may mask a decrease in cellular elements and thus in our GRm estimation.

Stress proteins of the HSP70 family did not show significant enhancement in *C. reniformis* transplanted to the polluted zone, in contrast to that reported for *C. crambe* (Agell et al. 2001, *Chapter 5*). Neither was the synthesis of stress proteins induced in the laboratory under high copper concentrations. We interpret this lack of response as due to a strong metabolic damage, because the lower survival rate at the polluted site indicates extreme adverse conditions for the species.

Clearance rates were significantly lower for the sponges incubated in the harbour. It seems that the higher copper concentration in the harbour reduce sponge filtration rates as it has been reported for cadmium (Olesen and Weeks, 1994). Lowering or stop of water pumping and the associated anoxia may explain the high sponge mortality at the polluted site when temperature exceeded 20˚C and oxygen concentration in water decreased. A continued reduction in pumping may result in cell starvation, which has been reported to induce cell apoptosis (Nickel and Brümmer, 2003). The consequent decrease in cellular elements may be responsible for the increase in the collagen/cell rate observed in the sponges transplanted to the harbour. The decrease in pumping rates may also explain the lack of copper accumulation in the specimens at the polluted site. Accordingly, this species accumulates copper at a

lower concentration (both inside and outside the harbour) than other sponges (e. g. *Crambe crambe*, Cebrian et al., 2003, *Chapter 2* and *3*) and ascidians (e.g. *Clavellina lepadiformis*, De Caralt et al., 2002) in the same habitat. The latter species are presumably more resistant to pollution and may withstand higher concentrations of metals in their tissues.

Our results show that *Chondrosia reniformis* does not denote gradual ecological changes in its morphological, or biochemical responses and therefore it cannot be considered a fast biological monitor. Conversely, this species can be classed as a "sensitive organism" since copper pollution causes changes in its physiology, which may lead to death. The effects of pollution in sensitive benthic organisms such as this sponge may represent a "watch alarm" that can help to prevent major changes at the community level if the adverse conditions are prolonged.

Acknowledgements

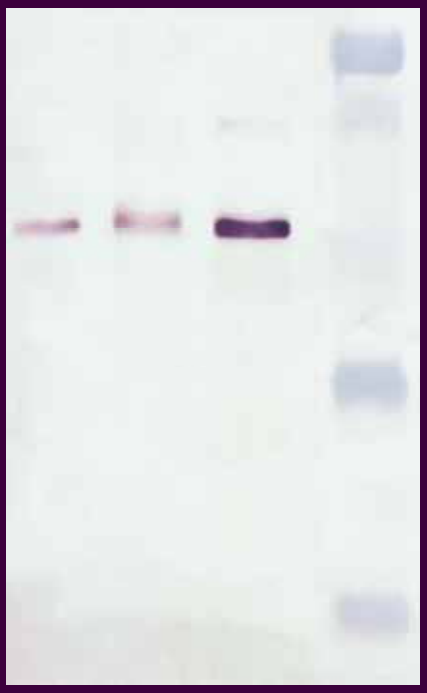
We thank the S.C.T. of the University of Barcelona for heavy metal analyses and X. Turon for his help with the statistical analyses. S. de Caralt and S. López-Legentil helped us with the fieldwork and A. Sánchez from IIQAB-CSIC with image analysis. The flow cytometry facility was supplied by CMIMA with the assistance of M. Ribes.

Resum

Capítol 4

Respostes de l'esponja mediterrània *Chondrosia reniformis* a la contaminació per metalls pesants.

S'han estudiat els efectes de la contaminació per metalls en l'esponja *Chondrosia reniformis*, trasplantant esponges d'una zona control no contaminada a una zona contaminada principalment per coure. No s'han observat efectes de la contaminació en el creixement i forma de l'esponja, inducció de les proteïnes d'estrès i acumulació de metalls pesants. Per contra, després de 4 mesos, les esponges trasplantades a la zona contaminada han experimentat una disminució en les taxes de filtració, un augment en el contingut de col·lagen i una supervivència més baixa. Això confirma un fort efecte de la contaminació en aquesta esponja. Les condicions ambientals de la zona contaminada semblen induir una reducció dràstica de les taxes de bombeig de l'esponja, procés que indirectament provoca una reducció de l'alimentació i la conseqüent disminució dels elements cel·lulars, comportant un increment de la proporció de col·lagen. El resultat final és una alta mortalitat de l'esponja.



Chapter 5

Does stress protein induction by copper modify natural toxicity in sponges?

Chapter 5

Does stress protein induction by copper modify natural toxicity in sponges? [1]

Abstract

Crambe crambe is a toxic Mediterranean sponge, which inhabits the sublittoral rocky bottoms, including some contaminated habitats. We investigated whether contamination by copper induced stress proteins in *C. crambe*, and whether such stress might alter the production of chemical defenses. The monoclonal antibody used cross-reacted with two heat-shock proteins of 54 and 72 kDa. Both proteins were induced to a greater or lesser extent by copper contamination. HSP54 accumulated more than HSP72, which, in contrast, appeared to respond faster and be less persistent. In a field experiment, we found a higher accumulation of HSP54 in individuals naturally inhabiting a copper-contaminated site than in those transplanted to this site four months earlier. In contrast, HSP72 was only significantly induced in the individuals transplanted to the contaminated site. In the laboratory, both proteins were induced by copper 30 µg/L but inhibited at 100 µg/L**.** The highest mean values of HSP54 and HSP72 corresponded to the sponges, which showed the lowest mean values of toxicity. Thus, toxicity and production of HSP displayed opposite trends, which seems to indicate a preferential investment in cell repair at the expense of toxic molecules under stress conditions.

[1] Agell G, Uriz MJ, Cebrian E, Martí R (2001). Does stress proteins induction by copper modify natural toxicity in sponges? Environ. Toxicol. Chem. 20 (11) : 2588-2593.

Introduction

Organisms that live in environments exposed to stressful conditions produce protection responses at ecological (Brown, 1997), organism (Menge and Sutherland, 1987) and cellular (Lindquist, 1986; Morimoto et al., 1990) levels. A number of behavioral, morphological and physiological traits minimize the probability that tissues and cells will be exposed to critically high levels of natural stressors. However, despite these adaptations, organisms may still produce metabolic responses that prevent cell damage when avoidance traits are insufficient and cells are exposed to particularly high levels of stressors. One well-conserved defense response involves the induction of heat-shock or stress proteins (Atkinson and Walden, 1985; Parsell and Lindquist, 1990; Sanders, 1993), which protect organisms from the damage produced by exposure to heat, Ultra-violet light, trace metals and xenobiotics (Sanders, 1990). The main groups of these proteins have been categori**z**ed by their molecular mass, and families of 60, 70 and 90 kDa are the most frequent and well-known. Heat-shock proteins (HSP) may be both constitutive and inducible (Lindquist, 1986; Morimoto et al., 1990). Constitutive forms modulate protein folding, transport, and repair (Parsell and Lindquist, 1993; Sanders, 1993). When a stressor induces their synthesis, they also protect proteins from denaturation and aggregation, and facilitate protein repair and degradation of harmful aggregates (Lindquist, 1986; Sanders, 1993; Coleman et al., 1995).

Both constitutive and environmentally inducible stress proteins may play a relevant role in the ecology of benthic sessile organisms, which cannot move away from the stress source. One of the current sources of stress in aquatic organisms is associated with sublethal levels of chemical contamination. The stress response appears to be linked to the species uptake and metabolism, which vary according to the organism and the contaminant considered (Parsell and Lindquist, 1994).

Sponges have rarely been explored for heat-shock protein expression after exposure to environmental stressors. Their induction by heat has been studied in *Suberites domuncula* and *Geodia cydonium* (Bachinski et al., 1997; Koziol et al., 1997; Krasko et al., 1997). As for their induction by contaminants, the non-ionic organic fraction from a polluted river, some PCBs, and Cadmium have been assayed in *Ephydatia fluviatilis* (Müller et al., 1995), *Geodia cydonium* (Wiens et al., 1990), and *Suberites domuncula* Müller et al., 1998), respectively.

Sponges produce an array of secondary metabolites, which display bioactivity against cells and microorganisms (Martin and Uriz, 1993). *Crambe crambe* is one of the most widespread and best defended chemically sponges in western Mediterranean (Uriz et al., 1992; Becerro et al., 1995). It produces a series of toxic molecules (Berlinck et al., 1990) with antibacterial (Becerro et al., 1994), antilarval and deterrent (Uriz et al., 1996) effects. Its toxicity may be induced by environmental factors (Becerro et al., 1995).

We report here the induction by copper of two stress proteins belonging to the HSP70 and HSP60 families in *C. crambe*. Sparse but healthy individuals of *C. crambe* naturally occur and even reproduce in copper contaminated habitats such as small harbors (authors pers. obs.). We also examine the effect of contamination on *C. crambe*'s chemical defenses, as measured by its natural toxicity, in parallel to the induction of stress proteins.

Material and Methods

Animals and study site

We conducted experiments both in the field and in the laboratory. For the field experiment the control and polluted areas were located as close to each other as possible to minimize sponge damage and stress during transport. We found the appropriate zones in the Blanes sublittoral (western Mediterranean). The control site was a vertical west-facing rocky wall from 4 to 10 m in depth. The polluted selected site was on the inner side of the Blanes harbour breakwater, 500 m from the control zone, and consisted of a vertical concrete wall with similar facing and depth to the control site. Consequently, both walls received similar incident light, as measured by a LiCor sensor. Other physical and chemical variables, such as sedimentation rates, nutrients, particulate organic matter, Polycyclic Aromatic Hydrocarbons, and trace metals in the water at both sites were monitored monthly. Most variables behaved similarly in both zones, except copper concentration in the water and organic matter in the sediment, which were significantly higher at the contaminated site, and water movement that was lower at the polluted site.

Field experiment

A total of forty sponges were taken at random from the control zone by SCUBA diving. The sponges were collected with their corresponding rocky substrate and transplanted to the new site within one hour of collection. To control for possible damage or stress during transplantation, twenty of these specimens were transplanted within the control site. The other twenty specimens were placed in separate polystyrene bowls underwater, and transported in seawater to the polluted site. The sponges were glued to the new substratum using a two-component non-toxic epoxy resin (IVEGOR ®). Another twenty specimens were chosen at random at the control site, labeled, and left untouched to control for handling stress. Four months after transplantation, all the specimens were collected, placed in plastic bowls underwater, transported to the laboratory at 17 ºC (sea temperature), immediately scraped from the substrate and stored at –80 ºC for further analysis. A set of native sponges from the contaminated site was also collected at the end of the experiment.

Laboratory experiment

Although copper was the only significant contaminant detected at the polluted site, a laboratory experiment was run to verify whether copper and not other uncontrolled factors affected HSP expression and toxicity of sponges in the field experiment. Fifteen randomly selected individuals of *C. crambe* were taken from the control site and transported to the laboratory as described above. The experiment, which was run at constant temperature (17 ºC) and natural light/dark photoperiod, lasted for five days. Two treatments consisting of copper 30 $\mu g \cdot l^{-1}$ (approximate concentration recorded at the polluted zone) and 100 $\mu g \cdot l^{-1}$, and a control were set up. We used individual polystyrene tanks (five replicates per treatment) with 2 L of 0.2 μm filtered, aerated seawater. The copper solution ($CuCl_2$/seawater) was freshly made up and changed daily to maintain copper nominal concentrations. Seawater in the controls was also changed daily. At the end of the experiment, samples were scraped from the substrate and stored frozen at –80ºC until analysis.

HSP analysis

Samples were hand homogenized, in an ice bath, in two volumes (w/v) of a calcium and magnesium-free solution (pH 7.3) containing 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl supplemented with 1 mM dithiothreitol (DTT), phenylmethylsulfonide (PMFS) 1 mM Tripsin Inhibitor and 1 % Igepal Ca-630 (Sigma-Aldrich Chemical, St. Louis, MO, USA). Homogenates were centrifuged at 15,000 x *g* at 4ºC for 1 hour, and the supernatants were stored at –80 ºC. Total protein content was determined by the method of Lowry et al. (Lowry et al. 1951) with BSA as standard. Frozen supernatants were slowly thawed in an ice bath. They were then boiled for 5 minutes after the addition of SDS-PAGE sample buffer (Laemli, 1970).

1-D electrophoresis: Samples of equal amounts of protein (50 μg) were loaded onto 10 % polyacrylamide running gels with 4 % stacking gels. Western blot: gels

were electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using 20 mM tris-HCl, 15 mM glycine, 20 % methanol (v/v) as transfer buffer. The membranes were blocked with TBS containing 0.5 % gelatin, 0.2 % Tween 20 and 0.1 % sodium azide for 30 minutes. The membranes were then probed with a 1:2,500 dilution of monoclonal antibody directed against bovine brain HSP70 (Sigma-Aldrich Chemical, St. Louis, MO, USA). We selected this antibody because it was reported to cross-react with HSP70 in different invertebrates such as rotifers (Cochrane et al. 1991), amphipods (Werner and Nagel, 1997), mollusks (Sanders and Martin, 1993), and sponges (Bachinski et al. 1997; Müller et al. 1998). Blots were rinsed twice in tris buffered saline solution (TBS) and then blocked with 1 % bovine serum albumin (BSA) in TBS containing 0.5 % gelatin and 0.2 % Tween-20 for 30 minutes. The membranes were then incubated for 1 hour in a 1:30,000 dilution of alkaline phosphatase-conjugated goat antimouse IgG (Sigma-Aldrich Chemical, St. Louis, MO, USA). Excess of secondary antibody was removed by two washes in 0.2 % Tween 20 in TBS. Finally, to visualize the bound sites of the antibody, the substrates p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used. One replicate of each control and treatment was assayed in the same Western blot for comparison. Western blots were scanned with Bio-Rad Fluor-S^{TM} Multilmager equipment, and the band density was quantified using the software Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). Thus, semiquantitative data on protein amounts are provided since it was not possible to use pure proteins as an internal standard.

Toxicity analysis

We used a standardized toxicity test on the sponge crude extract to measure the sponge chemical bioactivity, instead of quantifying and testing secondary metabolites separately. This procedure guaranteed detection of the possible synergistic effects among the many active metabolites described in *C. crambe* (Berlinck et al., 1990; 1992). We used the Microtox® (Microbics, Carlsbad, CA, USA) bioassay (Kaiser and Ribo, 1988), which is based on measurements of bioluminescence of the deep-sea bacterium *Vibrio fisheri* (= *Photobacterium phosphoreum*). The Microtox® results correlate with those of other, more ecologically relevant assays (Kaiser and Ribo, 1988; Becerro et al., 1995) and this test performs best in terms of accuracy and repeatability (Becerro et al., 1995). Furthermore, a good correlation has been found between toxicity and the amount of bioactive metabolites (Martí et al., 2003). Toxic molecules from the sponge samples were extracted with methanol (Martí et al., 1999). Crude methanol extracts, homogeneously resuspended in seawater (through sonication) were assayed at an initial concentration of 100 µg/mL relative to sponge dry weight. Microtox® analyzes four decreasing concentrations (a dilution factor of 2 was used) per sample, and a control. Light readings were taken after incubation for 5 min at 15 ºC, and the concentration that resulted in a 50% decrease in light production (EC50) was calculated.

Specimens, both native and those transplanted to the polluted zone for four months, accumulated copper at a similar concentration of about 200 $\mu g \cdot g^{-1}$. The copper within the sponges was expected to become toxic during sample manipulation for the Microtox assay (extraction and dissolution of the crude extract in low salinity water). Thus, in order to determine the natural toxicity of the sponge, independent of that due to the copper, we ran in parallel two sets of assays using five samples from the control site. The methanol extract of each sample was divided into two subsamples. One was analy**s**ed as it was while copper ($CuCl_2$) was added to the other to obtain a mean copper concentration similar to that found in the samples from the contaminated site.

Data analysis

Data from the field and laboratory experiments (HSP and toxicity values), which met the assumptions of parametric procedures, were analyzed by one-way ANOVA. The relationship between toxicity in both natural and copper-enriched samples was assessed by regression analysis.

Results

Heat shock proteins

The monoclonal antibody raised against bovine brain HSP70 (mAb) that we used cross-reacted immunologically in *Crambe crambe* with two heat shock proteins of 54 and 72 kDa (Fig. 5.1A and Fig. 5.2). However, HSP54 was expressed to a greater extent than HSP72 in all the samples. Indeed, HSP72 was hardly detected in controls, sponges naturally occurring in the contaminated site (Fig. 5.1A), and sponges incubated in 100 $\mu g \cdot l^{-1}$copper in the laboratory experiment (Fig.5.2). This protein appears to be more induced in the short term than HSP54, which, in contrast, accumulated more in the long term.

Field experiment

In the field experiment, we compared the expression of heat shock proteins among sponges naturally occurring in the polluted site, untouched individuals from a reference site (handling stress control), individuals transplanted from the control site to both control (transplant control) and polluted sites. The one-way ANOVA detected a significant effect of the contamination on HSP54 expression ($F=4.12$, $p=0.01$). *Post-hoc* tests indicated significantly ($p<0.05$) higher levels of HSP54 in specimens found in the contaminated habitat than in both controls or in specimens transplanted to the polluted site (ca. 2-fold the handling stress control).

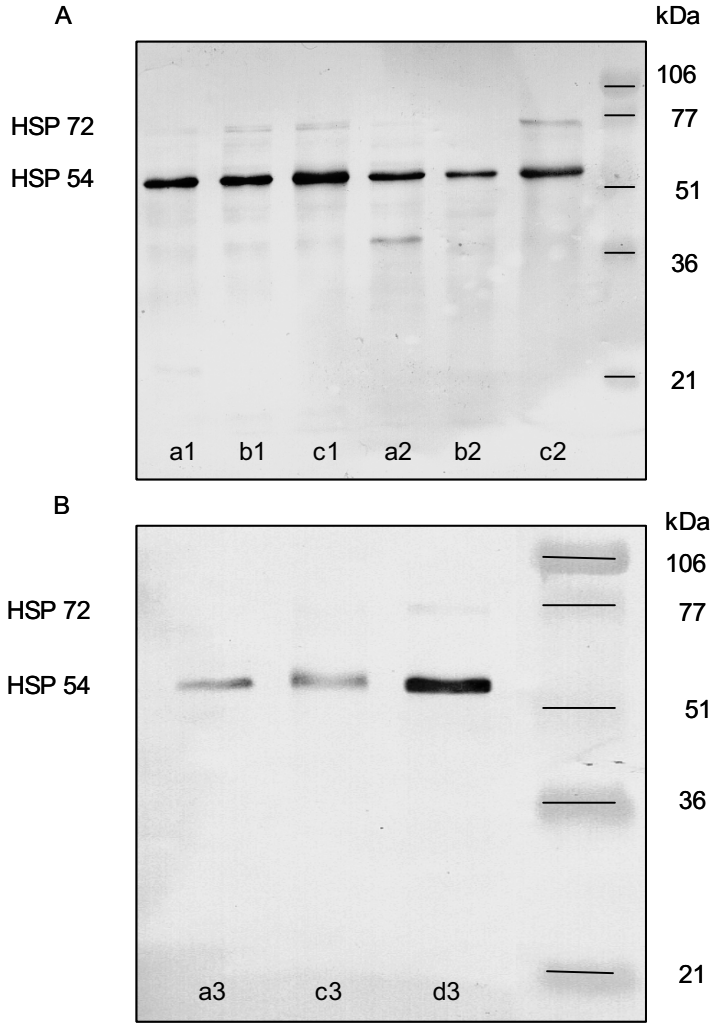


Figure 5.1. A) Western blot of *Crambe crambe* HSP proteins from the field experiment (N = 12): (a) handling stress controls; (b) transplant controls; (c) sponges transplanted to the polluted site for four months. Bands on the right lane are molecular weight markers, which were run in parallel. The bands corresponding to HSP54 and HSP72 are indicated on the left. B) Western blot of *C. crambe* HSP proteins in individuals from the handling stress control (a), transplanted to the polluted site (c) and specimens naturally occurring in the polluted site (d).

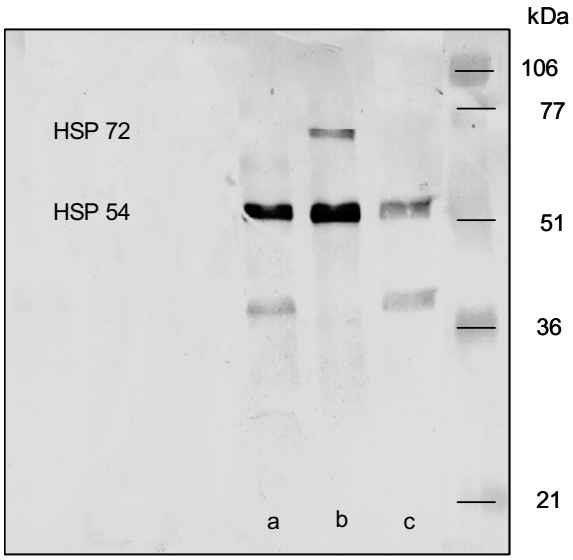


Figure 5.2 Western blot of *C. crambe* HSP proteins from the laboratory experiment (N = 5): (a) control; (b) individuals incubated in the presence of 30 mg/L copper; (c) individuals incubated in the presence of 100 mg/L copper. Bands in the right lane are molecular weight markers, which were run in parallel. The bands corresponding to HSP54 and HSP72 are indicated on the left.

Individuals transplanted to the contaminated zone showed higher protein levels than controls although no significant differences were found due to individual variability (Fig. 5.3). As for the HSP72, there was a significant effect of contamination on protein expression (one-way ANOVA, $F = 17.9$, $p < 0.001$**)** (Fig. 5.3). **T**his effect was due to the presence of a greater amount of protein (ca. 3-fold the handling stress control) in individuals transplanted to the polluted site for four months than in the two controls and the native sponges in the contaminated zone. No induction of HSP proteins due to sponge manipulation (transplant effect) was detected (Fig.5.3).

