Joana Pedroso de Lima Cabral de Oliveira

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Universidade de Coimbra

Rocky shore macroinvertebrate assemblages as indicators of sewage pollution

Joana Pedroso de Lima Cabral de Oliveira

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CONTENTS

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Rocky shores are under high human pressure, being daily exposed to several anthropogenic impacts such as nutrient enrichment or chemical pollution due to sewage discharges. In order to prevent further deterioration of coastal areas the search for efficient indices and indicators has become one of the hot topics in today's scientific publication. However, a detailed knowledge of community responses to anthropogenic impacts is essential to sustain those indices. Therefore, the main goal of the present thesis was to to understand how sewage pollution may affect intertidal macroinvertebrate assemblages from rocky shores, and it can be divided into two key points. First, to examine patterns such as abundance, species richness, structure and secondary production of the macrofauna assemblages exposed to sewage discharges. Second, compare the response of macrofauna assemblages to sewage discharges in Atlantic and Mediterranean shores, in order to search for similar patterns.

The first key point was explored in the chapter 2 of this thesis. This chapter aims to provide a comprehensive assessment of sewage effects on macroinvertebrate intertidal assemblages at three heights on the shore (littoral fringe, sublittoral fringe, and eulittoral) using an asymmetrical design. Sewage pollution changed the environmental variables and the abundance of macroinvertebrates, being *Mytilus galloprovincialis, Melarhaphe neritoides* and *Chthamalus montagui* the species most responsible for the dissimilarities observed. Effects were different on the three intertidal levels: community structure changed at the sublittoral fringe; suspension-feeders abundances and species richness increased at the eulittoral; no differences were detected at the littoral fringe. Furthermore, the results confirm that the presence of sewage discharges tended to benefit suspension feeders, and that the sensitive species were replaced by opportunistic ones. Regarding secondary production, it was higher in the impacted area, but mostly due to the production of tolerant species and suspension feeders.

Sewage pollution can also result in increasing levels of trace elements in the environment which can pose serious risks both to wildlife and human health. This highlights the importance of study the role of sewage discharges in the contamination of aquatic systems, especially in rocky shores, since part of our food resources is directly collected on coastal waters. Chapter 3 aims to understand if sewage pollution is contributing to the accumulation of trace elements on edible species (*M. galloprovincialis, Patella ulyssiponensis* and *Phorcus*

lineatus). It was found evidence of arsenic contamination due to the sewage discharges. With this in mind, the concentrations of arsenic on intertidal rocky shore macroalgae exposed to contaminated sewage discharges were measured. The results showed significantly higher concentrations of arsenic near the sewage discharges in all the species (*Asparagopsis armata*, *Codium* sp., *Plocamium cartilagineum* and *Ulva* sp.) except *Saccorhiza polyschides*. At the same time, it was assessed the potential of the studied species as bioindicators. At species level, the limpets (*P. ulyssiponensis*) seemed the best bioindicator for trace elements, while *A. armata* seemed to be the best choice to detect arsenic contamination.

Finally, the second key point was explored in the chapter 4. This chapter assesses the potential of eulittoral assemblages in representing an effective indicator of environmental perturbations related to sewage discharges across different environmental and geographical contexts (Atlantic shores, Mediterranean shores and Atlantic island shores). Multivariate analyses showed that, in all shores, the structure of eulittoral invertebrate assemblages did not differ significantly between reference and impacted areas. However, limpet populations of the genus *Patella* were significantly affected by sewage discharges. The responses differed across Atlantic and Mediterranean shores, probably due to within-species heterogeneities of ecological traits. Additionally, in the Atlantic island shore, an outfall has been decommissioned from the original beach (due to civil movements to save a surf spot), which has provided an ecological opportunity to assess also the recovery dynamics of the community. After the remove of the outfall, the dominant species of the eulittoral assemblage appeared to recover. However, in more detail it was possible to observe that littorinids populations had still not fully recovered.

This thesis contributes with important information to understand the response of intertidal macroinvertebrate assemblages to sewage pollution. The identification of all species to the lowest taxonomic level possible, and the sampling along all vertical levels, allowed a comprehensive knowledge of the effects of sewage discharges on macrofauna assemblages from rocky shores. Furthermore, a first attempt to evaluate and compare the responses of rocky shore macrofauna assemblages to sewage pollution in different geographical areas was carried out. Eulittoral assemblages from Atlantic and Mediterranean shores were studied, and the results seem to suggest that limpets of the genus *Patella* may be effective in evaluating the environmental status of coastal systems in relation to human impacts, such as sewage discharges.

As zonas costeiras estão sujeitas a intensa pressão humana, sendo o enriquecimento orgânico e de nutrientes, com origem em descargas de águas residuais, um dos principais problemas. Atualmente, procurando prevenir a contínua deterioração das zonas costeiras, a comunidade científica tem concentrado esforços na procura de índices e indicadores eficazes. Contudo, de forma a alcançarmos sustentadas ferramentas de gestão, é indispensável, antes do mais, adquirir um conhecimento detalhado das respostas das comunidades biológicas aos diferentes impactos antropogénicos, como o são, por exemplo, as descargas de águas residuais.

Nesse sentido, a presente tese como objetivo principal compreender os efeitos daquelas descargas nas comunidades de macroinvertebrados da zona intertidal da costa rochosa, podendo ser dividida em dois pontos principais: no inicial, quisemos estudar os padrões de abundância, riqueza específica, estrutura da comunidade e produção secundária das comunidades de macroinvertebrados expostos a descargas de águas residuais; no segundo, procurou-se comparar as respostas das comunidades intertidais expostas a este foco de perturbação em diferentes bioregiões (Atlântico e Mediterrâneo), por forma a encontrar padrões de resposta semelhantes.

O primeiro objetivo desta tese foi explorado no capítulo 2, que pretende contribuir para um melhor conhecimento dos efeitos da presença de descargas de águas residuais nas comunidades de macroinvertebrados, ao longo dos diferentes andares da zona intertidal. A presença das descargas de águas residuais alterou os parâmetros ambientais, bem como a abundância de macroinvertebrados, sendo *Mytilus galloprovincialis*, *Melarhaphe neritoides* e *Chthamalus montagui* as principais espécies responsáveis pelas diferenças observadas. Diferentes efeitos foram, entretanto, observados nos três andares: na franja sublitoral a estrutura da comunidade foi alterada; no eulitoral aumentou a riqueza específica bem como a densidade de espécies filtradoras; na franja litoral não foram observadas diferenças. Ademais, os resultados demonstraram que a presença de descargas de águas residuais beneficia as espécies filtradoras e, ainda, que espécies sensíveis foram substituídas por espécies tolerantes. A produção secundária da comunidade foi também superior junto ao foco de poluição, mas este incremento deveu-se, essencialmente, aos níveis de produção de espécies tolerantes e filtradoras.

Paralelamente, as descargas de águas residuais podem também contribuir para a contaminação dos sistemas aquáticos por metais e metalóides, com sérios riscos tanto para a vida selvagem como para a saúde humana. O capítulo 3 visa, assim, compreender se as descargas de águas residuais contribuem para a acumulação destes contaminantes em espécies comestíveis (*M. galloprovincialis*, *Patella ulyssiponensis* e *Phorcus lineatus*). Os resultados demonstraram que há contaminação por arsénio na área perturbada, tendo sido medidas as suas concentrações em algas intertidais também expostas às descargas de águas residuais contaminadas. Foram encontradas concentrações superiores perto das descargas em todas as espécies, exceto *Saccorhiza polyschides*. Ao mesmo tempo, foi avaliado o potencial das espécies estudadas como bioindicadores: as lapas (*P. ulyssiponensis*) parecem ser o melhor bioindicador para metais e metalóides, enquanto *Asparagopsis armata* se evidencia como a melhor opção para detetar a contaminação por arsénio.

O segundo objetivo foi analisado no capítulo 4, onde se avaliou o potencial das comunidades do andar eulitoral como indicador da presença de descargas de águas residuais em diferentes contextos ambientais e geográficos (Atlântico, Mediterrâneo e Insular Atlântico). A análise multivariada mostrou que, em todas as costas estudadas, a estrutura das comunidades não variou de forma significativa entre locais perturbados e não perturbados. Contudo, as populações de lapa (do género *Patella*) foram marcadamente afetadas pela presença do foco de poluição. As respostas diferiram entre as comunidades do Atlântico e do Mediterrâneo, presumivelmente devido às características ecológicas das diferentes espécies encontradas. Entretanto, na costa Insular Atlântica, a remoção de um emissário proporcionou a oportunidade ecológica para se avaliar a recuperação da comunidade: as espécies dominantes parece terem-no alcançado, mas observou-se o contrário para as populações de litorinideos.

Nesta tese, a identificação de todos os organismos ao nível da espécie, bem como a amostragem ao longo de todos os andares intertidais, permitiu um conhecimento abrangente dos efeitos das descargas de águas residuais sobre as comunidades da costa rochosa. Além disso, foi realizada uma primeira tentativa para avaliar e comparar as respostas das comunidades de macrofauna em diferentes áreas geográficas. Comunidades do eulitoral foram estudadas em costas do Atlântico e do Mediterrâneo, e os resultados sugerem que as lapas do género *Patella* podem ser eficazes na avaliação do estado ecológico dos sistemas costeiros expostos a descargas de águas residuais.

CHAPTER 1

General Introduction

Keywords

Rocky shore • Intertidal • Macrofauna assemblage • Sewage • Trace elements • Ecological indicators

General Introduction

1.1 The rocky shore environment

1.1.1 Tides

Rocky shores have fascinated marine biologists and ecologists for many years. Between the sea and the land, within a few meters, can be found one of the most rich and dynamic areas in the world. Nevertheless, the sharp environmental gradient that characterized the intertidal area creates a challenging habitat for marine species. There are mainly two environmental gradients: a vertical gradient defined by tides, and a horizontal gradient caused by wave action (Hawkins and Jones, 1992; Nybakken and Bertness, 2005; Little et al., 2010).
Tides are the periodic rise and fall of the sea, and are produced by the gravitational attraction of the Sun and the Moon on the Earth, and the centrifugal force from the rotating Earth-Moon system. Because the Moon is much closer to the Earth than the Sun, it is the main regulator of the Earth tides. Figure 1.1a shows the interactions between Earth (rotating on its own axis every 24 hours) and Moon, responsible for the daily rise and fall of the tides.

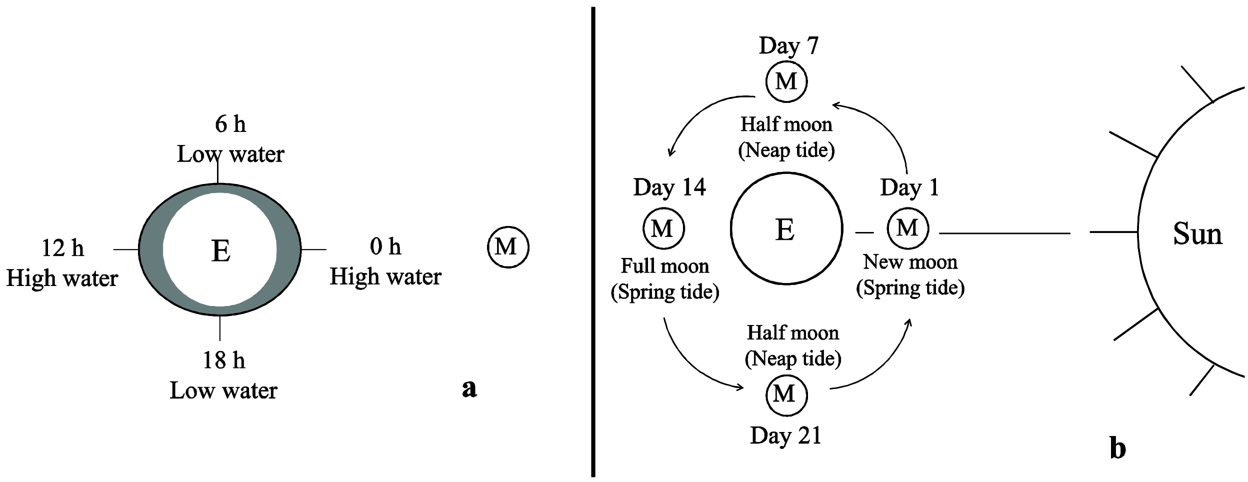

Figure 1.1 Daily and monthly tidal cycles (*Modified from Hawkins and Jones, 1992*).

Figure 1.1b illustrates the relative positions of the Earth, Sun and Moon based on a monthly cycle (approximately 28 days). The gravitational forces act together when the Sun, Moon and

Earth are aligned. This occurs at times of new and full Moon and results in tides with a large range, the spring tides. At times of half Moon, the Sun and Moon are at right angles to one another, and their forces are in opposition. Thus, the gravitational pull on the surface of the Earth is lower, and tides have a smaller range, the neap tides. Seasonal changes in the tidal amplitude are produced by differences in the declination of the Sun. The elliptic orbit of the Earth around the Sun, during the course of one year, also affects the range of the tides. In March and September, when the Sun is closest to the Earth its gravitational pull is greatest. This results in very large spring tides, the spring and autumn equinoctial tides. The opposite is observed in June and December, in the solstices, with lower spring tidal amplitudes (Hawkins and Jones, 1992; Nybakken and Bertness, 2003; Fish and Fish, 2011; Crothers, 2012).

Other factors, such as variations in the water depth, oceanic currents or the configuration of the coastline may have a marked impact on the type of tide and the tidal range along the world's shores. Figure 1.2 shows the global tidal variations.

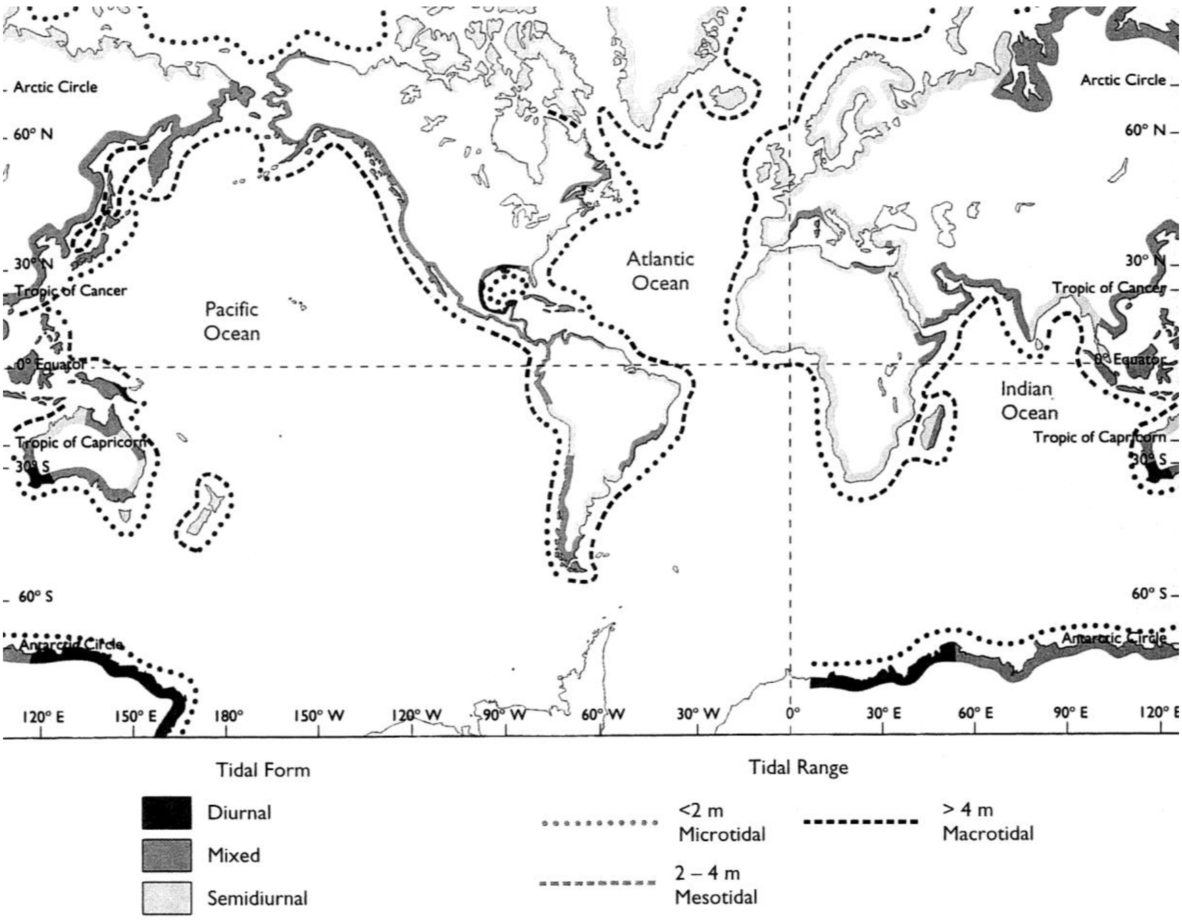

Figure 1.2 Global tide variations (*From Pinet, 1999*).

The most common tides in Europe are semidiurnal, with two high tides and two low tides each day. However, there are three tidal patterns: semidiurnal, diurnal (one high tide and one low tide daily) and mixed tides (vary irregularly twice a day).

It is also important to consider the differences in tidal range, the vertical distance between high and low tide. The tidal ranges vary from region to region (Fig. 1.2) and are normally divided into microtidal with less than 2m, mesotidal between 2 and 4m and macrotidal with more than 4m (Pinet, 1999; Fish and Fish, 2011).

All models are simplifications of the real world. However, given this simplification it is possible to predict tidal variations, which is essential to work in rocky shores. Tidal heights are measured in relation to a conventional level (chart datum), which corresponds to the lowest astronomic tide. Specific tidal levels above this chart datum may be useful reference points for shore ecologists (Fig. 1.3). There are the mean values for high and low water of spring and neap tides (MHWS, MLWS, MHWN, MLWN), and the average of these four heights, the mean tidal level (MTL). Also, the extreme spring tide levels are referenced, as the highest (EHWS) and the lowest (ELWS) tides of the year (Little et al., 2010; Fish and Fish, 2011).

1.1.2 Zonation: patterns of species distribution

Species that live in the rocky shores have to adapt to the sharp environmental gradient existent in this habitat. The vertical gradient, defined by the tides, produces periods of submersion and emersion, leading to contrasting conditions of temperature, salinity or desiccation. The horizontal gradient, caused by wave action, may have direct effects on species dislodgement or indirectly affect feeding or reproduction (Hawkins and Jones, 1992; Little et al., 2010).

One of the most striking characteristics of rocky shores around the world is the horizontal banding, or zonation, of organisms that live there. Although the easy recognition of this pattern, it is more difficult to explain its causes. Both physical and biological factors are responsible for this phenomenon. The upper limits are typically set by two abiotic factors: desiccation and temperature. The lower limits are explained by biotic factors, such as competition, predation or grazing (Lewis, 1964; Nybakken and Bertness, 2005; Fish and Fish, 2011).

From all the existent classifications (Stephenson and Stephenson, 1949, 1972; Lewis, 1964; Pérès and Picard, 1964), in this thesis, was adopted the terminology used by Lewis (1964). According to this author there are three major intertidal zones: littoral fringe, eulittoral and sublittoral fringe (Fig. 1.3). These zones are not defined by reference to the tidal levels but regarding biological factors, such as the distribution of specific organisms or groups of organisms. The littoral fringe, in the splash zone, is characterized by the presence of *Melarhaphe neritoides* and encrusting lichens. This level is the one with fewer species, and thus the most uniform. However, the variation in size of the littoral fringe is considerable and dependent of wave action. The eulittoral zone is daily exposed to the rise and fall of tides. This level contains more species and individuals than the littoral fringe, and it is generally characterized by the presence of barnacles, mussels and limpets. Finally, the sublittoral fringe shows the most stable environmental features, because is only emerse in spring tides. This is the level with higher densities and species richness, and it is dominated by large macroalgae, supporting a great diversity of fauna (Fish and Fish, 2011; Lewis, 1964).

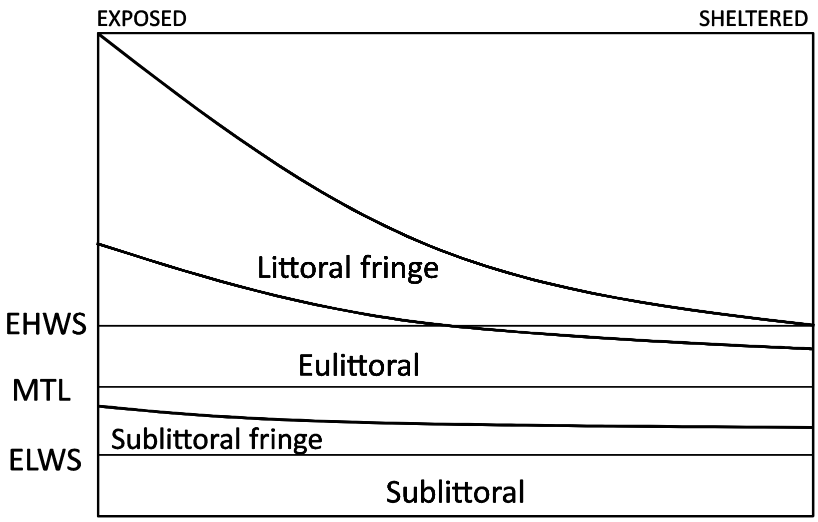

Figure 1.3 Diagram illustrating the terminology used for the main zones of rocky shores (Modified from *Lewis, 1964*).

The zonation patterns change with wave exposure, and also with latitude. Figure 1.3 illustrates the zonation patterns in exposed and sheltered shores. In exposed shores the upper limit of the littoral fringe is raised by several meters, as a result of wave spray being carried far up the beach. Moreover, the exposure can also affect the type of species present in the shore, due to different tolerances to wave action. In sheltered shores the eulittoral is dominated by large macroalgae, while barnacles and limpets are present in low numbers. The littoral fringe is narrow and *Littorina saxatilis* may be present. In exposed shores the eulittoral is covered by barnacles and mussels, and limpets are also found in large numbers. The littoral fringe is very broad and *Melarhaphe neritoides* is extremely abundant. Additionally, differences between exposed and sheltered shores are observed in the sublittoral fringe too, with the composition of algal assemblages changing accordingly to the species tolerance to wave action.

There are, also, geographical differences in the species distribution that will influence the patterns of zonation. For instance, diversity tend to increase towards the tropics and decrease towards the poles. Factors like temperature and the amount of radiant energy can change the productivity which associated with interactions at the community level (e.g., algae-grazer) may produce this latitudinal gradient (Hawkins and Jones, 1992).

1.2 Human impacts on rocky shores

Coastal areas are exposed to a wide range of anthropogenic impacts. Sewage discharges are among the most common anthropogenic impacts on rocky shores, resulting in organic and nutrient enrichment (Crowe et al., 2000; Arévalo et al., 2007; Halpern et al., 2008). The sewage can be treated in the wastewater treatment plants, where it receives primary (gross solids removed), secondary (organic matter removed) or tertiary (nutrients and bacteria removed) treatments. After the treatments, the effluents are usually discharged directly into the shore, or at some distance from the shore through pipeline systems. In Europe, the majority of sewage treatment plants have only primary and secondary treatment (http://epp.eurostat.ec.europa.eu), so nutrient enrichment and bacteria concentrations can become a major environmental problem leading to the deterioration of marine ecosystems. Moreover, sewage discharges from industrialized or urbanized areas usually contain trace elements. Although trace elements on aquatic systems may have natural sources, it is important to distinguish those from man-induced inputs. Therefore, the analysis of trace elements is an essential task to assess the potential environmental and human health risk associated with sewage discharges (Pinet, 1999; Alvarez et al., 2002; Maceda-Veiga et al., 2013).

Several legislative forces at European scale, such as the Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC) have been developed aiming the protection and management of the European aquatic ecosystems. Both directives are based on the same ecological concept: assessing the health of the aquatic ecosystems by determining the deviation from the expected reference conditions and, in case of degradation, intervene to recover a good ecological status. The assessment should be based in physico-chemical parameters and biological indicators, such as fish, phytoplankton, macroalgae or benthic invertebrates.