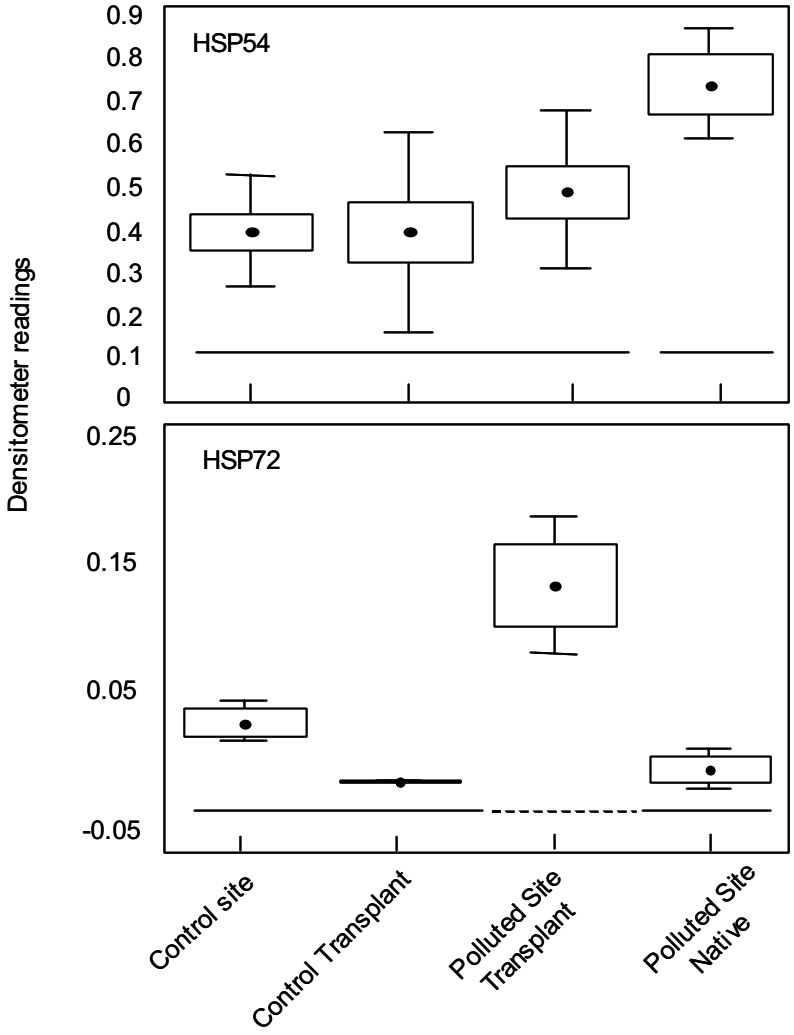


Figure 5.3. Densitometer readings of the HSP54 and HSP72 bands in sponges from the field experiment. Protein amounts, which proved not significantly different in a Tukey test were joined by horizontal lines. The dash line underlines the treatment that significantly differs (Tukey test) from those underlined by a continuous line. Boxes and vertical bars are standard errors and standard deviations, respectively.

Laboratory experiment

When we measured the effect of copper at 30 $\mu g \cdot l^{-1}$ and 100 $\mu g \cdot l^{-1}$ in the laboratory, both HSP54 and HSP72 were expressed to some extent (Fig. 5.4). The one-way ANOVA on treatment did not show significant differences in HSP54 expression ($F = 1.22$, $p = 0.34$). In contrast, HSP72 was only expressed in the presence of 30 $\mu g \cdot l^{-1}$copper (Fig.5.4).

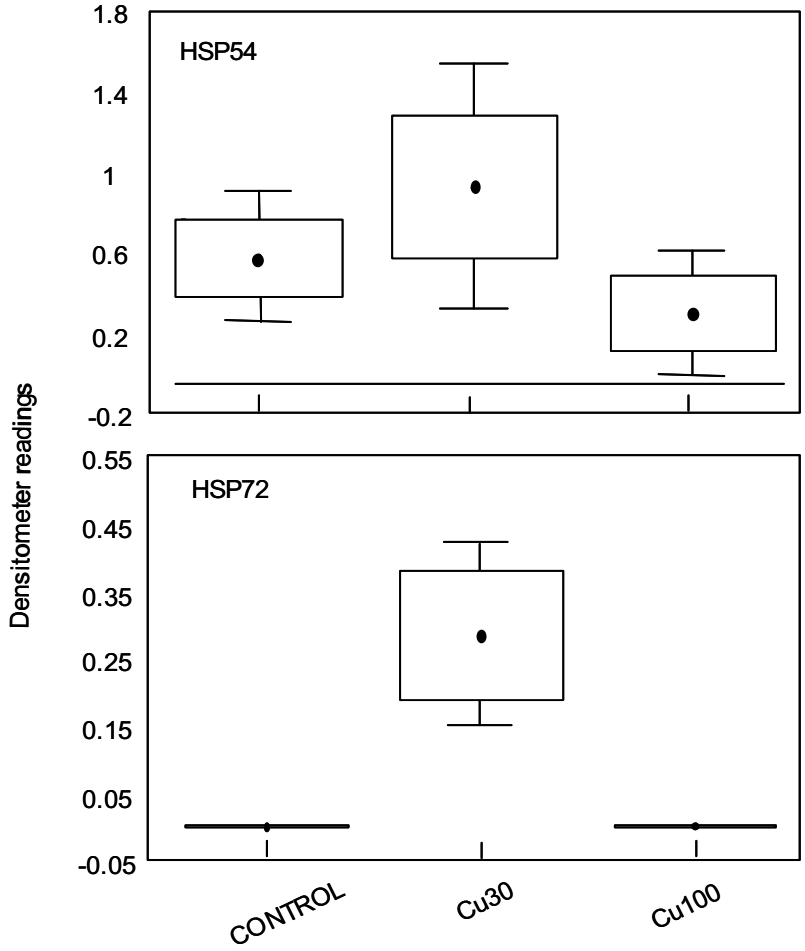


Figure 5.4. Densitometer readings of the HSP54 and HSP72 bands in sponges from the laboratory experiment. Amounts of protein that proved not significantly different in a Tukey test were joined by horizontal lines. Boxes and vertical bars are standard errors and standard deviations, respectively.

Toxicity

When we assayed individuals from the control site with and without adding copper, toxicity was consistently higher in the copper-enriched samples. The correlation between the toxicity values of samples with and without copper was high (r = 0.9) and significant ($p < 0.01$), so we were able to fit a regression equation (Fig. 5.5). We then used this equation to correct the initial values of toxicity obtained for transplanted and native samples at the contaminated site.

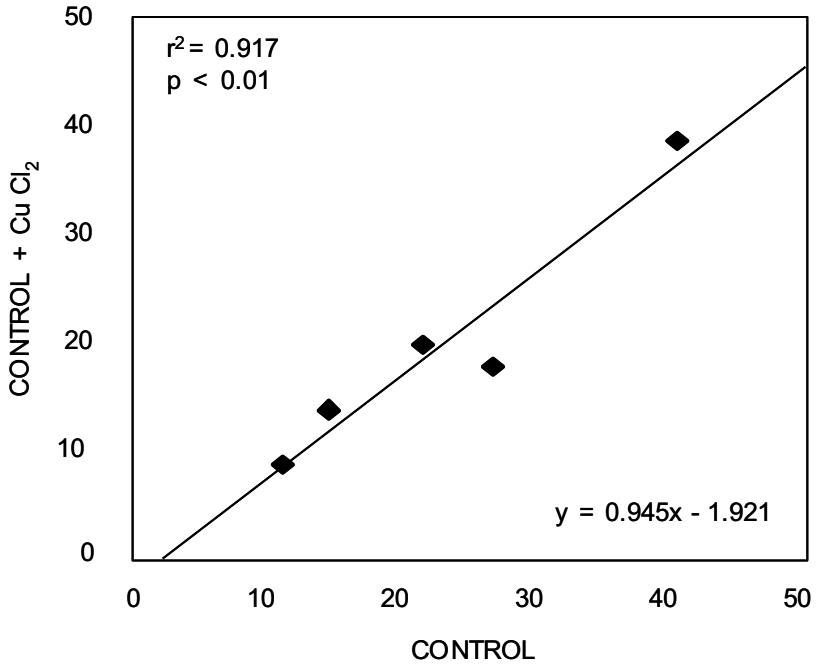


Figure 5.5. Regression line between natural toxicity (EC50) of five individuals of *C. crambe* from the control site and toxicity of the same individuals after the addition of copper (see methods). This equation was used to correct the EC50 values obtained for samples from the polluted site (transplants and native individuals).

Sponges naturally occurring at the polluted site were significantly less toxic and thus showed higher EC50 using the Microtox® assay ($p < 0.001$) than both individuals transplanted to the contaminated zone and controls (Fig.5.6A). When we analyzed the results of the transplant experiment, separately, the EC50 values were significantly higher (one-way ANOVA, $F = 15.55$, $p < 0.001$), and thus, toxicity was lower in individuals transplanted to the contaminated zone for four months than in both the

absolute and transplant controls (Fig. 5.6B). No differences were found between untouched individuals and those transplanted to the control zone ($p > 0.05$). Consequently, transplantation by itself had no effect on sponge toxicity (Figs. 5.6A and B).

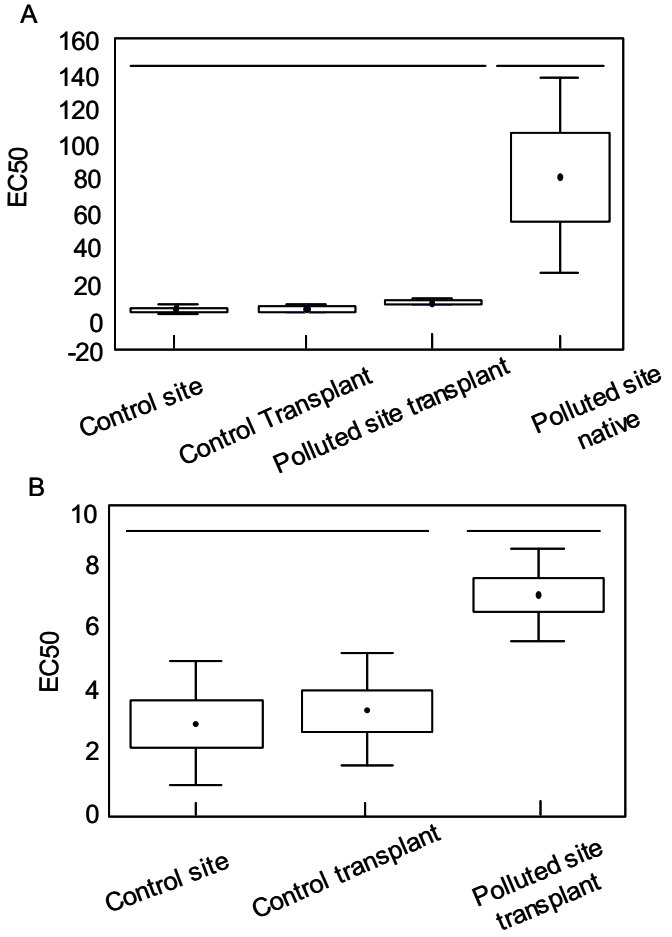


Figure 5.6. A) Toxicity values (EC50) in sponges from the field experiment and those naturally occurring at the polluted site. B) Separate representation of toxicity values (EC50) in sponges from the field experiment to highlight differences among treatments (unnoticed in Fig. A due to a larger Y axis scale). Values, which proved not significantly different in a Tukey test were joined by horizontal lines. Boxes and vertical bars are standard errors and standard deviations, respectively.

Discussion

This is the first time that induction of stress proteins by copper has been measured in sponges, and the first time that a protein of the HSP60 family has been reported in these invertebrates.

The monoclonal antibody used, which was raised against bovine brain HSP70 (mAb), immunologically cross-reacted with both the HSP72 and HSP54 kDa proteins. This antibody is known to cross-react with proteins of different molecular weights: 70 and 72kDa proteins in mammals and crustaceans, a 78kDa in bivalves and echinoderms (Sanders et al., 1994), and both 78 and 72 kDa proteins in the clam *Ruditapes decussata* (Solé et al., 2000). Yet, the cross-reaction of a specific antibody with proteins of two different HSP families is remarkable and had no been reported to date in sponges.

HSP54 and HSP72 proteins were expressed under copper contamination in the sponge *C. crambe*. However, HSP54 accumulated more than HSP72 in the long term, which appears to respond faster and be less persistent. In our field experiment, we found a higher accumulation of HSP54 protein in individuals of *C. crambe* inhabiting the copper contaminated site during their whole life (i.e more than five years, authors pers. obs.) than in those transplanted to the contaminated site for four months. Native specimens appeared healthier and reproduced more than transplanted individuals (Cebrian et al. 2003, Chapter 3), which indicates an acquired copper tolerance.

Correlation has been reported between the amount of stress proteins expressed and the ability to survive subsequent, more severe stress, which would otherwise be lethal to the organism (Lindquist, 1986; Sanders, 1993). That is, tolerance to copper appears to be enhanced in *C. crambe* as long as expression of HSP54 protein is high. In contrast, HSP72 was almost negligible in *C. crambe* specimens inhabiting the contaminated site while it was induced in the individuals transplanted to this site. This protein seems to be mainly induced in *C. crambe* while HSP54 may be both constitutive and inducible.

In the laboratory experiment, both proteins seem to be induced to a certain extent by 30 µg/L of copper but inhibited at 100 $\mu g \cdot l^{-1}$although only for HSP72 the differences were significant. Inhibition of the HSP production has also been reported above 200 $\mu g \cdot l^{-1}$copper for the seaweed *Enteromorpha intestinalis* (Lewis et al., 1998), and 50 $\mu g \cdot l^{-1}$for rotifers (Cochrane et al., 1991). This effect has been ascribed both in plants and animals to metabolic damage.

Expression of HSP was slightly higher in the laboratory experiment than in the field at comparable nominal concentrations of copper. It has been reported that similar nominal concentrations of heavy metals produce stronger noxious effects in organisms when they are assayed in the laboratory than in the field (Solé et al., 2000).

This may be due to metal binding to the organic matter, which is more abundant at sea and may reduce the bioavailability of active chemical species.

Although HSP54 appeared to be constitutive in *Crambe crambe*, its expression was significantly induced after long-term exposure to copper (specimens living at the contaminated zone). As for the amount of this protein, no significant response was observed after five days or four months of exposure to copper in the laboratory and in the field experiment, respectively. Persistence of the induced stress proteins has been considered a good basis for using stress proteins as biomarkers of exposure. Consequently, members of HSP60, which are produced and operate in the mitochondria (Ostermann et al., 1989), appear to be suitable biomarkers in this sponge but only for chronic copper contamination as it has been previously reported for other organisms (Sanders and Martin, 1993).

In contrast, HSP72 was quickly induced after both 5-days of exposure to copper in the laboratory and four months at sea. This protein appears to be a better potential biomarker of isolated contamination events.

Toxicity and production of HSP54 and HSP72 proteins displayed opposite trends. The highest mean values of HSP54 (native individuals) and HSP72 (individuals transplanted to the contaminated site) corresponded to the lowest mean values of toxicity (see Figs. 6A and B). This may be due to a preferential investment in cellular repair under stress. The production of both HSP proteins and chemical defenses are considered to be metabolically costly (Feder el al,. 1992; Fargeström et al., 1987). Thus, the resources available to the sponge must be partitioned among protein repair, production of chemical defenses, and other biological functions (Uriz et al., 1995). Taken together, these data indicate that, with copper contamination, investment in protein repair appears to be critical and, thus, may be enhanced at the expense of toxic molecules. However, other chemically defended invertebrates and plants need to be studied to ascertain the extent of this metabolic behavior.

Acknowledgements

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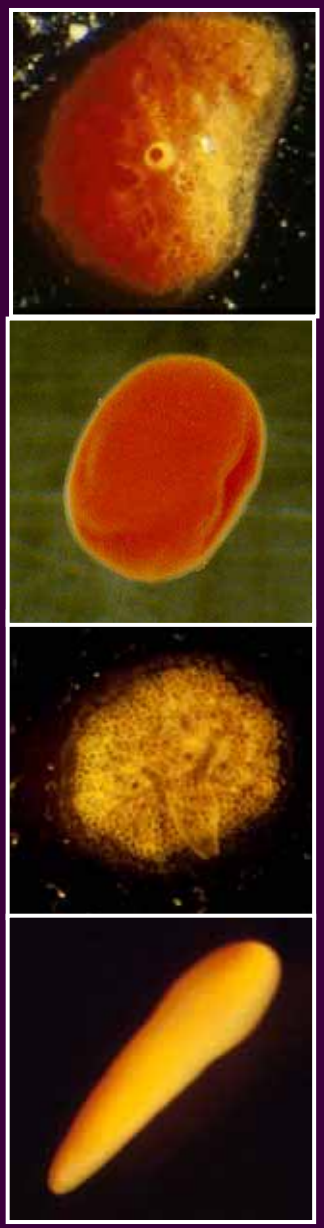
Capítol 5

Es produeixen canvis en la toxicitat natural de les esponges quan hi ha una inducció de les proteïnes d'estrès?.

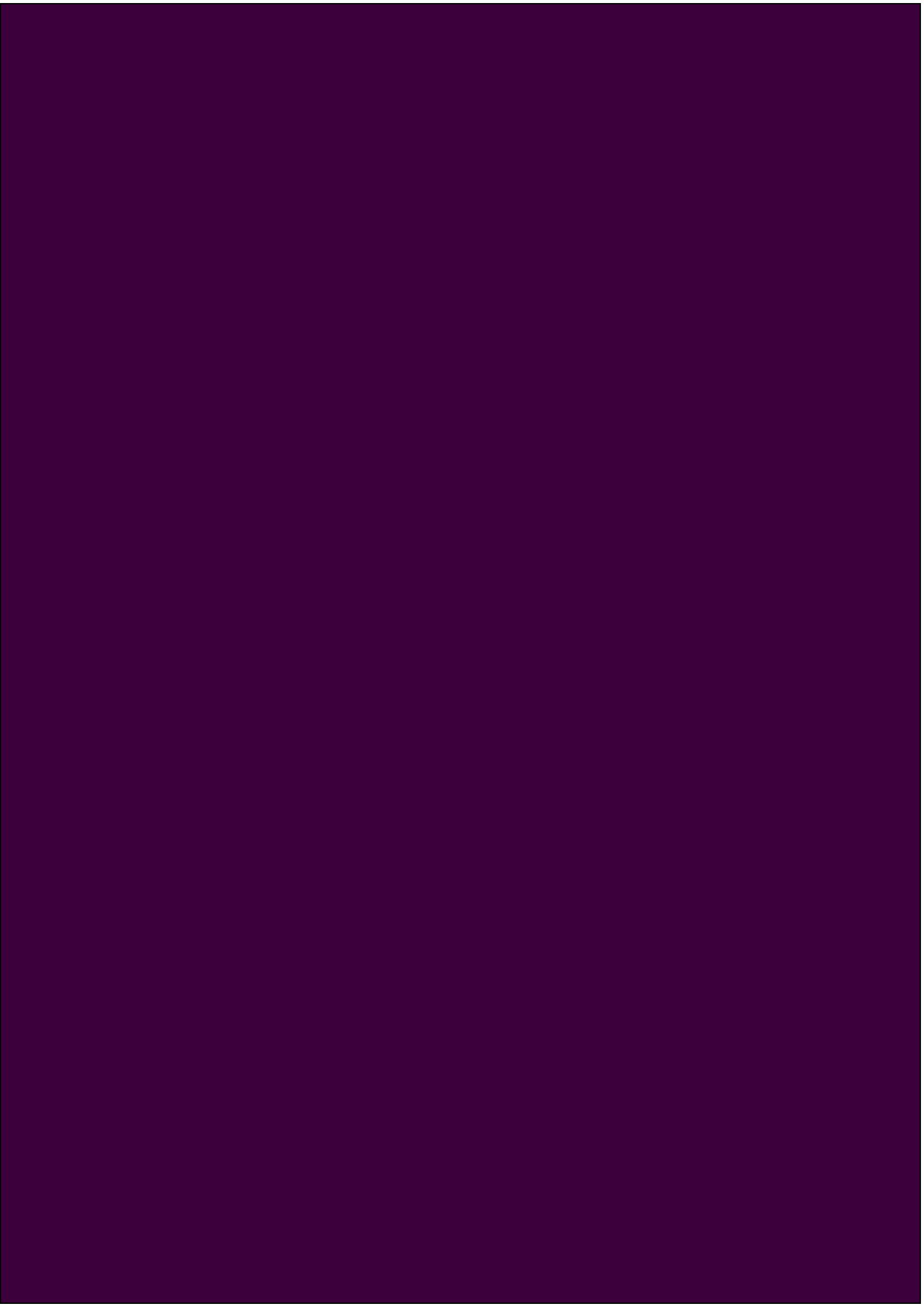
Crambe crambe és una esponja mediterrània que produeix substàncies tòxiques i que habita en els fons rocosos sublitorals, inclosos alguns hàbitats moderadament contaminats. En aquest estudi, s'ha investigat si la presència de coure produeix estrès en l'esponja, mesurant-lo mitjançant la quantificació de proteïnes d'estrès (HSP), i si aquest estrès altera simultàniament la producció de defenses químiques (bioactivitat) a l'esponja. Dos tipus d'HSP diferents, de 54 i 72 Kda respectivament, van ser induïdes en més o menys mesura per la contaminació per coure. L'HSP54 és mes abundant que l'HSP72, la qual, en canvi, va respondre amb molta rapidesa encara que va ser poc persistent. En l'experiment de camp, s'ha trobat una major concentració d'HSP54 en els individus que viuen de forma natural a la zona contaminada que en els individus trasplantats allà durant 4 mesos. En canvi, només l'expressió d'HSP72 ha estat induïda en els individus que han estat trasplantats a la zona contaminada. A l'experiment al laboratori, ambdues proteïnes han estat induïdes pel coure a concentracions de 30 µg/L, mentre que han estat inhibides a concentracions de 100 µg/L de coure. Els valors més alts d'HSP54 i HSP72 els presenten les esponges que han mostrat els valors més baixos de toxicitat. Segons això, la toxicitat i la producció de les HSP estan negativament correlacionades y segueixen, per tant, tendències oposades. La conclusió és que, en condicions d'estrès, les esponges inverteixen prioritàriament en mecanismes de reparació cel·lular a expenses de la síntesis de molècules tòxiques.

Part C



Chapter 6

Contrasting effects of heavy metals on larval settlement and juvenile survival in sponges.



Chapter 6

Contrasting effects of heavy metals on larval settlement and juvenile survival in sponges. [1]

Abstract

Metals contaminate sediments and waters of coastal areas threatening early stages of invertebrate development. Effects on these stages may largely determine the decline and even disappearance of invertebrate populations in polluted environments. Our study aimed to determine the possible influence of metals (Cu and Cd) on larval settlement and consecutive survival of two widespread sponges of the Mediterranean: *Crambe crambe* and *Scopalina lophyropoda*. Larvae of both species were exposed to Cu and Cd for a short period during one week, and settlement and following (6-month) survival of juveniles were monitored. Short exposures to copper and cadmium at the concentrations used did not affect *C. crambe* settlement compared with SW control, and no effect on consecutive survival of juveniles was observed. In contrast, short pulses of copper and cadmium at the concentrations used enhanced *Scopalina lophyropoda* settlement and did not affect the consecutive survival of juveniles with respect to SW controls. The present study provides the only available data on toxicity of copper and cadmium on sponge larval settlement.

[1] Cebrian E, Uriz MJ. Contrasting effects of heavy metals on larval settlement and juvenile survival in sponges. Aquatic Toxicol. *In press*.

Introduction

Significant amounts of pollutants have been released to marine and estuarine environments during the last few decades. Among them, metals are contaminating sediments and waters in coastal areas (Palanques et al., 1998; Puig et al., 1999). These contaminants may have great toxic effects and can be accumulated in organisms through the trophic chains, making them a significant threat to the sustainability and fitness of marine ecosystems (Brown and Ahsnullah, 1971; Berthet et al., 1992; Canesi et al., 1999).

Copper is one of the metals most abundant in the Mediterranean sublittoral areas (Tankere and Statham 1996) with harmful effects on marine invertebrates described (Ahsanullah and Florence 1984, Reichelt- Brushett and Harrison 2000, Negri and Heyward 2001, Viant et al., 2002). The main known sources of copper are the antifouling paints that are being used on small (under 25 m length) vessels (Adair 1987, Claisse and Alzien, 1993), sewage discharges (Bryan et al., 1987; Vale and Harrison 1994) and fungicides and herbicides used in coastal agricultural crops (Clark et al., 1993, Cheadle et al., 1999). Cadmium is another relevant pollutant from industrial discharge and is highly toxic to a variety of aquatic animals (e. g., Eisler, 1985; Selck, 1998; Au et al., 2001).

Sponges are among those invertebrates that can be frequently found in zones subjected to moderate levels of metals and hydrocarbons (Carballo et al., 1996; Carballo and Naranjo, 2002; Cebrian et al., 2003; Pérez, 2005). Although moderate pollution may seriously affect adult sponge physiology (Olensen and Weeks, 1994; Cebrian et al., 2003), some species can survive by using several mechanisms for neutralising the potentially noxious metals. The main mechanisms are accumulation of a non- or less toxic form of metals in the tissues (Féral et al., 1979; Zahn et al., 1981; Patel et al., 1985; Verdenal et al., 1990; Hansen et al., 1995; Cebrian et al., 2003; Pérez et al., 2005), induction of stress proteins (HSP family) (Agell et al., 2001), and binding by metallothionein or metallothionein-like proteins (Schröder et al., 2000; Berthet et al., 2005). However, data concerning toxicity of metals on marine larvae is, in general, scarce and no studies on the effects that these contaminants may produce on early phases of the sponge life cycle (larvae and settlers), have been conducted until now.

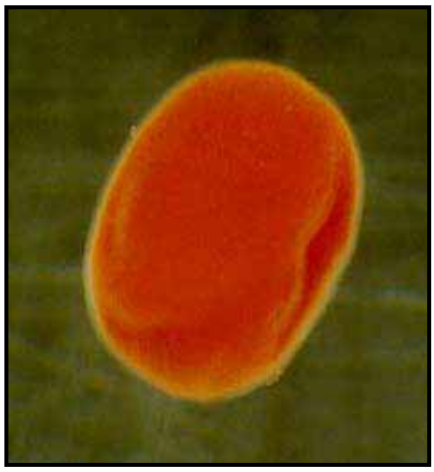
Sponge populations mainly rely on their larval settlement stages for maintenance (e. g. Blanquer, A., Uriz, M.J., Caujapé-Castells, J., unpublished data). Those early stages may be more sensitive to pollutants than adults, as has been reported for other invertebrates (Connor, 1972; Martin et al., 1981; reviewed in His et al., 1999). Sublethal effects of metals may have drastic repercussions at an ecological level when they alter biological processes of the organisms that indirectly affect to successive populations. For instance, a pollutant may kill half of the individuals of a

species population with little or no ecological significance, whereas a pollutant that does not kill organisms but retards their development may have a considerably higher ecological impact (Moriarty, 1983). Thus, sensitivity of larvae and juveniles to low levels of pollution may largely determine a subtle decline and even disappearance of sponge populations in polluted environments. Consequently, knowledge on the pollution effects on adult sponges is not sufficient for a full understanding of its ecological influence, but effects on sponge larvae and juveniles have also to be taken in consideration in identifying hidden threats to sponge populations. Hence, our study was aimed to determine the possible influence of heavy metals (Cu and Cd) on larval settlement and following survival of two representative sponge species of the Mediterranean (*Crambe crambe* and *Scopalina lophyropoda*).

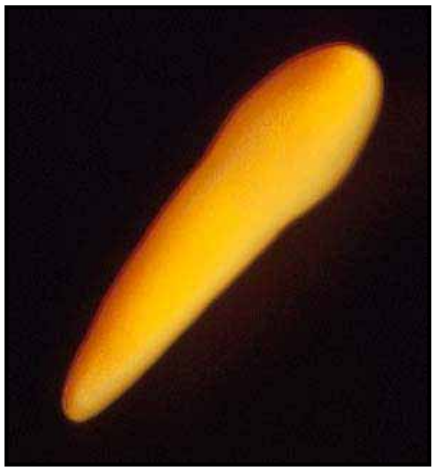
Materials and methods

Larvae collection

The species selected for the study were *C. crambe* and *S. lophyropoda*, two common encrusting sponges in western Mediterranean (Boury- Esnault, 1971; Uriz et al., 1992).



Crambre crambe



Scopalina lophyropoda

Both species reproduce during July-August at the study site (Blanes sublittoral, NE Spain, 41°40'37"N, 2°47'30"E), and release their parenchymella larvae to the water simultaneously (Uriz et al., 1998). Thus, larvae of both species coexist for a couple of months in the surroundings of adult populations. *C. crambe* larva is a type II parenchymellae (according to Mariani et al., 2005) with uniform dark orange to red

colour. *S. lophyropoda* larva is an elongated, type I, parenchymella (Mariani et al., 2005), with uniform bright orange colour.

Larvae were obtained from ripe specimens of *C. crambe* and *S. lophyropoda*. Specimens of both species were collected by scuba diving at Santa Anna Point, (Blanes sublittoral, NE Spain, 41°40'37"N, 2°47'30"E) a relatively unpolluted area, and transported to the laboratory in hermetic plastic bags. Larval release often occurred spontaneously during transport or consecutive acclimation in the laboratory.

Bioassays

Effects of Cu^{2+} and Cd^{2+}

Assays were carried out in sterile polystyrene culture tanks (500 ml). Five replicate tanks were used for each toxicant concentration as well as for seawater controls. Ten larvae of each species per container were used in the metal exposures. The treatments used were: copper (30 $\mu g \cdot l^{-1}$) as cupric chloride ($CuCl_2$) and cadmium (5 $\mu g \cdot l^{-1}$) as cadmium chloride ($CdCl_2$). Controls consisted of 0.2 μm filtered seawater (SW) from a non-polluted area. Metal concentrations in the seawater controls were below the threshold levels detectable by ICP-MS. We selected these particular concentrations because they fit in the range of the concentrations that can be found in polluted coastal waters (Cotté-Krief et al., 2002; Accornero et al., 2004).

Exposures were conducted in two steps in order to assess larval settlement and juvenile survivorship, respectively.

In the first step, experiments were conducted for 7 days to assess whether low concentrations of Cu^{2+} and Cd^{2+} affected larval settlement. We quantified settlement by directly counting the number of settlers per tank at each time point. We counted as a settler the stage at which larvae were irreversibly attached to the substratum to initiate the metamorphosis. Settlement in our controls occurred from 1 to 7 days after the release as has been reported for larvae of the two species (*C. crambe* and *S. lophyropoda*) in the field (Uriz, 1982; Uriz et al., 1998; Maldonado and Uriz, 1999; Uriz et al., 2001), which indicates that our experiments had appropriate conditions.

In a second step, the water of controls and treatments of the previous experiment was changed by clean (non-filtered) seawater and juvenile survivorship was monitored weekly during the first month and then after 6 months.

In the first-step experiments, water was changed daily (in both treatments and controls) to minimise changes in the concentration of pollutants with time due to metal absorption on the container walls (Batley et al.. 1999). In the second-step experiment, the seawater that was unfiltered in order to supply some natural food to the sponges was changed twice a week. Temperature (20-22ºC) and the day/night cycle were similar to that of the sponge habitat. The concentrations referred to in the experiments

are nominal concentrations, not concentrations available to the organisms, which was not determined.

Data treatment

Differences between treatments were analysed by means of multivariate analysis of variance (MANOVAR), since data do not meet the assumptions for ANOVAR (i.e. sphericity Mauchly's test), but met Box-test requirements (Von Ende, 1993).

The results of the short-term and long-term experiments were analysed by means of MANOVAR separately for each species. One-way ANOVAs were also used to analyse differences on larval settlement for treatment at each observation time. The Tukey-test was used for post-hoc comparisons.

Data met the assumptions of normality and homogeneity of variances as assessed by the Kolmogorov-Smirnov and Barlett tests, respectively. All the analyses were performed using Statistica 6.0 package.

Results

The effect of metals on larval settlement was different between species. At the first step experiment, copper and cadmium did not affect larval settlement in *C. crambe* ($p=0.307$; Fig. 6.1A) but both metals enhanced larval settlement in *S. lophyropoda* ($p< 0.05$; Fig. 6.1B) (Table 6.2). During the first 3 days of the experiment, larvae of *S. lophyropoda* behaved similarly in treatments and seawater control (Tukey $p> 0.05$), but from day 4 on, Cu^{2+} treatment significantly enhanced settlement (Tukey $p<0.01$). Cd^{2+} also significantly enhanced settlement from day 5 on. At the end of the experiment, the percentage of settlers was significantly different between treatments themselves and between each treatment and seawater controls, being the highest in the Cu^{2+} treatment (Tukey $p<0.05$; Fig. 1B).

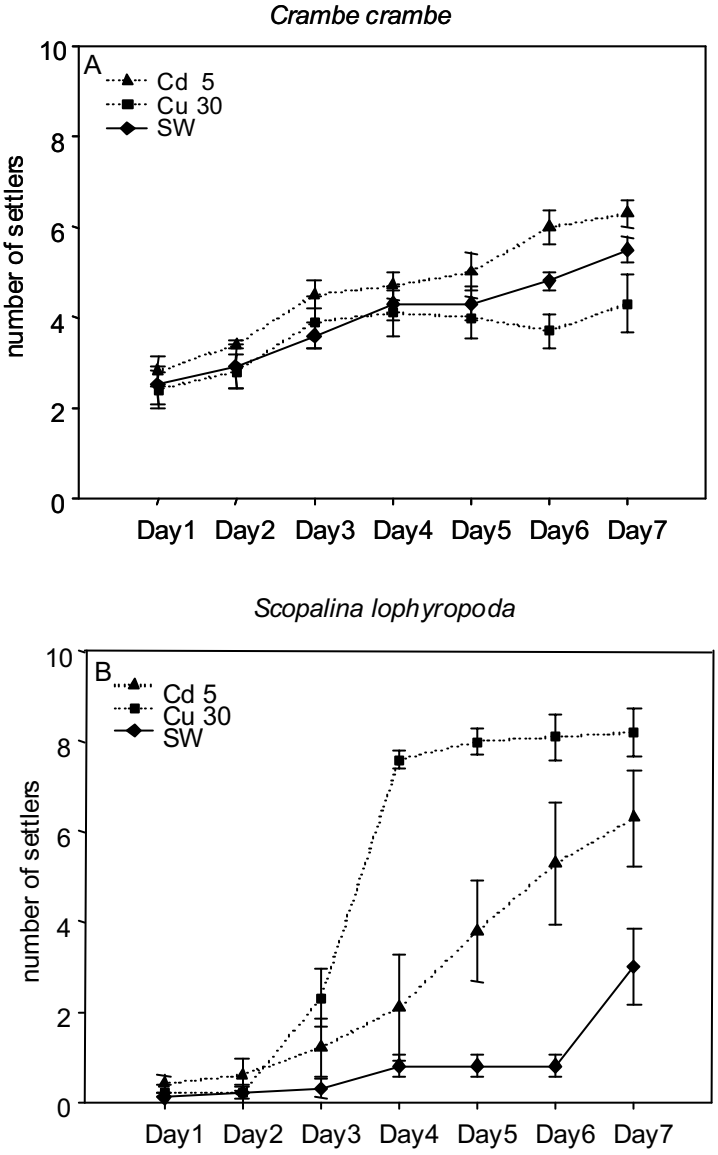


Figure 6.1. Settlement of *Crambe crambe* (A) and *Scopalina lophyropoda* (B) larvae treated with copper (30 $\mu g \cdot l^{-1}$) and cadmium (5$\mu g \cdot l^{-1}$) as a function of time. Vertical bars are standard errors. Values are from 5 replicate tanks per treatment with 10 larvae per tank.

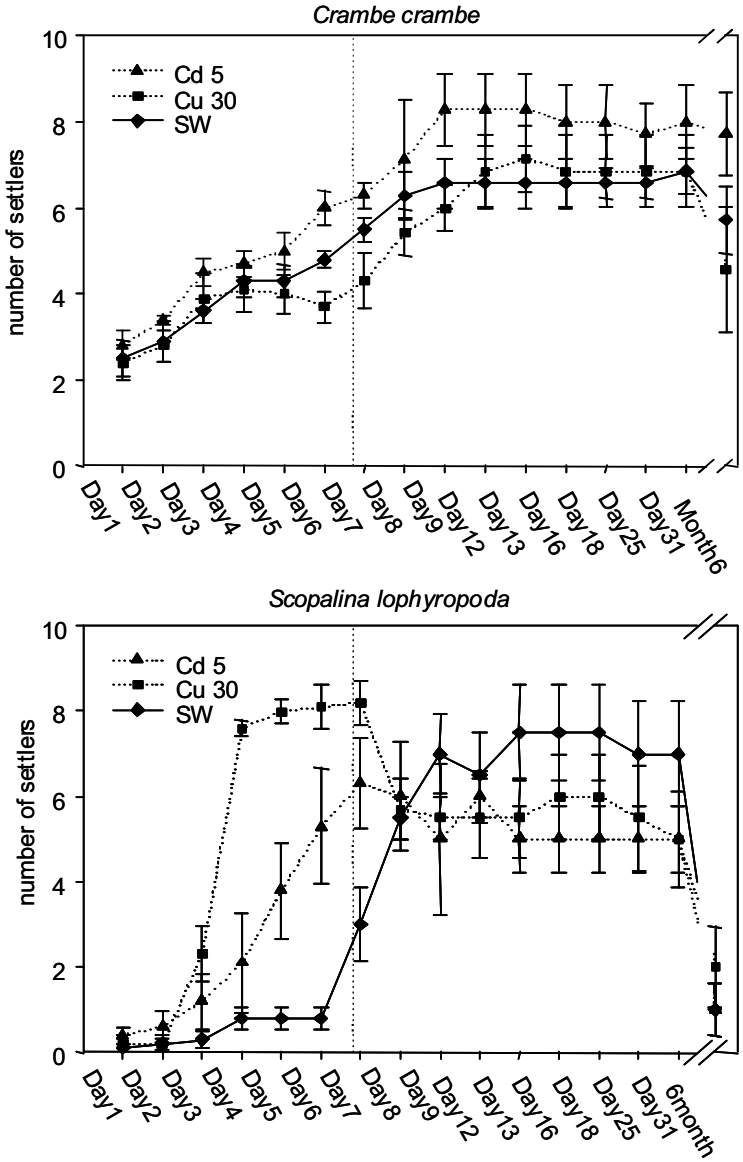


Figure 6.2. Survival of *Crambe crambe* (A) and *Scopalina lophyropoda* (B) juveniles, previously incubated under Cu and Cd (concentrations as in Figure 1), during the long term experiment. Vertical dashed lines separate the exposure period with the metal (first-step experiment) from that of maintenance in seawater (second-step experiment). Vertical bars are standard errors. Values are from 5 replicate tanks per treatment with 10 larvae per tank.

The survival trend with time of the *C. crambe* and *S. lophyropoda* juveniles during the long-term experiment is represented in Figure 6.2A and B. The multivariate analysis of variance indicated no significant differences (p= 0.487) between treatment and seawater control were found for *C. crambe* juveniles (Table 6.2). The percentage of *C. crambe* survivors was more or less constant from day 12 to the end of the experiment (6 months later) (Fig.6.2A). As for *S. lophyropoda*, juveniles showed no significantly different survival between treatments and control (p = 0.867; Table 6.2) from day 8 to the end of the experiment (Fig. 6. 2B). The percentage of survivors ranged from 50 to 60% in the treatments and from 50 to 77% in the controls after 1 month. After 6 months, however, the percentage decreased to ca.10-20% in both treatment and control

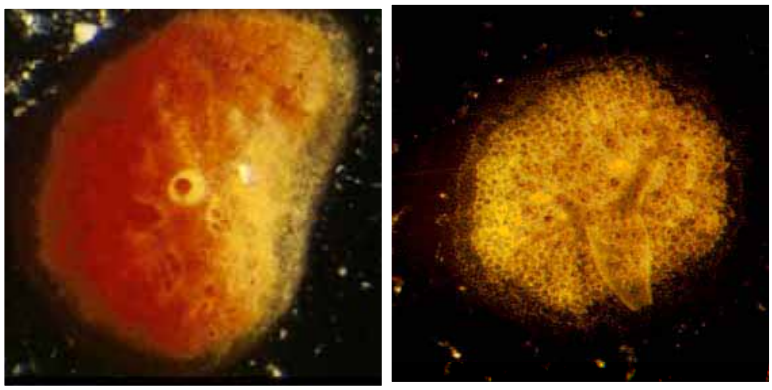
	Wilks lamda value	F	p
Crambe crambe			
First step			
treatment	0.520	1.28	0.307
time	0.095	31.42	0.000
treatment and time	0.373	2.11	0.096
Second step			
treatment	0.607	0.942	0.487
time	0.680	1.563	0.258
treatment and time	0.867	0.244	0.955
Scopalina lophyropoda			
First step			
treatment	0.118	6.39	0.001
time	0.080	38.20	0.000
treatment and time	0.059	10.39	0.000
Second step			
treatment	0.795	0.404	0.867
time	0.673	1.617	0.246
treatment and time	0.436	1.717	0.169

Table 6.2. MANOVAR results of the effects of cadmium and copper on each species (*Crambe crambe* and *Scopalina lophyropoda*) at the first- and second- step experiment respectively.

Discussion

This study approaches for the first time the effects of short exposures of heavy metals on sponge larval settlement, and analyses whether these short exposures have any consequence on the consecutive survival of juveniles.

The effects of metals (Cu and Cd) on larval settlement vary depending on the sponge species: copper and cadmium at the concentration assayed did not affect *C. crambe* settlement, but enhanced *S. lophyropoda* settlement, compared with SW controls. These results may seem at first glance surprising, since early developmental stages of invertebrates have been reported to be more responsive to toxicants than adults (His et al., 1999). Accordingly, there are some reports of harmful effects of Cu and Cd on invertebrate larvae (Wu et al., 1997; Reichelt-Brushett and Harrison, 2000), and metamorphosis (Negri and Heyward, 2001), although the concentrations assayed in the cited studies were much higher than those used in our experiments.



Crambe crambe settler *Scopalina lophyropoda* settler

On the other hand, the enhancement of *S. lophyropoda* settlement is in agreement with that of previous studies reporting hormesis (positive effects of toxicants at low concentrations) for metals (Stebbing, 1982; Beiras & His 1994). For example, Ng & Keough (2003) found that exposure to 100 $\mu g \cdot l^{-1}$ of copper accelerated bryozoan larval attachment. Furthermore, like for *C. crambe* larvae, there are also many other examples of no significant noxious effects of Cu and Cd on larval settlement, metamorphosis and post-metamorphic stages of invertebrates (e.g. Peckenick et al., 2001; Bellas et al., 2001; Blidberg, 2004,). As for the possible mechanisms involved in the observed induction of settlement, it has been reported that bivalent metals such as Cu and Cd may penetrate larval cells via transition channels (Gill and Epple, 1992), leading to increased of intracellular Ca^{2+} release (Marchi et al., 2004). Since an extracellular supply of calcium induces cell aggregation

in sponges (Philp, 1997), the reaggregation and recognition processes of cells, involved in larval settlement, are promoted in presence of Cu and Cd (Chapter 8).

The early settlement of *S. lophyropoda* larvae induced under Cu and Cd might favour juvenile survival, since the length of the larval period is shortened and larval reserves may be redirected to juvenile maintenance. Accordingly, it has been shown that juveniles originated from short-lived larvae survived better and grew faster than long-lived larvae (Maldonado and Young, 1999).

It has been reported that stresses experienced by larvae under low concentrations of pollutants may affect juvenile performance in a variety of taxonomic groups (reviewed by Pechenick et al. 1998). Our results showed, for both species, no significant differences in survival between settlers coming from larvae previously treated with Cu or Cd and those maintained in SW control. Thus, we can conclude that the concentrations of copper and cadmium used did not exert any effect on consecutive survival and growth of the sponge juveniles.

The present results are in accordance with those of Peckenik *et al*., 2001, where individuals surviving even the most toxic treatment, once transferred to clean seawater, performed as well after metamorphosis as control individuals.

To summarise, copper and cadmium at the concentrations assayed did not affect *C. crambe* settlement compared with SW control neither exerted any effect on the consecutive survival of juveniles. On the other hand, copper and cadmium at the concentration used enhanced *S. lophyropoda* settlement but did not affect the consecutive survival of juveniles.

The present study provides the only available data on the toxicity of copper and cadmium on sponge larval settlement. Nevertheless, our *in vitro* experiments need to be confirmed by further *in situ* approaches, since environmental variables may influence in several ways and strengths the impacts of these pollutants on sponge populations.

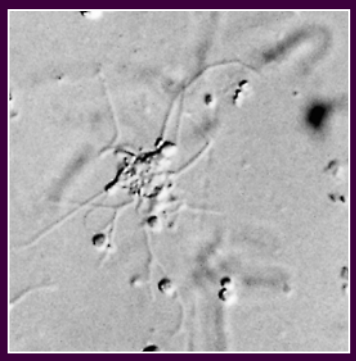
Acknowledgements

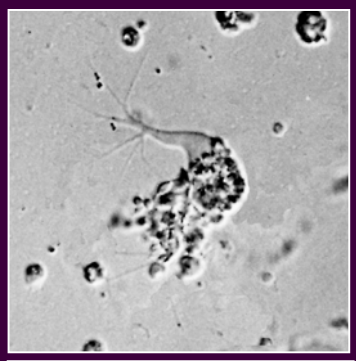
We thank R. Martí and G. Agell for helping in larval collection and experimental procedures. This research was partially funded by grants from the CE (SPONGES project COOP-CT-205-017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265 /MAR).

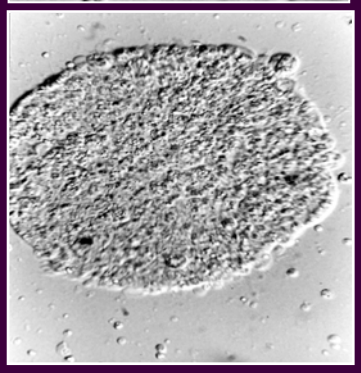
Capítol 6

Efectes contradictoris dels metalls pesants en l'assentament larvari i posterior supervivència dels juvenils d'esponges.