Benthic invertebrates are used worldwide to assess the status of marine ecosystems. They have been proved to respond to several types of anthropogenic impacts, from physical disturbance to chemical contamination or nutrient enrichment (Teixeira et al., 2010 and references therein). Moreover, their role as ecological indicators is widely accepted due to their sedentarism, long lives, easy sampling, and to the existence of extensive literature on their distribution in specific environments and on their response to different environmental stresses (Borja and Dauer, 2008; Dauvin et al., 2010; Fano et al., 2003; Simboura and Zenetos, 2002).

Previous studies have already approached the effects of sewage discharges on rocky shore communities. Littler and Murray (1975) studied the effect of sewage pollution on Californian intertidal rocky shores. There was a decrease in species richness and total cover. These authors also observed changes in the community structure, with sewage favouring suspension feeders and omnivores, and also a replacement of sensitive species by tolerant ones.

In the Australian coasts the effects of sewage pollution, as well as the recovery of subtidal and intertidal assemblages after decommission of the outfalls was extensively studied (Fairweather, 1990; Chapman et al., 1995; Roberts, 1996; Underwood and Chapman, 1996; Roberts et al., 1998; Roberts and Scanes, 1999; Archambault et al., 2001; Bishop et al., 2002). In general it was observed a decrease in fauna diversity and a dominance of ephemeral green algae at the intertidal (Fairweather, 1990). The closure of that inshore outfall, replaced by a deep-water ocean outfall, lead to recovery studies in the area. Studies on the recovery of the intertidal assemblages found no evident effects of sewage on eulittoral, but an increase in the number of species and decrease of green algae at the sublittoral fringe (Archambault et al., 2001). Similarly, in the same area, studies after the closure of the inshore outfall did not found any evidence of sewage effects at the subtidal (Chapman et al., 1995; Underwood and Chapman, 1996). In other Australian shore, the opening of the nearshore ocean outfall (20m), provided the opportunity to carry out a before/after sampling design. Although there were no differences in total cover or number of species, a marked shift in the structure of the assemblage was observed, with the replacement of algae and sponges by silt and ascidians (Roberts et al., 1998). Finally, evidence for the importance of sampling at different spatial scales was revealed with the dissimilar effects of sewage on the intertidal limpet *Patelloida latistrigata* at different spatial scales (Bishop et al., 2002). These authors also observed an increase of ephemeral green algae and opportunistic species in the intertidal assemblages near the sewage discharges.

In the Argentinean coasts, sewage effects were studied on a rocky intertidal community dominated by the bivalve *Brachidontes rodriguezi*. The sensibility of *B. rodriguezi* to sewage pollution led to the absence of this species in the polluted areas. As a consequence, other species increase in abundance due to the lack of competition for food and space. Therefore, the diversity was higher in the polluted areas (López-Gappa et al., 1990, 1993). Moreover, these authors also suggested that sewage discharges increase the cover of ephemeral green algae and the abundance of tolerant species, such as *Mytilus* spp. and *Balanus* spp.. Although these studies had some sampling design problems, due to the existence of only one impacted area and one reference area, the results obtained were confirmed by further studies (Vallarino et al., 2002). It was observed a change in the community structure, with a decrease of sensitive species (*B. rodriguezi* and *Syllis prolixa*) and an increase of opportunistic polychaetes (*Capitella capitata* and *Boccardia polybranchia*) near the sewage discharges (Vallarino et al., 2002; Elías et al., 2006).

In the Italian Mediterranean coasts the effects of sewage were studied in subtidal rocky shores communities. There was no effect on total cover or species richness, but it was observed an increase of species typical of organically polluted waters (Terlizzi et al., 2002). Concerning meiofauna, there was a decrease in the number of species but not in total abundances. Also, community structure changed and was observed a decrease of nematods and syllids and an increase of hydrozoans (Fraschetti et al., 2006). Regarding mollusc assemblages, there was an increase in the abundances near the sewage discharges, due to greater number of juveniles.

Although no effect was evident in the number of species, there were significant differences in molluscan species composition (Terlizzi et al., 2005a).

More recently, the effect of sewage treatment upgrade was studied in Cantabrian shores. The improvement from primary to secondary treatment of sewage seemed to have no effect at the eulittoral. However, a significant effect was observed at the sublittoral, being the main changes a decrease of the suspensivore polychaete *Spirorbis pagenstecheri* and an increase of *Patella ulyssiponensis* (Bustamante et al., 2012). Recent studies have attempt to find similar patterns on intertidal assemblages from several shores affected by sewage discharges (Atalah and Crowe, 2012; O'Connor, 2013). Atalah and Crowe (2012) suggested that molluscan assemblages seem to be good indicators of sewage pollution. Near the outfalls the number of species and total abundances decrease, and the assemblages were different. Species like *Rissoa parva*, *Gibbula umbilicalis*, *Nucella lapillus* or *Tricolia pullus* appeared to be sensitive to sewage pollution, while species such as *Mytilus galloprovincialis*, *Odostomia* sp. or *Littorina littorea* were favoured by the organic enrichment (Atalah and Crowe, 2012). However, O'Connor (2013) found no consistent responses of intertidal assemblages to the presence of sewage discharges. Although the effects of sewage were evident, and mussels, limpets and green algae appeared to be always more abundant near the outfalls, these conclusions were not statistically robust. Moreover, it was clear that there was a greater effect of spatial and temporal variations in the structure of the assemblages, when compared with the sewage effect (O'Connor, 2013).

As explained above, different patterns have been observed when assessing the effects of sewage discharges. Consequently, it is necessary to study in detail these communities in order to find common responses, which are essential to establish adequate environmental risk management.

1.3 General objectives

The main purpose of this thesis is to understand how sewage pollution may affect intertidal macroinvertebrate assemblages from rocky shores, and it can be divided into two key points. First, to examine patterns such as abundance, species richness, structure and secondary production of the macrofauna assemblages exposed to sewage discharges. Second, compare the response of macrofauna assemblages to sewage discharges in Atlantic and Mediterranean shores.

Three specific objectives were defined in order to achieve the goals outlined above:

(i) To examine the effects of sewage pollution on intertidal macroinvertebrate assemblages, across all vertical zonation levels, to assess the consistency of patterns;

(ii) To evaluate if sewage discharges are a source of trace element contamination and to assess the risk for human health;

(iii) To assess the potential of eulittoral macroinvertebrate assemblages as indicators of sewage pollution, comparing the response of macrofauna assemblages in Atlantic and Mediterranean shores.

CHAPTER 2

Understanding the effects of sewage pollution on intertidal macroinvertebrate assemblages

Chapter 2 is published in the form of a manuscript as:

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Understanding the effects of sewage pollution on intertidal macroinvertebrate assemblages

2.1 Introduction

Half the world's population lives along the coastline and consequently the habitats located in those areas are under great human pressure. This includes the entry of a great variety of toxic contaminants from agricultural, industrial and urban activities (Little et al., 2010). One of the main sources of the organic and nutrient enrichment in coastal areas is sewage discharges (e.g. Arévalo et al., 2007). In Europe, sewage can receive primary (gross solids removed), secondary (organic matter eliminated) or tertiary (nutrients and bacteria removed) treatment, prior to being discharged directly into the shore, or at some distance from shore, through pipeline systems (http://epp.eurostat.ec.europa.eu). Sewage outfalls discharging near rocky shores may have effects on the subtidal and intertidal hard bottom communities located nearby.

The responses of rocky shore intertidal benthic invertebrate assemblages to sewage pollution are poorly understood (Johnston and Roberts, 2009; Dauvin et al., 2010; Bustamante et al., 2012). Nevertheless there are several papers focusing on intertidal populations of polychaetes (Dauer and Conner, 1980; Elías et al., 2006; Jaubet et al., 2011), molluscs (Bishop et al., 2002; Terlizzi et al., 2005a; Vallarino et al., 2006; Atalah and Crowe, 2012) or crustaceans (Calgano et al., 1998; De-la-Ossa-Carretero et al., 2010). But studies dealing with the effect on the entire intertidal benthic community are very scarce (Littler and Murray, 1975; López-Gappa et al., 1990, 1993; Archambault et al., 2001; Klein and Zhai, 2002). Moreover, several of those studies do not have the most appropriate sampling design to detect human disturbances (e.g. using only one reference site). Furthermore, in rocky shores the species are distributed in bands, creating a vertical zonation, where physical and biotic factors diverge, and communities differ in terms of species richness and composition (Hawkins and Jones, 1992; Little et al., 2010). The previous studies have only focused on one of the tidal level (López-Gappa et al., 1990, 1993; Klein and Zhai, 2002). Finally, previous research was mainly based on visual census, which has the disadvantage of underestimating species with smaller dimensions (Littler and Murray, 1975; Archambault et al., 2001). Nevertheless, previous studies have already pointed out important results, such as the structural and functional changes of the community, and the replacement of sensitive species by opportunistic ones.

In view of the current concern to include functional approaches in the environmental impact assessment, the effects of sewage pollution can also be studied by assessing ecosystem function (Elliott and Quintino, 2007). Secondary production is a measure of ecosystem functioning, as it combines both static and dynamic components of a population's ecological performance (Dolbeth et al., 2012). Secondary production (production by heterotrophic organisms) is the amount of organic matter or energy incorporated in a given area per time unit (Dolbeth et al., 2012). The patterns of secondary production are affected by life history characteristics such as body mass, recruitment, age, life span, taxonomy and trophic status, and population biomass and density (Cusson and Bourget, 2005). As such, measures of secondary production are representative of population fitness in the system and can add more information to diversity and density parameters about ecosystem changes and, ultimately, on the food provision delivered by an ecosystem (Benke and Huryn, 2010; Dolbeth et al., 2012). The importance of incorporating secondary production to assess the impacts of sewage pollution has been recognized for freshwater (Whiles and Wallace, 1995; Woodcock and Huryn, 2007; Faupel et al., 2012) and estuarine ecosystems (Dolbeth et al., 2011, 2012). However, little is known for the rocky shores habitats.

With all this in mind, the aim of this chapter is to study the effects of sewage pollution on the diversity, abundance, structure and secondary production of macrofaunal assemblages compared with reference areas not exposed to this human induced disturbance. This study was carried out across all vertical zonation levels (littoral fringe, eulittoral and sublittoral fringe) to assess the consistency of patterns.

2.2 Materials and methods

2.2.1 Study site and sampling procedures

The study was conducted in Peniche peninsula (Fig. 2.1), on the central western Portuguese coast. In the peninsula, a sewage treatment plant was constructed in 1998. The outfall releases secondary treated effluent. It serves a human population of 40,000 and discharges the effluent directly into the intertidal area of the rocky shore.

The lack of pre-impact data led to the choice of an ACI (after control/impact) experimental design (Chapman et al., 1995; Glasby, 1997). Consequently, three sampling areas were selected: one impacted area, near the sewage discharges (Imp) and two reference areas (R1 and R2) to account for the natural differences among undisturbed areas. All sampling areas had comparable environmental conditions, with regard to slope, orientation, wave exposure and type of substrate (Fig. 2.1).

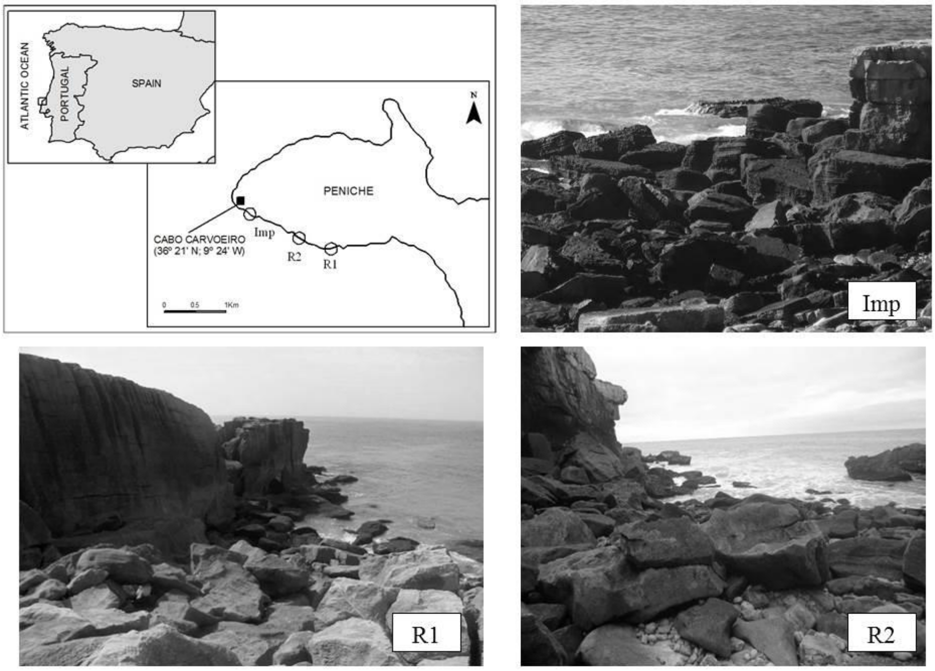


Figure 2.1 Map of Peniche peninsula, western coast of Portugal, showing the location and photos of the sampling areas: Imp – impacted area; R1 and R2 – reference areas.

In the intertidal Portuguese coast (European temperate latitudes) there is a characteristic zonation pattern: littoral fringe, eulittoral and sublittoral fringe (Boaventura et al., 2002a). The littoral fringe is characterized by the presence of *Melarhaphe neritoides* and encrusting lichens; the eulittoral zone is dominated by barnacles and mussels; and the sublittoral fringe is dominated by red algae in central and southern regions (Boaventura et al., 2002a). In agreement, in each sampling area the three levels were sampled. For each intertidal zonation level, five quadrats (12x12cm) were randomly selected and then organisms were collected by scraping using a spatula and a chisel. In total 45 samples were collected during each field trip in February, April, July and November 2010. Since the studied area presents a temperate climate four sampling dates were chosen in order to account for the temporal variation. It was not our goal to study the differences between seasons but to capture the natural yearly variation of the community's response. During the sampling programme in all areas, environmental variables (dissolved oxygen, temperature, salinity and pH) were measured in the seawater and water samples were collected for laboratory determination of nutrients concentrations, total suspended solids (TSS) and bacteria (total coliforms).

2.2.2 Laboratory procedures

All material collected from scrapings were sieved through a 500μm mesh and all individuals were identified to the lowest taxonomic level possible. All individuals were counted and weighted to determine density and biomass (estimated as ash free dry weight, AFDW). To estimate AFDW individuals were dried at 60 ºC for four days, weighted, ignited in a muffle furnace at 450 ºC for eight hours, and reweighed to determine the ash content. These determinations were made using the entire animals including the shells. Water samples were filtered (Whatman GF/F glass-fibre filter) and stored frozen at -18 ºC until analysis. Analyses followed the standard methods described in Limnologisk Metodik (1985) (for ammonia and phosphate), in Strickland & Parsons (1968) (for nitrate and nitrite), and ESS Method 340.2 (for total suspended solids). Water samples (250 mL) for the quantification of total coliforms bacteria were collected in parallel to those obtained for physical and chemical analyses. Samples were collected in sterile polycarbonate flasks filled to capacity, sealed with gas-tight rubber stoppers and immediately placed on ice until processing in the laboratory (3h later). Total coliforms in the water samples were determined using the membrane filtration technique. A twenty-fold dilution was used for the water samples from the impacted area. The samples were cultivated onto CHROMOCULT® coliform agar (Merck), with an incubation period of 24h at 37 ºC. At the end of this period CFU were counted.

2.2.3 Data analysis and secondary production estimates

A distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was carried out separately for each intertidal level (littoral fringe, eulittoral and sublittoral fringe) to test for differences in density and biomass of the invertebrate assemblage structure between impacted and reference areas. The model consisted of two factors: Time (4 levels, random, orthogonal) and Location (1 impacted and 2 reference areas, fixed, orthogonal). In both cases, the design was asymmetrical (Underwood, 1994) due to the presence of a single impacted area. Therefore, the Location term, and all terms involving Location, was partitioned into two portions: the 1-degree-of-freedom contrast of Imp-v-Rs and the variability between reference areas (Rs). The same partitioning was performed for the residual variability for observations within Imp (Res Imp) within Rs (Res Rs). Appropriate denominators for F ratios were identified from expected mean squares and tests were constructed following the logic of asymmetrical design (see Terlizzi et al., 2005b). All analyses were based on Bray–Curtis similarity on squared-root transformed data, and each term in the analysis was tested by 4999 random permutations of appropriate units. To visualize multivariate patterns, differences in community structure among treatment levels were visualized by principal coordinate (PCO) analyses on the basis of Bray-Curtis similarities. Species classes found at each intertidal level are displayed as vectors in the PCO plots.

The total number of species (S) was calculated for each observation unit, using the DIVERSE routine contained in the PRIMER statistical package. Univariate permutational analyses of variance (Anderson, 2001) were carried out on several variables using the same experimental design as described above for the multivariate analyses. The variables were: number of species, total faunal density, density of taxonomic classes (Gastropoda, Bivalvia, Crustacea, Polychaeta and Polyplacophora) and abundance of *Corallina* spp. Univariate analyses were performed using PERMANOVA, with Euclidean distances as the measure of similarity. For all statistical tests, the significance level was set at $p \leq 0.05$. All calculations were performed using the PRIMER v 6 software package (Clarke and Gorley, 2006).

For the present assessment secondary production was estimated with Cusson and Bourget (2005) empirical model:

$$\log P = 0.45 + 1.01 \times \log \bar{B} - 0.84 \times \log L - 0.09 \times \bar{w}$$

where P is the annual production (kJ.m^{-2}.year^{-1}), $\bar{B}$ is the annual mean biomass (kJ.m^{-2}), $\bar{w}$ is the annual mean individual mass (kJ.individual^{-1}), and L is the life span (years). Data on $\bar{B}$ and $\bar{w}$ were obtained by averaging the biomass obtained in the four sampling occasions, as representative of annual mean biomass. For the application of the model, information on the species life span was collected from the literature (http://www.marlin.ac.uk, www.nhm.ac.uk, http://www.genustraithandbook.org.uk), and all species were also assigned to a functional feeding group (Appendix A) for a discrimination of production per feeding guild. For rare species or the ones with no information on life span, an average life span value inferred from similar species traits was considered (after recommendation from Cusson and Bourget (2005). Biomass was converted into energy (kJ) using weight- to-energy provided in Brey (2001). A single value of annual production is obtained for each sampling area and level.
The secondary production distribution within the macroinvertebrate assemblage in the three sampling areas (Imp, R1 and R2) and zonation levels (littoral fringe, eulittoral and sublittoral fringe) was explored using a two-way crossed ANOSIM with no replicates. Similarities in the production data were calculated as the Bray-Curtis coefficient after square-root-transformation of the raw data to scale down the scores of the very productive species (Clarke and Warwick, 2001). Non-metric Multidimensional Scaling (nm-MDS) was performed subsequently to clarify the patterns and similarity percentages obtained from CLUSTER analysis were overlaid in the plot. All the analyses were done using PRIMERv6 and PERMANOVA+ routines (Anderson et al., 2008).

2.3 Results

2.3.1 Environmental variables

In general the environmental parameters were markedly different when comparing the sampling areas (Table 2.1). The impacted area presented higher seawater temperature and lower dissolved oxygen, salinity and pH. Nutrient concentrations, especially ammonia and phosphate, and total suspended solids (TSS) were also higher near the sewage discharges.

Table 2.1 Variation (mean ± SD) of environmental parameters in the three sampling areas (R1 and R2 – reference areas; Imp – impacted area).

		R1	R2	Imp
Seawater temperature (ºC)		15.4±1.8	15.9±1.8	18.4±1.7
Dissolved oxygen (mg/L)		9.35±0.5	9.38±0.8	6.89±3.5
Salinity		35.9±0.3	35.9±0.3	27.9±7.1
pH		8.01±0.2	8.07±0.1	7.87±0.3
Nutrients	**Ammonia (NH_4)**	0.07±0.03	0.04±0.03	1.1±1,0
	Phosphate (PO_4)	0.02±0.01	0.02±0.01	2.5±2.3
	Silica (Si)	0.6±0.3	0.5±0.4	1.6±1
	Nitrite (NO_2)	0.02±0.01	0.02±0.01	0.1±0.01
	Nitrate (NO_3)	0.07±0.06	0.08±0.06	0,15±0,13
Total suspended solids/L		24.8±12,5	13.2±7.4	61.3±26.4
Total coliforms (UFC/100mL)		98	202	>300

2.3.2 Intertidal macroinvertebrate assemblage abundance

2.3.2.1 Littoral fringe

At the littoral fringe only the gastropod *Melarhaphe neritoides* and (occasionally) the isopod *Ligia oceanica* were found. PERMANOVA analysis did not detect any significant differences in macrofauna assemblage density between impacted and reference areas, but only a significant temporal variability (Table 2.2). The same was observed for biomass, with no differences between impacted and reference areas (Table 2.2).

Table 2.2 PERMANOVA results on density and biomass of littoral fringe assemblages. Significant results are given in bold.

		Density			Biomass		
Source of variability	**df**	**MS**	***F***	*P*	**MS**	***F***	*P*
Time = Ti	3	7960.5	8.044	**0.0002**	2.0261	3.428	**0.0202**
Location = Lo	2	420.3	0.325	0.7392	0.9126	1.743	0.2612
Imp-v-Rs	1	834.9	0.729	0.4458	0.1331	0.132	0.8150
Rs	1	5.77	0.004	0.8574	1.6921	43.38	**0.0322**
Ti x Lo	6	1293.2	1.307	0.2728	0.5237	0.886	0.5132
Ti x Imp-v-Rs	3	1145.8	1.150	0.3416	1.0083	1.737	0.1740
Ti x Rs	3	1440.5	1.860	0.1444	0.0390	0.060	0.9844
Res	48	989.6			0.5911		
Res Imp	16	1419.9			0.4722		
Res Rs	32	744.5			0.6506		

Melarhaphe neritoides was found along all the intertidal area, and not only at this level. For that reason, the population was studied along all tidal height (Fig. 2.2). In the impacted area *M. neritoides* was more abundant, but the individual biomasses were lower.

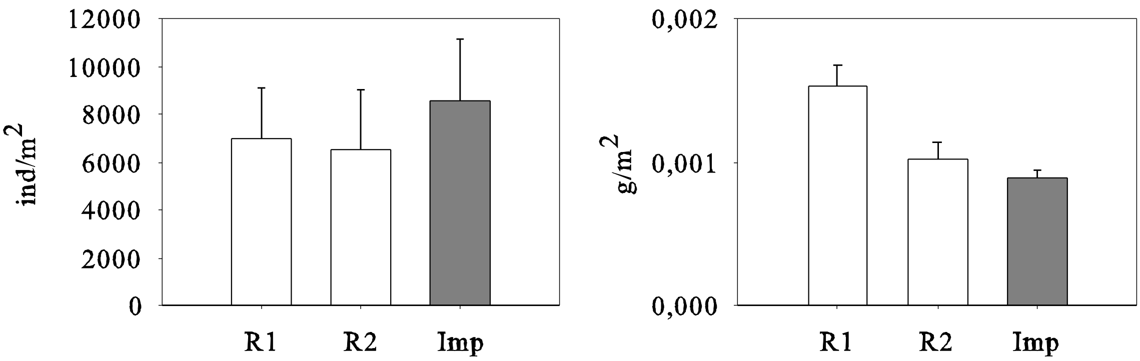


Figure 2.2 *Melarhaphe neritoides* density (ind/m^2±SE) and individual biomass (g/m^2±SE) in the three sampling areas. R1 and R2 – reference areas; Imp – impacted area.

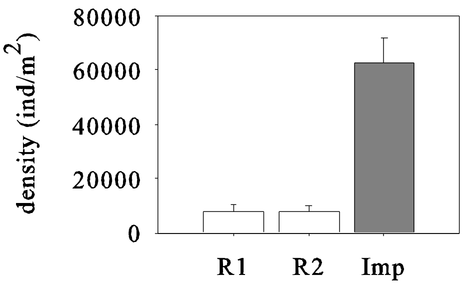
2.3.2.2 Eulittoral

The eulittoral assemblages differed significantly between impacted and reference areas, and those differences were consistent through time (Table 2.3). The same was observed for biomass (Table 2.3).

Table 2.3 PERMANOVA results and pair-wise comparisons on density and biomass of eulittoral assemblages. Significant results are given in bold.

Source of variability	df	Density			Biomass		
		MS	*F*	*P*	MS	*F*	*P*
Time = Ti	3	2647.9	3.073	**0.0014**	1331.30	3.7826	**0.0004**
Location = Lo	2	18126	10.118	**0.0052**	9506.0	12.347	0.0058
Imp-v-Rs	1	32674	12.002	**0.0250**	18083.0	13.228	**0.0268**
Rs	1	3579.3	4.158	0.0550	929.06	5.3781	0.0550
Ti x Lo	6	1791.5	2.079	**0.0064**	769.87	2.1874	**0.0036**
Ti x Imp-v-Rs	3	2722.2	2.979	**0.0020***	1367.0	3.8756	**0.0002***
Ti x Rs	3	860.8	0.802	0.5834	172.75	0.7153	0.6656
Res	48	861.6			351.96		
Res Imp	16	438.5			572.89		
Res Rs	32	1073.1			241.49		
Pair-wise*		Feb: p=**0.0028**; Apr: p=**0.0016**; Jul: p=**0.0008**; Nov: p=**0.0002**			Feb: p= **0.0002;** Apr: p= **0.001** Jul: p= **0.0006;** Nov: p= **0.0002**		

Similarly, total faunal density was superior in the impacted area (Fig. 2.3). Those differences were statistically significant (Table 2.4), and were observed in all sampling times, except in February. Regarding species richness (Table 2.4) it can be observed a significant interaction Imp-v-Rs, indicating that the number of species at the eulittoral was higher near the sewage discharges (Fig. 2.3).



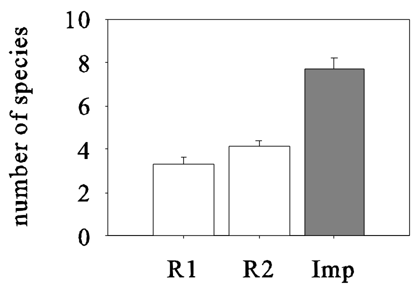


Figure 2.3 Changes in density (ind/m^2±SE) and number of species in the eulittoral. R1 and R2 – reference areas; Imp – impacted area.

Table 2.4 PERMANOVA results and pair-wise comparisons on total faunal density and number of species for the eulittoral. Significant results are given in bold.

Source of variability	df	Total faunal density MS	*F*	*P*	Number of species MS	*F*	*P*
Time = Ti	3	489.5	5.187	**0.0046**	0.483	4.636	**0.007**
Location = Lo	2	7343.1	10.233	**0.015**	5.155	29.514	**0.003**
Imp-v-Rs	1	14627	10.383	0.0542	9.859	35.405	**0.0274**
Rs	1	59.25	2.248	0.2352	0.451	6.363	0.1002
Ti x Lo	6	717.6	7.603	**0.002**	0.175	1.677	0.1434
Ti x Imp-v-Rs	3	1408.8	15.692	**0.0002***	0.278	2.556	0.0622
Ti x Rs	3	26.35	0.2436	0.865	0.071	0.640	0.5882
Res	48	94.38			0.104		
Res Imp	16	66.87			0.091		
Res Rs	32	108.14			0.111		
Pair-wise*		Feb: p=0.333; Apr: p=**0.0012**; Jul: p=**0.0004**; Nov: p=**0.0008**					

Consistent with the PERMANOVA results, the PCO plot shows a clear separation between impacted and reference areas samples for eulittoral (Fig. 2.4).

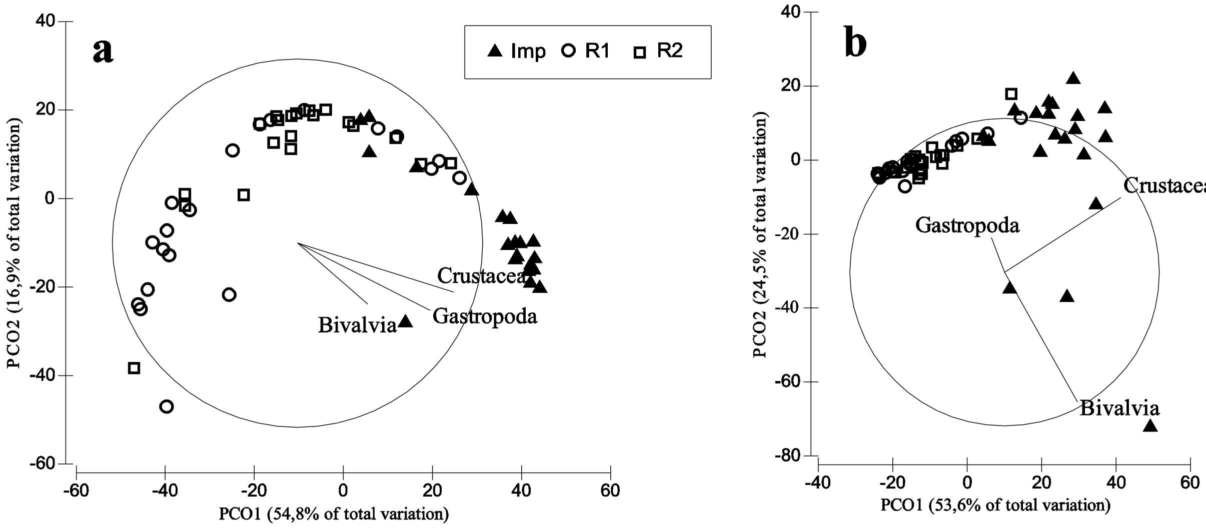


Figure 2.4 Principal Coordinates Ordination (PCO) plots at both impacted (filled symbols) and reference areas (empty symbols) for eulittoral assemblages for density (**a**) and biomass (**b**).

In Table 2.5 it can be observed that those differences were consistent along all sampling dates, except in February for Gastropoda and Crustacea. Regarding biomass (Fig. 2.4b), Crustacea and Bivalvia seem to be more abundant in the impacted area, while Gastropoda shows higher values in the reference areas.

Table 2.5 PERMANOVA results and pair-wise comparisons on species classes of eulittoral assemblages. Significant results are given in bold.

		Gastropoda		Bivalvia		Crustacea	
Source of variability	**df**	***F***	*P*	***F***	*P*	***F***	*P*
Imp-v-Rs	1	5.806	0.1156	21.484	**0.0298**	8.090	0.0766
Ti x Imp-v-Rs	3	34.808	**0.0002***	3.671	**0.0180***	9.317	**0.0006***
Pair-wise*		Feb: p=0.2324		Feb: p=**0.008**		Feb: p=0.887	
		Apr: p=**0.001**		Apr: p=**0.0004**		Apr: p=**0.0026**	
		Jul: p=**0.0008**		Jul: p=**0.0042**		Jul: p=**0.0004**	
		Nov: p=**0.0002**		Nov: p=**0.0006**		Nov: p=**0.0006**	

It is also important to highlight the patterns observed for the species found in lower densities at the eulittoral (Table 2.6).

Table 2.6 Density (ind/m^2±SE) of the most common species found at the eulittoral.

Specie	R1	R2	Imp
Littorina saxatilis	0	0	73±28
M. neritoides	1906±806	2809±1136	31778±5908
Patella spp.	694±101	649±61	264±36
Lasae adansoni	0	0	1101±354
Mytilus galloprovincialis	122±72	38±18	2203±839
Campecopea hirsuta	14±8	0	236±90
Chthamalus montagui	5052±1945	4451±983	25875±3666
Dynamene sp.	0	0	90±49
Hyale perieri	21±7	118±23	410±98
Hyale pontica	0	0	45±45

The gastropod *Littorina saxatilis* and the bivalve *Lasae adansoni* only appeared in the impacted area. Also among crustaceans, all species presented higher abundances near the sewage discharges, and some of these were observed only in the impacted area (*Dynamene* spp., *Tanais dulongii*, *Hyale pontica*). The limpets *Patella* spp. (Fig. 2.5) showed higher densities in the reference areas, but lower individual biomasses. In other words, individuals of *Patella* spp. were more abundant in the reference areas, but were bigger near the sewage discharges.

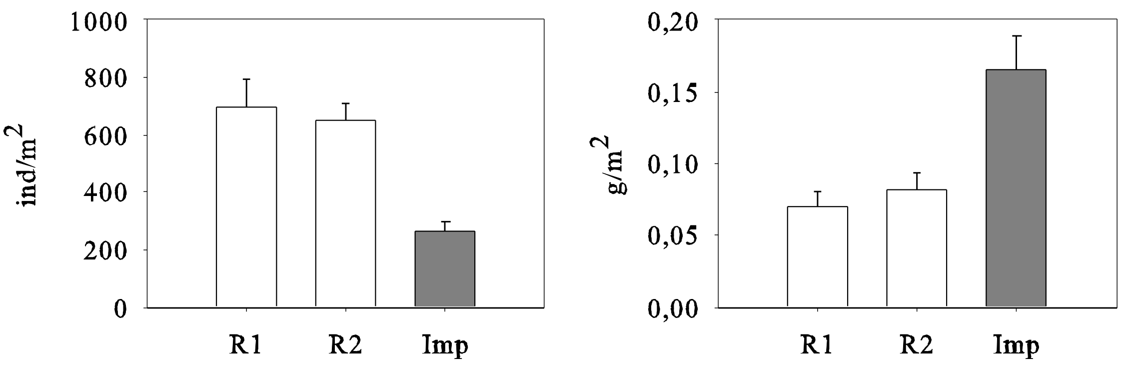


Figure 2.5 *Patella* spp. density (ind/m^2±SE) and individual biomass (g/m^2±SE) in the three sampling areas. R1 and R2 – reference areas; Imp – impacted area.

Finally, Figure 2.6 shows the contribution by feeding guilds for density and biomass.

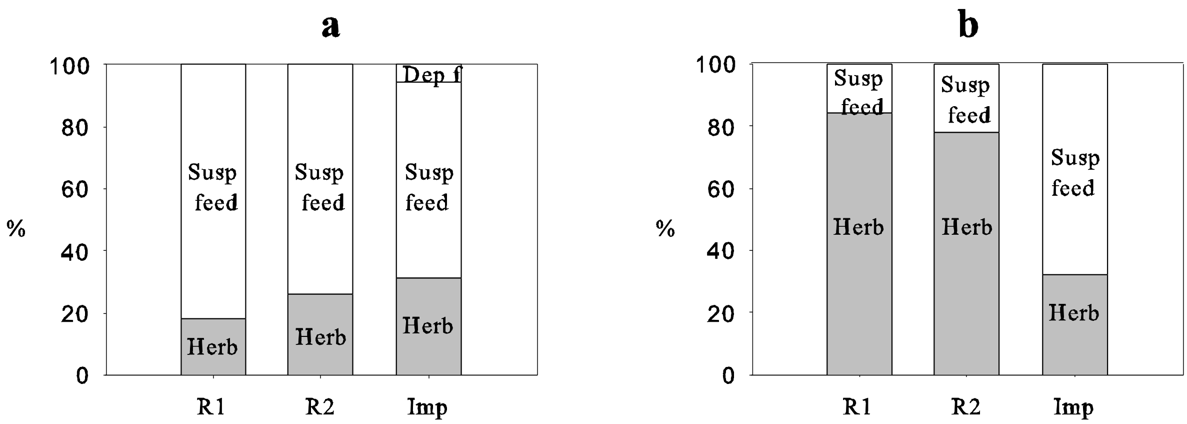


Figure 2.6 feeding guilds contribution (%) for density (**a**) and biomass (**b**), at the eulittoral in the three sampling areas: R1, R2 – reference areas; Imp – Impacted area.

Herb- Herbivores; Susp feed – Suspension feeders; Dep f – Deposit feeders.

As can be seen, at the eulittoral, the feeding guild composition for density (Fig. 2.6a) is similar in the three areas, with a dominance of suspension feeders. On the contrary, considering biomass (Fig. 2.6b), the feeding guild composition changed. There was a dominance of herbivores in the reference areas, replaced by a higher percentage of suspension feeders in the impacted area (Fig. 2.6).

2.3.2.3 Sublittoral fringe

The sublittoral fringe presented higher densities and species richness (Fig. 2.7).

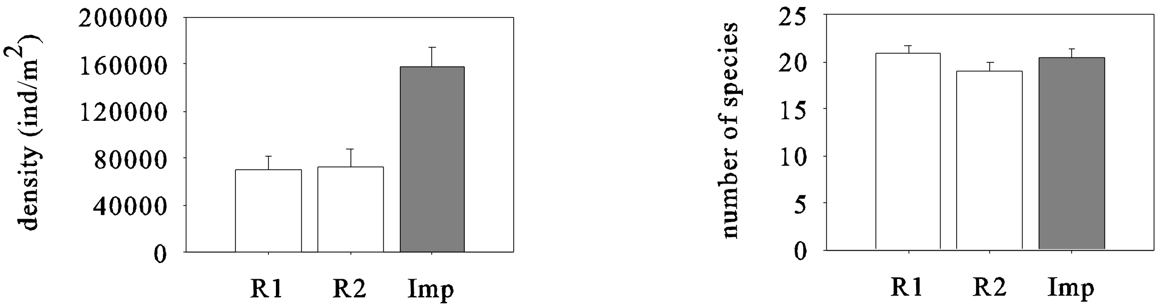


Figure 2.7 Changes in density (ind/m^2±SE) and number of species in the sublittoral fringe. R1 and R2 – reference areas; Imp - impacted area.

This level was dominated by the red algae *Corallina* spp., which was more abundant near the sewage discharges (Ti×Imp-v-Rs: F = 0.092, P = 0.9622; Imp-v-Rs: F = 189.9, P = 0.0308). Concerning the macrofauna, the assemblages differed significantly between impacted and reference areas, and those differences were consistent through time (Table 2.7). The same was observed for biomasses (Table 2.7). Moreover, for total faunal density there were only significant differences in February and April, while species richness was similar in all sampling areas (Table 2.8).

Table 2.7 PERMANOVA results and pair-wise comparisons on density and biomass of sublittoral fringe assemblages. Significant results are given in bold.

Source of variability	df	Density			Biomass		
		MS	***F***	*P*	**MS**	***F***	*P*
Time = Ti	3	8887.8	11.935	**0.0050**	6682.3	5.9970	**0.0002**
Location = Lo	2	12125	4.160	**0.0050**	11236	3.6895	**0.0078**
Imp-v-Rs	1	21449	6.186	**0.0278**	19815	5.9757	**0.0276**
Rs	1	2801.4	1.186	0.3816	2656.9	0.9575	0.4322
Ti x Lo	6	2914.7	3.914	**0.0002**	3045.4	2.7330	**0.0002**
Ti x Imp-v-Rs	3	3467.3	3.951	**0.0002***	3315.9	2.6747	**0.0002***
Ti x Rs	3	2362.1	2.499	**0.0004**	6800.7	4.7932	**0.0002**
Res	48	744.7			1114.3		
Res Imp	16	343.3			505.2		
Res Rs	32	945.4			1418.8		
Pair-wise*		Feb: p=**0.0014**; Apr: p=**0.0004**; Jul: p=**0.003**; Nov: p=**0.0006**			Feb: p= **0.0058;** Apr: p= **0.001;** Jul: p= **0.003;** Nov: p= **0.001**		

Table 2.8 PERMANOVA results and pair-wise comparisons on total faunal density and number of species for the sublittoral fringe. Significant results are given in bold.

Source of variability	df	Total faunal density			Number of species		
		MS	***F***	*P*	**MS**	***F***	*P*
Time = Ti	3	381.3	5.486	**0.0034**	1.449	7.617	**0.0040**
Location = Lo	2	1907.4	2.583	0.1462	0.311	0.898	0.4524
Imp-v-Rs	1	3814.7	2.638	0.1960	0.047	0.172	0.6926
Rs	1	0.0086	0.0002	0.9168	0.575	1.369	0.4118
Ti x Lo	6	738.31	10.623	**0.0002**	0.346	1.820	0.1120
Ti x Imp-v-Rs	3	14461.1	21.939	**0.0002***	0.273	1.295	0.2832
Ti x Rs	3	30.52	0.4087	0.744	0.419	1.835	0.1504
Res	48	69.50			0.190		
Res Imp	16	59.16			0.113		
Res Rs	32	74.67			0.229		
Pair-wise*		Feb: p=**0.0004**; Apr: p=**0.0002**; Jul: p=0.0614; Nov: p=0.141					

The PCO plot (Fig. 2.8) showed that the community structure changed along the sampling areas. Considering density (Fig. 2.8a), there was more Bivalvia, Crustacea and Polychaeta in the impacted area, while Gastropoda were more common in the reference areas. Regarding biomass (Fig. 2.8b), Bivalvia were associated to the impacted area, while Polyplacophora seems related with both reference areas.

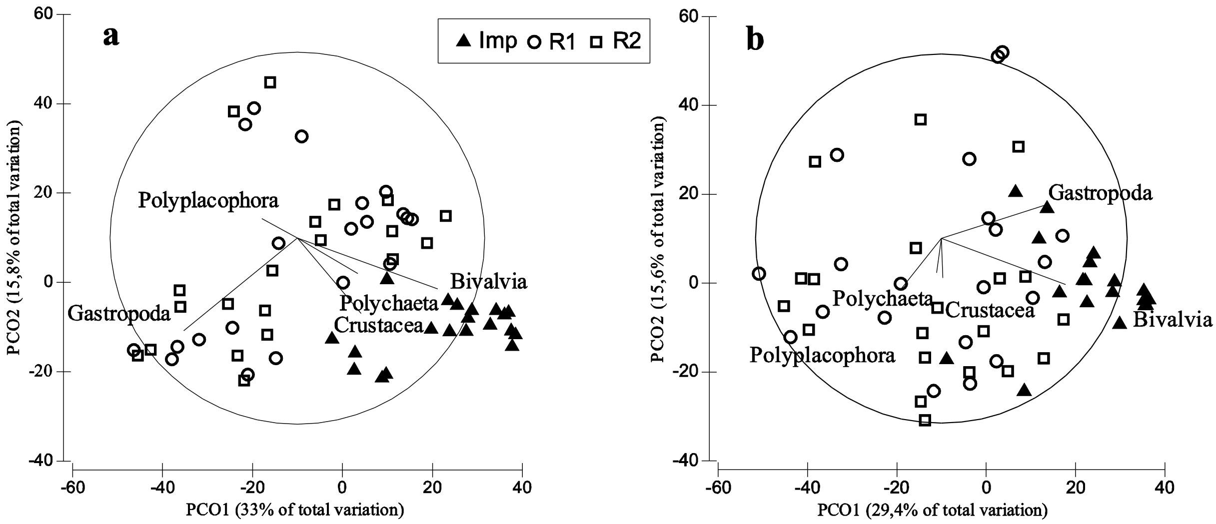


Figure 2.8 Principal Coordinates Ordination (PCO) plots at both impacted (filled symbols) and reference areas (empty symbols) for sublittoral fringe assemblages for density (**a**) and biomass (**b**).

The differences between impacted and reference areas were statistically significant for all the classes, and consistent in the majority of the sampling dates (Table 2.9). In addition, as can be seen in Table 2.10, there was a change in the dominant species. While *M. galloprovincialis* was the most abundant species in the impacted areas, *Rissoa parva* was the dominant species in the reference areas.

Table 2.9 PERMANOVA results and pair-wise comparisons on species classes of sublittoral fringe assemblages. Significant results are given in bold.

	Gastropoda		**Bivalvia**		**Crustacea**		**Polychaeta**		**Polyplacophora**	
Source of variability	***F***	*P*	***F***	*P*	***F***	*P*	***F***	*P*	***F***	*P*
Imp-v-Rs	1.324	0.3428	7.185	0.094	9.616	0.071	1.310	0.3286	12.03	0.0576
Ti x Imp-v-Rs	14.51	**0.0002***	13.26	**0.0002***	15.75	**0.0002***	7.826	**0.0008***	3.745	**0.0162***
Pair-wise*										
	Feb: p=**0.0004**		Feb: p=**0.001**		Feb: p=**0.0026**		Feb: p=0.1354		Feb: p=**0.016**	
	Apr: p=**0.0096**		Apr: p=**0.0008**		Apr: p=**0.002**		Apr: p=**0.0002**		Apr: p=**0.0006**	
	Jul: p=**0.0112**		Jul: p=**0.0014**		Jul: p=**0.0012**		Jul: p=**0.0122**		Jul: p=0.0796	
	Nov: p=**0.0018**		Nov: p=0.353		Nov: p=**0.0008**		Nov: p=**0.0378**		Nov: p=**0.005**	

Table 2.10 Biomass (g/m^2±SE) of *Corallina* spp. and density (ind/m^2±SE) of the most common species found at the sublittoral fringe.

Species	R1	R2	Imp
Corallina spp.	918±105	721±126	1398±125
Barleeia unifasciata	1903±481	1420±336	5889±1514
Gibbula umbilicalis	45±15	21±10	45±14
Nucella lapillus	59±23	42±14	56±31
Odostomia eulimoides	101±77	69±33	0
Patella spp.	66±26	45±22	514±113
Rissoa parva	29472±10775	32990±12265	2653±910
Skeneopsis planorbis	5674±1514	2854±778	1208±432
Tricolia pullus	472±239	410±159	122±48
Lasae adansoni	278±183	0	2906±601
Modiolus modiolus	10427±2322	10205±2755	10281±1038
Musculus costulatus	819±203	375±105	1538±313
Mytilus galloprovincialis	15823±4349	19944±5468	109250±17727
Campecop lusitanica	101±60	90±64	7±5
Dynamene sp.	170±37	233±73	4243±1010
Hyale perieri	819±219	288±127	2028±595
Hyale pontica	28±15	10±10	170±130
Idotea balthica	14±11	97±56	0
Idotea pelagica	118±36	111±41	722±208
Ischyromene lacazei	63±34	38±35	934±390
Pirimela denticulata	42±12	149±38	6.9±48
Tanais dulongii	306±114	56±19	1618±474
Eulalia viridis	69±20	35±12	160±35
Perineris cultrifera	97±44	97±37	17±10
Platynereis dumerilli	35±15	42±14	87±38
Sabellaria alveolata	94±32	52±19	233±124
Syllis amica	448±135	115±59	42±25
Syllis gerlachi	132±94	156± 75	0
Nematoda	108±78	76±56	483±141
Nemertinea	6.9±5	0	2170±1226
Acanthochitona crinita	347±108	677±212	42±20
Acantho fascicularis	590±105	979±198	111±40
Echinoidea	38±17	76±33	0
Ophiuridea	17±14	63±28	0

Several species of gastropods were present at the sublittoral fringe (Table 2.10). Some species were equally abundant along all sampling areas (as *Nucella lapillus* or *Gibbula umbilicalis*). However, the majority were mainly present at the reference areas, namely *Rissoa parva*, *Skeneopsis planorbis* or *Tricolia pullus*. On the contrary, bivalves (as *Mytilus galloprovincialis*, *Musculus costulatus* or *Lasae adansoni*) were more abundant near the sewage discharges, with the exception of *Modiolus modiolus* (Table 2.10). Regarding crustaceans, *Hyale perieri*, *Idotea pelagica*, *Dynamene* spp. and *Tanais dulongii* showed higher abundances near the sewage discharges (Table 2.10). Nevertheless, other species seemed to prefer the reference areas (*Pirimela denticulata*, *Campecopea lusitanica*). The same pattern was also observed for the polychaetes (Table 2.10): *Eulalia viridis* or *Sabellaria alveolata* were more abundant in the impacted area and *Perinereis* spp. or *Syllis gerlachi* were more abundant in the reference areas. Finally, nematoda and nemertinea seemed to prefer the impacted area, while Polyplacophora were more abundant in the reference areas (Table 2.10). Other classes, although with lower numbers, like echinoidea and ophiuridea were only observed in the reference areas.

Finally, Figure 2.9 shows the contribution by feeding guilds for density and biomass. As can be seen, at the eulittoral, the feeding guild composition for density (Fig. 2.9a) changed. There was a dominance of suspension feeders in the impacted area, while in the reference areas the percentage of suspension feeders and herbivores was even. On the contrary, considering biomass (Fig. 2.9b), the feeding guild composition was similar in the three areas, with a dominance of suspension feeders.