Els metalls pesants contaminen en gran mesura els sediments i les aigües de les zones costaneres, amenaçant les primeres fases del desenvolupament de molts invertebrats. Alteracions en aquestes fases, poden determinar la decadència i fins i tot la desaparició de poblacions d'invertebrats marins en ambients contaminats. En aquest capítol hem volgut determinar la influència que poden tenir els metalls pesants (Cu i Cd) en l'assentament larvari i la supervivència posterior (6 mesos) dels juvenils de dues espècies d'esponges mediterrànies (*Crambe crambe* i *Scopalina lophyropoda*). Per això, les larves d'ambdues espècies s'han sotmès a concentracions de Cu i Cd durant una setmana. Polses curts de coure i cadmi no han afectat l'assentament de *Crambe crambe* i tampoc la posterior supervivència dels juvenils. En canvi, les mateixes exposicions de coure i cadmi han afavorit l'assentament larvari de *S. lophyropoda*, sense afectar, però, la posterior supervivència dels juvenils.

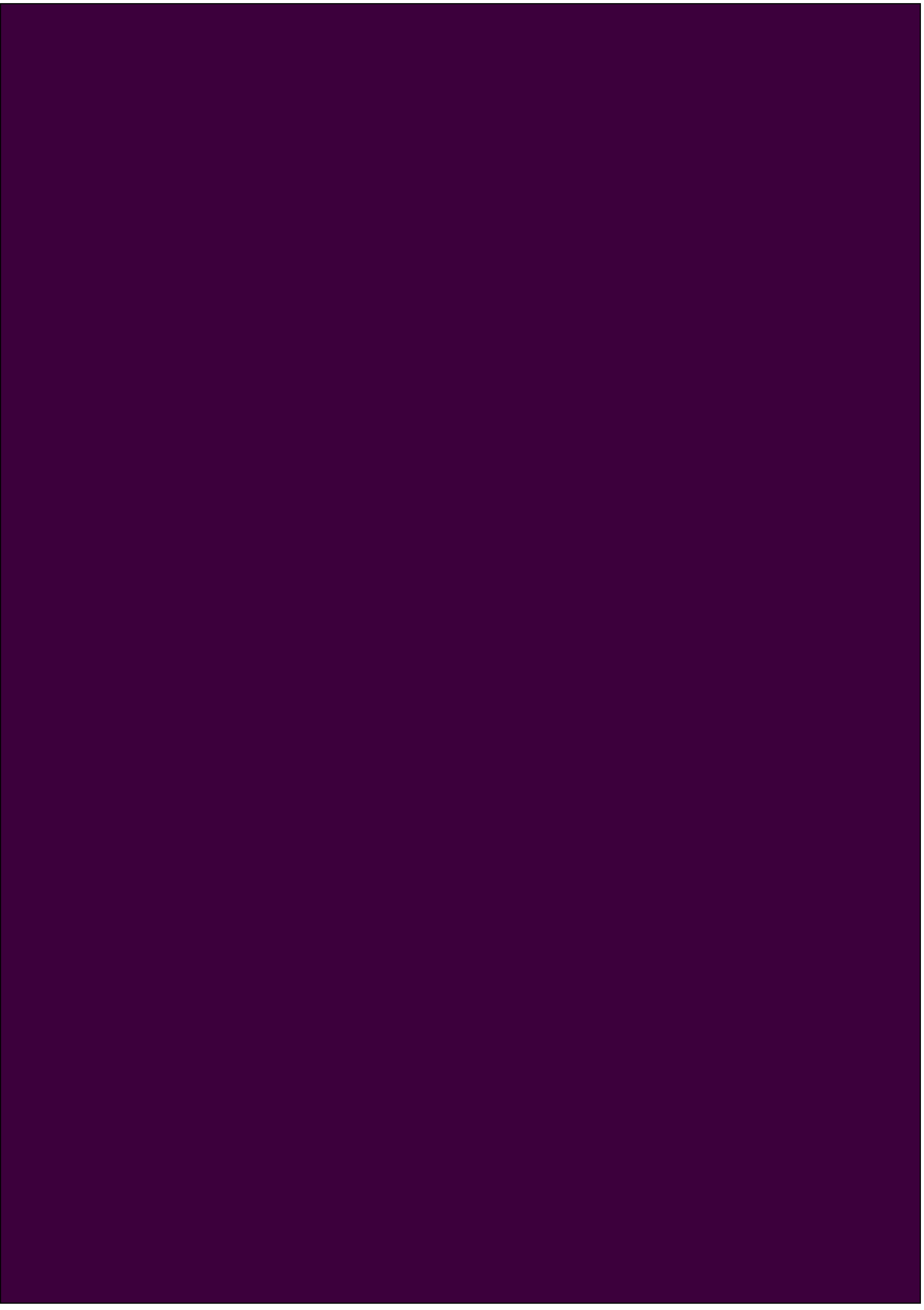






Chapter 7

Contrasting effects of heavy metals on sponge cells behaviour.



Chapter 7

Contrasting effects of heavy metals on sponge cells behaviour.[1]

Abstract

Mediterranean coastal areas are highly contaminated by heavy metals, which have been reported to produce harmful effects in marine organisms. Sponges are particularly vulnerable to water-borne metals because they are able to process large amounts of water. Dissociated sponge cells can move in response to external stimuli, and the cell body changes shape through production of pseudopodia and phylopodia. We studied for the first time the effects of heavy metals (cadmium and copper) on motility and aggregation of isolated sponge cells. Cell shape was assessed by using several shape indices. The two metals studied induced changes on cell shape. Copper and cadmium enhanced pseudopodia formation and cell motility. On the other hand, the two metals enhanced cell aggregation at the concentrations assayed. Our results show that sponge cells respond to metal pollution in different ways and that these responses can be assessed by calculating several shape indices.

[1] Cebrian E, Uriz MJ. Contrasting effects of heavy metals on sponge cells behaviour. Arch. Environ. Contam. Toxicol. *In press*.

Introduction

Coastal marine and estuarine environments have long been used as disposal areas for industrial and mining wastes. They are also subjected to transient toxicant releases that may occur in the form of spills or runoff from agricultural and urban areas, as well as antifouling biocides. As a result, Mediterranean coastal areas are highly contaminated by heavy metals (Palanques et al. 1998; Puig et al. 1999), which produce harmful effects in marine organisms due to their toxicity and bioaccumulation thought the trophic chains. Among heavy metals, copper is one of the most abundant with well-known harmful effects on marine invertebrates (Ahsanullah and Florence, 1984; Reichelt- Brushett and Harrison, 2000; Negri and Heyward, 2001). Cadmium is another relevant pollutant from industrial discharge and is highly toxic to a variety of aquatic animals (e. g., Eisler, 1985; Selck et al., 1998; Au et al., 2001).

Sessile benthic invertebrates are especially susceptible to heavy metal pollution because of their suspension-or filter-feeding habitat and their reduced motility, which prevens them to scape from toxicants released to a given area (Naranjo et al. 1998; Carballo and Naranjo, 2002; Rosenberg et al. 2004; Perez et al. 2005). Sponges are particularly vulnerable to water-borne metals because they are able to process large amounts of water (Reiswig, 1971; Turon et al., 1997; Ribes et al., 1999). On the other hand, sponges have been proposed as a heavy metal biomonitors because they have a high capacity for accumulating heavy metals (Patel et al., 1985; Olesen and Weeks, 1994; Hansen et al., 1995, Pérez et al. 2005) and because they experience morphological, biological, physiological and biochemical changes when they are submitted to metal contamination (Agell et al., 2001; Philp, 1999; Schröder et al., 2000; Cebrian et al., 2003,2006; Berthet et al. 2005).

In this way, a set of recent studies have tackled the effects of heavy metals on different sponge species and at different organization levels: molecular (Agell et al. 2001; Cebrian et al., 2006), physiological (Cebrian et al., 2003) and population level (Cebrian et al., *submitted*), and at different stages of the sponge life cycle (larvae, settlers, juveniles and adults, Cebrian and Uriz, 2007 a, b). These studies have reported contrasting effects depending on the exposure time, the metal and the species considered and the life-cycle stage. While those heavy metals appear to be noxious for adults (Cebrian et al. 2003; 2006) they seem to be innocuous or even beneficial for larvae and settlers (Cebrian and Uriz, 2007a). For instance, copper inhibited growth and reproduction in *Crambe crambe* adults, but it did not affect larval settlement. However, in other sponge species (*Scopalina lophyropoda*) copper and cadmium enhanced settlement.

During larval settlement and metamorphosis, an extensive reorganization process occurs, which implies movement, self-recognition and aggregation of cells. Thus, alterations in one or several aspects of the cell behaviour can have important effects on settlement processes. To understand the effects that low concentrations of heavy metals produced on *Scopalina lophyropoda* settlement, we searched for

changes in shape, motility and aggregation in *Scopalina lophyropoda* cells submitted to short pulses of copper and cadmium.

Dissociated sponge cells crawl in response to external stimuli, and the cell body changes shape through production of pseudopodia and phylopodia (Stossel, 1994). We used these changes on shape, assessed through shape indices, to analyse cell motility in sponge cells under the effects of copper, mercury and cadmium.

Moreover, heavy metals may alterate Ca^{2+} homeostasis (Marchi et al., 2004; Verbost et al., 1989; Viarengo et al., 1994) and therefore affect calcium-induced aggregation (Philp, 1999). Concretely, it has been reported that these contaminants can affect the viability of the cell-to-cell aggregation in other invertebrates (Auffret and Oubella, 1997) and sponges (Philp, 1999). Thus, we also studied the effects of copper and cadmium on cell aggregation by measuring the number and size of aggregates in metal incubated cells.

This is the first study that attempts to investigate the effects of heavy metals on sponge cell behaviour as a proxy for biological effects at the settlement and early post-settlement stages. While most previous studies on invertebrate cells have used extremely high concentrations that may ever be experienced by the organisms in the field, the present study attempts to analyse cellular responses to metal pulses at more realistic concentrations, which could be found in polluted sites. The responses at the cellular level may have the potential to anticipate changes at higher levels of biological organization (Cajaraville et al., 2000).

Materials and Methods

The effects of heavy metals on the sponge cell behaviour (i.e. cell motility and aggregation) were assessed by shape indices analyses. As cell motility generally involves the production of pseudopodia and phylopodia, it can be indirectly measured by increases in cell irregularity.

The study was carried out with a suspension of cells isolated from *Scopalina lophyropoda*, which is a widespread species with patchy distribution in the Mediterranean sublittoral, growing on vertical rocky walls. *S. lophyropoda* was collected from 5 m depth of the Blanes sublittoral (NE Spain, 41°40'37"N, 2°47' 30"E), placed in bowls underwater and transported to the laboratory. Once in the laboratory, the sponges were placed in sterile plastic bowls. Experiments were performed within 2 h of sample collection. For obtaining dissociated cells, a small sample of tissue was placed in 20 ml of filtered seawater and submitted to mechanical stirring for 5 min.

For studying the effects of heavy metals, the sponge cells were incubated for three hours in copper (30 and 100 $mg \cdot l^{-1}$) and cadmium (5 and 10 $mg \cdot l^{-1}$). The metals

were incorporated as solutions of cupric chloride ($CuCl_2$) and cadmium chloride ($CdCl_2$). Controls consisted in cells incubated in filtered seawater from a non-polluted area, where metal concentrations in the seawater were below the threshold levels detectable by ICP-MS. Temperature was maintained at 14 ºC during the incubation. Aliquots of 20 ml of cell suspension of each treatment were dispensed onto a microscope slide. Slides were observed through a Leitz (Wild HSP 52) light microscope with a camera for image recording. A number of microscope fields (N=30) were photographed from each slide.

Pictures were digitalised; the number of cells was counted and their perimeter and area were measured with the program NIH image for Macintosh. Cell shape was approached from three shape indices, which were calculated:

Circularity index (C) (Turon and Becerro, 1992)

C= As/Ap

where As=area of sponge patch, and Ap=area of a circle with perimeter equivalent to that of sponge patch; a value of 1 represents a perfect circle, while 0 is approached as the outline becomes more irregular due to the presence of pseudopodia, which is an indicator of cell motility.

Directional index (D) (Becerro et al., 1994)

D = 1- (P/M)

where M= length of maximum straight line through two cell points, and P=length of maximum straight line perpendicular to M. This index measures cell elongation. A value of 0 indicates a perfect circle; 1 is approached as directionality increases, which denotes directional movement.

Convolution index (Cv) (Becerro et al., 1994)

Cv = 1-(Pc/Ps)

Where Pc = perimeter of an ellipse, with an area and directional growth index equivalent to those of the cell. This index is a measure of peripheral irregularity. A value of 0 indicates a perfect ellipsoid; 1 is approached as irregularity of the border increases, as it happens when cells move.

Aggregation index

An index of aggregation was calculated as the percentage of grouped cells among the total number of cells in a microscope field.

Data analysis

Differences between treatments were analysed by means of one-way ANOVA (Statistica 4.1 package). The Tukey-test was used for post-hoc comparisons. Assumptions of normality and homogeneity of variances were examined using the Kolmogorov-Smirnov and Barlett tests, respectively. Variables were rank-transformed (Conover and Iman, 1981; Potwin and Rolf, 1993) prior to the analysis when assumptions were not fulfilled.

Results

Cadmium

Cadmium added to seawater had a significant effect on cell circularity, directionality and convolution (Table 7.1). Circularity index was significantly lower (Tukey-test, $p<0.05$) in cells at 10 $\mu g \cdot l^{-1}$ Cd^{2+} than those maintained in seawater. By contrast, directional growth and convolution increased in cells cultured with cadmium (Fig.7.1 B and C).

Treatment	Variable	Factor	DF	F	*p*
Cadmium ($\mu g \cdot l^{-1}$)	Circularity	Treatment	2	5,61	0,004
		Error	307		
	Directionality	Treatment	2	7,09	0,000
		Error	307		
	Convolution	Treatment	2	5,34	0,005
		Error	307		
	Aggregation	Treatment	2	56,39	0,000
		Error	220		
Copper ($\mu g \cdot l^{-1}$)	Circularity	Treatment	2	3,77	0,025
		Error	149		
	Directionality	Treatment	2	4,76	0,009
		Error	149		
	Convolution	Treatment	2	1,62	0,200
		Error	149		
	Aggregation	Treatment	2	5,22	0,007
		Error	80		

Table 7.1: One-way ANOVA for treatments (Cadmium and copper) in shape indices of *Scopalina lophyropoda* cells.

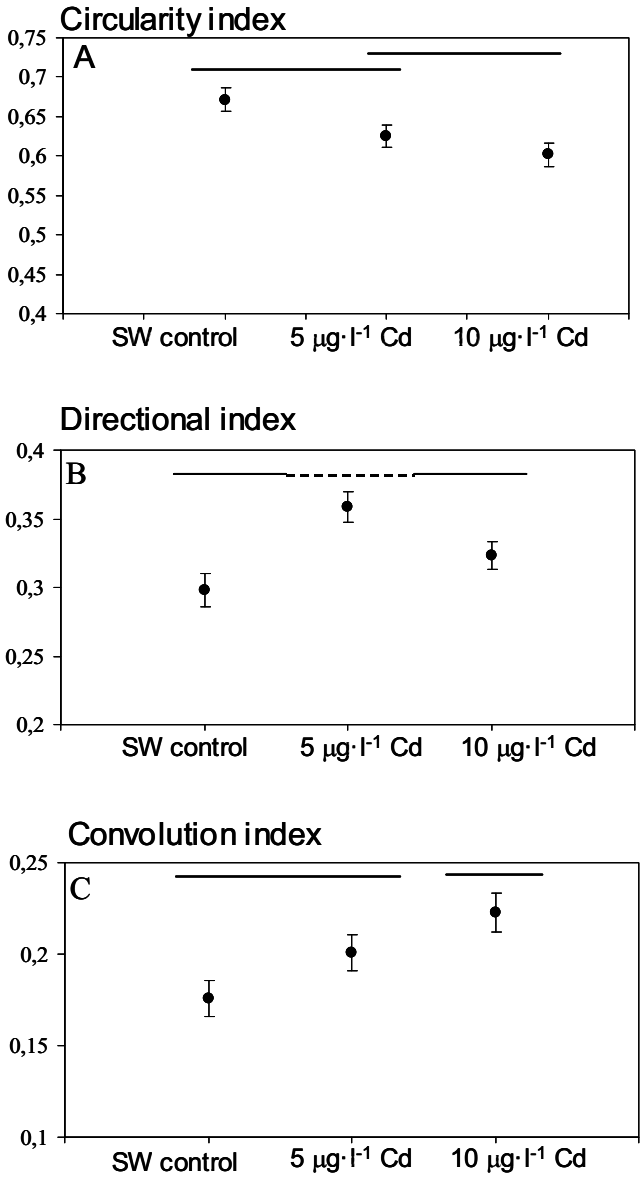


Figure 7.1: Shape indices of *Scopalina lophyropoda* cells incubated in cadmium and SW control: perimeter/area (A); circularity (B); directionality (C) and convolution (D). Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Directional movement of cells at 5 $\mu g \cdot l^{-1}$ Cd^{2+} was significantly higher than in those at 10 $\mu g \cdot l^{-1}$ Cd^{2+} and in SW control (Tukey-test, $p<0.05$), while convolution was significantly higher in cells at 10 $\mu g \cdot l^{-1}$ Cd^{2+} than at 5 $\mu g \cdot l^{-1}$ Cd^{2+} and sea water (Tukey-test, $p<0.05$).

Cadmium increased aggregation of *S. lophyropoda* cells. The aggregation index was significantly higher in cells incubated under the two cadmium treatments than in SW controls (Table 7.1; Fig. 7.2).

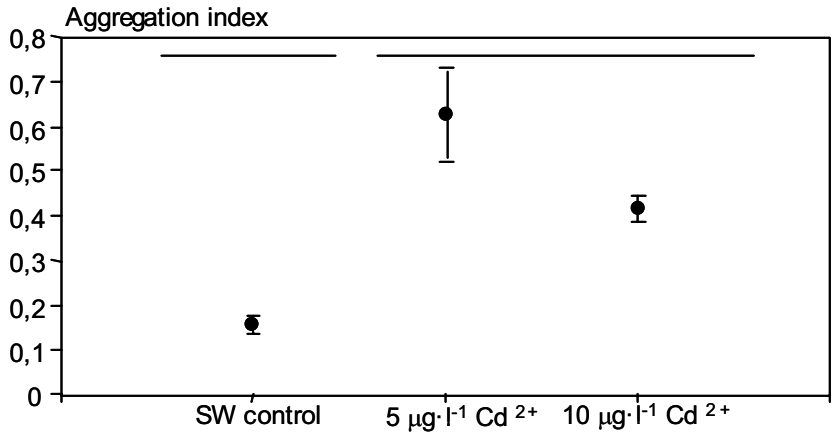


Figure 7.2. Aggregation index of *Scopalina lophyropoda* cells, incubated in cadmium and SW control. Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Copper

Copper at the higher concentration assayed (100 $\mu g \cdot l^{-1} Cu^{2+}$) (Tukey-test, $p<0.05$) had a significant effect on the cell circularity index, which was significantly lower than that of cells in seawater and at 30 $\mu g \cdot l^{-1} Cu^{2}$ (Table 7.1; fig. 7.3A). Copper at the lowest concentration assayed (30 $\mu g \cdot l^{-1} Cu^{2+}$) had a significant effect (Tukey-test, $p<0.05$) on the directional index (D) of the *S. lophyropoda* cells, being significantly higher (Tukey-test, $p<0.05$) in cells at 30 $\mu g \cdot l^{-1} Cu^{2+}$ than in cells at 100 $\mu g \cdot l^{-1} Cu^{2+}$ and in seawater control (Table 7.1; Fig. 7.3B). No effects of copper were observed on the convolution index for any of the concentrations assayed (Table 7.1; Fig.7.3C).

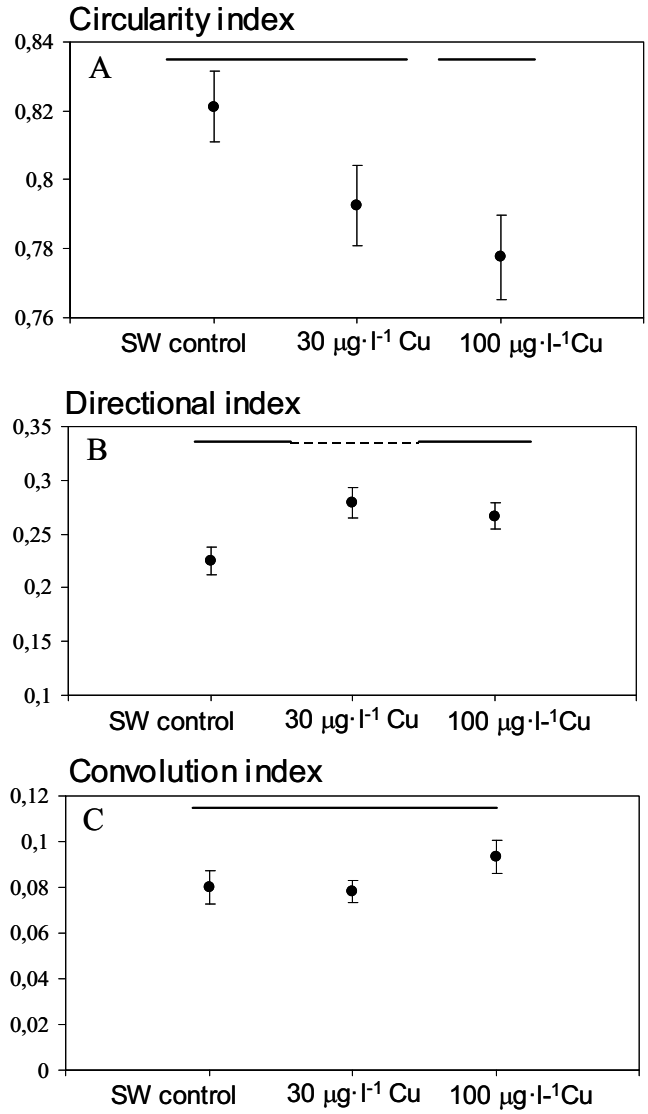


Figure 7.3. Shape indices of *Scopalina lophyropoda* cells incubated in copper and SW control: circularity (A); directionality (B) and convolution (C). Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey-test, are joined by horizontal lines.

Cell aggregation was significantly enhanced in cells cultured at 100 $\mu g \cdot l^{-1} Cu^{2+}$ (Table 7.1; Fig. 7.4) with respect to control cells. But the analysis failed to detect differences were found between treatments and between cells at the lower concentration of copper and seawater control.