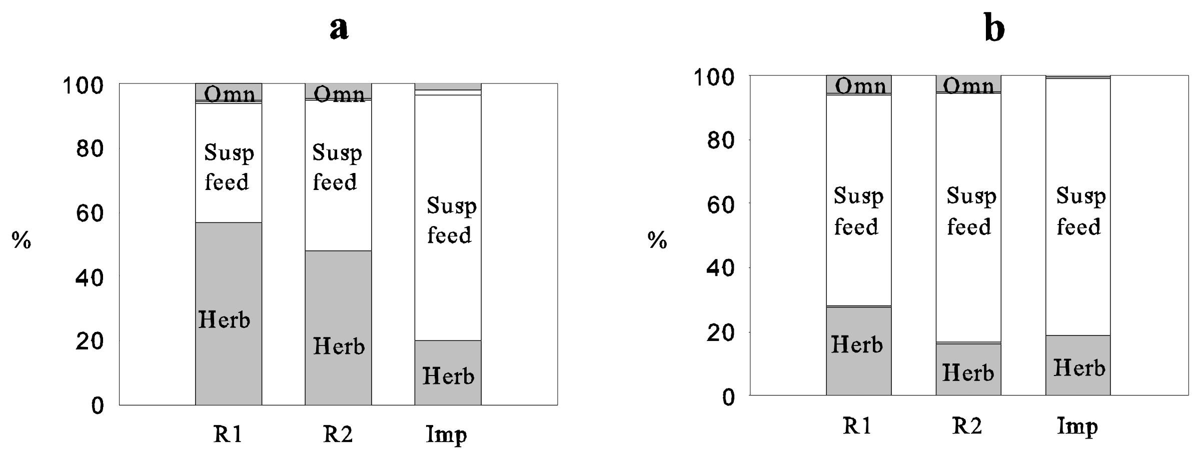


Figure 2.9 feeding guilds contribution (%) for density (**a**) and biomass (**b**), at the sublittoral fringel in the three sampling areas: R1, R2 – reference; Imp – Impacted area. Herb- Herbivores; Susp feed – Suspension feeders; Omn – Omnivores.

2.3.3 Secondary production estimates

Secondary production was studied for each zonation level at each of the three sampling areas (Fig. 2.7). The highest benthic community production was observed in the impacted area for the eulittoral and sublittoral fringe, while the lowest values were consistently observed in the littoral fringe, independently of the impact (Fig. 2.7a). The littoral fringe was dominated by *Melarhaphe neritoides* in all the three areas. However, different species contributed to the overall community production among the tidal levels, with fewer species contributing to overall community production in the reference areas in the eulittoral (Fig. 2.7b). Moreover, a change in the dominant productive species was observed, being *Patella* spp. the dominant in the reference areas and *Chthamalus montagui* in the impacted one.

At the sublittoral fringe, an opposite pattern was found with fewer species contributing for the production levels near the sewage discharges (Fig. 2.7b). In the reference areas, the dominant contributors to the total secondary production were *Rissoa parva*, *Modiolus modiolus* and *Mytilus galloprovincialis*, while in the impacted area about 50% of the community production was due to *M. galloprovincialis* alone (Fig. 2.7b). These differences in the species composition and respective contribution for community total production were statistically significant for the tidal levels (ANOSIM, $R = 1$, $p<0.05$), but not between impacted and reference areas (ANOSIM, $R = -0.03$, $p>0.05$). However, a separation in the nm-MDS was visible when comparing reference and impacted areas at the eulittoral and sublittoral fringes (only 40% similarity, Fig.2.8).

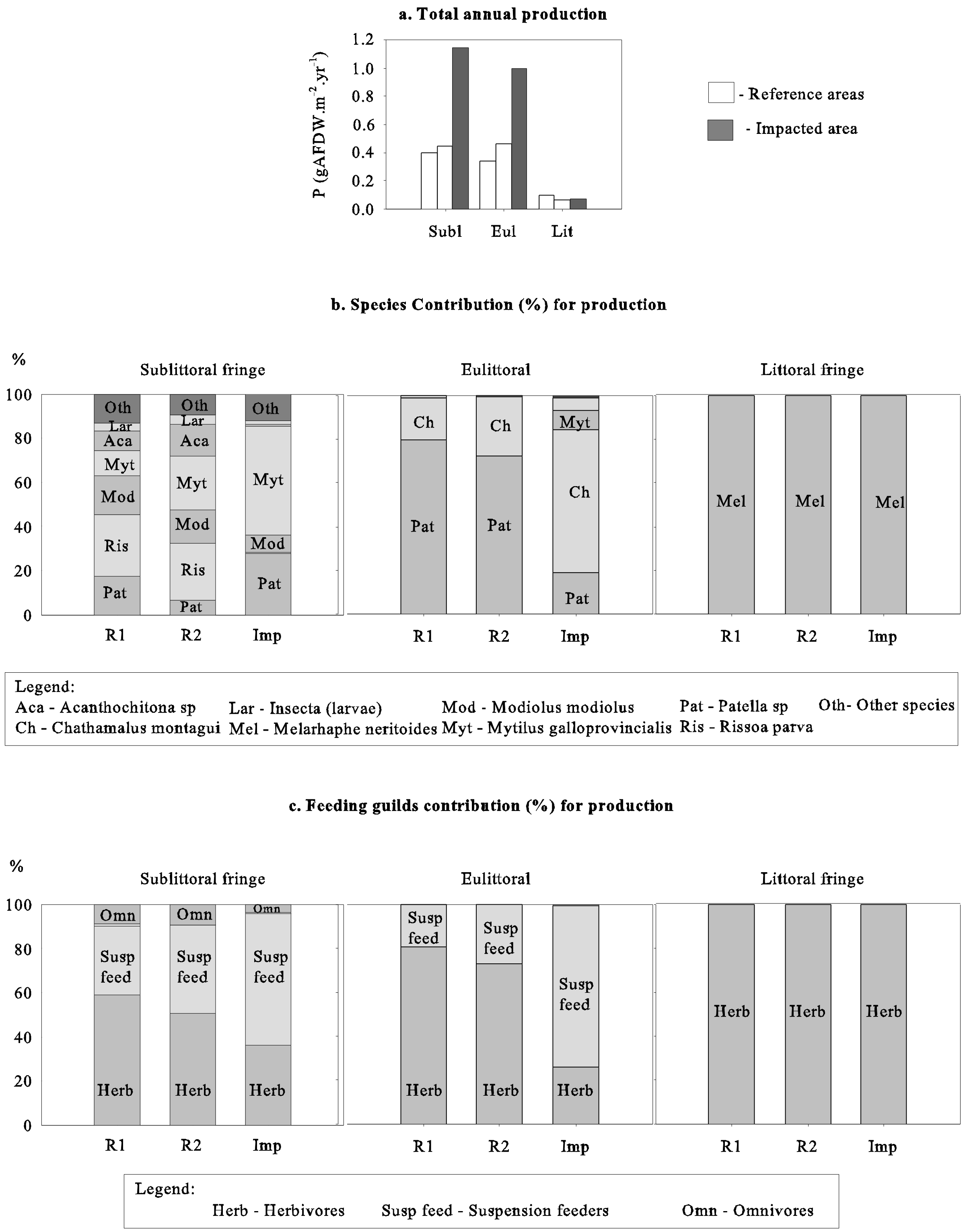


Figure 2.7 a. Total community annual production at the sublittoral fringe, eulittoral and littoral fringe in the three sampling areas. **b.** Species contribution (%) for production and **c.** feeding guilds contribution (%) to production in each level in the three sampling areas. R1, R2 – reference areas; Imp – Impacted area.

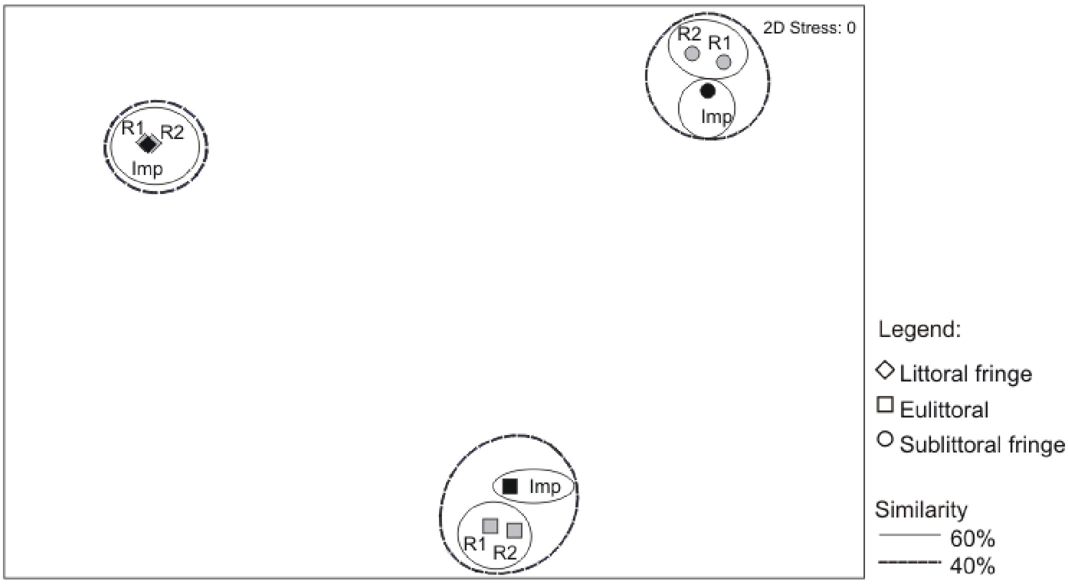


Figure 2.8 Two dimensional nm-MDS ordination plot of macroinvertebrate assemblage production for the reference (R1 and R2) and impacted (Imp) areas at the sublittoral fringe, eulittoral and littoral fringe, with indication of the 40% and 60% similarity groups.

Regarding the feeding guilds, differences were also observed between impacted and reference areas. Near the sewage discharges the feeding guild that most contributed for total production was the suspension feeders, while in the reference areas were the herbivores the group that most contributed for production levels (Fig. 2.7c). When comparing tidal levels, feeding guilds composition for the sublittoral and eulittoral was similar, dominated by herbivores and suspension feeders and a smaller percentage of omnivores, especially at the sublittoral. A single feeding guild was found at the littoral fringe, the herbivore *Melarhaphe neritoides*.

2.4 Discussion

2.4.1 Environmental variables

The effects of the sewage discharges in the environmental variables have already been noticed in previous studies. Roberts et al. (1998) and Elías et al. (2009) detected an increase in the nutrient concentration and suspended solids. López-Gappa et al. (1990, 1993) observed an increase in the seawater temperature and total coliforms and a decrease in pH, salinity and dissolved oxygen values in Argentina shores. As a rule, during the sewage treatment gross solids are eliminated from the effluents (primary treatment). Afterwards organic matter is removed (secondary treatment) and finally bacteria and nutrients are taken out of the effluents (tertiary treatment). However, the sewage treatment plant near our sampling area is only prepared to secondary treatment, and consequently the observed increase in the sea water temperature and the decrease in dissolved oxygen, salinity and pH become expectable. Likewise, the lack of tertiary treatment also explains the higher concentrations of total suspended solids, total coliforms and nutrients near the sewage discharges. Concerning nutrients, the higher concentrations were found for ammonia and phosphate, which is also expectable, since domestic and industrial effluents are the main contributors for the eutrophication of marine waters.

2.4.2 Macroinvertebrate assemblage density

2.4.2.1 Littoral fringe

The littoral fringe is the level more distant from the sewage discharges, which can explain the lower effects observed in abundance and species richness. Two species were found at this level: the gastropod *Melarhaphe neritoides* and the isopod *Ligia oceanica*. *L. oceanica* was only found in the impacted area. This isopod is a mobile omnivore that feeds on particulate organic matter and detritus (Fish and Fish, 2011; Littler and Murray, 1975) which might explains its higher abundance near the sewage discharges. On the contrary, no differences were found for *M. neritoides* between sampling areas. However, to assess the effect of sewage discharges on *M. neritoides* population it is necessary to study the population along all tidal height, and not only at the littoral fringe. Planktonic larvae settle on the lower levels of rocky shores and then start moving upshore, resulting in a shell size gradient (Cabral-Oliveira et al., 2009). The results obtained in this work confirmed previous findings (Cabral-Oliveira et al., 2009) where *M. neritoides* density was higher in the impacted area as a result of massive settlement. Also in this work was found a higher number of juveniles at eulittoral near the outfall. The higher concentration of nutrients near sewage discharges will lead to a larger quantity of microalgae on the rocky surfaces, on which *M. neritoides* feed. This could be attractive to the settling of *M. neritoides* larvae. However, during its life span a significant mortality leads to similar density values at the littoral fringe and to bigger individuals in the reference areas. This reduced size of *M. neritoides* in the impacted area is most probably related to the greater densities found, in turn leading to greater competition for food.

2.4.2.2 Eulittoral

Previous studies (Archambault et al., 2001) suggested that sewage discharges have little impact in organisms that live at the eulittoral. However, different conclusions can be drawn with the present work.

At the eulittoral the higher abundances found in the impacted area were explained by the larger number of gastropods (*Melarhaphe neritoides*), bivalves (*Mytilus galloprovincialis*) and crustaceans (*Chthamalus montagui*). *M. galloprovincialis* and *C. montagui* had higher densities near the sewage discharges, at all levels. Both species are filter-feeders and space occupiers (Hawkins and Jones, 1992). Attending to the higher amount of suspended solids near the sewage discharges it was expectable to find higher densities where food availability is higher.

Concerning the so called “not dominant species”, at the eulittoral, the higher abundances of the bivalve *Lasae adansoni*, the isopods *Campecopea hirsuta* or *Dynamene* spp., and the small periwinkles *Littorina saxatilis* near sewage discharges can be due to the higher amount of empty barnacle’s cases, which are the habitat of those species (Fish and Fish, 2011). This led to an increase in species richness in the impacted area. Previous authors (Magurran and McGill, 2010) have already pointed out that periodic disturbance might increase biodiversity by adding more resources to the habitat and by promoting the coexistence of species adapted to different conditions.

The limpets *Patella* spp. were more abundant in the reference areas, which possibly explains the higher contribution of herbivores in that areas. But the individuals were bigger near the sewage discharges. These results are consistent with earlier studies (Tablado and López-Gappa, 2001) that observed greater sizes and growth rates near the sewage discharges. Intraspecific competition normally occurs in the rocky intertidal environment when space or food resources are not enough or when recruitment occurs in high densities leading to crowding (Boaventura et al., 2002b). Due to the organic enrichment near the sewage discharges, the competition should be mainly for space. The higher abundance of juveniles near the sewage discharges promotes competition for space, and only a small number of individuals survive and become adults. Nevertheless those adults grow more (than the adults from the reference areas) due to the higher availability of food. Another important issue to be taken into account is the higher sea water temperature observed near the sewage discharges that can also promote the growth of this species.

2.4.2.3 Sublittoral fringe

At the sublittoral fringe were found the highest number of species, and also the higher abundances. Although the number of species was similar in all sampling areas there were qualitative differences in the species present in the impacted and reference areas.

In the impacted area the dominant class was Bivalvia, which can be explained by the feeding mode. Being filter-feeders it was expectable to find higher densities near the sewage discharges, rich in suspended solids.

Regarding the other taxonomic groups (Gastropoda, Crustacea, Polychaeta and Polyplacophora) there were species that seem to prefer the reference areas, and others that were more abundant in the impacted area. Even so, the majority of gastropods and chitons seem to prefer the reference areas, while Crustacea and Polychaeta seem to prefer the impacted area. Polyplacophora, like most gastropod species, seem to prefer non-polluted habitats. Earlier studies (Airoldi, 2003; Terlizzi et al., 2005a) have advanced the possible explanation that changes in the sedimentation rate due to the increase of suspended solids near the sewage discharges can have negative effects on gastropods. Atalah and Crowe (2012) have also found consistent differences in molluscan assemblages related with nutrient enrichment, which suggests that those assemblages can be potential indicators of pollution in coastal areas.

Concerning marine worms, the result was expected, since other studies on polychaetes assemblages have also observed significant greater density, biomass and average number of species near the sewage discharges (Anger, 1975; Dauer and Conner, 1980; Pearson and Rosenberg, 1978). Nematoda and nemertinea normally increase its abundance near sewage discharges due to their ability to exploit the food resources available (Fraschetti et al., 2006 and references therein). Due to that, these groups are classified as tolerant by Borja et al. (2000).

Crustaceans are generally considered as very sensitive to pollution (De-la-Ossa-Carretero et al., 2010 and references therein). The organic content and oxygen availability are some of the factors used to explain the sensitivity of crustacean species. In this study these characteristics were also found near the sewage discharges. However, in the present study, the majority of crustacean species seem to prefer the impacted area. The most common crustaceans found at the sublittoral fringe were *Hyale perieri*, *Dynamene* spp. and *Tanais dulongui*. Earlier studies already found higher densities of these amphipods and tanaids species near sewage discharges (Adami et al., 2004; Kalkan et al., 2007). The higher abundances near sewage are probably explained by the higher amount of *Corallina* sp. (Fish and Fish, 2011) acting as refuge and increasing the heterogeneity of the substrate. Despite all these, some crustacean species were more abundant at the reference areas (e.g. *Pirimela denticulata*, *Campecopea lusitanica*).
Some other classes only appeared in the reference areas, like echinoidea and ophiuroidea. This pattern was somehow expected since these groups are known to be very sensitive (Borja et al., 2000).

2.4.3 Secondary production estimates

The secondary production of the macroinvertebrate community was higher near the sewage discharges for the sublittoral and eulittoral zones, but no differences were found at the littoral fringe, most probably because the littoral fringe is the level locating further away from the sewage discharges. Differences in the community production composition were only statistically significant between tidal levels, however there was a discrimination between reference and impacted areas (>40% similarity). This increase in production due to nutrient enrichment has also been observed before, as intermediate levels of nutrient enrichment in relatively impoverished systems can increase primary production and subsequently secondary production (Nixon and Buckley, 2002; Singer and Battin, 2007). However, it is important to identify the source of the production, in particular the species responsible for that increase. It is assumed that an increase of secondary production does not necessarily represent a healthier ecosystem (Dolbeth et al., 2012). At the sublittoral fringe and eulittoral, there were differences in the species that most contributed to total community production. Near the sewage discharges, at the eulittoral, *Patella* spp. was replaced by *Chthamalus montagui*. The sublittoral fringe in the impacted area was dominated by the bivalve *M. galloprovincialis*, with a considerable decrease in species such as *Rissoa parva* and *Acanthochitona* spp. The dominant species in the impacted area, *C. montagui* and *M. galloprovincialis* are tolerant species to nutrient enrichment and trace elements (Borja et al., 2000, http://www.marlin.ac.uk), while *R. parva* and *Acanthochitona* spp. are sensitive (Terlizzi et al., 2005a; Atalah and Crowe, 2012) and *Patella* spp. is moderately sensitive to salinity decreases (http://www.marlin.ac.uk). In fact, *M. galloprovincialis* has been associated to moderate-poor environmental quality status and *Patella* spp. to high-good status in coastal rocky assemblages (Díez et al., 2012). So, although there was a general community production increase in the impacted area, this increase was due to the tolerant species in detriment of the sensitive ones, a pattern that has already been notice for another habitats under stress due to nutrient enrichment (Dolbeth et al., 2003; Singer and Battin, 2007) and contaminants (Whiles and Wallace, 1995; Woodcock and Huryn, 2007).

The feeding guild composition also changed in response to the sewage impact. A previous study of the macrobenthic trophic structure from Portuguese rocky shore areas (Boaventura et al., 1999) found that filter feeders were numerically dominant at shallower depths, being replaced by detritivores at the deepest areas, while in the intermediate areas both herbivores and carnivores became abundant. In the present study, where feeding guilds were quantified by production and not by density, the eulittoral and sublittoral fringe were dominated by herbivores, followed by suspension (or filter) feeders in the reference areas. In the impacted area, suspension feeders were the main contributor for the total community production. This dominance of suspension feeders may be related with the higher amount of suspended solids near the sewage discharges and therefore higher food availability for suspension feeders. Previous studies pointed out that suspension feeders are an example of optimal foraging in marine context (Gili and Coma, 1998), and that in favourable conditions (nutrient enrichment and tolerance to the changes in the environmental parameters) species belonging to this feeding guild can achieve extremely high abundances. For the reference areas, at the eulittoral, the herbivores contribution to production was higher, while suspension feeders such as *C. montagui* decreased considerably, probably due to lower food resources and decreasing temperature (Riley, 2002). This decrease of barnacle production might have decrease competition for space, enabling herbivore species such as *Patella* spp. to attain higher production levels.

Based on all stated above the presence of sewage discharges seem to: (i) change the environmental variables; (ii) increase the total densities and biomasses of macroinvertebrates, being *Mytilus galloprovincialis*, *Melarhaphe neritoides* and *Chthamalus montagui* the species most responsible for the dissimilarities; (iii) have a different impact in the three intertidal levels. At the sublittoral fringe the community structure was changed. At the eulittoral suspension-feeders abundances and species richness increased. At the littoral fringe no effect was observed; (iv) change the feeding guilds moving forward the dominance of suspension feeders; (v) promote a qualitative change by the replacement of sensitive species by tolerant ones; (vi) increased the community secondary production, but mostly due to the production of tolerant species.

CHAPTER 3

Sewage pollution as a source of trace element contamination and the example of arsenic accumulation

Chapter 3 is submitted in the form of a manuscript as:

Cabral-Oliveira J, Pratas J, Mendes S, Pardal MA (2013) Trace elements in edible rocky shore species: effect of sewage discharges. Submitted to Human and Ecological Risk Assessment.

Cabral-Oliveira J, Coelho H, Pratas J, Mendes S, Pardal MA (2013) Macroalgae as bioindicators of arsenic contamination on coastal areas. Submitted to Scientia Marina.

Sewage pollution as a source of trace element contamination and the example of arsenic accumulation

3.1 Introduction

The aquatic systems are the ultimate deposit for several types of substances released intentionally or involuntarily by human activities. Among all, the coastal areas suffer the most severe impacts, due to the higher human pressure existent in those areas (Kennish, 1997). Sewage treatment plants play an important role in preserving the water quality around the world. However, the sewage treatment plants fail to remove several contaminants such as PAHs, drugs or trace elements (Maceda-Veiga et al., 2013). Trace elements can be subdivided into two categories: (i) essential trace elements (e.g., cobalt, copper, iron, manganese) which are necessary to the metabolism at low concentrations but may be toxic at high concentrations; and (ii) non-essential trace elements (e.g., arsenic, cadmium, lead) which generally are not required for metabolic functions being toxic even at low concentrations (Kennish, 1997). Trace elements are potentially harmful because they can be accumulated in living tissues and biomagnified through the food-chain. The accumulation and toxicity of trace elements on marine organisms will depend on physico-chemical factors, like dissolved element concentrations, temperature and salinity, and biotic factors such as reproductive and nutritional state (Maceda-Veiga et al., 2013). This potential toxicity to marine organisms and persistence in the environment can pose serious environmental and health risks, that are important to monitor (Alvarez et al., 2002).

Arsenic (As) is a widely distributed element in marine ecosystems and it can enter into the environment through both natural and anthropogenic processes. The main anthropogenic sources of this contaminant are domestic and industrial wastewaters, fuel combustion, mining and agricultural pesticide production (WHO, 2010). The toxicity of As is highly dependent on its chemical form, but since it can be harmful to humans even at low concentrations (Mieiro et al., 2012), it has been ranked as number one in the top ten most hazard substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2011). The main adverse effects reported to be associated with ingestion of arsenic in humans are nausea, vomiting, and diarrhea (low levels) and skin lesions, cancer, cardiovascular diseases (long-term ingestion).

Therefore, the potential toxicity of As can pose serious environmental and health risks, that are important to monitor.

Molluscs and macroalgae have been widely recognized as good bioindicators (Rainbow, 1995; Conti and Cecchetti, 2003; Cravo et al., 2004; Ballesteros et al., 2007). Soft tissues of marine molluscs may accumulate trace elements from the environment (water, sediment and/or food) (Cravo and Bebianno, 2005) and generally are not able to regulate these contaminants in their bodies (Díaz et al., 1992). Within molluscs, species like *Mytilus galloprovincialis* (O'Leary and Breen, 1997; Szefer et al., 2002; Duarte et al., 2012) and *Patella ulyssiponensis* (Cravo and Bebbiano, 2005 and references therein) are often used as bioindicators of trace element contamination. Studies using gastropods like *Phorcus lineatus* are scarce (Nicolaidou and Nott, 1990; O'Leary and Breen, 1997; Cubadda et al., 2001) being its potential as a bioindicator species controversial. Intertidal rocky shore macroalgae have been considered suitable bioindicators of water quality due to their sedentarism, easy sampling and taxonomic identification, worldwide distribution and capacity to respond to several anthropogenic impacts (Rainbow, 1995; Ballesteros et al., 2007). More specifically, macroalgae have high affinity for several trace elements and revealed sensitivity to environmental changes in As concentrations in the seawater (Chaudhuri et al., 2007). Macroalgae seemed the most suitable bioindicator of trace elements because, in contrast with marine fauna, they take up the trace elements directly from the water column (Rodriguez-Figueroa et al., 2009; Benkdad et al., 2011), responding exclusively to the contaminant levels in the seawater. The concentrations of As in intertidal macroalgae have already been the focus of previous studies. Some obtained baseline data to evaluate inter-site and inter-sample differences (Chaudhuri et al., 2007; Brito et al., 2012), others examined the As concentrations in industrialized coastal areas (Benkdad et al., 2011). Other studies aimed to assess As speciation in natural conditions (Slejkovec et al., 2006; Llorent-Mirandes et al., 2010). Also, Maher and Clarke (1984) have compared the total As concentrations found in macroalgae collected from clean and contaminated areas due to anthropogenic activities.

In addition to the choice of good bioindicators, it is also important the selection of an adequate sampling design. The majority of the previous studies compared the accumulation of trace elements between one reference area and one contaminated area, which could lead to problems of pseudoreplication. In ecology, the necessity to find methods able to detect anthropogenic impacts led to the development of BACI (Before-After, Control-Impact) procedures. This method argues that the use of multiple sampling times, before and after the disturbance, is essential to avoid temporal confounding (Chapman et al., 1995; Glasby, 1997). However, in many cases, pre-impact data are not available. In order to overtake this problem, Underwood (1991) developed a beyond-BACI design (ACI design) where the use of two or more control areas helps to distinguish between natural variability that characterises assemblages and variability-induced by a particular form of disturbance, such as sewage discharges.

In this chapter there were two main objectives. The first is to understand if sewage discharges are a source of trace element contamination. For this purpose, the accumulation of trace elements by edible molluscs was compared between sewage-impacted area and reference areas. It was found evidence of arsenic contamination due to the sewage discharges. With this in mind, the second goal is to compare As accumulation by macroalgae from impacted and reference areas. At the same time, it was assessed the potential of the molluscan species as bioindicators of trace element contamination and of the macroalgae species as bioindicators of As contamination.

3.2 Materials and methods

3.2.1 Study site and data collection

The study was conducted in Peniche peninsula (Fig. 2.1), on the central western Portuguese coast, as described in chapter 2.

The mollusc and the algae specimens were collected randomly by hand on the intertidal shore in November 2010, then stored in polythene bags and frozen at -20 ºC until trace elements analysis. All specimens of the same studied molluscan species (*Patella ulyssiponensis*, *Phorcus lineatus* and *Mytilus galloprovincialis*) and macroalgae species (*Plocamium cartilagineum*, *Asparagopsis armata*, *Codium* spp., *Ulva* spp. and *Saccorhiza polyschides*) belonged to the same size class in order to avoid misinterpretations due to size-dependent accumulation trends, as previously reported (e.g. Szefer et al., 2002, Cravo and Bebbiano, 2005).

At all sampling sites, water samples were collected for laboratory determination of As concentrations, due to the contamination observed in the mollusc tissues. Water samples were filtered (Whatman GF/F glass-fibre filter, 0.45µm) and stored frozen at -18 ºC until analysis.

3.2.2 Laboratory procedures

At the laboratory, molluscs' soft tissues were removed from their shells and prepared for analysis. The mollusc species were selected because they are popular edible-species in many countries, highlighting the importance to evaluate the associated risk to human health. The shell height was measured and the soft tissues were accurately weighed in dry, pre-cleaned Teflon digestion vessels. Approximately 0.5 g of fresh soft body tissues was digested using 5mL of HNO_3 65% and 2mL of H_2O_2 30%.

Five species of algae were chosen due to their different morphological and physiological characteristics and also because they are common species in the Atlantic shores. In laboratory the macroalgae were washed with seawater (collected in the respective sampling area) and epiphytes were eliminated manually. Algae were accurately weighed in dry, pre-cleaned Teflon digestion vessels. Approximately 1g of fresh algal sample was digested using 8mL of HNO_3 65% and 2mL of H_2O_2 30%.

The vessels were sealed and placed in the microwave chamber (Multiwave 3000 – Anton Paar) for digestion. The digests were then diluted with nanopure-distilled water and filtered. The analytic determinations of As, Cd, Co, Cu, Pb and Ni were performed using an Atomic absorption spectrophotometer (Thermo scientific SOLAAR M2), with graphite furnace and autosampler. For Zn, Fe, Mn, the analytical determinations were performed by using atomic absorption spectrophotometer 2380 Perkin-Elmer.

The accuracy and precision of the analytical methodology for the trace elements determinations were assessed by replicate analysis of certified reference materials (CRM-National Research Council Canada), namely DOLT – 4 (dogfish liver) for molluscs samples. For algal samples, certified references (Virginia tobacco leaves CTA-VTL-2, Polish Certified Reference Material) were also used to data quality control.

The recovery efficiency depended on the respective element and was: As (96.8%), Cd (92.8%), Co (98.4%), Cu (94.9%), Fe (92.0%), Ni (97.4%), Pb (98.8%) e Zn (90.6%). The detection limits for trace elements were (in µg. g^{-1}): 0.01 for As; 0.001 for Cd; 0.005 for Co, Cu, Pb and Ni; 0.2 for Zn; 0.75 for Fe; and 0.5 for Mn.

The determination of As concentrations on the seawater followed the methods described in Bermejo-Barrera et al. (1998).

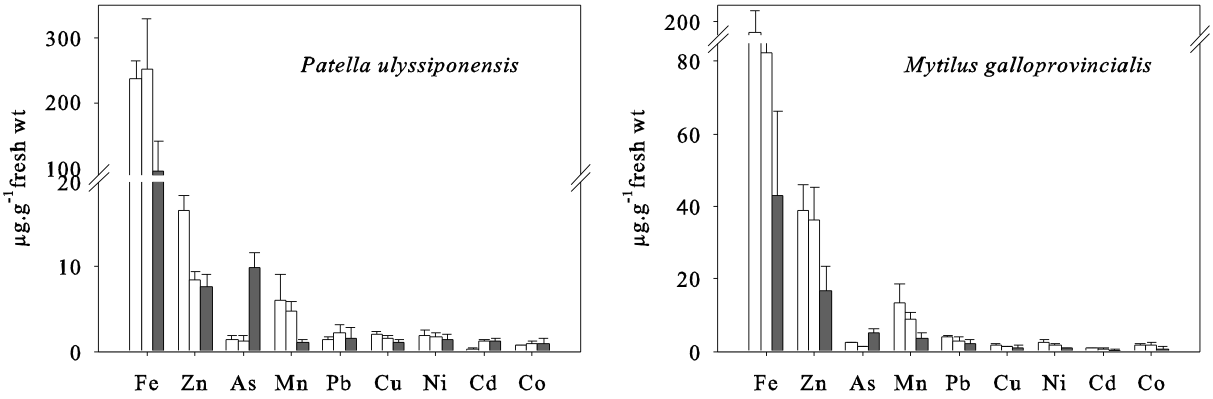
3.2.3 Data analysis

In order to understand if the presence of sewage discharges changed the physical and chemical parameters in the seawater of the three sampling areas, a principal component analysis (PCA), based on correlations, was performed using the CANOCO version 4.5 package (ter Braak and Smilauer, 1998). Three-way analysis of variance (ANOVA) was performed to assess whether trace element concentrations varied significantly among species, trace elements and areas. Post hoc pairwise comparisons tests (Tukey's HSD or Dunnett's multiple comparison of group means) were employed to determine significant differences between species, trace elements and areas. A two-way analysis of variance (ANOVA) with replication was used to assess the differences between sampling areas and species, for As concentrations (Zar, 1996). All data were checked for normality and homoscedasticity. For all statistical tests, the significance level was set at $p<0.05$. All calculations were performed with IBM SPSS Statistics 19.0.

3.3 Results

3.3.1 Trace element contamination in molluscs

Data on soft tissue concentrations of As, Co, Cd, Cu, Fe, Mn, Ni, Pb and Zn of *Patella ulyssiponensis*, *Phorcus lineatus* and *Mytilus galloprovincialis* from impacted and reference area are presented in Fig. 3.1. For all the measured elements significant inter and intra-specific variations were observed, being the concentrations of Fe, Zn and Mn consistently the highest in the reference areas for all the three species (Fig. 3.1). However, in the impacted area the pattern has changed due to the higher concentrations of As, that become the third (*M. galloprovincialis*), second (*P. ulyssiponensis*), and first (*P. lineatus*) element more abundant (Fig. 3.1).



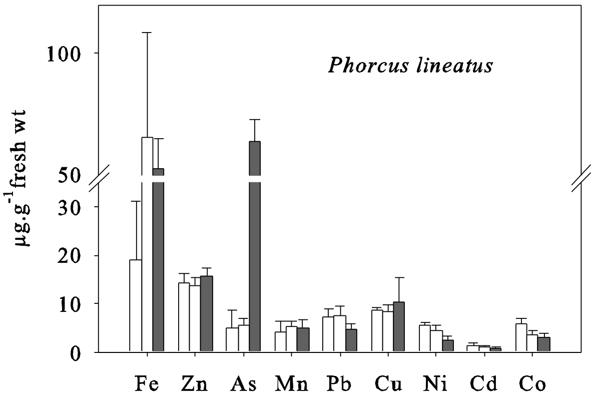


Figure 3.1 Concentration (µg.g^{-1}fresh weight) of trace metals (mean±SD) in soft tissues of all the studied species:

- reference areas

- impacted area

Regarding the simultaneous analysis of the factors (species, trace elements and sampling areas), statistically significant differences were detected (Table 3.1).

Table 3.1 Three-way ANOVA analysis testing for differences between species (Sp), trace elements (TrEl) and sampling areas (Ar). Significant results are given in bold.

Source	d.f.	SS	MS	*F*	*P*
Species (Sp)	2	9624.20	4812.10	20.62	**0.00**
Trace elements (TrEl)	8	459777.35	57472.17	246.31	**0.00**
Area (Ar)	2	4365.94	2182.97	9.36	**0.00**
Sp x TrEl	16	172263.00	10766.44	46.14	**0.00**
Sp x Ar	4	12040.58	3010.14	12.90	**0.00**
TrEl x Ar	16	54872.26	3429.52	14.70	**0.00**
Sp x TrEl x Ar	32	58547.54	1829.61	7.84	**0.00**
Error	324	75601.31	233.34		

This indicates that the changes in the inter-species concentrations vary with the trace elements measured, and simultaneously across the different sampling areas. Additionally, statistical differences were found between both reference areas and the impacted area (R1=R2≠Imp). Therefore, this suggests that the presence of sewage discharges had influence on the bioavailability of trace elements on intertidal molluscs (Table 3.2). In particular, it is possible to observe that only *P. ulyssiponensis* showed significant differences in the trace element concentrations among both reference areas and the impacted area. Concerning the trace elements, As and Fe were the elements which concentrations differed among both reference areas and the impacted area. Moreover, As concentrations were higher near the sewage discharges, while Fe concentrations were higher in the reference areas, particularly for *P. ulyssiponensis* (Fig. 3.1).

Table 3.2 Pairwise comparisons, testing species and trace element concentrations on the three sampling areas: R1, R2 – reference areas; Imp – impacted area. Significant results are given in bold.

	Imp vs R1	Imp vs R2	R1 vs R2
M. galloprovincialis	**0.000**	0.062	**0.002**
P. ulyssiponensis	**0.000**	**0.000**	0.876
P. lineatus	**0.003**	0.138	0.129
	Imp vs R1	Imp vs R2	R1 vs R2
As	**0.000**	**0.000**	0.969
Cd	1.000	0.969	0.969
Co	0.828	0.917	0.910
Cu	0.988	0.937	0.949
Fe	**0.000**	**0.000**	0.221
Mn	0.406	0.582	0.779
Ni	0.768	0.857	0.909
Pb	0.806	0.810	0.996
Zn	0.220	0.571	0.507

3.3.2 Physico-chemical parameters

Due to the fact that the As was the only element that showed significant differences between impacted and reference areas, it was important to understand if the As contamination was also noticed in the seawater.

As a dimension-reducing technique, the PCA results led to two principal components that accounted for nearly 98.5% and 1.5% (first and second axes, respectively) of the overall variability of the data (Fig. 3.2). The other components were neglected because they did not provide significant additional explanation to the data.

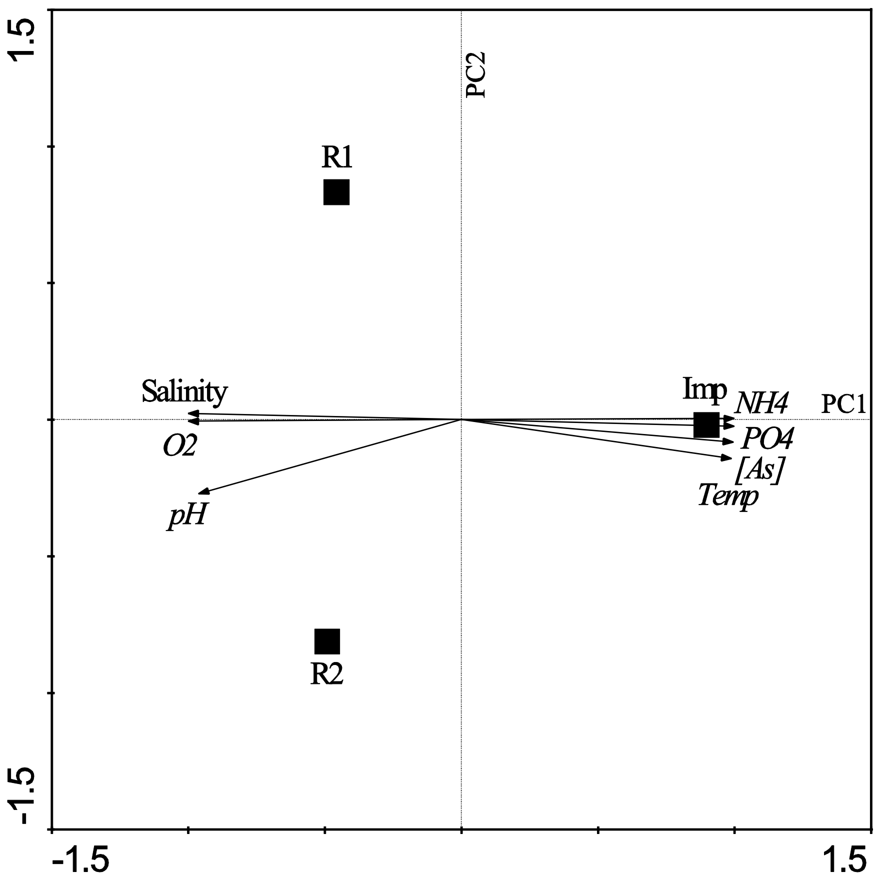


Figure 3.2 Principal component plot for reference (R1 and R2) and impacted (Imp) areas.

The first principal component was clearly associated with salinity, dissolved oxygen, pH, seawater temperature, nutrients and As concentrations (Fig. 3.2). From the projection of the PCA (Fig. 3.2), sampling areas appear distributed along an impacted gradient, with the reference sites (R1 and R2) presenting high values of salinity, dissolved oxygen and pH in contrast with the impacted area, which was characterized by high temperatures and concentrations of As and nutrients.

3.3.3 Arsenic contamination in macroalgae

Since As was found to be statistically different and in higher concentrations near the sewage discharges (both in mollusc soft tissues and seawater), total As concentrations in the intertidal macroalgae species from reference and impacted areas were analysed and are presented in the Fig. 3.3.

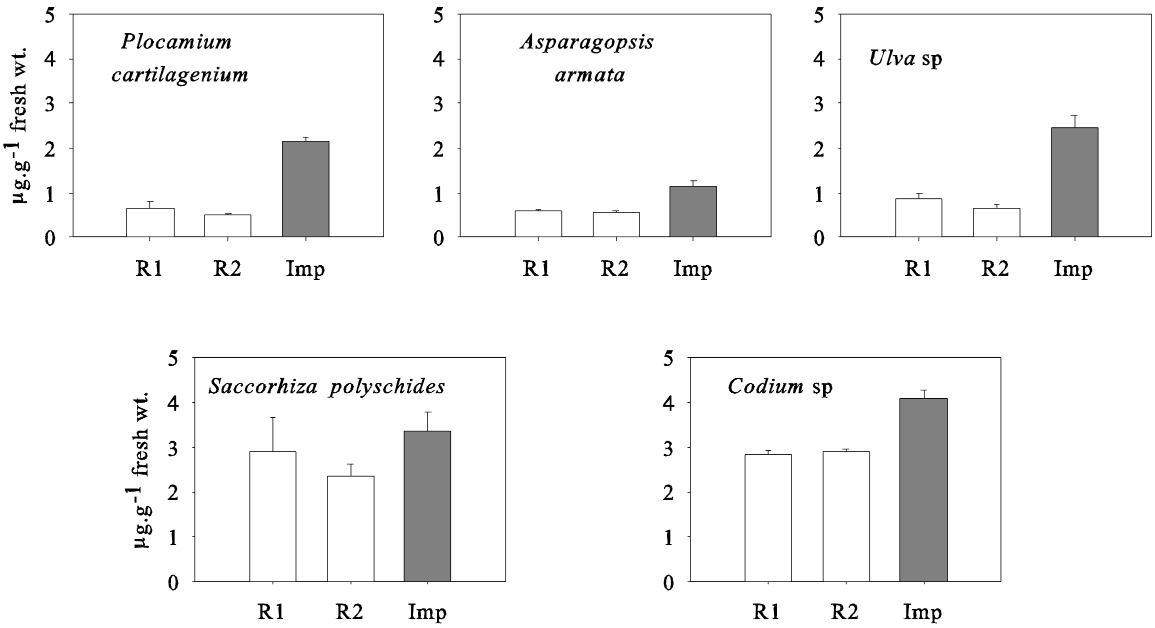


Figure 3.3 Arsenic concentrations (mean±SD fresh weight) in the five macroalgae species studied, for all sampling areas: R1, R2 – reference areas; Imp – impacted area

The As concentrations increased in the order: *Asparagopsis armata* < *Plocamium cartilagenium* < *Ulva* sp. < *Saccorhiza polyschides* < *Codium* sp. (Fig. 3.3). The two-way ANOVA revealed significant differences in species among areas (Table 3.3). Moreover, the interaction between species and sampling areas was also significant. In addition, multiple-comparison tests showed that the presence of sewage discharges changed the As concentrations (R1=R2≠Imp) in all the studied species, with the exception of the phaeophyta *S. polyschides* (Table 3.3).

Table 3.3 ANOVA analysis and multiple-comparison tests for As concentrations among species and for all the studied areas: R1, R2 – reference areas; Imp – impacted area.

Source of variation	d.f.	SS	MS	*F*	*P*
Species (Sp)	4	76.454	19.113	277.805	**0.000**
Area (Ar)	2	23.195	11.597	168.561	**0.000**
Sp x Ar	8	3.788	0.474	6.883	**0.000**
Error	60	4.128	0.069		

Multiple comparisons	Imp vs R1	Imp vs R2	R1 vs R2
Plocamium cartilagenium	**0.000**	**0.000**	0.327
Asparagopsis armata	**0.001**	**0.001**	0.879
Codium sp	**0.000**	**0.000**	0.741
Ulva sp	**0.000**	**0.000**	0.172
Saccorhiza polyschides	**0.007**	**0.000**	**0.001**

3.4 Discussion

3.4.1 Trace element contamination in molluscs

The presence of trace elements on aquatic systems can be estimated by analysing water, sediments and marine organisms. Chemical analyses of the water fail to inform about the bioavailability of trace elements and sediments are rarely available in rocky shores (Campanella et al., 2001; Guerra-Garcia et al., 2010). Consequently, the use of organisms as bioindicators seems the most appropriated approach in studies undertaken in rocky shores. Thus, in this study were used three mollusc species: *Patella ulyssiponensis*, *Phorcus lineatus* and *Mytilus galloprovincialis*. All these species are easy to identify and worldwide distributed, available for sampling throughout the year and large enough to provide adequate tissue for (individual) analysis, which makes them potential good bioindicators (Rainbow, 1995). Moreover, in order to evaluate the associated risk to human health, all the selected species are used for direct human consumption in many areas of the planet.

The accumulation of trace elements on mollusc soft tissues was compared among impact and reference areas. In both reference areas, for all the species, the elements found in higher concentrations were Fe, Zn and Mn. Those essential elements are metabolically important, and are normally found in high concentrations in marine molluscs (Cravo and Bebianno, 2005). However, in the impacted area the pattern has changed due to the higher concentrations of As, in all the studied species. The industries that exist near the impacted area may be responsible for this As contamination. In fact, the only elements whose concentrations have changed with the presence of sewage discharges were Fe and As. Concentrations of Fe were higher in the reference areas, especially in *P. ulyssiponensis*. Cravo and Bebianno (2005) have already found high variability of Fe concentrations in limpets, which is in agreement with the present work. On the contrary, for all the studied species, the As concentrations were higher near the sewage discharges. This contaminant is ranking as number one in the top ten most hazard substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2011), even if its toxicity is highly dependent on its chemical form. Besides, it is known that As may be harmful to humans even at low concentrations when ingested over a long time period (Mieiro et al., 2012). For this reason, although the concentrations found in this study (Table 3.4) are not high in comparison with other polluted areas (Klarić et al., 2004; Guerra-García et al., 2010), it seems that sewage from industrial areas might be a source of As contamination, and may pose serious human health problems.

Table 3.4 Arsenic concentrations ($\mu g.g^{-1}$ *fresh weight ● dry weight) found in different study areas.

	Mytillus spp.	*Patella* spp.
Klarić et al., 2004	2.0-16.4●	-
Guerra-García et al., 2010	11.1-21.7●	10.5-27.9●
Present work	0.9-6.5*	0.2-13.6*

It is also important to acknowledge the inter- and intra-specific variation in the accumulation of trace elements. This evidence is in agreement with previous studies, which have already verified that the accumulation of trace elements was not consistent, varying with location and taxa (Mayer-Pinto et al., 2010). Moreover, it seems that closely related species could exhibit different accumulation strategies, and even individuals of the same species can show a great range of concentrations for each contaminant (e.g. Campanella et al., 2002; Szefer et al., 2002). This can be justified by several abiotic (e.g. salinity, temperature, pH) and biotic (e.g. body size, sex, physiological conditions, reproductive status) parameters that can affect elements bioavailability and accumulation in the soft tissues of molluscs (Szefer et al., 2002; Cravo and Bebianno, 2005). Although this inter- and intra-specific variation, molluscs are widely considered good bioindicators of trace element contamination. More specifically, in this study *P. ulyssiponensis* seemed to be the best bioindicator. This is in accordance with previous studies that suggested limpets as good bioindicators of sewage pollution (Bishop et al., 2002; Espinosa et al., 2007; Atalah and Crowe, 2012), as well as of trace element contamination (Cravo et al., 2004; Cravo and Bebianno, 2005).

Finally, in order to assess the potential risk for human health, the concentrations of trace elements found in this study were compared with the European advisory guidelines for commercialized seafood products. However, given that the European regulations exist only for bivalve molluscs, it was only possible to compare the concentrations obtained for *M. galloprovincialis* with the maximum required by law. According to the Commission Regulation (EC) nº 1881/2006 the maximum concentration allowed in commercialized bivalve molluscs is 1.5 $\mu g.g^{-1}$ fresh weight of lead and 1.0 $\mu g.g^{-1}$ fresh weight of cadmium. In this work the maximum observed for lead was 4.6 $\mu g.g^{-1}$ fresh weight and for cadmium 1.4 $\mu g.g^{-1}$ fresh weight in both references and impacted areas. These values were relatively low when compared with other studies undertaken near polluted areas (Szefer et al., 2002). Nevertheless, the concentrations of Pb and Cd exceeded the threshold limits set by the EU legislation, which may pose serious risks both to wildlife and human health.