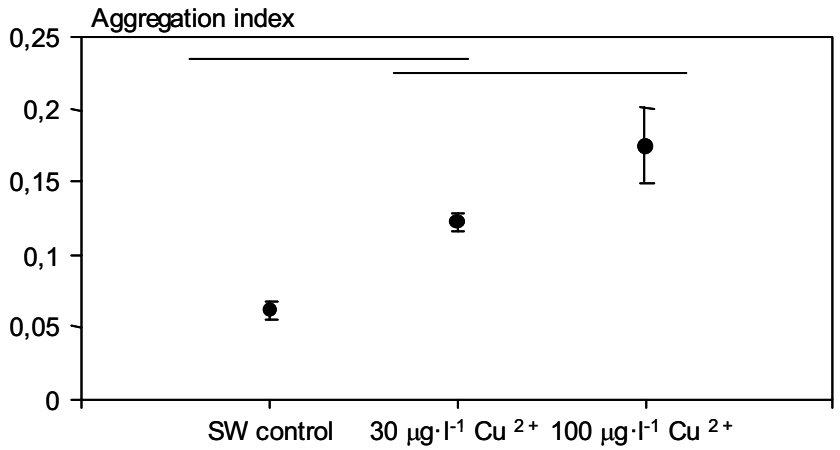


Figure 7.4. Aggregation indices of *Scopalina lophyropoda* incubated in copper and SW control. Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Discussion

Sponge populations in metal polluted environments can bioaccumulate metals (e.g. Cebrian et al. 2003, 2006), which may impair cell functions. In the present study, we have shown that copper and cadmium cause morphological cellular changes and affect cell aggregation in *Scopalina lophyropoda* after 3 h of metal incubation. Changes in cell shape and motility, which are likely related with changes in the cytoskeleton (White 1974, Syversen et al. 1984), have also been reported to occur in cells of other invertebrates submitted to heavy metals (Burlando et al. 2000; 2003).

S. lophyropoda cells responded differently to the various metal concentrations assayed. Both metals caused an increase of the irregularity and directional shape of sponge cells by enhancing pseudopodia/phylopodia formation, which indicate cell movement. In line with our results, it has also been reported that cadmium and copper at low concentrations significantly increase shape irregularity (Gómez-Mendikute and Cajaraville, 2003) and pseudopodia production in other invertebrate cells by promoting phagocytosis (Cheng and Sullivan 1984; Coles et al. 1995; Pile et al. 1999;

Olabarrieta et al. 2001). Conversely, at higher concentrations or during longer incubation times (e.g. at 100ppm Cd^{2+} during 24h or at 5ppm Cu^{2+} for one month), the effects of copper and cadmium appear to be harmful for invertebrate cells, as they inhibit cytoskeleton production and the cells adopt a round shape with no cell extensions (Cheng and Sullivan, 1984).

Heavy metals have been reported to modify cell calcium homeostasis, which results in a series of cytotoxic mechanisms that affect the cytoskeleton (Cima et al., 1998) causing a spherical shape of cells, but it does not seem to occur in sponge cells under copper and cadmium, likely due to the low metal concentrations or the short exposition time used in our experiments.

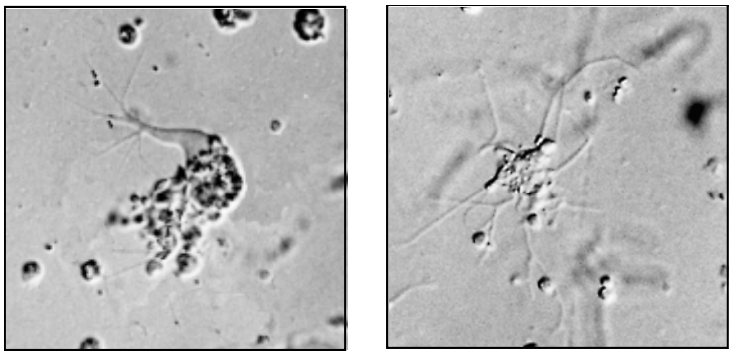


Figure 7.5. Examples of pseudopodia and phylopodia formation on *Scopalina lophyropoda* cells incubated under short pulses of copper and cadmium.

Actually, most pollutants enter marine waters in short pulses at moderate concentrations, conditions that we aimed to reproduce in the present study. However, a noxious effect of copper and cadmium at higher concentrations or longer exposition cannot be discarded.

Copper and cadmium enhanced cell aggregation in *S. lophyropoda* at the concentrations assayed (30 and 100 $\mu g \cdot l^{-1}$ copper and 5 and 10 $\mu g \cdot l^{-1}$ cadmium). We suggest that an increment of the cytosolic calcium concentration, which is required for cell aggregation in sponges (Dunham et al., 1983; Weissmann et al., 1985), was induced by the heavy metals through an alteration of calcium homeostasis (e. g. Burlando et al., 2000; Pourahmad and O'Brien, 2000). However, much higher concentrations (10 ppm) of copper and cadmium may be toxic, since they have been reported to inhibit cell aggregation in oyster haemocytes, while intermediate concentrations (1ppm) did not affect cell aggregation at all (Auffret and Oubella, 1997).

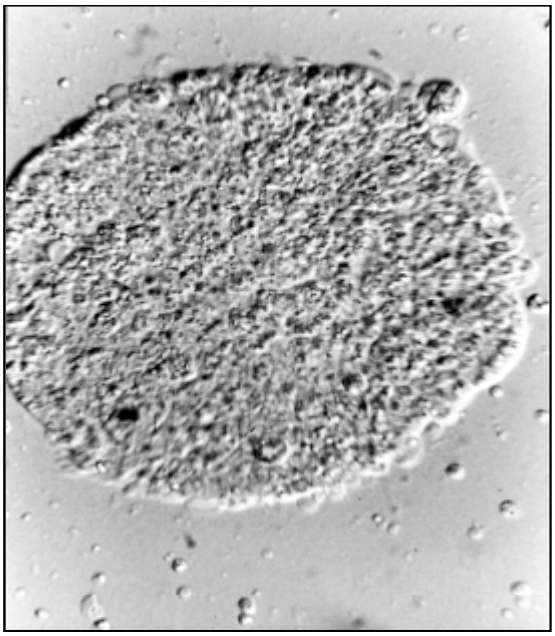


Figure 7.6: *Scopalina lophyropoda* cell aggregates induced by heavy metals.

To summarise, the two metals studied induced changes of different sign on cell shape. Moderate concentrations of copper and cadmium enhanced pseudopodia formation, cell motility and cell aggregation.

Our results show that sponge cells respond to metal pollution and that these responses can be detected by calculating several shape indices. These effects at a cellular level may anticipate changes that would occur at higher levels of biological organisation, such as organisms and populations. The study of effects at the cellular level add to the available methods for early detection of harmful pollution-related effects, which is desirable for designing adequate management strategies and to prevent future outbreaks in marine ecosystems.

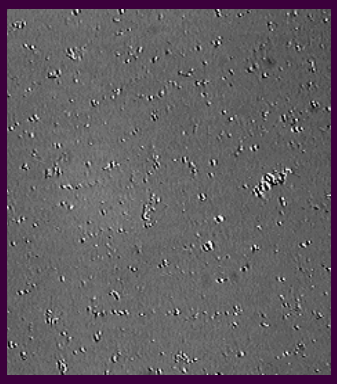
Acknowledgements

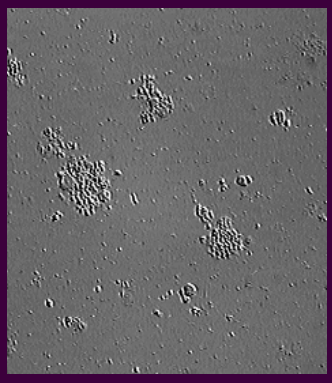
We thank R. Martí and G. Agell for helping in sampling and experimental procedures. This research was partially funded by grants from the CE (SPONGES project COOP-CT-205-017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265 /MAR).

Capítol 7

Efectes contradictoris dels metalls pesants en el comportament cel·lular de les esponges.

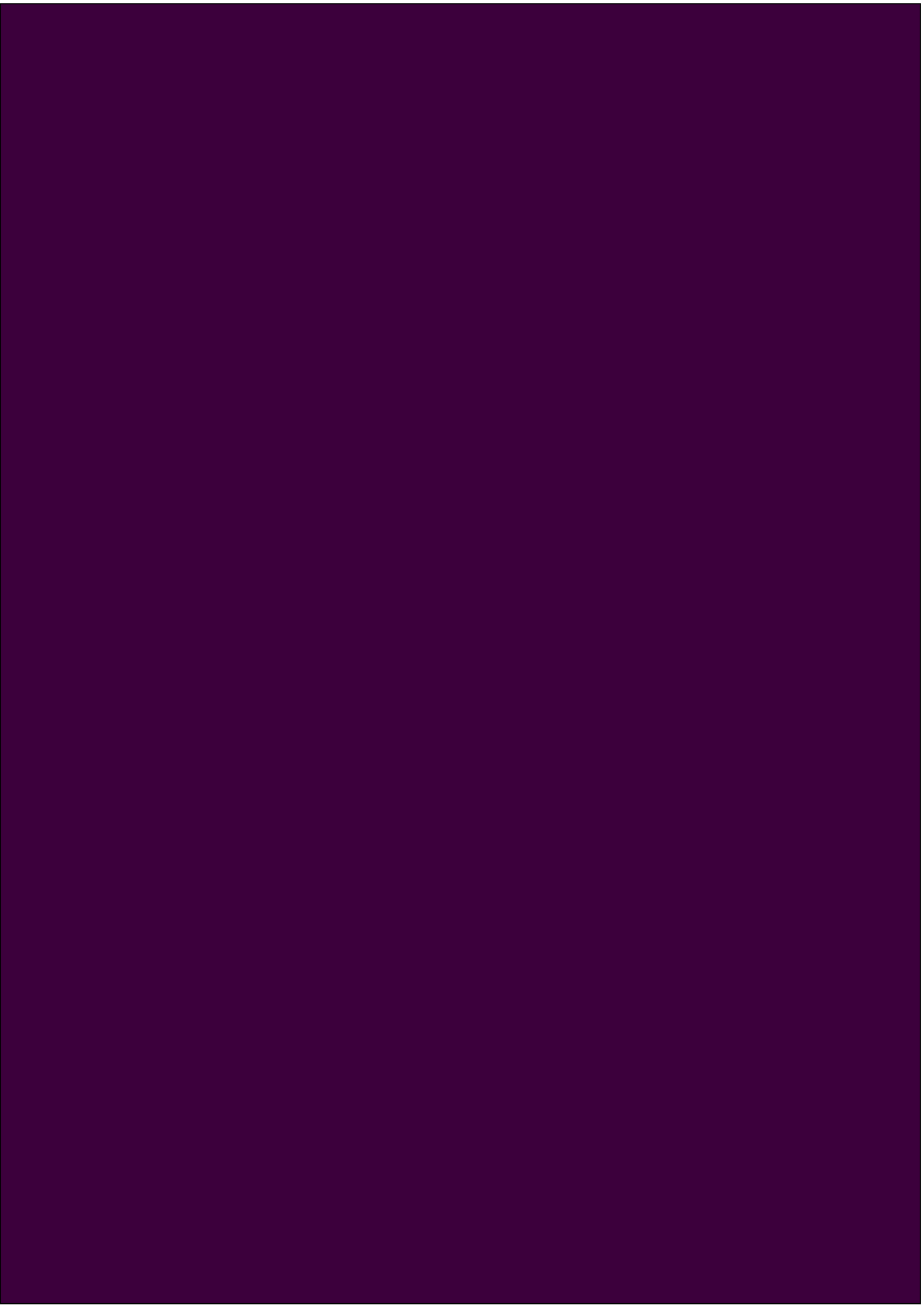
Les esponges processen i filtren grans quantitats d'aigua. Això les fa particularment vulnerables als metalls del medi. En aquest sentit, destaquen els efectes nocius del coure i el cadmi, els quals estan àmpliament descrits en altres invertebrats marins. En aquest capítol estudiem l'efecte d'aquests dos metalls en l'agregació cel·lular i en diversos índexs de forma - els quals donen una idea de la motilitat de les cèl·lules. Els dos metalls estudiats afecten a la forma i a l'agregació de les cèl·lules de les esponges. Concentracions de coure i cadmi afavoreixen la formació de pseudòpodes i la motilitat cel·lular. De la mateixa manera, els dos metalls estudiats afavoreixen l'agregació cel·lular. Aquests resultats ens mostren que les cèl·lules responen als metalls i que dites respostes poden ser mesurades a partir de diversos índexs de forma.





Chapter 8

Do heavy metals play an active role in sponge cell behaviour in absence of calcium? Consequences in larval settlement



Chapter 8

Do heavy metals play an active role in sponge cell behaviour in absence of calcium? Consequences in larval settlement.[1]

Abstract

Moderate concentrations of copper and cadmium can have positive effects on sponges by enhancing cell aggregation and larval settlement. The cellular events underlying these effects are not fully understood, but it is known that cell aggregation needs of an extracellular supply of calcium, which in absence of external calcium, may also be provided by the release of internal calcium stores. In order to known the mechanisms by which Cd^{2+} and Cu^{2+} affected sponge cells and larvae and whether the alteration of calcium homeostasis is involved in such effects, we incubated *Scopalina lophyropoda* cells for three hours and larvae for seven days in calcium- free seawater (CFSW), seawater (SW), and CFSW with copper (Cu CFSW) and cadmium (Cd CFSW). Both metals seem to play an active role in calcium dependent processes: sponge cells incubated in Cd CFSW and Cu CFSW, aggregated as much as those in SW control. Similarly, Cd and Cu first accelerated larval settlement but toxic effects of both metals were evidenced after a longer exposition (five days), leading to settler mortality. Thus, although the effect of these two heavy metals may be positive in the short term, long term exposition or higher concentrations may alter cell functions and affect sponge viability and fitness.

[1] Cebrian E, Uriz MJ. Do heavy metals play an active role in sponge cell behaviour in absence of calcium ? Consequences in larval settlement. J. Exp. Mar. Biol. Ecol. *In press.*

Introduction

It is widely recognised that moderate levels of some pollutants that are apparently innocuous for adult invertebrates can negatively affect the physiology of their juvenile and larval stages (Rinkevich and Loya, 1977; His et al., 1999). Among pollutants, heavy metals have been reported to produce contrasting effects on early life stages depending on the metal concentration and the time of exposure. Copper and cadmium at a high concentration are toxic for cells (i.e. Auffret and Oubella, 1997) and larvae (Wu et al., 1997; Reichelt-Brushett and Harrison, 2000), and alter larval metamorphosis (Negri and Heyward, 2001). Conversely, the same pollutants, at low concentrations, are reported to have positive effects on invertebrate cells and larvae. Moderate concentrations of copper and cadmium seem to significantly increase pseudopodia production in cells (Gómez-Mendikute and Cajaraville, 2003), promoting phagocytosis (Cheng and Sullivan, 1984; Coles et al., 1995; Pipe et al., 1999; Olabarrieta et al., 2001). Similarly, moderate concentrations of copper accelerate attachment of a bryozoan larva (Ng and Keough, 2003).

In sponges, exposition to copper and cadmium are harmful against adults (Cebrian et al., 2003, 2006, *Chapter 3 and 4*) while short pulses of the same metals improved cell aggregation and fastened larval settlement (*Chapter 6 and 7*). The cellular events underlying these effects are not fully understood, but available studies indicate that both metals can affect the cell calcium homeostasis (Verbost et al., 1989; Viarengo et al., 1994; Marchi et al. 2004).

During larval settlement and metamorphosis of sponges, an extensive reorganization process occurs, which implies movement, self-recognition and aggregation of cells (Weismann et al., 1985). Three components have been considered necessary for cell aggregation in sponges to occur (Dunham et al., 1983): a proteoglycan complex (aggregation factor- MAF), a cell surface receptor, and Ca ions. Furthermore, the “aggregation factor” (MAF) promotes cell adhesion only in presence of Ca^{2+} (e.g. Müller and Zahn, 1973; Jumblatt et al., 1980).

Bivalent heavy metals can enter the cells via Ca^{2+} channels (Verbost et al., 1987; Roesijadi and Unger, 1993) promoting the release of cytosolic calcium (Pourahmad and O’Brien, 2000; Marchi et al., 2004), which may be responsible for an enhancement of cell aggregation and, indirectly for, speeding up larval settlement, as observed in *Scopalina lophyropoda* cells and larvae submitted to short pulses of Cu^{2+} and Cd^{2+} (Chapter 6 and 7).

In order to learn more about the mechanisms by which Cd^{2+} and Cu^{2+} may enhance cell aggregation and larval settlement in sponges, and whether the alteration of calcium homeostasis induced by heavy metals is involved in such effects, we incubated *Scopalina lophyropoda* cells and larvae in calcium- free seawater under copper and cadmium, and monitored the resulting effects.

Materials and Methods

The study was carried out with both larvae and a cell suspension of *Scopalina lophyropoda*, which is a Mediterranean sponge species with a patchy distribution along the rocky sublittoral. *S. lophyropoda* was collected from the Blanes coast (NE Spain, 41°40'37"N, 2°47'30"E), placed in bowls underwater and transported to the laboratory. Larvae release often occurred spontaneously during the transport or posterior acclimation in the laboratory. The *S. lophyropoda* larva is an elongated parenchymella (type I, according to Mariani et al., 2005), uniform, bright orange in colour. For obtaining dissociated cells, a small sample ($3mm^3$) of tissue was placed in 20 ml. of filtered seawater and submitted to mechanical stirring for 5 min till an hyaline solution was obtained indicating sponge cell dissociation.

Cells Bioassays

For studying the possible effects of heavy metals in cell aggregation in absence of calcium, the sponge cells were incubated for three hours at 14 ºC, in calcium- free seawater (CFSW), 0.2 mm filtered seawater from a metal unpolluted area (Pinedo, 1998) (SW control), and CFSW contaminated with copper (30 $\mu g \cdot l^{-1}$) and cadmium (5 $\mu g \cdot l^{-1}$). Three replicates per treatment were arranged. Metal concentrations in the seawater controls were below the threshold levels detectable by ICP-MS.

Aliquots of 20 ml of cell suspension of each treatment were dispensed onto a microscope slide. Slides were observed through a Leitz (Wild HSP 52) light microscope with a coupled camera for image recording. A number of microscope fields (N=30) were photographed from each slide. Pictures were digitalised and the number of cells and clusters of cells was counted with the program NIH image for Macintosh. Aggregation index, defined as the percentage of cell clusters among the total number of cells in a field, was calculated for each treatment.

Larvae bioassays

Assays were carried out in polystyrene culture tanks (500 ml). Five replicate tanks with 10 larvae each were used for each treatment as well as for seawater controls. We assessed settlement by direct counting the number of settlers per tank at each point time. We counted as a settler the stage at which larvae were irreversibly attached to the substratum to initiate the metamorphosis. Settlement of larvae in the SW controls took place from 1- 7 days after release as it has been reported in field studies (Uriz, 1982; Uriz et al., 1998; Maldonado and Uriz, 1999), which indicates appropriated conditions in our experiments. Treatments for this experiment were: 0.2 mm filtered seawater (SW) used as an absolute control, calcium free seawater (CFSW) and CFSW with copper (30 $\mu g \cdot l^{-1}$) (hereafter Cu CFSW) and cadmium (5 $\mu g \cdot l^{-1}$) (hereafter

Cd CFSW). The number settlers at each observation time was standardised with respect to the initial number of larvae (time=0).

Data analyses

Differences in cell aggregation between treatments (only one observation time) were also analyzed by means of one-way ANOVA. The Tukey test was used for post-hoc comparisons.

Differences in the percentage of settlers between treatments with time were analyzed by multivariate analysis of variance MANOVA of reapeted measures, since data do not met the assumptions for ANOVAR (i.e. sphericity Mauchly's test) but comply with those of the Box-M test (homogeneity of the variance/covariance matrix) (Von Ende, 1993). To analyze differences between treatments for each observation time, we used one-way ANOVA. Data met the assumptions of normality and homogeneity of variances required for the ANOVA-MANOVAR (Kolmogorov-Smirnov and Barlett tests, respectively). All the analyses were performed using Statistica 6.0 package.

Results

Cells

In absence of calcium cell aggregates were rare and were formed by a few cells (Fig. 8.1A). In contrast, in SW control and Cd CFSW and Cu CFSW treatments, cell aggregates were numerous and larger in size (Fig. 8.1B, C and D). Significant differences in cell aggregation were found between treatments and SW control (ANOVA, $p<0,05$). Cd CFSW, Cu CFSW and SW significantly increased (Tukey-test, $p<0.05$) cell aggregation compared with CFSW (Fig. 8.2A, B) (Table 8.1).

One-way ANOVA	SS	Degr. free	MS	F	p
Cadmium assay	0.254	2	0.187	6.43	0.003
Copper assay	0.385	2	0.192	12.87	0.000

MANOVAR	Wilks- value	F	p
Treatment	0.398	2.029	0.023
Time	0.736	2.685	0.101
*Treatment*Time*	0.306	4.043	0.004

Table 8.1. ONE-WAY ANOVA results of the treatment effect on sponge cell aggregation. MANOVAR results of the treatment effect (copper, cadmiun CFSW and SW) on sponge larval settlement.

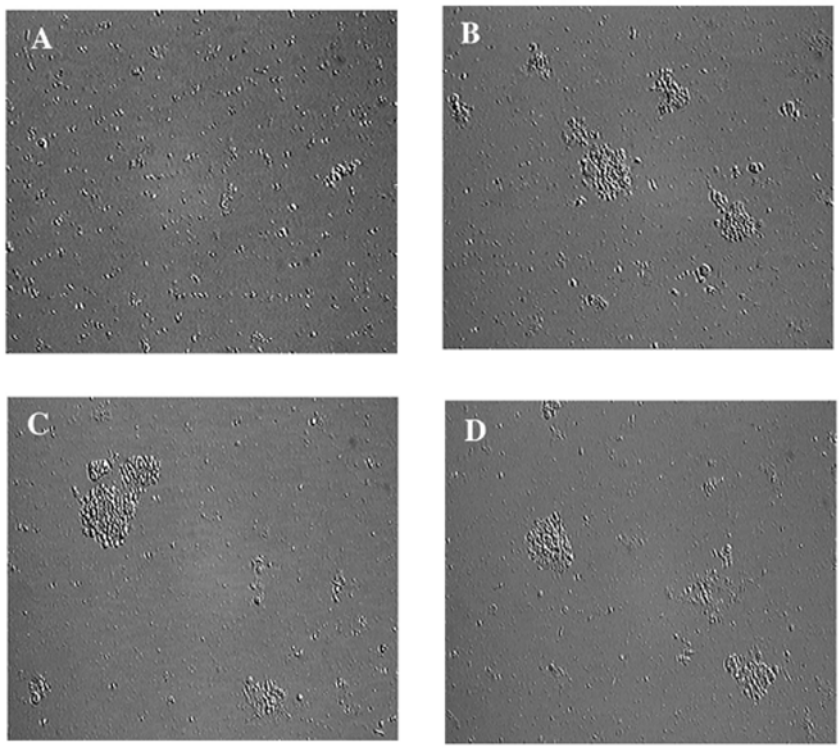


Figure 8.1. Some representative pictures of *S. lophyropoda* cells in the different experimental treatments (optic microscopy). A) Isolated cells in CFSW treatment after 3 h. B) Cell aggregates in Cd CFSW treatment C) in Cu CFSW and D) in sea water after 3h.