3.4.2 Physico-chemical parameters

Sewage pollution is normally associated with organic and nutrient enrichment (Arévalo et al., 2007; Cabral-Oliveira et al., 2009). However, contaminants like trace elements can also be associated with this source of pollution, especially when the sewage treatment plants receive industrial effluents (Alvarez et al., 2002). In this study, the presence of sewage discharges seems to change the environmental variables. Results suggested that sewage discharges increased seawater temperature, and decreased salinity, pH, dissolved oxygen and the nutrient (ammonia and phosphate) loads in the seawater increased (already focus in chapter 2) and also increased the trace element concentrations, such as As, in the seawater. Previous studies (Chaudhuri et al., 2007) have found As concentrations in the seawater between 21.0±9.8µg/L and 47.6±16.9µg/L. In this study similar concentrations were found in the reference areas (R1=21.5µg/L and R2=19.9µg/L) but near the sewage discharges the concentrations were higher (187.4µg/L), thus confirming the As contamination in that area. Therefore, it seems that the sewage discharges in our case-study are a source of As contamination, probably due to the industries present in the area (e.g. shipyards, metal smelting).

3.4.2 Arsenic contamination in macroalgae

The response of algal communities to nutrient and organic enrichment is well known, with a decrease in the number of species and a reduction of stratification due to the disappearance of large perennial species, replaced by opportunistic ones (May, 1985; Soltan, 2001; Terlizzi et al., 2002; Arévalo et al., 2007). However, the impact of As contamination due to sewage discharges and the effects on the As accumulation by intertidal macroalgae has not yet been assessed.

In this study, near the sewage discharges, along with higher As concentrations in the seawater, was also noticed significantly higher concentrations of As in all the macroalgae studied, except for *Saccorhiza polyschides*. The possibility to identify the samples affected by As contamination with respect to their distance to the contaminantion source (impacted against reference areas) attest the use of macroalgae species as suitable bioindicators. The only exception was the phaeophyta *S. polyschides*.

Brown algae generally accumulate the highest concentrations of As (Slejkovec et al., 2006; Brito et al., 2012). Moreover, Maher and Clarke (1984) found that *Cystoseira* spp. and *Sargassum* spp. accumulate more As in polluted areas (exposed to mining activities) than in clean areas. However, the phaeophyta used in this study (*S. polyschides*) have shown significant differences between all the sampling areas, and consequently do not seem a suitable bioindicator of As contamination. This supports the idea that methods based on indicator-species may be better than those based on functional-form groups (Arévalo et al., 2007), due to the variations in the species responses to the contamination.

Regarding chlorophyta, both species (*Ulva* sp. and *Codium* sp.) responded to the higher As concentrations in the seawater near sewage discharges. Previous studies (Slejkovec et al., 2006) have found baseline values for *Ulva* spp. (1.35±0.07 μg.g^{-1} fresh wt) slightly superior to those found in the reference areas of this study (0.76±0.16 μg.g^{-1} fresh wt). However, there is no agreement in the response of this species to As contamination. Some studies found similar As accumulations in clean and polluted areas (Maher and Clarke, 1984), while others noticed the opposite (Chaudhuri et al., 2007), which raises some questions about the use of *Ulva* sp. as bioindicator of As contamination. About *Codium* sp., to our best knowledge, this is the first attempt to compare the As accumulation among clean and polluted areas. Nevertheless previous studies (Llorent-Mirandes et al., 2010) have also found higher As concentrations in *Codium* sp. than in *Ulva* sp. (27.7±2.9μg.g^{-1} and 5.3±0.8μg g^{-1}, respectively), such as in the present study. On the other hand, green algae rocky cover changes seasonally (Archambault et al., 2001; Bishop et al., 2002) and Rainbow (1995) suggests that a good bioindicator of trace elements should be available for sampling throughout all year. Therefore, chlorophyta may not be the most suitable bioindicator to As contamination.

Concerning rhodophyta, both species (*Asparagopsis armata* and *Plocamium cartilagineum*) responded to the higher As concentrations in the seawater near sewage discharges. However, those species belong to different ecological status groups (ESG). Marine benthic macroalgae can be divided in two groups: EGS I (late successional) and ESG II (opportunistic). ESG I comprises thick or calcareous thallus macroalgae, with low growth rates and long life cycles. ESG II includes sheet-like and filamentous macroalgae species with high growth rates and short life cycles. *P. cartilagineum* is perennial (ESG I) while *A. armata* is an opportunistic (ESG II) species (Orfanidis et al., 2001). Those groups are associated with alternative ecological states: ESG I with pristine and ESG II with degraded ecological states. Moreover, the use of these functional groups has already been tested to water quality (Orfanidis et al., 2001) and more recently to detect pollution (Díez et al., 2003). This information can be useful in the selection of appropriated bioindicators. A suitable bioindicator for trace element contamination should be present in both disturbed and undisturbed areas, to allow comparisons between areas. Therefore, species from ESG II may be more appropriate as bioindicators for As contamination. Consequently, from all the studied species, *A. armata* seems to be the best bioindicator for As contamination in coastal areas.

In conclusion, the effect of sewage pollution on the accumulation of trace elements on intertidal molluscs seems to suggest that: (i) trace element accumulation by molluscs can be affected by the presence of sewage discharges (in our case-study As, due to the type of industries near our sampling areas); (ii) limpets (*P. ulyssiponensis*) seemed the best bioindicator for trace elements; (iii) Cd and, especially, Pb exceed the legislation values concerning human consumption. Moreover, although the recommendations merely focus on Cd, Pb and Hg, this study suggests the importance of include As in European legislation to prevent human health concerns; (iv) macroalgae species seem to be suitable bioindicators of As contamination in coastal areas, with the exception of *Saccorhiza polyschides*. The information obtained from previous studies, together with the ecological characteristics of each species lead to the proposal of *Asparagopsis armata* as the best bioindicator for As contamination in temperate Atlantic coastal areas

CHAPTER 4

Can eulittoral rocky shore macroinvertebrate assemblages be used as an indicator of sewage pollution?

Chapter 4 is submitted in the form of a manuscript as:

Cabral-Oliveira J, Bevilacqua S, Terlizzi A, Pardal MA (2013) Comparing Atlantic and Mediterranean shores: testing the ability of eulittoral invertebrate assemblages to detect the effects of sewage discharges in coastal areas. To be submitted.

Cabral-Oliveira J, Pardal MA (2013) Sewage discharges in oceanic islands: effects and recovery of eulittoral macrofauna assemblages. Submitted to Environmental Management.

Can eulittoral rocky shore macroinvertebrate assemblages be used as an indicator of sewage pollution?

4.1 Introduction

Coastal areas are under high human pressure, being daily exposed to several anthropogenic impacts such as sewage discharges (Crowe et al., 2000; Halpern et al., 2008; Arévalo et al., 2007). These anthropogenic impacts seem to affect the ecosystem functioning and resilience, threatening the supply of ecosystems services provided by coastal areas (de Groot et al., 2012; Pinto et al., 2012). Ecosystem services are the benefits (essential services and goods) for human population that derive, directly or indirectly, from ecosystem functions (Constanza et al., 1997; Pinto et al., 2012). They can be divided into four categories: provisioning, regulating, cultural and supporting. Provisioning includes the products obtained directly from the ecosystem, such as food resources. Regulating refers to the benefits obtained from the ecosystem processes, for example, when rocky shores act like a buffer for wind, water and waves (disturbance regulation). Cultural involve the nonmaterial benefits like cognitive and aesthetic experiences. Finally, supporting consist in all the benefits necessary for the production of all the other ecosystem services (de Groot et al., 2012; Pinto et al., 2012). Important ecological attributes, such as, biodiversity and community structure can be included in this last category and influence the provision of others ecosystem services. Tourism and production of goods and human welfare are some of the economic activities that rely on a good ecosystem quality (Pinto et al., 2012; Liquete et al., 2013).

Previous studies have already showed that sewage discharges may change the abundances and structure of intertidal assemblages (Littler and Murray, 1975; Archambault et al., 2001; Bishop et al., 2002), but there is no consensus about the effect on the species richness. Polychaetes assemblages increase their abundances near the sewage discharges, due to opportunistic species (Dauer and Conner, 1980). Crustaceans and bivalves filter-feeders (e.g. Balanidae, Mytilidae) have been reported to be more abundant near the sewage discharges due to the higher amount of suspended solids (López-Gappa et al., 1993; Pinedo et al., 2007).

On the contrary, in gastropods assemblages a general decrease in abundance and the disappearance of sensitive species near sewage discharges is known (Terlizzi et al., 2005; Atalah and Crowe, 2012).

Eulittoral assemblages of intertidal rocky shores are often exposed to sewage discharges, and to the potential ensuing perturbations of environmental conditions (Ashton and Richardson, 1995; Bustamante et al., 2012; Fraschetti et al., 2006; Garaffo et al., 2012; Smith, 2000). Such assemblages generally do not have many species, are accessible to sample, and the species present have widespread distributions and are easy to identify at high taxonomic resolution. These features make eulittoral assemblages potentially cost-effective in discerning between disturbed and undisturbed environmental conditions across different environmental contexts and geographical regions. Nevertheless, to our knowledge no attempt has been made to understand whether such assemblages are a suitable indicator of human impacts related to sewage discharges, able to consistently respond over large spatial scales. Two tasks were carried out in order to fulfil this gap, and are described in this chapter. The first task compared the response of eulittoral assemblages to sewage discharges in different bioregions (Atlantic and Mediterranean shores). Eulittoral assemblages and also the most common and abundant taxa (i.e. barnacles, *Melarhaphe neritoides*, *Patella* spp.) were studied in impacted (near an outfall) and reference areas in both bioregion, in order to assess the consistency of patterns. The second task aimed to examine the response of eulittoral assemblages to the presence of sewage pollution in insular ecosystems (Azorean archipelago). Moreover, in our sampling area, an outfall has been decommissioned from the original beach (due to civil movements to save a surf spot) and relocated in another area. This has provided an ecological opportunity to assess also the recovery dynamics of the community one year after the cessation of the sewage disposal.

4.2 Materials and Methods

4.2.1 Study areas

4.2.1.1 Atlantic and Mediterranean shores

The study areas were located on the central Western Portuguese shore (E Atlantic) and on the SE Italian shore (Mediterranean Sea) (Fig. 4.1). In the Mediterranean Sea the mean temperature of surface waters varies between 14-16 ºC in winter and 22-28 ºC in summer.

Tidal amplitudes are small (max 50 cm). Evaporation greatly exceeds precipitation and river runoff, so the Mediterranean Sea is characterised by very high (about 38‰) salinity (Barale, 2008). The impacted area was characterized by the presence of an outfall. The outfall on the Mediterranean shore releases secondary treated effluent, and serves a human population of 30,000 inhabitants.

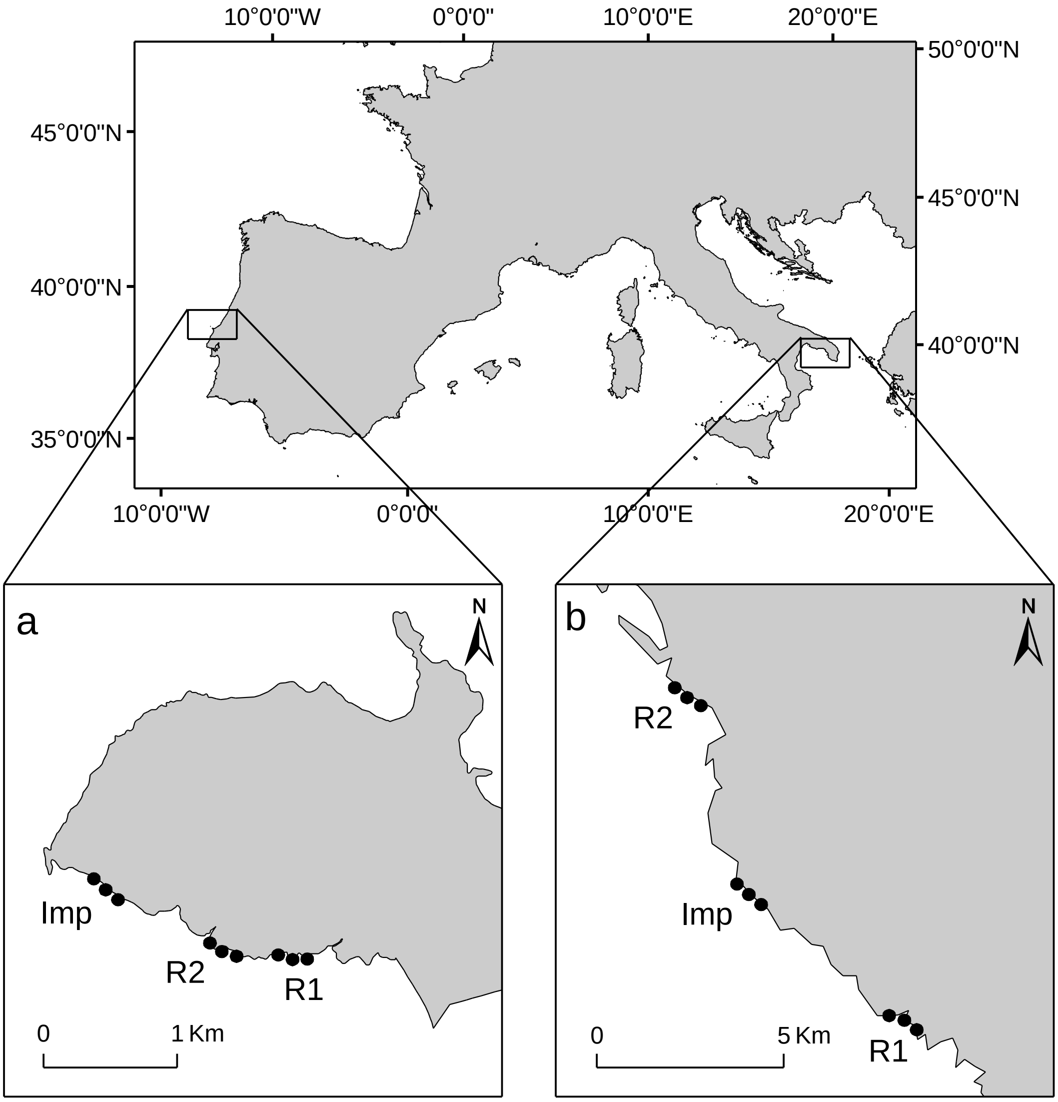


Figure 4.1 Study areas in Portugal (a) and Italy (b). Imp = impact, R1, R2 = reference areas. Three sites (●) were chosen in each area.

On the Atlantic shores the temperature of the sea surface shows marked seasonality, varying between 13 and 15 ºC during winter, and reaching 20-22 ºC during summer. The tidal regime in the Portuguese shores is semidiurnal. The extreme tidal range of spring tides is approximately of 3.5 to 4 m. Surface salinity is relatively constant, ranging between 35-36‰ (Boaventura et al., 2002a). The impacted area was characterized by the presence of an outfall. The outfall on the Portuguese shore releases secondary treated effluent, and serves a human population of 40,000.

4.2.1.2 Atlantic island shores

The sampling was undertaken in Terceira Island in the Azorean archipelago. The Azorean Archipelago is situated between 37º to 40º N and 25º to 31º W, in the middle of the Atlantic Ocean. It comprises 9 volcanic islands, organized into 3 separate groups: eastern, central and western. The islands are surrounded by deep water (≈1000m) and the coastline topography is complex, with steep cliffs alternating with rocky shores (Wallenstein and Neto, 2006; Martins et al. 2008, 2010). Weather is typically variable, with considerable cloudiness and some rainfall occurring in every season. Mean annual air and water temperatures are 13 ºC and 16 ºC, respectively in winter, and 17 ºC and 19 ºC, in summer (Morton et al., 1998). Terceira (382 km^2) is the easternmost island of the central group and the third-largest of the archipelago (Fig. 4.2). Most of Terceira's coastline is boarder by cliffs of low to moderate elevation and often interrupted, especially along the eastern and southern coasts, by lower areas, more protected from waves (Morton et al., 1998). The shores can be very exposed to wave action, and tidal range never exceeds 2m (Wallenstein and Neto, 2006; Martins et al. 2008, 2010). The impacted area was characterized by the presence of an outfall in operation discharging effluents directly on the rocky shore in the intertidal zone. The outfall releases secondary treated effluent, and receives domestic and industrial discharges. The post-impacted area is characterized by a decommission outfall that has ceased operation in November 2011.

4.2.2 Sampling design

In all study areas (Atlantic shore, Mediterranean shore and Atlantic Island shore) the assemblages were sampled at the eulittoral, middle zone of the shore, daily covered and uncovered by tides, and dominated by barnacles and mussels. The upper limit is set by the presence of barnacles, while the lower limit is marked by the top of the filamentous red or brown algae (Lewis, 1964).

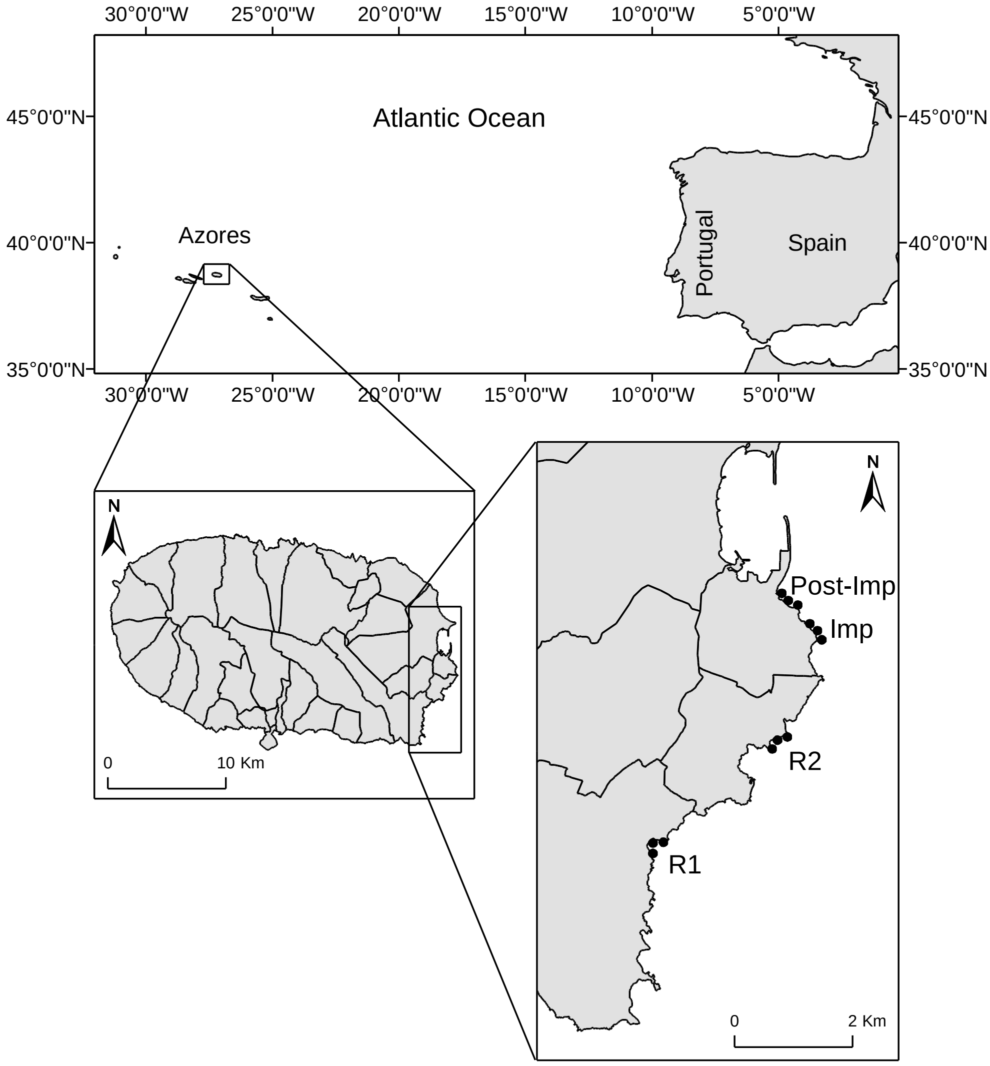


Figure 4.2 Location of the sampling areas. Imp = impact area (outfall in operation); Post-Imp=post-impacted area (decommissioned outfall); R1, R2 =reference areas. Three sites (●) were chosen in each area.

At the Atlantic and Mediterranean shores the sampling was undertaken on two random dates between June and September 2012, in three locations: the outfall (Imp) and two reference areas (R1 and R2) (Fig. 4.1). At the Atlantic Island the sampling was undertaken in April

2013 in four locations: one outfall in operation (Imp), one decommissioned outfall (Post-imp) and two reference areas (R1 and R2) (Fig. 4.2). All locations presented comparable environmental conditions (slope, orientation, wave exposure and type of substrate).
In all study areas (Atlantic shore, Mediterranean shore and Atlantic Island shore), three sites (approximately 100 to 300 m apart) were selected in each location, and at each site five randomly-located quadrats (20 × 20 cm) were sampled. The percent cover values of algae and the number of animals was estimated in the field, for each quadrat. The percent cover of organisms filling less than 1/4 square was set at an arbitrary value of 0.5 (Dethier et al., 1993). Final values were expressed as percentages (algae) and number of individuals (invertebrates).

4.2.3 Statistical analyses

4.2.3.1 Atlantic and Mediterranean shores

A distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was carried out separately for each region (Atlantic and Mediterranean shores) to test for differences in the invertebrate assemblage structure between impacted and reference conditions. PERMANOVA was also employed to test for differences in the structure of algal assemblages between impacted and reference conditions only for Mediterranean shores, as for Atlantic shores algal stands characterized almost exclusively the outfall location (see Results). The model consisted of three factors: Time (Ti, 2 levels, random), Location (Lo, 3 levels, random), Site (Si(Lo), 3 levels, nested in Lo, random). In both cases, the design was asymmetrical (Underwood 1994) due to the presence of a single impacted area. Therefore, the Location term, and all terms involving Location, was partitioned into two portions: the 1-degree-of-freedom contrast of Imp-v-Rs and the variability between reference areas (Rs). The same partitioning was performed for the Site term (Si(Imp) and Si (Rs)) and for the residual variability for observations within Imp (Res Imp) within Rs (Res Rs). Appropriate denominators for *F* ratios were identified from expected mean squares and tests were constructed following the logic of asymmetrical design (see Terlizzi et al., 2005b). All analyses were based on Bray–Curtis similarity on untransformed data, and each term in the analysis was tested by 4999 random permutations of appropriate units. To visualize multivariate patterns, non-metric multidimensional scaling (nMDS) ordinations were done on the basis of a Bray–Curtis similarity matrix. The analysis was performed using the computer programs DISTLM.exe and PERMANOVA.exe (Anderson 2004, 2005). A SIMPER analysis on Mediterranean algal assemblages was also conducted to identify taxa most contributing to the observed patterns. The analysis was performed using the PRIMER v 6 software package (Clarke and Gorley, 2006).

Asymmetrical analysis of variance (ANOVA) based in the same model described above, was carried out separately for each region (Atlantic and Mediterranean) to test for differences in the densities of barnacles, limpets (i.e. *Patella* spp.) and littorinids (i.e. *Melarhaphe neritoides*) between Imp-v-Rs. ANOVA was also employed to test for differences in algae cover between the Atlantic and the Mediterranean shores in each sampling date individually. The model consisted of three factors: Region (Re, 2 levels, fixed), Location (Lo(Re), 3 levels, nested in Re, random), Site (Si(Lo(Re)), 3 levels, nested in Lo, random). Asymmetrical ANOVA was carried out for both regions to test for differences in total algal cover between Imp-v-Rs, using the same experimental design described above for single taxa. Prior to analyses, the assumption of homogeneity of variances was checked using Cochran's *C*-test and data were appropriately transformed, whenever required. The analyses were carried out using the computer program GMAV 5 (University of Sydney, Australia).

4.2.3.2 Atlantic island shores

A distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was carried out separately for each outfall (outfall in operation and decommissioned outfall) to test for differences in the invertebrate assemblage structure between outfall and reference conditions. The model consisted of two factors: Location (1 impacted and 2 reference areas, fixed, orthogonal) and site (3 levels, nested in location, random). In both cases, the design was asymmetrical (Underwood, 1994) due to the presence of a single impacted area, as described above. All analyses were based on Bray–Curtis similarity on squared-root transformed data, and each term in the analysis was tested by 4999 random permutations of appropriate units. To visualize multivariate patterns, differences in community structure among treatment levels were visualized by principal coordinate (PCO) analyses on the basis of Bray-Curtis similarities.

Asymmetrical analysis of variance (ANOVA) based in the same model described above, was carried out to test for differences in the densities of barnacles (*C. stellatus*), limpets (*P. candei*) and littorinids (*T. striatus* and *M. neritoides*) between Imp-v-Rs. Prior to analyses, the assumption of homogeneity of variances was checked using Cochran's *C*-test and data were appropriately transformed, whenever required. All calculations were performed using the PRIMER v 6 software package (Clarke and Gorley, 2006).

4.3 Results

4.3.1 Atlantic and Mediterranean shores

PERMANOVA did not detect any significant differences in the multivariate structure of eulittoral invertebrate assemblages between impact and reference areas for both the Atlantic and the Mediterranean shores (Tables 4.1 and 4.2).

Table 4.1 PERMANOVA results on Atlantic shore assemblages. Significant results are given in bold and *P*-values in italic are from Monte Carlo asymptotic distribution.

Source of variability	df	SS	MS	Pseudo-F	P(perm)
Time=Ti	1	9727.0	9727.0	4.022	***0.047***
Location=Lo	2	3785.2	1892.6	0.762	0.711
Imp-v-Rs	1	1637.9	1637.9	0.491	*0.743*
Rs	1	2147.3	2147.3	0.799	0.612
Site(Lo)=Si(Lo)	6	31725.0	5287.4	1.330	0.282
Si(Imp)	2	6779.6	3389.8	1.930	0.269
Si(Rs)	4	24945.0	6236.2	1.226	0.373
Ti×Lo	2	4836.5	2418.2	0.608	0.704
Ti×Imp-v-Rs	1	2019.0	2019.0	0.605	0.665
Ti×Rs	1	2817.5	2817.5	0.554	0.592
Ti×Si(Lo)	6	23862.0	3977.0	2.822	**0.000**
Ti×Si(Imp)	2	3513.7	1756.9	1.031	0.395
Ti×Si(Rs)	4	20348.0	5087.1	4.032	**0.000**
Res	72	101460.0	1409.2		
Res Imp	24	40892.0	1703.8		
Res Rs	48	60568.0	1261.8		

Table 4.2 PERMANOVA results on Mediterranean shore assemblages. Significant results are given in bold and *P*-values in italic are from Monte Carlo distribution.

Source of variability	df	SS	MS	Pseudo-F	P(perm)
Time=Ti	1	9406.8	9406.8	0.962	*0.482*
Location=Lo	2	74098.0	37049.0	3.730	**0.022**
Imp-v-Rs	1	15497.0	15497.0	2.163	*0.115*
Rs	1	58601.0	58601.0	3.164	0.137
Site(Lo)=Si(Lo)	6	10651.0	1775.2	0.294	0.991
Si(Imp)	2	5266.7	2633.3	0.186	0.932
Si(Rs)	4	5384.3	1346.1	0.677	0.825
Ti×Lo	2	19549.0	9774.7	1.620	0.199
Ti×Imp-v-Rs	1	1745.0	1745.0	0.621	*0.557*
Ti×Rs	1	17804.0	17804.0	8.960	**0.017**
Ti×Si(Lo)	6	36212.0	6035.3	5.152	**0.000**
Ti×Si(Imp)	2	28263.0	14132.0	10.495	**0.000**
Ti×Si(Rs)	4	7948.7	1987.2	1.833	**0.023**
Res	72	84346.0	1171.5		
Res Imp	24	32317.0	1346.6		
Res Rs	48	52029.0	1083.9		

In both cases, analyses showed a significant Ti×Si(Lo) interaction, highlighting a high significant spatial and temporal variability at the scale of sites. Assemblages from Mediterranean shores also showed a significant variability among locations (Table 4.2). Such patterns were clearly illustrated in the corresponding nMDS plots (Fig. 4.3).

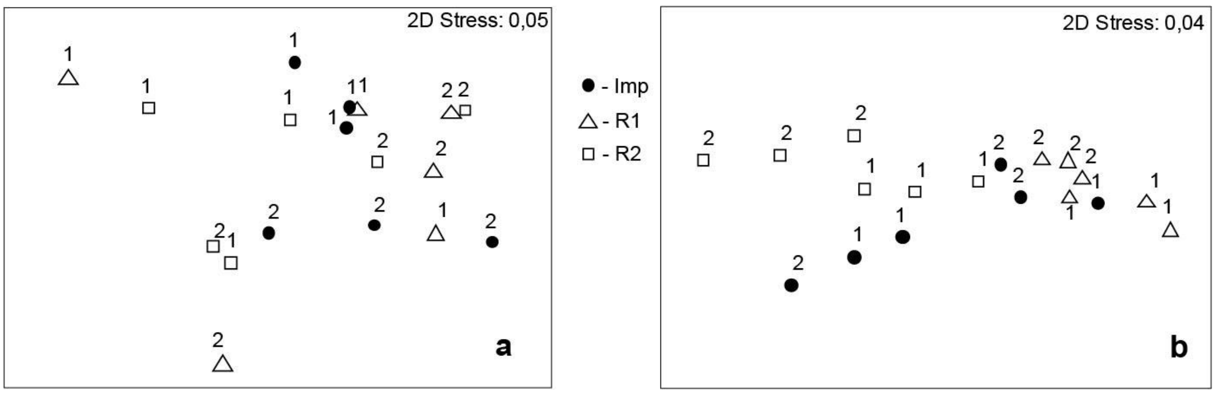


Figure 4.3 Non-metric multidimensional scaling ordination (nMDS) of site centroids for Atlantic (**a**) and Mediterranean (**b**) shores. Numbers indicate the time of sampling.

Figure 4.4 shows the densities of the dominant species at the eulittoral. As can be seen there was no differences between impacted and reference areas Rs for barnacles and *M. neritoides* neither for Atlantic nor for Mediterranean shores. On the other hand, for limpets *Patella* spp. there were differences in both shores. For the Atlantic, the mean abundance of *Patella* spp. was lower at the impact than in reference areas whilst the opposite pattern was found for the Mediterranean shore (Fig. 4.4).

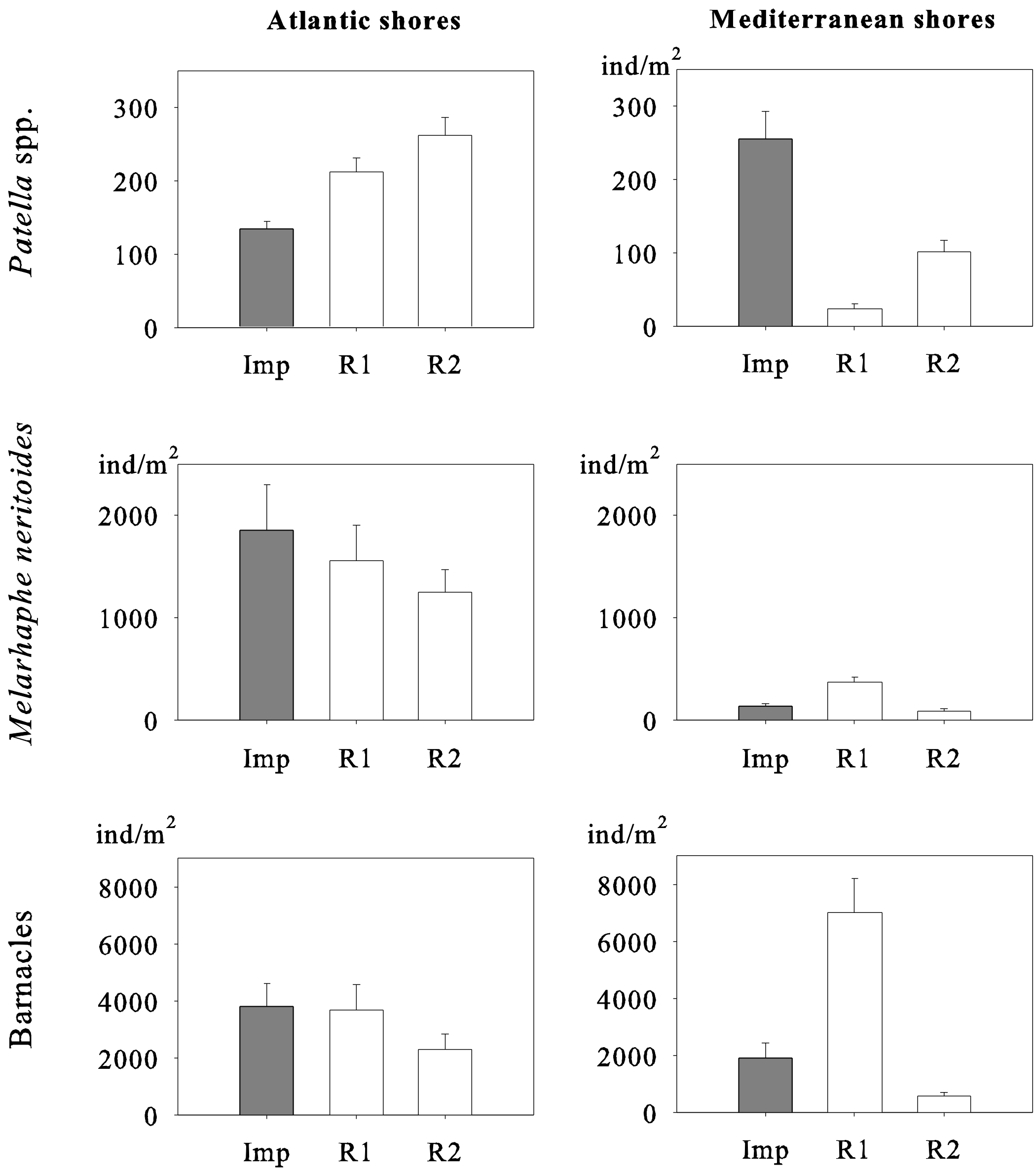


Figure 4.4 Density (ind/m^2±SE) of *Patella* spp., *Melarhaphe neritoides* and barnacles in Atlantic and Mediterranean shores. Imp – impact; R1, R2 – reference areas.

ANOVA on average abundance also revealed no significant differences in Imp-v-Rs for barnacles and *M. neritoides* neither for Atlantic nor for Mediterranean shores (Table 4.3). In contrast, *Patella* spp. showed a significant effect of sewage discharges that was consistent in time for both shores (Table 4.3).

Table 4.3 Asymmetric ANOVA testing for differences between impacted (Imp) and reference areas (Rs) (see text for further details). Significant results are given in bold.

		Barnacles		*M. neritoides*		*Patella* spp.	
	Source	*F*	*P*	*F*	*P*	*F*	*P*
Atlantic	Imp-v-Rs	0.276	0.605	0.192	0.660	11.052	**0.003**
	Ti×Imp-v-Rs	0.013	0.919	2.013	0.156	1.495	0.230
Mediterranean	Imp-v-Rs	0.890	0.508	2.558	0.114	5.865	**0.016**
	Ti×Imp-v-Rs	1.059	0.303	0.411	0.523	0.363	0.548

The analysis of graphs in Fig. 4.5 indicated the trends of algal cover from time 1 to time 2 in both shores. It can be observed a decrease in algal cover from time 1 to time 2 in both shores. Similarly, ANOVA on total algal cover showed a significant effect of region for time 2 but not for time 1, indicating high temporal variability of differences in eulittoral algal stands between Atlantic and Mediterranean shores (Table 4.4). Significant high spatial variability at the scale of sites and locations was also detected (Table 4.4). Differences between reference and impacted areas were only observed for time 1.

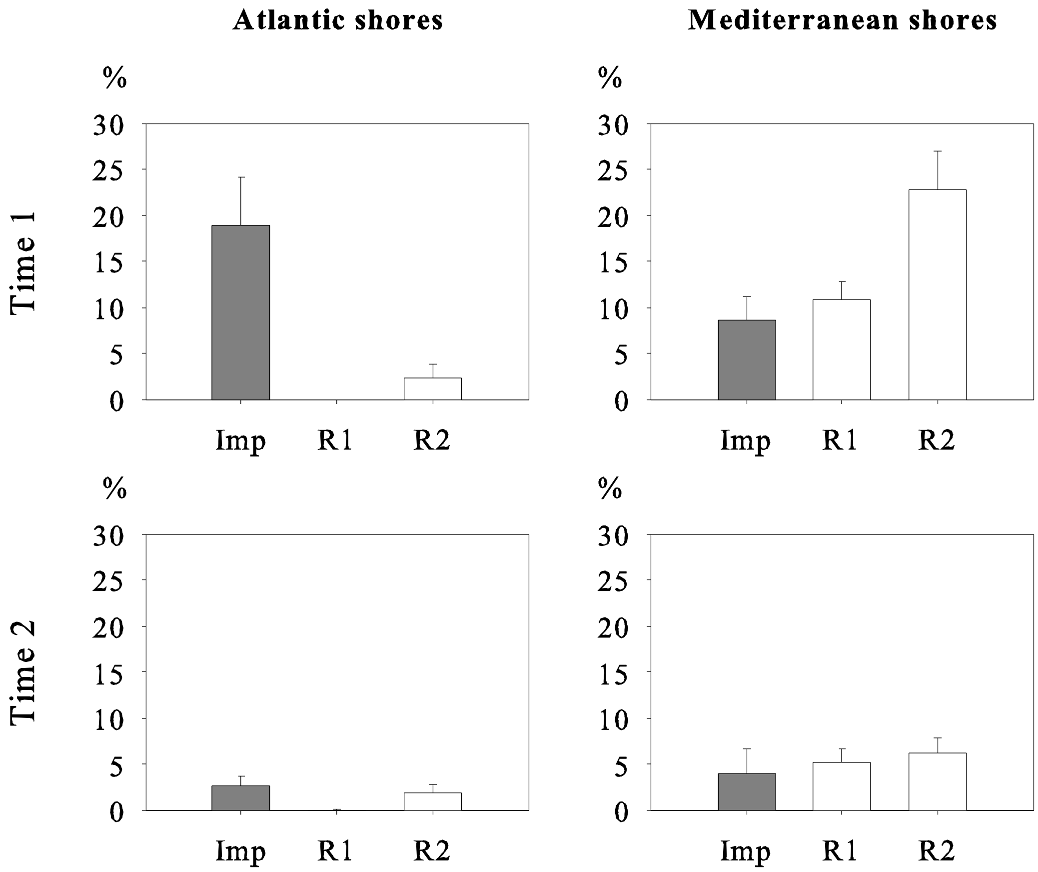


Figure 4.5 Total algal cover (%) in Atlantic and Mediterranean shores in both sampling dates. Imp – impact; R1, R2 – reference areas.

Table 4.4 ANOVA testing for differences in cover algae between regions: Atlantic and Mediterranean shores. Significant results are given in bold.

		Time 1			Time 2		
Source	df	MS	*F*	*P*	MS	*F*	*P*
Region=Re	1	42.860	1.68	0.265	19.810	9,590	**0.036**
Location(Re)=Lo(Re)	4	25.508	5.40	**0.010**	2.066	1,220	0,353
Site(Re(Lo))=Si(Re(Lo))	12	4.728	3.08	**0.002**	1.694	3,440	**0.001**
Res	72	1.533			0.493		

PERMANOVA on algal assemblages for Mediterranean shores highlighted a significant Ti×Imp-v-Rs interaction (F = 3.288, P = 0.03), indicating that structure of algal assemblages differed between Imp-v-Rs although not consistently in time. SIMPER analysis showed that such differences were mostly due to the fact that reference areas were characterized by encrusting and filamentous rhodophytes, whereas filamentous green algae were more abundant at the outfall location (Table 4.5).

Table 4.5 SIMPER analysis on Mediterranean algal assemblages.

		Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Time 1	Rhodophyta	2.60	10.27	37.35	1.23	43.78	43.78
	Phaeophyta	0.13	4.17	26.07	0.97	30.56	74.34
	Chlorophyta	3.67	1.12	16.18	0.69	18.96	93.30
Time 2	Rhodophyta	2.47	3.83	74.53	2.31	76.28	76.28
	Chlorophyta	0.63	0.10	21.74	0.67	22.25	98.53

For Atlantic shores, ANOVA on total algal cover showed a significant Ti×Imp-v-Rs interaction, indicating a significant effect of impact that varied in time (F = 66.502, P = 0.000). In time 1, algae were quite abundant at the impact but almost absent from reference areas, whereas in time 2 a strong reduction of algal cover at the impact area occurred (Fig. 4.5). For Mediterranean shores, ANOVA did not detect any significant differences between Imp-v-Rs (Ti×Imp-v-Rs: F = 0.348, P = 0.596; Imp-v-Rs: F = 3.383, P = 0.162), although algae were generally less abundant at the impact when compared to reference area (Fig. 4.5).

4.3.2 Atlantic island shores

4.3.2.1 Outfall in operation: effects on eulittoral assemblages

For both, outfall in operation and decommissioned outfall, the results of PERMANOVA analyses testing for spatial differences between impacted and reference areas were summarised in Table 4.6. At the assemblage level, the multivariate analyses showed significant differences only at location level, and even so there was no significant differences (although the borderline values) between the impacted area and the average of the reference areas.

Table 4.6 PERMANOVA testing for differences in eulittoral assemblage structure. Significant results are given in bold.

		Outfall in operation			Decommissioned outfall		
Source of variability	**df**	**MS**	***F***	***P***	**MS**	***F***	***P***
Location = Lo	2	3057.3	4.505	**0.027**	1260.3	1.560	0.192
Outfall-v-Rs	1	5620.4	11.628	0.053	2026.4	3.180	0.054
Rs	1	494.2	0.642	0.800	494.2	0.642	0.792
Site(Lo)	6	678.7	1.185	0.298	808.1	2.025	**0.015**
Si(Outfall)	2	497.8	0.599	0.720	885.9	2.856	**0.020**
Si(Rs)	4	769.2	1.734	0.078	769.2	1.734	0.083
Res	36	572.6			399.1		
Res Outfall	12	830.8			310.2		
Res Rs	24	443.5			443.5		

Table 4.7 Asymmetric ANOVA testing for differences between impacted (Imp) and reference areas (Rs). Significant results are given in bold.

		Patella candei			*Chthamalus stellatus*			*Tectarius striatus*			*Melarhaphe neritoides*		
Source of variability	**df**	**MS**	***F***	***P***	**MS**	***F***	***P***	**MS**	***F***	***P***	**MS**	***F***	***P***
Location = Lo	2	122.86	19.52	**0.0042**	209.65	2.09	0.2008	4.80	0.26	0.8132	97.34	3.68	0.0912
Imp-v-Rs	1	221.91	30.80	**0.0002**	403.51	5.98	**0.0148**	8.15	0.54	0.4782	182.99	5.60	**0.0248**
Rs	1	23.80	2.77	0.2060	15.79	0.14	0.6950	1.45	0.14	0.4782	11.69	0.34	0.7952
Site(Lo)	6	6.30	0.91	0.4974	100.46	1.58	0.1780	18.43	1.22	0.3116	26.47	0.77	0.5996
Si(Imp)	2	1.72	0.26	0.7320	68.49	0.84	0.4650	35.09	1.36	0.2816	10.47	0.27	0.7842
Si(Rs)	4	23.80	2.77	0.2060	116.45	2.14	0.1078	10.09	1.05	0.4018	34.47	1.09	0.3814
Res	36	6.89			63.47			15.05			34.29		
Res Imp	12	6.61			81.67			25.89			39.34		
Res Rs	24	7.04			54.36			9.63			31.77		

The structure of the benthic assemblages was represented in Fig. 4.6, showing a slight separation between assemblages from impacted and reference areas. Also, in the PCO plots (Fig. 4.6) was more evident the variability among sites than among sampling areas.

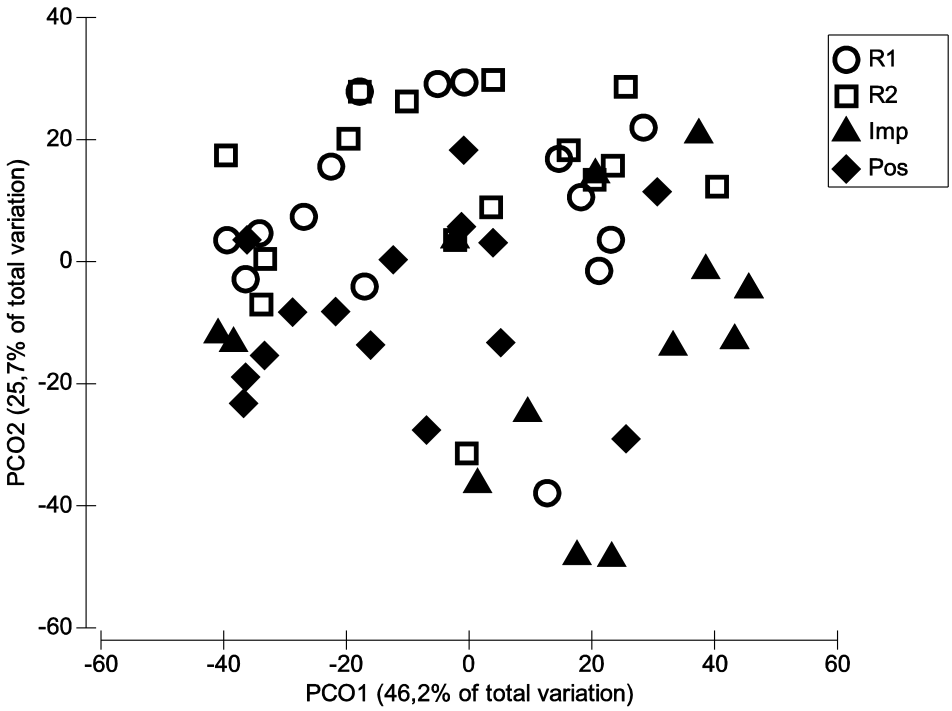


Figure 4.6 Principal Coordinates Ordination (PCO) plots at the outfall in operation (filled triangle), decommissioned outfall (filled diamond) and reference areas (empty symbols) on the basis of Bray-Curtis similarities of the square-root transformed data.

The same analyses were performed for the most dominant species: *Patella candei*, *Chthamalus stellatus*, *Tectarius striatus* and *Melarhaphe neritoides*. Results were reported in Fig. 4.7 and Table 4.7 Significant differences were found between reference and impacted areas for all species, except for *T. striatus* (Table 4.7).

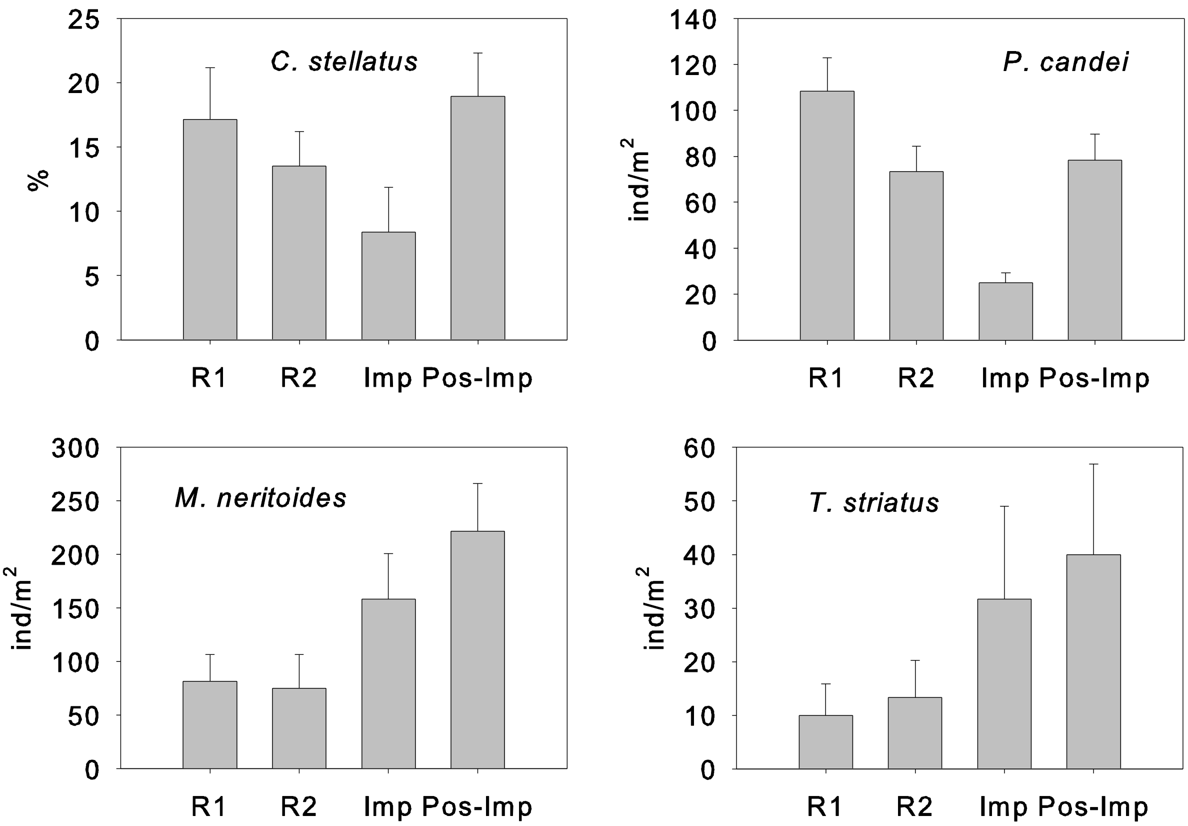


Figure 4.7 Cover (%) and density (ind/m^2±SE) of the dominant species in the eulittoral assemblages: *Chthamalus stellatus*, *Patella candei*, *Melarhaphe neritoides* and *Tectarius striatus*. Imp = impact area (outfall in operation); Post-Imp=post-impacted area (decommissioned outfall); R1, R2 =reference areas 1 and 2.