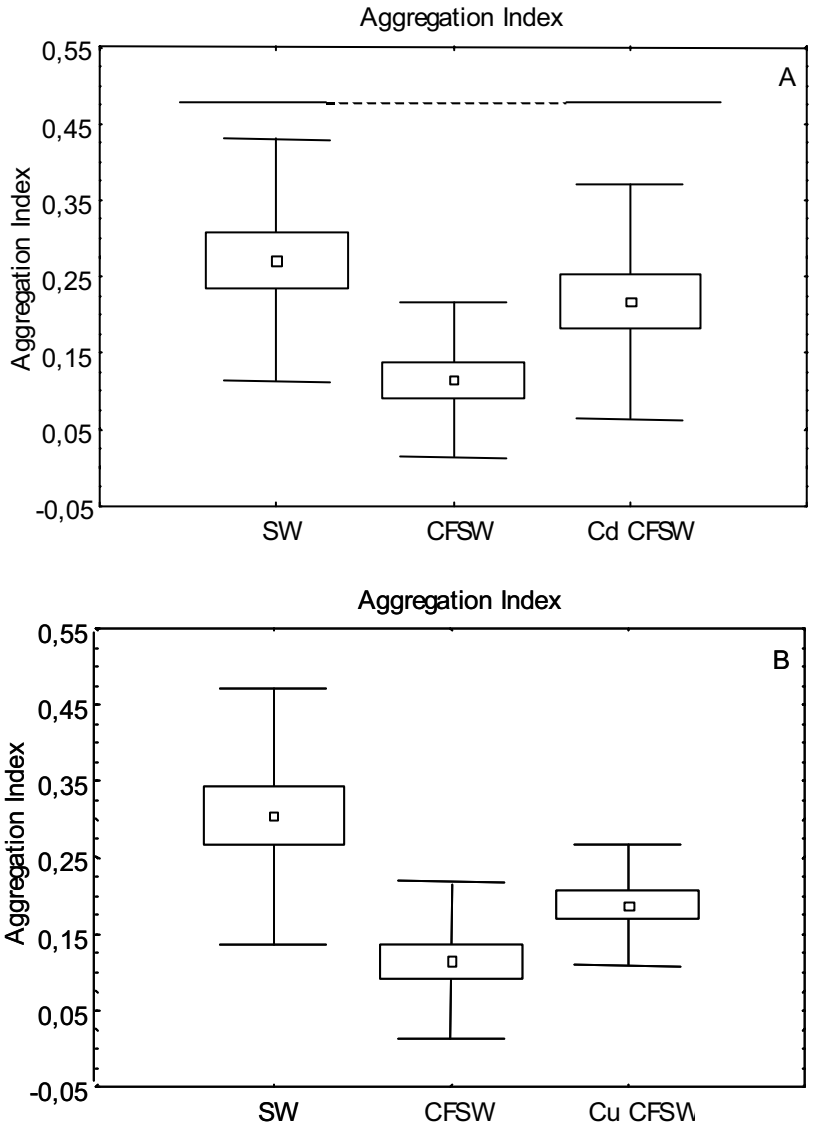


Figure 8.2. Aggregation index of *Scopalina lophyropoda* cells. A) Incubated in SW, CFSW and Cd CFSW. B) Incubated in SW, CFSW, and Cu CFSW control. Boxes represent standard errors; vertical bars are standard deviations. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines.

Larvae settlement

The time course of the percentage of settlers of *Scopalina lophyropoda* during the experiment (7 days) is represented in Fig. 8.3. The MANOVAR analysis showed that the treatments had a significant effect ($p<0.01$) on larval settlement (Table 8.1). At day 3, larvae started to settle in both Cd and Cu treatments and in SW control (Fig. 8.3) being the percentage of settlers similar in the three cases. In contrast, no larvae settled on day 3 from those incubated in CFSW, neither during the whole experiment (Fig. 8.3). However, on day 5, settlement increased in SW control, while settlers disappeared from Cu CFSW and Cd CFSW treatments due to post-settlement mortality. At the end of the experiment (day 7), settlement was significantly higher in SW control than in the heavy metals and CFSW treatments (ANOVA $p<0.05$).

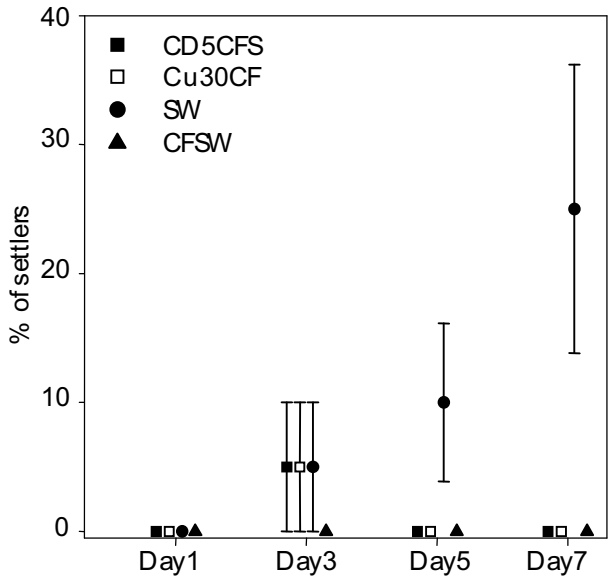


Figure 8.3. Time course of the percentage of *Scopalina lophyropoda* settlers under Cu ($30\mu \cdot l^{-1}$) CFSW, Cd ($5\mu \cdot l^{-1}$) CFSW, CFSW and SW control. Vertical bars are standard errors.

Discussion

Our results showed that cell aggregation and larval settlement are calcium-dependent processes, since they do not occur in cells and larvae maintained in CFSW. The failure to settle observed in CFSW cultured larvae was likely due to the incapacity of cells to recognise and join each other during the reorganisation process involved in metamorphosis (e.g. ectosome and choanosome formation). In contrast, larvae incubated in Cd CFSW and Cu CFSW settled at day 3 similarly to those in seawater control. Accordingly, while cells did not aggregate in CFSW, those maintained in Cd CFSW and Cu CFSW aggregated similarly to those in SW.

Cell aggregation in sponges occurs in response to an extracellular supply of calcium (Philp, 1997), which in absence of external calcium (i.e. CFSW medium) may also be provided by the release of internal calcium stores (Dunham et al., 1983). Bivalent heavy metals and calcium use the same transition channels (Gill and Epple, 1992) and may interact antagonically. Both metals may enter the cells by binding to sulfhydryl groups in proteins Ca-ATPases (Viarengo and Nicotera, 1991), therefore leading to alterations on Ca dependent signalling, such as an induction of the intracellular calcium release (Marchi et al., 2004).

CFSW prevented larval settlement in *S. lophyropoda*, whereas, at the beginning of the experiment, settlement was similar to that in SW control when Cu or Cd was added to CFSW. Cu^{2+} and Cd^{2+} ions may penetrate into the larval cells through calcium transport systems, inducing calcium ions release, which would allow cell aggregation and recognition during larval settlement. However, in the second week of the experiment, all settlers died in the heavy metal treatments. A concentration increase of both metal ions within larval cells with time might be responsible for the harmful effects observed in settlers incubated in Cd CFSW and Cu CFSW media.

In contrast, when cultured with Cd and Cu in natural seawater (Cebrian and Uriz, 2007a, *Chapter 6*), *Scopalina lophyropoda* larvae settled in higher numbers compared with SW controls, and no post-settlement mortality occurred. Consequently, it seems that in seawater, heavy metals do not exert the harmful effects observed in CFSW medium, probably because heavy metals and calcium compete for the same transition channels (Gill and Epple, 1992), which would prevent the massive entry of heavy metals, and their accumulation and toxic effects in cells.

To summarise, sponge cells incubated in Cd CFSW and Cu CFSW during 3 hours, aggregated similarly to those in SW control and significantly more than in CFSW. Likewise, Cd CFSW and Cu CFSW treatments fastened larval settlement in the short-term, but toxic effects of both metals were evidenced after longer exposition, which leaded to settler mortality. Thus, both metals seem to play an active role in calcium dependent processes, with no harmful effects evidenced at the very short

term, but with toxic effects at longer term, which may endure further consequences not only in cells, but also in all processes that encompass cell reorganization such as larval settlement. Long term expositions or higher concentrations of Cd and Cu may produce metal accumulation within cells, leading to impairment in cell functions, which in turn, will endure negative consequences on sponge health and fitness (Cebrian et al., 2003, 2006; *Chapters 3 and 4*).

Acknowledgements

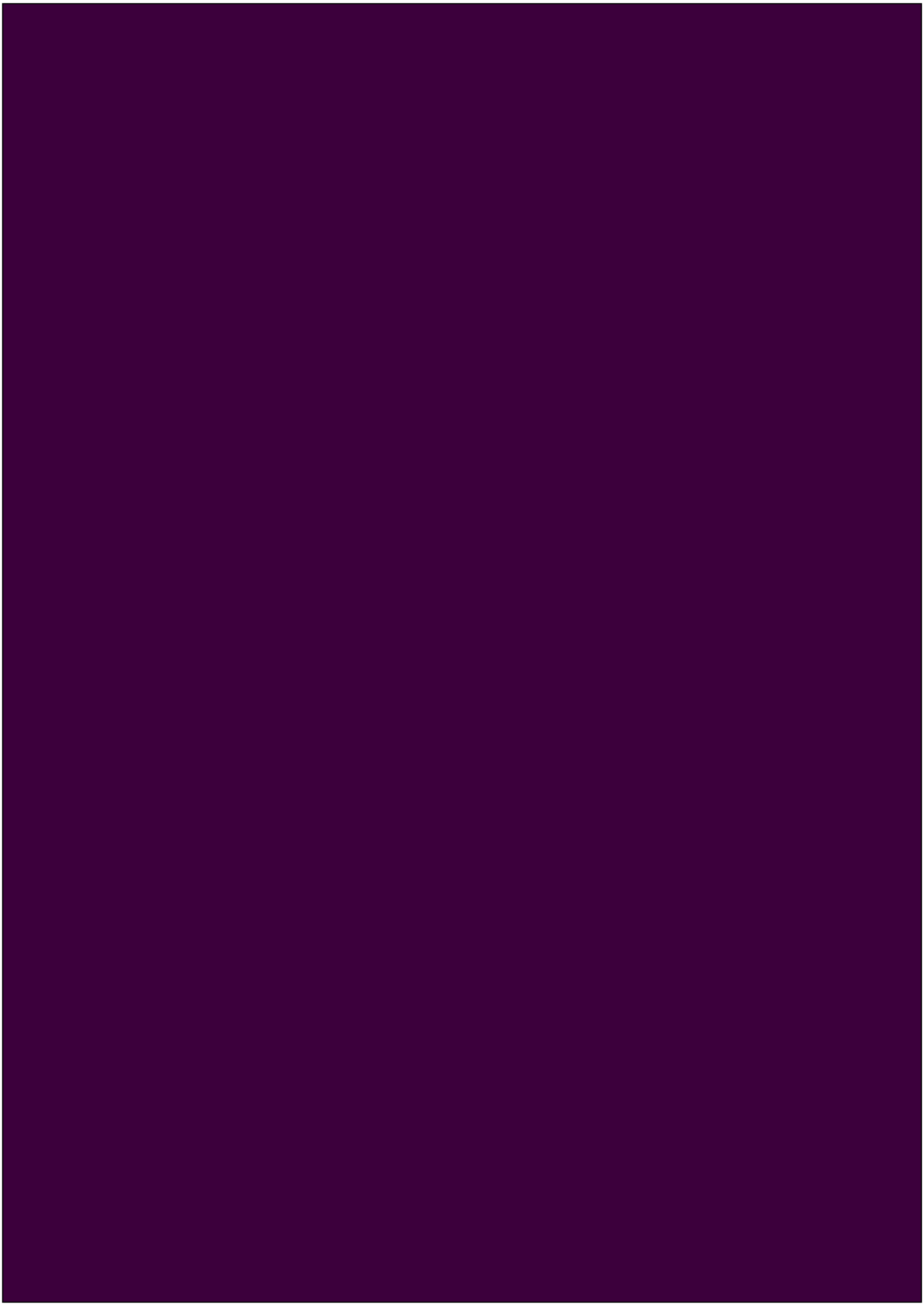
We thank R. Martí and G. Agell for help in sampling and experimental procedures. This research was partially funded by grants from the CE (SPONGES project COOP-CT-205-017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265 /MAR).

Capítol 8

Paper dels metalls pesants els processos cel·lulars en absència de calci. Conseqüències en l'assentament larvari.

Com hem vist en altres capítols (Capítols 6 i 7), concentracions moderades de coure i cadmi poden afectar positivament l'assentament larvari, l'agregació cel·lular i motilitat de les esponges. Tanmateix, els mecanismes cel·lulars, gràcies als quals es produeixen els efectes observats, no són del tot coneguts. Així és malgrat es coneix que l'agregació cel·lular necessita d'un aport de calci extracel·lular, el qual, en absència de calci extern, pot provenir de l'alliberament dels dipòsits de calci intracel·lular. A fi de conèixer millor el mecanisme pel qual Cd i Cu afecten positivament a les cèl·lules i larves de les esponges i, per analitzar com l'alteració de l'homeostasis del calci intervé en aquests efectes, hem incubat cèl·lules de *S. lophyropoda* durant tres hores i larves durant 1 setmana en presència de metalls al laboratori; els tractaments experimentals han estat: aigua de mar sense calci (CFSW), aigua de mar (SW), i aigua de mar sense calci contaminada amb coure (Cu CFSW) i cadmi (Cd CFSW). D'acord amb els resultats, sembla que els dos metalls juguen un paper actiu en els processos dependents de calci: les cèl·lules incubades en Cd CFWS i Cu CFSW s'agreguen igual que les incubades en aigua de mar malgrat que no disposen de calci. De la mateixa manera, tant el Cd com el Cu han accelerat l'assentament larvari en els primers moments, per bé que després de 5 dies d'incubació han provocat efectes tòxics que han acabat amb la mortalitat dels assentats. Per tant, podríem dir que, malgrat que a curt termini sembla que els metalls tinguin un efecte positiu, una exposició més prolongada o exposicions a concentracions més altes poden provocar alteracions en les funcions cel·lulars i comprometre la viabilitat i la fitness de les esponges.

General conclusions



General conclusions

- There are important differences in heavy metal accumulation by sponges. Differences in sponge morphology and aquiferous system and thus, filtration and clearance rates, as well as the different skeleton nature (mineral/or proteinaceous) probably account for the different accumulation patterns observed.
- Similarly, the mechanisms of metal uptake seem to vary depending on the trace metal and the species considered. In general, copper bioaccumulation fits a net accumulation strategy while lead concentration seems to be regulated in sponge tissues.
- *Crambe crambe* is capable to bioaccumulate copper and lead as a function of the surrounding bioavalailbe metal. *Phorbas tenacior* and *Dysidea avara* bioaccumulate copper but not lead, and *Chondrosia reniformis* bioaccumulate neither copper nor lead. Furthermore, *Crambe crambe* can reflect spatial and temporal differences of metal bioavailability in the Mediterranean marine environments. Consequently, among all species studied, only *C. crambe* offers advantages in the context of biomonitoring as it can provide information on the background levels of metals in the environment.
- *Crambe crambe* submitted to sublethal concentrations of heavy metals experiences several behavioral and physiological responses such as changes in shape, growth rates, reproduction output, and metal accumulation, which can be easily monitored.
- In contrast, *Chondrosia reniformis* does not denote ecological changes in its morphology, growth or metal accumulation as a function of metal concentration in the environment. Thus, it cannot be considered a heavy metal biomonitor, but a “sensitive organism” of heavy metal pollution, since moderate heavy metal pollution causes changes in its physiology, such as a decrease in the filtration activity, which may lead to death.
- Responses at a molecular level also differ according to the species considered. Whereas sublethal concentrations of copper do not induce HSPs expresion in *Chondrosia reniformis*, HSP54 and HSP72 proteins were expressed under copper contamination in *Crambe crambe*, being the first time that a protein of the HSP60 family has been reported in sponges. HSP72 seems to be mainly induced in *C. crambe* while HSP54 may be both constitutive and inducible.
- Differences in tolerance to heavy metal pollution between *C. crambe* and *C. reniformis*, can be explained by differences in HSPs expression. *C. crambe* induces HSP production and then can live in moderately polluted areas, while

C. reniformis do not enhance HSPs production under the same conditions and neither inhabit these moderately polluted areas. The lack of molecular response in *Chondrosia renifomis* may be due to a strong metabolic damage, and the consequent impairment of some physiological activities, such as filtration.

- *Crambe crambe*, subjected to sublethal heavy metal pollution, seems to invest preferentially in protein repair, as for example HSPs, at the expenses of the production of chemical defenses.
- Short pulses of copper and cadmium at the concentrations assayed do not affect *C. crambe* settlement neither exert any effect on posterior survival of juveniles. In contrast, short pulses of copper and cadmium enhance *Scopalina lophyropoda* settlement but do not affect posterior survival of juveniles.
- Heavy metals can induce changes on cell shape in a very short time. Copper and cadmium enhanced pseudopodia and phylopodia formation and thus, cell motility. Furthermore, both metals enhanced cell aggregation.
- Detection of heavy metal effects on sponges at a cellular level, by calculating several shape indices, can be en easy way to anticipate changes that would occur at higher levels of biological organisation.
- Effects of copper and cadmium on sponge cells and larval settlement seem to be related to an active role of both metals in calcium dependent processes. Harmful effects are not evident at the very short term, since cell aggregation and larval settlement is promoted. However, longer expositions or higher concentrations of heavy metals may produce metal accumulation within cells, leading to cell impairment, which consequently, may result in a failure of larval settlement.

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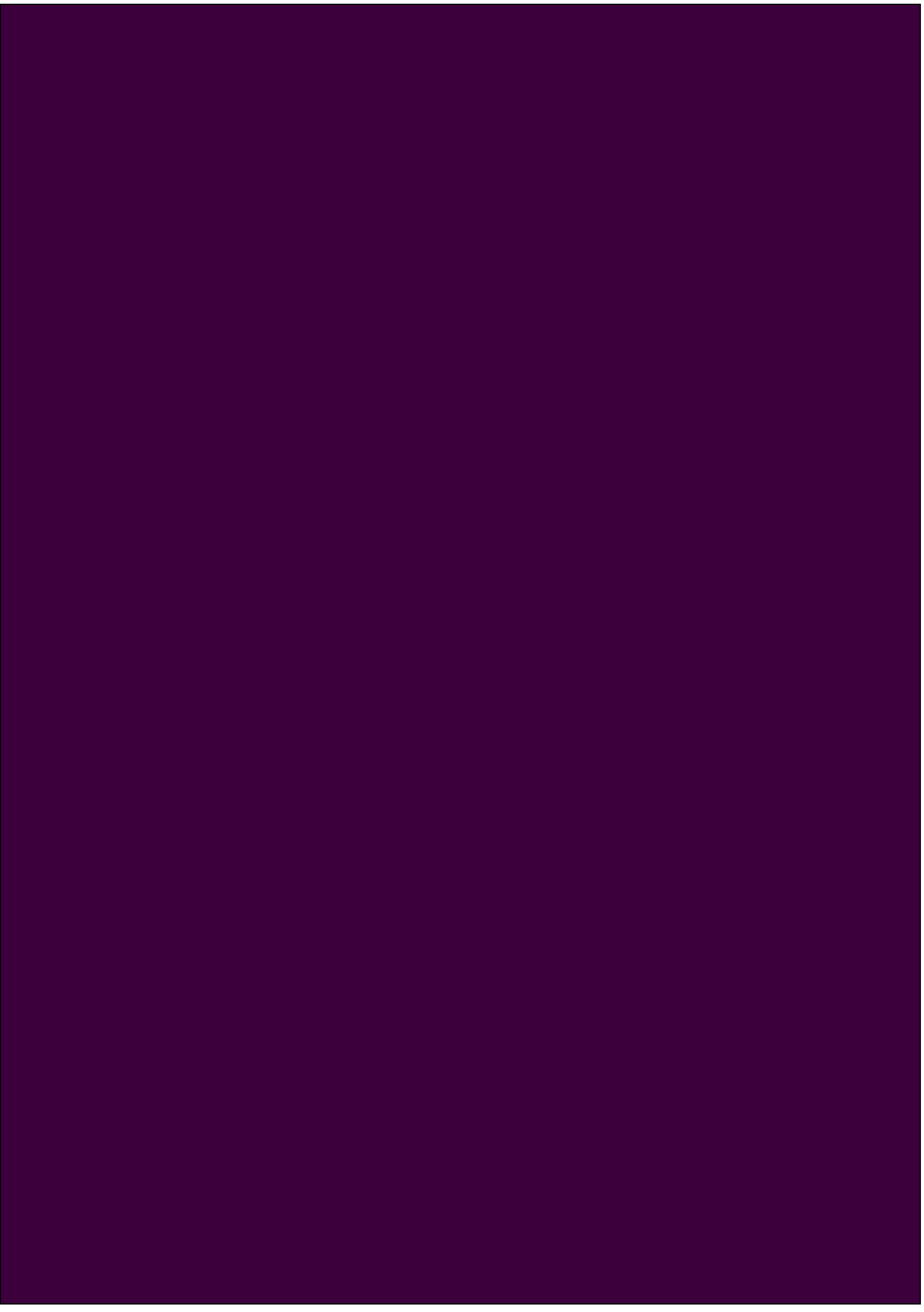

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Introducció general

Els ecosistemes marins i particularment les zones costaneres, estan sotmesos de manera inexorable a un creixent grau d'estrès. Això és degut principalment al continu desenvolupament urbà al llarg de totes les costes, l'exposició continuada a substàncies perilloses, la pesca extensiva i la sobrepesca, la destrucció de l'hàbitat i la introducció continuada d'espècies al·lòctones, així com d'altres amenaces per a la biodiversitat marina (Wells, 1999). Els efectes de dites pertorbacions són a vegades evidents al suposar la desaparició d'espècies i l'empobriment de tot l'ecosistema. Això no obstant, més sovint la transformació dels ecosistemes es dóna de forma molt més subtil i en molts casos només és detectada per pocs especialistes. En aquest sentit, avui en dia es pot considerar que no existeix cap exemple d'ecosistema prestí (e. g. Jackson, 2001).

El mar Mediterrani representa un "punt calent" de biodiversitat marina, amb espècies que es distribueixen per un gran nombre de comunitats (Bianchi and Morri, 2000; Occhipinti-Ambrogi and Savini, 2003; Boudouresque, 2004). Aquestes comunitats no escapen, però, de la tendència general de declivi de la biodiversitat en tots els ambients marins. En aquest ordre de coses, durant les últimes dècades, destaca l'impacte de gran quantitat de contaminants provinents d'activitats antropogèniques abocats a les costes del Mediterrani, amenaçant l'estabilitat de les seves comunitats i la supervivència de moltes espècies sensibles.

Entre aquests contaminants, tenen una especial importància els metalls pesants, els quals són responsables en gran part, de la contaminació dels sediments i les aigües de les costes, tant industrialitzades com agrícoles (Palanques et al., 1998; Puig et al., 1999). Els metalls són contaminants conservatius, no susceptibles a l'atac bacterià, o si ho són, és a molt llarg termini, el que a efectes pràctics, representa addicions permanents en els ecosistemes marins. Per tant, representen una amenaça real per a la sostenibilitat i la salut del medi marí (Brown and Ahsnullah, 1971; Berthet et al., 1992; Canesi et al., 1999).

Existeixen moltes proves dels efectes de la contaminació pels metalls pesants en animals. En aquest sentit, existeixen alguns exemples documentats dels impactes de contaminació aguda per metalls sobre espècies que habiten els fons marins. En aquests casos, els metalls provoquen canvis dràstics en la composició i la distribució espaial de les espècies de les comunitats sublitorals (p. e. Diaz-Castaneda et al., 1989). Per bé que el més comú és que els metalls pesants s'alliberin en els ecosistemes costaners en baixes concentracions, produint efectes subletals en els organismes que només s'observen a nivell de l'ecosistema a més llarg termini.

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Els nivells de metalls pesants en els ambients marins són mesurats en funció de les concentracions que presenten a l'aigua, sediments i biota. La quantificació dels metalls pesants a la columna d'aigua és difícil de dur a terme fruit de les limitacions de la metodologia disponible per detectar les baixes concentracions en què els metalls es troben normalment presents a l'aigua de mar. A més, les concentracions de metalls dissolts a l'aigua varien considerablement en el temps; per exemple, amb els cicles de marea, els aports d'aigua dolça o l'estacionalitat.

Als sediments els metalls pesants s'acumulen a majors concentracions que a l'aigua, el que facilita la seva quantificació. Val a dir, a més, que determinar els metalls pesants en els sediments permet un major grau d'integració temporal. No obstant això, l'anàlisi dels metalls pesants en els sediments també presenta inconvenients. D'una banda, la quantitat de metalls depèn en gran mesura de les característiques del sediment. D'altra banda, com així també ocorre quan hom analitza l'aigua, només proporcionen la quantitat total i no pas la fracció que és realment disponible per als organismes vius (Rainbow, 1995).

Davant d'aquests dos mètodes, s'ha proposat la utilització d'organismes vius (biomonitors) com a mesura indirecta de l' abundància de metalls pesants en el medi (Rainbow and Phillips, 1993; Phillips and Rainbow, 1993; Rainbow, 1995), la quantitat de metalls als teixits dels animals reflecteix doncs, la proporció de metall realment disponible pels organismes, la qual és la veritable responsable dels seus potencials efectes nocius (Connell et al. 1999). És per això, que utilitzar organismes com a mesura dels nivells de metall biodisponible en els sistemes marins, es considera una bona elecció en els estudis de biomonitoratge.

A l'hora de triar les especies adients per dur a terme un biomonitoratge, i interpretar el significat de les concentracions de metalls al seus teixits, cal tenir en compte alguns aspectes: en primer lloc, un biomonitor apropiat ha de ser capaç de reflectir les concentracions presents en l'ambient, això és, ha d'acumular metalls pesants en funció de la concentració present en l'ambient; en segon lloc, l'acumulació de metalls varia entre les espècies, fins i tot entre les que viuen en un mateix hàbitat (Hare, 1992; Phillips and Rainbow, 1993); i en tercer lloc, s'ha de considerar que un organisme pot acumular diferentment en funció del teixit que hom analitza, del seu estat fisiològic (reproducció, creixement, regressió, fitness), estació de l'any i del metall considerat. Per tant, les variacions temporals d'acumulació de metalls pesants en un organisme poden ser resultat, no només de les diferències estacionals de metall biodisponible, sinó també de l'estat fisiològic de l'organisme. Així doncs, el significat d'una concentració de metall donada en un organisme en un cert moment, no es pot interpretar en termes absoluts, sinó que depèn de totes les variables mencionades. El coneixement de les estratègies d'acumulació i de l'estat fisiològic d'una espècie ens permetran determinar la significació d'una quantitat de metall mesurada en els seus teixits (Rainbow, 1995). És per això, que abans de plantejar-se

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qualsevol estudi de biomonitoratge, els esforços teòrics i metodològics haurien de dirigir-se a ampliar el coneixement en tots els aspectes abans mencionats.

Efectes subletals dels metalls pesants en els invertebrats bentònics

La literatura exposa molts exemples en torn a la quantificació de metalls en una llarga llista d'organismes i en múltiples localitats. Aquests estudis són molt valuosos per determinar les fluctuacions de les concentracions de metalls pesants a la biota i permetent identificar les zones contaminades. La seva limitació és, però, que no faciliten informació sobre els efectes ecològics/biològics que produeixen. En aquest sentit, es fa palesa la necessitat d' obtenir informació sobre les relacions entre la bioacumulació i els efectes.

Entre tots els efectes que poden causar els metalls pesants en la biota marina, els que tenen repercusions a nivell de població han estat els més descuidats per part de les investigacions en ecotoxicologia (Depledge and Hopkin, 1993). Generalment, els efectes dels metalls pesants en les poblacions impliquen la pèrdua d'individus, el que es sovint detectable a una escala temporal de varis dies o mesos. Davant d'això, es considera que la millor forma de detectar canvis ecotoxicològics en les poblacions de forma efectiva en un termini raonablement curt, és estudiant les alteracions que es produeixen en els diferents nivells d'organització biològica, com ara el molecular, cel·lular, fisiològic i poblacional. Cal conèixer com els contaminants alteren les taxes de creixement, la fecunditat i longevitat dels individus, els ritmes biològics i els mecanismes de defensa, tant cel·lulars com mol·leculars (Gray, 1979; Moriarty, 1983; Depledge, 1984). Fins ara, però, els estudis dels efectes dels metalls pesants en molts invertebrats s'han adreçat a investigar aspectes concrets de la biologia dels organismes, com el creixement (Tewari et al. 2001; David, 2003; Yang and Wu, 2003) o la reproducció (Bhattacharya and Vadya, 1999; Daka and Hawkins, 2002), i són rars els que tracten d'analitzar els efectes dels metalls pesants a diferents nivells d'organització biològica.

La concentració d'un metall pesant en un organisme no dóna informació sobre els fluxes d'entrada i sortida ni l'abast dels efectes nocius. Aquests aspectes només es poden inferir a partir d'aproximacions experimentals, tant *in situ* com al laboratori. Degut a les dificultats inherents als experiments en el mar (e.g. interacció dels diferents factors ambientals i dificultat d'atribuir un effecte a un factor concret), la majoria dels estudis que tracten els efectes dels metalls pesants sobre els organismes s'han dut a terme al laboratori (e. g. Brown and Ahsnullah, 1971; Kobayashi, 1980; Rainbow et al., 1980; Rainbow and Wang, 2001). De totes formes, és difícil predir les respostes "naturals" a partir dels resultats obtinguts en condicions "artificials".

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Per la seva part, els escassos experiments de camp disponibles, acostumen a ser descriptius: avaluen sovint només els efectes de la contaminació a partir de mortalitats o canvis d'estructura observats en la comunitat. És així que quan detectem canvis en l'estructura de les comunitats, estem restringits a mesurar només els efectes letals de la contaminació. Ara bé, hom pot constatar que la majoria de contaminants són introduïts en els ambients marins en baixes concentracions i aquestes poden afectar les funcions biològiques de molts organismes sense arribar a matar-los (e.g. Newton and McKenzie, 1995). D'altra banda, els estudis experimentals de camp en torn els efectes nocius dels metalls pesants sobre els organismes bentònics rarament proporcionen resultats concloents. Davant d'això, caldria que els resultats de camp es confirmessin mitjançant experiments de laboratori en els quals els diferents factors poden ser aïllats, les variables controlades i els efectes atribuits a un factor concret. En resum, els estudis experimentals que combinen aproximacions tant al laboratori com *in situ* i que consideren diferents nivells d'organització biològica, són bàsics per a un millor coneixement dels efectes biològics, fisiològics i ecològics dels metalls pesants en els organismes.

Efectes subletals dels metalls pesants sobre cèl·lules, larves i juvenils d'invertebrats bentònics.

Els efectes subletals dels metalls pesants poden tenir repercussions molt dràstiques quan alteren processos biològics dels organismes que afecten indirectament a les successives poblacions. Per exemple, un contaminant pot provocar la mort a la meitat d'individus d'una població i tenir poca o cap significació ecològica, mentre que un contaminant que no mati cap organisme, però sí que retrasi el seu desenvolupament, pot tenir un impacte ecològic considerable (Moriarty, 1983).

En aquest terreny, s'han documentat efectes apartentment contradictoris dels metalls pesants sobre fases inicials dels desenvolupament i sobre cèl·lules d'invertebrats. En general, els efectes depenen de la concentració del metalls i del temps d'exposició. Altes concentracions poden ser tòxiques per cèl·lules (p.e. Auffret and Oubella, 1997), larves (p.e. Wu et al., 1997) i poden alterar la metamorfosis (Negri and Heyward, 2001). En canvi, els mateixos contaminants, però a concentracions més baixes, poden afectar positivament l'assentament larvari d'invertebrats (Ng and Keough, 2003), encara que els aspectes cel·lulars que determinen aquest augment de l'assentament no són del tot coneguts. En aquest ordre de coses, alguns estudis suggereixen, que els metalls pesants poden afectar a l'homeostasis del calci dins la cèl·lula (Verbost et al., 1989; Viarengo et al. 1994; Marchi et al., 2004), el que podria afectar l'agregació i reorganització cel·lular que es produeix durant l'assentament larvari (Burlando et al., 2000; Pourahmad and O'Brien, 2000).

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Per conseqüent, conèixer de manera extensiva els efectes dels metalls pesants en els adults no és suficient per adquirir una idea global de la seva rellevància ecològica, sinó que cal també entendre els efectes que els metalls poden tenir a les primeres fases del desenvolupament dels organismes i, en particular, cal comprendre els mecanismes cel·lulars que hi intervenen.

Biomarcadors de contaminació per metalls pesants en invertebrats bentònics

Un biomarcador es pot definir com "una mesura de fluids corporals, cèl·lules, o teixits que indiquin a nivell cel·lular o bioquímic la presència de contaminants o la magnitud de la resposta de l'hoste" als mateixos (Bodin et al. 2004). Una versió més generalista inclouria també mesures dels efectes fisiològics, de comportament i energètics (Ross et al., 2002; Magni et al., 2005).

Els biomarcadors han resultat ser unes eines molt útils a l'hora d'investigar l'exposició i els efectes dels contaminants en els organismes vius (Depledge and Hopkin, 1993). La taula 1 ens mostra una classificació d'alguns marcadors biològics en funció dels diferents nivells d'organització biològica, que poden ser utilitzats per determinar i monitoritzar efectes tòxics en els organismes vius. L'ús simultani de diferents biomarcadors que indiquin tant l'exposició als contaminants com els seus efectes tòxics a diferents nivells d'organització biològica ens proporcionarà informació del gran nombre de conseqüències que pot tenir la contaminació en el medi ambient.

Els marcadors fisiològics i de comportament ens donen informació dels efectes nocius de la contaminació a nivell d'organisme i de població, i com a consequència, a nivell de tota la comunitat (Taula 1). El creixement, la reproducció, el reclutament, les taxes de filtració, els canvis morfològics, els elements estructurals, són, entre d'altres, els biomarcadors més utilitzats que indiquen respostes fisiològiques a nivell d'individu i els quals poden repercutir tant en les poblacions com en les comunitats.

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Nivell d'organització	Tipus d'efecte	Marcador biològic	Tipus de marcador	Escala temporal
Mol·lècules biològiques	Respostes mol·leculars	Expressió gènica: regulació cel·lular i proteïnes de defensa/reparació: (e. g. HSPs i proteïnes capturadores de metalls)	Marcadors d'exposició	Segons
Cèl·lules	Efectes en teixits i cèl·lules	Agregació i motilitat cel·lular	Marcadors d'efectes	Minuts
Individus	Efectes fisiològics i malalties	Reducció del creixement		Hores
	Deteriorament de la capacitat reproductiva	Canvis en l'esforç reproductiu	Marcadors de canvis ecològics	Dies
	Mort	Respiració Taxes de filtració		
Poblacions	Disminució de la reproducció	Deformitats		
		Mort		
	Reducció en el nombre d'individus	Descens de la població		Mesos
	Descens de la població	Canvis en la densitat de població		
Comunitats	Possible extinció	Analisis de DNA	Marcadors de canvis evolutius	
Ecosistemes	Reducció de la biodiversitat	Canvis en els descriptors ecològics (e.g. biodiversitat, riquesa)		Anys

Taula 1. Alguns exemples de marcadors biològics utilitzats en estudis d'ecotoxicologia (Modificat d'Evenden and Depledge, 1997).

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Els marcadors fisiològics i de comportament ens donen informació dels efectes nocius de la contaminació a nivell d'organisme i de població, i com a conseqüència, a nivell de tota la comunitat (Taula 1). El creixement, la reproducció, el reclutament, les taxes de filtració, els canvis morfològics, els elements estructurals, són, entre d'altres, els biomarcadors més utilitzats que indiquen respostes fisiològiques a nivell d'individu i els quals poden repercutir tant en les poblacions com en les comunitats.

D'altra banda, la majoria de biomarcadors moleculars presenten una resposta molt més ràpida a l'exposició als tòxics que els fisiològics. Per tant, poden ser utilitzats com un primer marcador d'alarma en front de la contaminació. A més, aquest tipus de biomarcadors acostumen a donar informació de la naturalesa del contaminant, ja que molts d'ells són específics pels diferents contaminants (Depledge and Hopkin, 1993).

Els biomarcadors també es poden classificar en dues categories en funció del tipus d'informació que proporcionen:

1.- biomarcadors d'exposició: indiquen que un organisme ha estat en contacte amb una font de contaminació, però aquest pot continuar creixent o reproduint-se amb tota normalitat; aquests tipus de biomarcadors es poden entendre com una part del procés d'aclimatació a alteracions de les condicions del medi, i

2.- biomarcadors d'efecte: relacionen l'exposició a un contaminant amb canvis en la *fitness* (adaptació òptima) de l'organisme (Depledge, 1993) (Taula 2).

Biomarcadors d'exposició	**Biomarcadors d'efecte**
Acumulació	Potencial/Marge de creixement
Metalotionines	Reproducció
Processos oxidatius	HSPs
Citocrom P459	Taxes de filtració

Taula 2: Exemples de biomarcadors d'exposició i d'efecte.

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La majoria de marcadors bioquímics són més apropiats per indicar l' exposició a un tòxic que per indicar els efectes nocius en els sistemes ecològics (Depledge i Hopkin, 1993). En canvi, els fisiològics són millors biomarcadors d'efecte.

Com que cada tipus de biomarcador té els seus pros i contres, un estudi integrat que englobi biomarcadors bioquímics, fisiològics i de comportament, seria la millor aproximació per avaluacions generals. Així és perquè en la natura, les fonts i els nivells de contaminació són sovint incerts el que impedeix, predeterminar un o uns pocs marcadors adequats.

Les esponges com a biomonitors de metalls pesants

Durant els últims anys, s'han dut a terme un nombre considerable d'estudis referents al monitoratge biològic dels metalls pesants. Els organismes bentònics i sèssils semblen ser particularment apropiats pel biomonitoratge de contaminació local, ja que no poden fugir de les emissions de tòxics d'una determinada àrea (Rosenberg et al., 2004; Naranjo et al., 1998; Carballo and Naranjo, 2002). La majoria d'aquests estudis s'han fet amb invertebrats de fons tous (Ugolini et al., 2004; Usero et al., 2005). No obstant, malgrat la seva importància ecològica, els fons rocosos han estat objecte de menys interès. L'excepció la trobem en el cas dels musclos i cloïsses, els quals sí que han estat utilitzats en varis programes de biomonitoratge, com per exemple el "mussel watch" (Claisse, 1989; Jernelov, 1996).

Dels diversos organismes bentònics que habiten els fons rocosos, les esponges compleixen molts dels criteris desitjables que es demanen a un bon biomonitor: són organismes sedentaris, abundants, àmpliament distribuïts, de vida llarga, són disponibles durant tot l'any, són suficientment grans per proveir el teixit necessari per les anàlisis, resistents a la manipulació que requereixen molts dels experiments i són tolerants a les variacions dels paràmetres fisico-químics ambientals (Carballo et al., 1996). A més, les esponges són resistents o susceptibles als metalls pesants, si bé això varia notablement en funció de l'espècie o el metall considerat (Perez, 2001), i poden acumular tòxics en funció de la quantitat de metall present en l'ambient (Olesen i Weeks, 1994; Hansen et al. 1995).

Si bé la idoneïtat de les esponges com a biomonitors dels metalls pesants ha estat mencionada per varis autors (Patel et al., 1985; Hansen et al., 1995), aquestes no han estat utilitzades en estudis d'avaluació globals. Perez i col·laboradors han documentat la capacitat d'algunes espècies d'esponges per reflectir la contaminació per metalls pesants, tant a nivell espaial com temporal. No obstant això, com he dit

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abans, hi ha molts aspectes que s'han de considerar abans de que una espècie es pugui proposar de forma general com a biomonitor de metalls pesants. Abans, s'haurien de conèixer les respostes espècie-específiques d'acumulació de metalls pesants i les variacions espaials i temporals (a llarg i curt termini) en l'acumulació dels metalls pesants en funció de la concentració present al medi, a més dels efectes que aquests poden tenir en les espècies considerades.

D'altra banda, s'haurien de dur a terme estudis que tractin sobre els efectes subletals de concentracions de metalls pesants en les poblacions d'esponges a fi de predir les conseqüències ecològiques que pot tenir una determinada càrrega de metall. Fins a l'actualitat, hi ha pocs estudis dels efectes dels metalls pesants sobre les esponges (Pérez et al. 2005; Berthet et al. 2005). Per tant, un estudi dels efectes dels metalls pesants a varis nivells d'organització (des de les molècules a l'organisme), cosa que necessàriament implica l'ús d'un ampli rang de biomarcadors, milloraria el coneixement que tenim fins ara dels efectes dels metalls pesants sobre les esponges.

A nivell molecular, la idoneïtat de les proteïnes d'estrès ("Heat shock proteins - HSPs -, en anglès, sigles que utilitzem d'ara endavant) com a biomarcadors dels efectes adversos que poden tenir els metalls pesants, ha estat demostrada per altres invertebrats (Sanders, 1990). Pel cas de les esponges, les HSPs s'han estudiat com a resposta a l'exposició a diferents factors d'estrés, com la inducció per la fracció orgànica no iònica d'un riu contaminat, alguns PCBs i el cadmi (Wiens et al., 1990, Müller et al., 1995, Müller et al., 1998). De totes formes, la inducció de les proteïnes d'estrès envers la contaminació per metalls pesants *in situ* ha estat poc estudiada. A nivell d'organisme, fins a l'actualitat, no s'han fet estudis dels efectes dels metalls pesants en les esponges. Aquestes recerques haurien de considerar variables fisiològiques com el creixement, canvis en la morfologia, esforç reproductiu, supervivència i taxes de filtració, ja que són aquestes variables les que poden alterar l'estat de salut d'una espècie i, per tant, la dinàmica de població.

Els estudis dirigits a conèixer els efectes dels metalls pesants en les primeres fases del desenvolupament de les esponges són també particularment interessants. Així ho són perquè les poblacions d'esponges, com molts altres invertebrats, depenen pràcticament de l´èxit de l'assentament larvari per mantenir-se. És àmpliament reconegut que nivells moderats d'alguns contaminants que són aparentment innocus pels adults de molts invertebrats, poden afectar negativament la fisiologia de les fases larvàries i dels seus juvenils (Rinkevich and Loya, 1977; His et al., 1999). Per tant, la sensibilitat de les larves i juvenils davant dels nivells baixos de contaminació pot determinar en gran mesura una decadència subtil i fins i tot la desaparició de poblacions d'esponges que viuen en zones contaminades per metalls pesants. Durant l'assentament larvari i la metamorfosis de les esponges, es dóna una extensiva reorganització, la qual implica el moviment, el reconeixement i l'agregament cel·lular

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(Weisman et al., 1985), de manera que qualsevol modificació que puguin causar els metalls pesants en un o varis aspectes del comportament cel·lular, pot també causar alteracions en l'assentament de les esponges.

Per tant, encara que s'hagi demostrat la utilitat d'algunes espècies d'esponges en biomonitoratges de fons rocosos, són encara necessaris estudis sobre l'acumulació, mecanismes d'assimilació dels metalls pesants, variacions tant espaials com temporals de l'acumulació de metalls, per tal de poder utilitzar les esponges com a biomonitors de metalls pesants de forma general. De la mateixa manera, també són necessaris estudis sobre els efectes subletals que els metalls pesants poden tenir sobre les poblacions d'esponges, a efectes d'esbrinar les conseqüències ecològiques que pot tenir una contaminació donada.

En aquesta tesi doctoral s'estudien els efectes nocius que poden tenir els metalls pesants en les poblacions d'esponges, seguint la tendència actual a en els estudis d' ecotoxicologia que proposa utilitzar un ampli ventall d'aproximacions que impliquin varies metodologies, cadascuna de les quals focalitzada en un nivell d'organització biològica. Amb aquest fi el monitoratge, els experiments al mar mitjançant el trasplantament d'esponges i els experiments al laboratori, han estat les tècniques que s'han utilitzat, sota la consideració que la combinació de les diferents metodologies és el millor instrument per determinar la contaminació per metalls i els seus efectes en les poblacions bentòniques dels ambients marins costaners.

Després d'una revisió exhaustiva de la literatura ecotoxicològica, podem dir que en aquest estudi es presenta per primera vegada, una aproximació multidisciplinària, que engloba biologia, fisiologia, comportament larvari i cel·lular, inducció/inhibició mol·lecular, amb la finalitat d'estudiar els efectes de la contaminació per metalls en les esponges. De totes formes, part d'aquest estudi té les limitacions inherents al seu caràcter intensiu, les quals caldria mencionar: En els estudis experimentals, s'han hagut de seleccionar les espècies més adequades en cada cas, per diferents motius: e.g. presència en llocs contaminats, resistència a la manipulació, facilitat per obtenir cél·lules dissociades i/o larves. Igualment, per als estudis experimentals dels efectes a nivell mol·lecular, larvari, i cel.lular s'han seleccionat els metalls *a priori* més relevants en la zona d'estudi.

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Objectius i estructura de la tesi

L'objectiu general d'aquest estudi ha estat analitzar les respostes de les esponges a concentracions subletals de metalls pesants i determinar la seva utilitat com a biomonitors d'aquests metalls. En primer lloc, s'ha volgut conèixer la capacitat de les esponges per acumular metalls pesants tant a una escala temporal com espaial. En segon lloc, mitjançant l'ús de diferents biomarcadors, analitzar els efectes subletals dels metalls en les esponges, a diferents nivells d'organització biològica, des de les mol·lècules fins a les poblacions. S'han combinat experiments de camp i laboratori per tal d'entendre millor els models d'acumulació i els efectes en funció de la espècie i metall considerat.

Els objectius específics han estat:

- Estudiar l'acumulació de metalls pesants en diferents espècies d'esponges i en diferents localitats amb l'objectiu de determinar si són capaces de reflectir la quantitat de metall present en l'ambient. **Capítol 2.**

- Buscar una espècie d'esponja que sigui capaç de reflectir les variacions espaials i temporals dels metalls pesants disponibles en l'ambient, la qual seria una bona candidata com a biomonitor de metalls pesants. **Capítol 2.**

- Estudiar els efectes que poden causar concentracions subletals de metalls pesants sobre la fisiologia i biologia de les esponges. **Capítols 3 i 4.**

- Determinar si les HSPs poden ser bons marcadors mol·leculars de concentracions subletals de coure, estudiant experimentalment la seva inducció per coure, tant al laboratori com al mar. **Capítol 5.**

- Examinar els possibles efectes de concentracions subletals de coure sobre les defenses químiques (bioactivitat) de les esponges. **Capítol 5**.

- Estudiar els efectes de concentracions moderades de coure i cadmi sobre l'assentament i posterior supervivència de juvenils d'esponges. **Capítol 6.**

- Estudiar els efectes de concentracions moderades de coure i cadmi sobre la motilitat i l'agregació de les cèl·lules de les esponges. **Capítol 7.**

- Buscar explicacions a les alteracions de la motilitat i l'agregació cel·lular implicades en els processos d' assentament de les esponges, detectades en els anteriors capítols. **Capítol 8.**

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Aquest estudi ha estat dividit en tres parts principals, les quals tracten sobre (i) l'acumulació, (ii) els efectes dels metalls pesants sobre els adults i (iii) sobre les primeres fases dels desenvolupament, respectivament:

Part A: Esponges marines com biomonitors espaials i temporals de metalls pesants. Capítol 2.

En aquesta part s'ha estudiat l'acumulació de metalls entre diferents espècies i s'ha determinat la capacitat d'algunes esponges de reflectir les concentracions de metalls pesants presents en l'ambient. Aquesta part s'ha abordat mitjançant un monitoratge al llarg de la costa catalana. Entre les esponges més abundants del Mediterrani occidental s'han seleccionat 4 espècies: *Dysidea avara*, *Phorbas tenacior*, *Crambe crambe* i *Chondrosia reniformis*. Aquestes espècies presenten diferents característiques estructurals, que, *a priori*, semblen poder influenciar en les seves diferents capacitats de bioacumular metalls. Així mateix, s'ha comparat la concentració dels metalls en els teixits de les esponges amb la dels sediments a tres localitats amb diferents nivells de contaminació per metalls.

A continuació, de les espècies estudiades, s'ha escollit l' espècie *a priori* més idònia (*Crambe crambe*) a efectes d'analitzar la seva capacitat de reflectir les variacions de metalls pesants en el medi a una a escala espaial i temporal més àmplia.

Les variacions espaials s'han analitzat comparant l'acumulació de metalls en l'esponja seleccionada i en el sediment de 16 localitats al llarg de la costa catalana (NE Península Ibèrica, mar Mediterrani). Les estacions de mostreig s'han seleccionat amb la intenció que reflectissin els diferents graus d'impactes antropogènics que podem trobar a la zona. Les variacions temporals s'han analitzat mitjançant un mostreig mensual de l'acumulació de metalls en l'esponja en dues localitats diferents.

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Part B: Efectes de la contaminació per metalls pesants en dues espècies d'esponges mediterrànies (*Crambe cram*be i *Chondrosia reniformis*). Capítol 3, 4 i 5.

En aquesta part, s'han estudiat els efectes biològics i fisiològics de concentracions subletals de metalls pesants en adults de dues espècies d'esponges que presenten patrons d'acumulació oposats (veure Part A; capítol 2) i diferent organització estructural (esquelet mineral *vs.* col·lagen). Per assolir aquest objectiu, s'ha dut a terme un experiment de camp on especímens de les dues espècies han estat trasplantats des d'una zona control (no contaminada) a un lloc contaminat i s'han examinat les respostes de les esponges a diferents nivells d'organització biològica (des de l'individu fins les mol·lècules). S'han analitzat, d'una banda, variables a nivell d'organisme, com per exemple canvis en la morfologia, creixement, supervivència, contingut de col·lagen i taxes de filtració; i d'altra banda, variables a nivell molecular, com ara la producció de substàncies bioactives i la síntesis de proteïnes d'estrès (HSPs). Paral·lelament s'ha realitzat un experiment al laboratori, per tal de verificar que l'expressió de les HSPs és bàsicament deguda als efectes estressants del coure, i no a altres factors no controlats del lloc contaminat. Al laboratori a més d'analitzar l'expressió de les HSPs per coure, també es va estudiar com la contaminació i la inducció de HSPs afectava a la toxicitat de les esponges. Per saber si altres variables que no fossin la contaminació per metalls eren diferents al lloc contaminat i al lloc control podent influenciar els resultats dels experiments de camp, s'han caracteritzat les principals variables fisico-químiques (com els nutrients la matèria orgànica particulada, les taxes de sedimentació, la qualitat del sediment, l'hidrodinamisme i la irradiància) de l'àrea d'estudi (zona control i contaminada) durant el període que van durar el trasplantaments.

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Part C: Efectes dels metalls pesants en l'assentament larvari, la supervivència dels juvenils i el comportament cèl·lular de les esponges. Capítols 6, 7 i 8.

En aquesta part, s'ha examinat com nivells subletals de metalls pesants (Cu i Cd) afecten a les primeres fases del desenvolupament de les esponges, les quals, són per norma general, molt més sensibles a la contaminació que els adults. Per aquest etudi s'han seleccionat les espècies *Scopalina lophyropoda* i *Crambe crambe* ja que són espècies representatives de les esponges sublitorals mediterrànies i presenten un alliberament massiu de larves durant un període curt de temps, fet que permet la recol·lecció d'un nombre suficient de larves pels experiments. Les larves s'han sotmès a polsos curts de Cu i Cd al laboratori ; s'ha mesurat l'assentament larvari i la posterior supervivència dels juvenils després de 6 mesos. Com que l'assentament implica una intensa reorganització i reagrupació cel·lular, també s'han estudiat els possibles efectes de polsos curts de Cu i Cd en el comportament de les cèl·lules d'esponges, a fi d'entendre millor les respostes observades en l'assentament. Aprofitant els canvis de forma que pateixen les cèl·lules al desplaçar-se, s'han estudiat els efectes d'aquests dos metalls a partir de l'agregació cel·lular i diversos índex de forma. S'ha dut a terme un estudi addicional per tal d'investigar els mecanismes gràcies als quals el coure i el cadmi afecten positivament a les cèl·lules i larves de les esponges, i com l'alteració de l' homeostasis del calci provocada pels metalls pesants pot estar involucrada en aquests efectes. Per realitzar aquest estudi, s'han incubat cèl·lules i larves de *S. lophyropoda* en aigua de mar sense calci i amb concentracions de Cu i Cd i s'han monitoritzat els efectes. Les concentracions utilitzades en aquests experiments pretenen estar dins del rang de concentracions que es poden trobar en aigües costaneres contaminades.

Tres capítols d'aquesta tesi (capítols 3, 4 i 5), ja han estat publicats a *Mar. Pollut. Bull.*, *Environ. Pollut.,* and *Environ. Toxicol. Chem.*, respectivament. Altres 3 (capítols 6, 7 i 8) estan en prensa a *Aquatic toxicol.*, *Arch. Env. Cont. and Toxicol.* i *J. Exp. Mar. Biol. Ecol.* respectivament. El restant (capítol 2) està sotmès.

Resultats

Els següents paràgrafs resumeixen els principals resultats, recollits en els diferents capítols, obtinguts en aquesta tesi doctoral. Tots els capítols han estat publicats o estan en process de revisió en revistes internacionals.

Capítol 2: Les esponges com a biomonitors dels metalls pesants en mostrejos temporals i espaials en el Mediterrani nord-occidental: comparació multiespecífica.

La contaminació per metalls pesants ha augmentat dràsticament durant els últims 20 anys a les costes mediterrànies. En aquest treball, hem dut a terme un estudi comparatiu de l'acumulació de metalls entre quatre espècies d'esponges mediterrànies, a efectes de seleccionar-ne la més apropiada per ser utilitzada en estudis de monitoratge de metalls pesants en el medi marí. Els resultats obtinguts indiquen que la bioacumulació de coure s'ajusta a una estratègia d'acumulació neta, mentre que la concentració de plom sembla estar regulada en la majoria de les esponges estudiades. D'entre totes les espècies estudiades, només *Crambe crambe* bioacumula plom i coure en funció de la disponibilitat d'aquest metalls en l'ambient, el que la fa apropiada pels monitoratges. També s'ha comparat l'acumulació de metalls en *C. crambe* amb la dels sediments, amb l'objectiu d'examinar la seva capacitat com a bioindicador tant a escales espaials com a temporals. Els resultats indiquen que aquesta espècie proporciona informació dels diferents nivells de metalls pesants presents en l'ambient al llarg del temps. Més concretament, *C. crambe* és capaç de mostrar fluctuacions estacionals de la quantitat de metalls disponibles, les quals serien impossibles de detectar mitjançant els mostrejos clàssics (sediments).

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Capítol 3: Efectes subletals dels metalls en l'esponja mediterrània *Crambe crambe*: acumulació dels metalls i respostes biològiques.

S'han examinat els efectes de baixes concentracions de metalls pesants en el creixement, esforç reproductiu, morfologia i supervivència dels adults i en l'assentament larvari i supervivència post-asentament de la esponja *Crambe crambe*. Esponges d'una zona control, "*a priori* considerada neta", es van trasplantar tant en la mateixa zona (control del efecte transplantament) com a una zona contaminada propera. Després de 4 mesos, es van mesurar les variables mencionades a les esponges intactes de la zona neta, a les transplantades a mateixa zona, a les transplantades a la zona contaminada, i a les esponges que vivien de forma natural a la zona contaminada. També es van mesurar les principals variables ambientals (químiques i físiques) durant tot l'experiment a ambdues zones. A excepció de diferències puntuals en la quantitat de matèria orgànica particulada, silicats, nitrats i hidrodinamisme, la majoria de variables ambientals es van comportar de forma similar als dos llocs. En canvi, el coure a l'aigua, i la concentració de matèria orgànica en el sediment van ser significativament més altes a la zona contaminada. Aquest dos factors podrien estar implicats en els efectes observats: disminució del percentatge d'especímens amb embrions, morfologies més irregulars, i disminució de les taxes de creixement, en els individus trasplantats a la zona contaminada. Els individus que vivien a la zona contaminada i els trasplantats a aquesta zona durant 4 mesos, van bioacumular per terme mig 10 vegades més coure, 2,5 vegades més plom, i 3,2 vegades més vanadi que els de la zona control. Les diferències van ser fortament significatives en el cas del coure ($p<0.001$),lleugerament significatives ($p<0.05$) en el cas del plom (segurament degut l' alta variabilitat entre els especímens) i no significatives en el cas del vanadi ($p>0.05$). Per tant, podríem dir que *C. crambe* és un bon indicador de la contaminació per metalls ja que acumula metalls en altes concentracions. D' altra banda, les esponges del lloc contaminat van presentar un creixement menor, fecunditat i supervivència més baixes i un augment de la irregularitat en la forma que en alguns casos acabava en fissions. Per tant, els hàbitats contaminats per metalls pesants poden comprometre a mig/llarg termini, l'estructura i dinàmica de les poblacions de l'esponja.

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Capítol 4: Respostes de l'esponja mediterrània *Chondrosia reniformis* a la contaminació per metalls pesants.

S'han estudiat els efectes de la contaminació per metalls en l'esponja *Chondrosia reniformis*, trasplantant esponges d'una zona control no contaminada a una zona contaminada principalment per coure. No s'han observat efectes de la contaminació en el creixement i forma de l'esponja, inducció de les proteïnes d'estrès i acumulació de metalls pesants. Per contra, després de 4 mesos, les esponges trasplantades a la zona contaminada han experimentat una disminució en les taxes de filtració, un augment en el contingut de col·lagen i una supervivència més baixa. Això confirma un fort efecte de la contaminació en aquesta esponja. Les condicions ambientals de la zona contaminada semblen induir una reducció dràstica de les taxes de bombeig de l'esponja, procés que indirectament provoca una reducció de l'alimentació i la conseqüent disminució dels elements cel·lulars, comportant un increment de la proporció de col·lagen. El resultat final és una alta mortalitat de l'esponja.

Capítol 5: Es produeixen canvis en la toxicitat natural de les esponges quan hi ha una inducció de les proteïnes d'estrès?.

Crambe crambe és una esponja mediterrània que produeix substàncies tòxiques i que habita en els fons rocosos sublitorals, inclosos alguns hàbitats moderadament contaminats. En aquest estudi, s'ha investigat si la presència de coure produeix estrès en l'esponja, mesurant-lo mitjançant la quantificació de proteïnes d'estrès (HSP), i si aquest estrès altera simultàniament la producció de defenses químiques (bioactivitat) a l'esponja. Dos tipus d'HSP diferents, de 54 i 72 Kda respectivament, van ser induïdes en més o menys mesura per la contaminació per coure. L'HSP54 és mes abundant que l'HSP72, la qual, en canvi, va respondre amb molta rapidesa encara que va ser poc persistent. En l'experiment de camp, s'ha trobat una major concentració d'HSP54 en els individus que viuen de forma natural a la zona contaminada que en els individus trasplantats allà durant 4 mesos. En canvi, només l'expressió d'HSP72 ha estat induïda en els individus que han estat trasplantats a la zona contaminada. A l'experiment al laboratori, ambdues proteïnes han estat induïdes pel coure a concentracions de 30 µg/L, mentre que han estat inhibides a concentracions de 100 µg/L de coure. Els valors més alts d'HSP54 i HSP72 els presenten les esponges que han mostrat els valors més baixos de toxicitat. Segons això, la toxicitat i la producció de les HSP estan negativament correlacionades y segueixen, per tant, tendències oposades. La conclusió és que, en condicions d'estrès, les esponges inverteixen prioritàriament en mecanismes de reparació cel·lular a expenses de la síntesis de molècules tòxiques.

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Capítol 6: Efectes contradictoris dels metalls pesants en l'assentament larvari i posterior supervivència dels juvenils d'esponges.

Els metalls pesants contaminen en gran mesura els sediments i les aigües de les zones costaneres, amenaçant les primeres fases del desenvolupament de molts invertebrats. Alteracions en aquestes fases, poden determinar la decadència i fins i tot la desaparició de poblacions d'invertebrats marins en ambients contaminats. En aquest capítol hem volgut determinar la influència que poden tenir els metalls pesants (Cu i Cd) en l'assentament larvari i la supervivència posterior (6 mesos) dels juvenils de dues espècies d'esponges mediterrànies (*Crambe crambe* i *Scopalina lophyropoda*). Per això, les larves d'ambdues espècies s'han sotmès a concentracions de Cu i Cd durant una setmana. Polses curts de coure i cadmi no han afectat l'assentament de *Crambe crambe* i tampoc la posterior supervivència dels juvenils. En canvi, les mateixes exposicions de coure i cadmi han afavorit l'assentament larvari de *S. lophyropoda*, sense afectar, però, la posterior supervivència dels juvenils.

Capítol 7: Efectes contradictoris dels metalls pesants en el comportament cel·lular de les esponges.

Les esponges processen i filtren grans quantitats d'aigua. Això les fa particularment vulnerables als metalls del medi. En aquest sentit, destaquen els efectes nocius del coure i el cadmi, els quals estan àmpliament descrits en altres invertebrats marins. En aquest capítol estudiem l'efecte d'aquests dos metalls en l'agregació cel·lular i en diversos índexs de forma - els quals donen una idea de la motilitat de les cèl·lules. Els dos metalls estudiats afecten a la forma i a l' agregació de les cèl·lules de les esponges. Concentracions de coure i cadmi afavoreixen la formació de pseudòpodes i la motilitat cel·lular. De la mateixa manera, els dos metalls estudiats afavoreixen l'agregació cel·lular. Aquests resultats ens mostren que les cèl·lules responen als metalls i que dites respostes poden ser mesurades a partir de diversos índexs de forma.

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Capítol 8: Paper dels metalls pesants els processos cel·lulars en absència de calci. Conseqüències en l'assentament larvari.

Com hem vist en altres capítols (Capítols 6 i 7), concentracions moderades de coure i cadmi poden afectar positivament l'assentament larvari, l'agregació cel·lular i motilitat de les esponges. Tanmateix, els mecanismes cel·lulars, gràcies als quals es produeixen els efectes observats, no són del tot coneguts. Així és malgrat es coneix que l'agregació cel·lular necessita d'un aport de calci extracel·lular, el qual, en absència de calci extern, pot provenir de l'alliberament dels dipòsits de calci intracel·lular. A fi de conèixer millor el mecanisme pel qual Cd i Cu afecten positivament a les cèl·lules i larves de les esponges i, per analitzar com l'alteració de l' homeostasis del calci intervé en aquests efectes, hem incubat cèl·lules de *S. lophyropoda* durant tres hores i larves durant 1 setmana en presència de metalls al laboratori; els tractaments experimentals han estat: aigua de mar sense calci (CFSW), aigua de mar (SW), i aigua de mar sense calci contaminada amb coure (Cu CFSW) i cadmi (Cd CFSW). D'acord amb els resultats, sembla que els dos metalls juguen un paper actiu en els processos dependents de calci: les cèl·lules incubades en Cd CFWS i Cu CFSW s'agreguen igual que les incubades en aigua de mar malgrat que no disposen de calci. De la mateixa manera, tant el Cd com el Cu han accelerat l'assentament larvari en els primers moments, per bé que després de 5 dies d'incubació han provocat efectes tòxics que han acabat amb la mortalitat dels assentats. Per tant, podríem dir que, malgrat que a curt termini sembla que els metalls tinguin un efecte positiu, una exposició més prolongada o exposicions a concentracions més altes poden provocar alteracions en les funcions cel·lulars i comprometre la viabilitat i la fitness de les esponges.

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Conclusions

- Hi ha diferències importants en els patrons d'acumulació de metalls per les esponges. Diferències en la morfologia, en el sistema aqüífer, i per tant, en les taxes de filtració; així com diferències en la naturalesa dels esquelets (mineral o proteic) són probablement les responsables dels diferents patrons d'acumulació observats.

- Els mecanismes d'acumulació dels metalls també varien en funció del metall considerat. En general, la bioacumulació de coure s'ajusta a una estratègia d'**acumulació**, mentre que la concentració de plom sembla estar **regulada** en les esponges.

- Les diferents espècies analitzades acumulen en diferent grau d'eficàcia els metalls presents en el medi. L'espècie *Crambe crambe* és capaç de bioacumular coure i plom en funció de la quantitat disponible en el medi. *Phorbas tenacior* i *Dysidea avara* bioacumulen coure, però no plom; i *Chondrosia reniformis* no bioacumula ni coure ni plom.

- *Crambe crambe* és capaç de reflectir canvis espaials i temporals de la quantitat de metall disponible en els ambients marins Mediterranis. Així doncs, *C. crambe* ofereix avantatges dins del context del biomonitoratge perquè pot proporcionar informació dels nivells de metalls pesants en l'ambient.

- *Crambe crambe*, sota concentracions subletals de metalls pesants, experimenta canvis morfològics, fisiològics i de comportament, com ara són alteracions de forma, taxes de creixement, esforç reproductiu i acumulació dels metalls, els quals poden ser mesurades.

- En canvi, *Chondrosia reniformis* no experimenta canvis en la seva morfologia, creixement o acumulació de metalls en funció de la concentració dels metalls presents en l'ambient, per la qual cosa, no pot ser considerada un bon biomonitor. Es tracta d'un "organisme sensible" a la contaminació per metalls, ja que contaminacions moderades causen canvis en la seva fisiologia, com ara són la disminució de l'activitat filtradora, que freqüentment acaben comportant-li la mort.

- Les respostes a nivell molecular també difereixen en funció de les espècies considerades. Mentre que concentracions subletals de coure no indueixen l'expressió de HSPs a *Chondrosia reniformis*, les proteïnes HSP54 and HSP72 s'expressen en resposta a contaminació per coure a *Crambe crambe*.

Aquesta és la primera vegada que una proteïna de la família HSP60 s'ha trobat en esponges. La proteïna HSP72 sembla bàsicament induïble, mentre que HSP54 pot ser tant constitutiva com induïble.

- Les diferències en la tolerància a la contaminació per metalls pesants entre *C. crambe* i *C. reniformis* podrien estar relacionades amb les diferents expressions de les HSPs. *C. crambe* indueix la producció de HSPs, i per tant, pot viure en àrees contaminades ja que disposa d'un mecanisme reparador del possibles danys cel·lulars produïts pels metalls. Mentre que en les mateixes condicions, *C. reniformis* no indueix la producció de HSPs i, per conseqüent, no és capaç de viure en àrees contaminades. La falta de resposta a nivell molecular en *C. reniformis* pot ser imputable a un fort dany metabòlic, i al conseqüent deteriorament d'algunes activitats cel·lulars i fisiològiques, les quals es tradueixen en un fort detriment de les taxes de la filtració.

- *Crambe crambe*, sotmesa a una contaminació subletal per metalls pesants, sembla invertir els recursos disponibles preferentment en mecanismes de reparació de les proteïnes, com per exemple les HSPs, més que no pas en la producció de defenses químiques.

- Polsos curts de coure i cadmi, a les concentracions utilitzades, no afecten ni a l'assentament de les larves de *C. crambe* ni a la posterior supervivència dels juvenils. En canvi, polsos curts de coure i cadmi semblen afavorir l'assentament de les larves de *S. lophyropoda*, sense afectar a la posterior supervivència dels juvenils.

- Els metalls pesants poden induir canvis en la forma de les cèl·lules de les esponges. Coure i cadmi faciliten la formació de pseudòpodes, filòpodes i, per tant, la motilitat cel·lular. Tant el coure com el cadmi promouen l'agregació cel·lular.

- La detecció dels efectes dels metalls pesants sobre les esponges a nivell cel·lular, mitjançant varis índexs de forma, pot anticipar-nos canvis que afecten nivells més alts d'organització biològica, com serien els organismes i les poblacions.

- A les esponges, el coure i el cadmi semblen jugar un paper actiu en els processos cel·lulars depenents del calci. Això explicaria els efectes positius trobats en cèl·lules i larves d'esponges incubades amb aquests metalls. A curt termini, no s'evidencia cap efecte nociu. No obstant, en exposicions més llargues o a concentracions més altes, els metalls pesants podrien acumularse dins les cèl·lules comportant un perjudici per la supervivència i l'assentament larvari.