Both the cover of barnacles and the number of limpets were consistently lower in the impacted area (Fig. 4.7). On the contrary, the abundance of littorinids in the impacted area tended to be higher than in the reference areas (Fig. 4.7), even if there were only significant differences for *M. neritoides* (Table 4.7). Finally, regarding the limpet sizes it seemed that in the vicinity of the outfall the limpets were bigger than those found in the reference areas (Table 4.8). ANOVA on limpet sizes showed significant differences between impacted and reference areas (F = 22.08, P = 0.034), but not between reference areas (F = 0.19, P = 0.893).

Table 4.8 *Patella candei* sizes (cm) in the four sampling areas: Imp = impact area (outfall in operation); Post-Imp = post-impacted area (decommissioned outfall); R1, R2 = reference areas.

	Average	maximum	minimum
R1	18.8	29.5	12.4
R2	20.7	36.0	14.5
Imp	27.1	40.0	17.0
Post-imp	17.2	27.0	10.0

4.3.2.2 Decommissioned outfall: recovery of eulittoral assemblages

PERMANOVA did not detect any significant differences in the multivariate structure of eulittoral invertebrate assemblages between post-impacted and the average of the reference areas (Table 4.6). Analyses only showed a significant site-to-site variability in the post-impacted areas, highlighting the spatial variability within sites in that area (Table 4.6). Such patterns were clearly illustrated in the PCO plot (Fig. 4.6).

ANOVA on average species abundances has not revealed any significant differences between post-impacted and reference areas, except for *M. neritoides* (Table 4.9). Only for *T. striatus* significant variability at the scale of sites was noticed (Table 4.9) in the post-impacted area. The dominant species *C. stellatus* and *P. candei* seemed to recover after the cessation of the sewage disposal, increasing their densities to values similar to those from the reference areas (Fig. 4.7). On the other hand, for the littorinids the abundances remained higher even after the sewage discharges stopped (Fig. 4.7). Finally, it appeared that there was also a recovery in the limpet sizes in the post-impacted area since the values were similar to the ones registered in the reference areas (Table 4.8). Similarly, ANOVA did not detect any significant differences on limpet sizes between post-impacted and reference areas (F = 1.75, P = 0.254).

Table 4.9 Asymmetric ANOVA testing for differences between post-impacted (Post-imp) and reference areas (Rs) (see text for further details). Significant results are given in bold.

		Patella candei			*Chthamalus stellatus*			*Tectarius striatus*			*Melarhaphe neritoides*		
Source of variability	**df**	**MS**	***F***	***P***	**MS**	***F***	***P***	**MS**	***F***	***P***	**MS**	***F***	***P***
Location = Lo	2	13.87	1.94	0.2076	36.49	0.42	0.7016	24.47	0.40	0.8560	252.33	6.27	**0.0316**
Post-imp -v-Rs	1	3.94	0.53	0.4822	57.20	0.93	0.3402	47.48	3.10	0.0836	492.97	16.06	**0.0002**
Rs	1	23.80	2.77	0.2060	15.79	0.14	0.6950	1.45	0.14	0.4782	11.69	0.34	0.7952
Site(Lo)	6	7.16	1.02	0.4292	86.36	1.47	0.2272	60.96	7.53	**0.0002**	40.23	1.36	0.2634
Si(Post-imp)	2	4.31	0.62	0.5328	26.17	0.39	0.6690	162.71	32.45	**0.0008**	51.74	2.04	0.1642
Si(Rs)	4	23.80	2.77	0.2060	116.45	2.14	0.1078	10.09	1.05	0.4018	34.47	1.09	0.3814
Res	36	7.01			58.85			8.09			29.64		
Res Post-imp	12	6.94			67.83			5.01			25.40		
Res Rs	24	7.04			54.36			9.63			31.77		

4.4 Discussion

4.4.1 Atlantic and Mediterranean shores

The urgency to find efficient tools allowing to prevent the deterioration of coastal areas led to the search for indicators able to detect the effects of anthropogenic impacts on those habitats (Juanes et al., 2008).

The present results showed that, for both Atlantic and Mediterranean shores, the multivariate structure of eulittoral invertebrate assemblages did not seem to be affected by sewage discharges. Such findings are aligned with other studies that did not find any evident effect of sewage discharges in determining changes in assemblages, number and identity of species in the eulittoral zone (e.g. Archambault et al., 2001; Bustamante et al., 2012). Indeed, this intertidal zone is exposed daily to high level of natural disturbance caused by tidal regimes, desiccation, and wave action. Therefore, intertidal species might be better adapted to tolerate sharp environmental variations (Lüning et al., 1990), such as those potentially induced by sewage discharges (e.g. organic enrichment, freshwater inputs). However, previous research in the same areas of the present study, based on destructive sampling methods (chapter 2) have found differences in Atlantic eulittoral assemblages due to the presence of sewage discharges, with higher species richness and higher abundance of the dominant species. These results seem to suggest that an alternative explanation of the observed patterns might rely on the fact that visual estimates of invertebrate density, although effective for sessile and sedentary taxa, might not be adequate for motile and/or cryptic ones as well, thus leading to a restricted appreciation of assemblages response to environmental drivers.

The response of the dominant species were more relevant, especially *Patella* spp., whose abundances were significantly different between reference and impacted areas, both in Atlantic and Mediterranean shores. Atalah and Crowe (2012) have associated the presence of sewage discharges with changes in the abundance and structure of gastropod assemblages, highlighting the value of such assemblages as indicators of nutrient enrichment. Furthermore, previous studies on the effects of sewage pollution on limpets have noticed a reduction in the number of species near the outfall (e.g., Espinosa et al., 2007), suggesting that limpet species might be good indicators of nutrient enrichment associated to sewage effluents. Other studies, in contrast, reported a significant increase of limpets (*Patelloida lastritigata*) in intertidal areas impacted by sewage discharges with respect to unaffected areas (e.g. Bishop et al., 2002).

In this study populations of limpets of the genus *Patella* were demonstrated to be significantly affected by sewage discharges in both Atlantic and Mediterranean shores. However, responses varied across shores. In the Atlantic, limpet density was lower at the outfall than at reference areas, while the opposite pattern occurred in the Mediterranean. As limpets are herbivorous, it could be argued that such patterns might be due to difference in algal cover between the two regions and explained in terms of different food availability. Differences in algal cover between Atlantic and Mediterranean shores were, nevertheless, not consistent in time and, when present (i.e., time 1) they appeared to be very low. Moreover, average abundance of algae seemed comparable (time 2) or even higher (time 1) at the Atlantic than at the Mediterranean outfall, which is in contrast with the observed differences in limpet densities for Imp-v-Rs between the two regions. Interestingly, for both regions, algae seemed to show opposite patterns imputable to the outfall with respect to those exhibited by limpets, suggesting that differences in the algal cover were likely to be a consequence of differences in limpet density, and therefore of different grazing pressure, rather than causing them. However, algal assemblages were quite different comparing the outfall and reference areas in both shores. For instance, green filamentous algae were more abundant at the impact, while reference areas were mostly characterized by encrusting and filamentous red algae. This is a well-known outcome of sewage discharges in the intertidal zone that often leads to increased organic matter, which, in turn, triggers the proliferation of opportunistic green algae (Raffaelli and Hawkins, 1996). Several algal taxa that are likely to be facilitated by the presence of the outfall (such as cyanophyta, diatoms and opportunistic green filamentous Cladophorales) are the main dietary components of many limpet species (e.g. Della Santina et al., 1993). Therefore, a higher number of limpets at the impact areas would be expected as a consequence of the increased availability of food resources. This, nevertheless, does not exhaustively elucidate the opposite patterns of limpet densities at the outfall between Atlantic and Mediterranean shores.

A possible explanation of such contrasting patterns may rely on the fact that the species of limpets are different between the two investigated shores. Species belonging to the genus *Patella* reported for the central Portuguese shores are *P. depressa* and *P. ulyssiponensis* (Boaventura et al., 2002), with *P. depressa* being most common in the eulittoral zone (Silva et al., 2003). More species are present on Mediterranean shores: *P. rustica*, *P. ulyssiponensis*, *P. caerulea*, *P. ferruginea*, *P. nigra* (Giannuzzi-Savelli et al., 1994). *P. ulyssiponensis*, *P.*

rustica, and *P. caerulea*, in particular, are the most common species in the Mediterranean eulittoral zone (Della Santina et al., 1993; Simunovic, 1970). Such species might respond differently to sources of disturbance related to sewage discharges, such as nutrient enrichment or increased contaminants, showing different levels of tolerance. For instance, *P. caerulea* is more tolerant than the other species to environmental changes induced by sewage discharges (Espinosa et al., 2007), whereas *P. ulyssiponensis* may tolerate increased amount of pollutants (e.g., Bebianno et al., 2003). Therefore, discrepancies between the two shores concerning the effect of sewage discharges on patterns of limpet abundance might depend on the interplay between tolerance/sensitiveness and the response to resources availability of region-specific dominant species of the eulittoral zone.

4.4.2 Atlantic island shores

4.4.2.1 Outfall in operation: effects on eulittoral assemblages

The ecology of intertidal rocky shore communities in Atlantic islands has received far less attention than mainland coastlines, and to our best knowledge this study was the first attempt to understand the effect of sewage discharges on insular intertidal assemblages. Previous studies (Benedetti-Cecchi et al., 2003) have found differences between eulittoral assemblages from islands and the mainland, with higher spatial and temporal variance on islands, but with lower abundances of barnacles and limpets. Moreover, ecological processes such as patterns of dispersal or growth are probably different between islands and the mainland (Benedetti-Cecchi et al., 2003), giving rise to possible differences in the response of the assemblages to the sewage discharges.

In this study the sewage discharges seemed to have no significant effects on eulittoral invertebrate assemblages. This is in accordance with previous studies (e.g. Archambault et al., 2001; Bustamante et al., 2012) which have found no changes in the structure, abundance or species richness of eulittoral assemblages exposed to sewage discharges. These authors suggested that eulittoral assemblages were not affected since waves, tides and currents removed the effluents rapidly from the areas (Underwood and Chapman et al., 1996; Archambault et al., 2001). Furthermore, invertebrate assemblages from rocky shores are naturally highly variable and so it becomes more difficult to detect the effects of an impact on a multivariate analysis (Bustamante et al., 2012). However, other studies, based on destructive sampling methods (chapter 2) have found differences in eulittoral assemblages due to the presence of sewage discharges, with higher species richness and higher abundance of suspension-feeders. These results suggest that the observed patterns may be in part justified by the fact that visual estimates of invertebrate density could not be so sensible to detect more subtle effects of anthropogenic impacts.

The distribution pattern of the dominant species was modified by the presence of sewage discharges. There was a decrease in the cover of barnacles and in the number of limpets, and an increase in the abundance of littorinids. Previous studies have noticed that sewage discharges tended to beneficiate filter-feeders like barnacles (Hawkins and Jones, 1992). These higher densities are probably related with food availability, attending the higher amount of suspended solids near the sewage discharges. However, in this study, there was a decrease in the cover of barnacles near the sewage discharges. This may be due to the fact that the dominant species in Terceira coast is *Chthamalus stellatus*, known to be very sensitive to organic enrichment (Borja et al., 2000). Furthermore, it has already been observed that the recruitment of this species could be affected by physical disturbance (Martins et al., 2009), and perhaps the same occurs in the presence of sewage pollution. On the other hand, the higher amount of littorinids near the sewage discharges has already been noticed in previous works (Cabral-Oliveira et al., 2009). The higher concentration of nutrients near sewage areas will lead to a larger quantity of microalgae on the rocky surfaces, on which littorinids feed. This could be attractive to the settling of larvae, producing higher population densities. Finally, there were fewer limpets in the impacted area, but the individuals were bigger than those found in the reference areas. Previous studies on the effects of sewage pollution on limpets have suggested that patellidae species may be good indicators of nutrient enrichment related to sewage effluents (e.g. Bishop et al., 2002; Espinosa et al., 2007), and have associate this species to high-good status in coastal rocky assemblages (Díez et al., 2012). Nevertheless, other authors found that limpets may benefit from nutrient enrichment (Tablado et al., 1994; Rogers, 2003). These opposing responses could be due to differences between species, depending on within-taxon heterogeneities of ecological traits. The greater sizes and growth rates near the sewage discharges observed are also in agreement with previous studies (Tablado and López-Gappa, 2001). This could be explained with the higher availability of food, and also the higher sea water temperature observed near the sewage discharges, which may promote the growth of this species. The effects of sewage pollution on limpets will bring not only ecological but also socio-economic concerns, due to the commercial and recreational exploitation of *P. candei* in the Terceira Island. It could lead to human health concerns, due to the ingestion of contaminated limpets harvested in the vicinity of the outfall. Moreover, these traditional activities seemed to be a highly valued good to local population, and may have a strong local economic importance (Pinto et al., 2012). Therefore, a decreased abundance of this species may affect the socio-economic conditions of the local population.

4.4.2.2 Decommissioned outfall: recovery of eulittoral assemblages

The primary aim of the closure of the outfall was to prevent the effluent into beaches highly used by local surfers, in order to avoid human health issues. This objective has been succeeded, but it is also important to understand if the eulittoral assemblages had recovery. The multivariate analysis seemed to suggest a recovery of the system, and also the abundances of the barnacle *C. stellatus* and the limpet *P. candei* returned to values similar to the reference areas. In opposition, the behavior of the littorinids was not so expected, with the abundances remaining higher even after the sewage discharges ended. Previous studies on recovery of rocky shore assemblages after the cessation of sewage disposal have not reach similar conclusions. On the subtidal, some authors found neither evident effects of sewage discharges, nor recovery after cessation (Underwood and Chapman, 1996). Others registered changes in the structure of the assemblages, followed by a rapid recovery after the removal of the outfall (Roberts et al., 1998; Archambault et al., 2001). Birchenough and Frid (2009) described the effects after the cessation of sewage sludge in subtidal habitats, and have noticed two recovery phases. The first, immediately after-disposal phase, showed a localized increased in abundance and species richness. The second, after one year, where it was noticed that the system started to return to conditions similar to the ones observed in the reference areas. On the eulittoral, some studies found no changes in the assemblages due to the presence of sewage discharges (Archambault et al., 2001). Other studies examined the recovery of eulittoral assemblages after the improvement of sewage treatment (from primary to secondary level) and noticed a decrease in the cover of suspension feeders and an increase of the limpets *Patella ulyssiponensis*.

In the present study, at the assemblage level, there were no statistical differences between post-impacted and reference areas. However, it was observed a significant site-to-site variability in the post-impacted areas. Changes in small-scale variability have already been pointed out as a possible feature of environmental stress (Warwick and Clarke, 1993; Terlizzi et al., 2005b; Bustamante et al., 2012). This variability among sites may be due to differences in total cover or species richness, changes in a particular species, or changes in the taxonomic composition (Terlizzi et al., 2005b). In this case, they were probably related with *T. striatus* behavior, which also registered significant differences among sites in the post-impacted area. At species level, the recovery of limpets and barnacles was an important step to the recovery of the system. Barnacles are important species since provide shelter to several other species, while limpets are dominant grazers essential to balance the macroalgae growth (Little et al., 2010; Fish and Fish, 2011). Not so expected was the response of the littorinids after the cessation of sewage disposal. The recovery processes and the biological community composition are controlled by a variety of environmental influences (Birchebough and Frid, 2009). The response of the littorinids could be due to specific characteristics of the species (dispersal mechanisms, settlement, habitat preferences) or to differences in the ecological processes of this species in islands. Further research is necessary in order to fully understand the recovery processes of littorinids species.

Above all, the present study raises some questions about the potential of evaluations based on eulittoral assemblages to serve as a cost-effective assessment of sewage impact on rocky shores. Specific taxa, and especially limpets of the genus *Patella*, might be more effective in evaluating the environmental status of coastal systems in relation to main human impacts, such as sewage discharges, without the need of surveying the whole community, which would be more costly and time-consuming. However, the response of limpet assemblages seems to be different across the bioregions, depending on within-taxon heterogeneities of ecological traits, and thus deserving further research in order to ascertain their potential utilization as suitable environmental indicators over large spatial scales.

CHAPTER 5

General discussion

General discussion

5.1 Sewage disturbance on rocky coastal areas

Disturbance may be physical, chemical or biological, and can have natural or anthropogenic origin. It can change ecosystem processes and biodiversity patterns. The effects of disturbance can vary greatly, depending on the timescale (duration and frequency), spatial scale and intensity of disturbance, as well on the ability of the species present to respond to stress. The effects can range from total destruction, to the enhancement of local biodiversity. Sewage pollution is an anthropogenic chemical disturbance, due to the presence of substances/elements to which the community is not adapted (Magurran and McGill, 2010). Sewage is a mixture of organic matter, inorganic nutrients and microbes such as viruses and bacteria (Pinet, 2000). However, only in recent years the direct discharge of sewage into the sea has been questioned. Since then the sewage treatment methods have developed in order to prevent further deterioration of aquatic systems (Raffaelli and Hawkins, 1996)

5.1.1 Sewage effects on the abiotic environment

The potential adverse effects of sewage effluents are mainly due to eutrophication, toxicity, turbidity, sedimentation and decreases in salinity. The stress level from sewage discharges may vary considerably depending on the type and on the effluent treatment, as well as on the physical environmental conditions in which the effluent is discharged (Bustamante et al., 2012).

Sewage includes the waste-water produced by the community, which could have three different sources: domestic, industrial and rainwater (Seghezzo et al., 1998). The sewage treatment plants (STP) can involve three stages: primary, secondary and tertiary treatment. In the primary treatment the sewage is allowed to settle, and then the deposits (gross solids) and floating materials (e.g. oils, greases) are removed. The secondary treatment aims to degrade the biological content of the sewage. The majority of STP uses aerobic biological processes to eliminate the organic matter. The micro-organisms are then removed by sedimentation processes. Finally, in the tertiary treatment pathogenic organisms and nutrients are removed

(McKinney and Schoch, 1998). These different treatments gradually improve the effluent quality, and will be responsible by diverse impacts on the abiotic environment. Previous studies near untreated sewage discharges (Gappa et al, 1990; Elías et al., 2009) noticed an increase in temperature of the seawater, turbidity and total coliforms, and a decrease in pH, salinity and dissolved oxygen. Other authors when comparing secondary to primary treatment, found a decrease in suspended solids, nutrients and turbidity (De-la-Ossa-Carretero et al., 2011). To our best knowledge, there is no study comparing secondary and tertiary treatment effects on abiotic parameters in rocky shores. However, a decrease in nutrients and bacteria would be expectable.

In this study the environmental parameters suffer changes with the presence of sewage discharges, especially in salinity, oxygen and nutrients (NH_4 and PO_4). Although not statistically significant other parameters also change (seawater temperature, pH, total coliforms). This occurs because the discharges are not continuous, and due to the dilution of the waves and tides, the values of standard deviation are higher, which prevent the differences to be statistically significant. In reality, with secondary treatment some of these parameters should not be so affected by the presence of sewage discharges. However, this could be explained by the type of effluents received.

The flow rate and composition of the effluents which enter in the STP seem to vary significantly, depending on several factors, such as, economic aspects, social behavior, type and number of industries located in the collection area, climatic conditions or water consumption (Seghezzo et al., 1998). The Urban Waste-Water Treatment Directive (91/271/EEC), which aims to prevent the adverse effects of sewage on the environment, draws attention to the industrial waste waters. Industrial effluents should be subject to a pre-treatment also due to the presence of contaminants, such as PAHs or trace elements.

The major anthropogenic emissions of trace elements eventually end up in waste-water. If the STP are not prepared to remove these elements (generally by chemical precipitation processes), the trace element contamination of marine ecosystems may pose serious environmental and health risks (Baysal et al., 2013).

The analysis of trace elements is essential to assess the potential environmental and health risk associated with the sludge and sewage discharges coming from STP. Zinc, copper, nickel, cadmium, lead, mercury and chromium are the principal elements limiting sludge recycling to agricultural land (Álvarez et al., 2002). Also, sewage discharged to aquatic systems can pose health problems to the organisms and, finally, to humans. For example, seafood (such as the species studied in this thesis: *Mytilus galloprovincialis*, *Phorcus lineatus*, *Patella* spp) may represent risk for human health since they can accumulate trace elements from the aquatic environment and magnify them up the food chain (Maceda-Veiga et al., 2013). Finally, trace metal contamination may as well be very useful as an index of human exposure (Conti and Finoia, 2010).

In our sampling area, as already stated in chapter 2, the sewage discharges appear to be a source of arsenic (As) contamination, probably due to the industries present in the area (e.g. shypyards and metal smelting). According to the Agency for Toxic Substances and Disease Registry (ATSDR, 2011) the number one on the priority list of hazardous substances is As. The toxicity of As in organisms depend on its concentration and speciation, being inorganic arsenic species generally more toxic than organoarsenic species (Rahman et al., 2012). Although the information obtained from total arsenic determination is not enough to assess the toxicological risk in the environment, it can give an important contribute on contamination risks and help choosing potential good bioindicators. From all the studied species in this work, *Patella* spp., representative of the macrofaunal group and *Asparagopsis armata* representative of the macroalgae group seem to be the best bioindicators for trace element contamination.

5.1.2 Effects on the intertidal macroinvertebrate assemblages

5.1.2.1 What to measure

Contamination by the presence of unnatural chemicals is not synonymous with pollution, which implies the effects of, or responses to, such contamination (Bishop et al., 2002). The presence of sewage discharges is responsible for the modification of several environmental parameters (chapter 1 and 2), which will probably lead to changes in the biological communities. How can these effects on the biological communities be measured?

Sewage discharges can have severe effects on the structure and functioning of the marine ecosystems (chapter 2). In this section several biological metrics and their ability to detect the effects of disturbance will be discussed.

The impact of sewage pollution in marine habitats has been studied by assessing the response of the biological assemblages. One of the ways to measure responses from biological communities is by comparing species richness. However there is not a consistent response of diversity to sewage pollution: in some cases it might increase (López-Gappa et al., 1993), decrease (Littler and Murray, 1975; Archambault et al., 2001) or have no clear effect, as previously mentioned in chapter 2. In Peniche shores the species richness response to sewage discharges change along the tidal levels, increasing at the eulittoral, and decreasing at the sublittoral fringe. Changes in species richness seem to depend on the type and intensity of disturbance and on the original community of the study area (Whomersley et al., 2010). Moreover, species richness have the advantage of being simple to interpret and easy to calculate, but it could be insensitive to subtle changes (Magurran and McGill, 2010).

Other studies have analysed the effects of sewage pollution on the patterns of density and biomass of biological assemblages. However, there is not a homogeneous response either. In some cases the presence of sewage discharges increased the abundances (Littler and Murray, 1975), while in others decreased (López-Gappa et al., 1990, 1993; Archambault et al., 2001). In our study, the density and biomass increased near the sewage discharges both at eulittoral and sublittoral fringe.

The most consistent result was observed on the effects of sewage on the community composition and structure (Littler and Murray, 1975; Chapman et al., 1995; Fraschetti et al., 2006; among others), but the method requires taxonomic expertise and it might be difficult to compare studies due to different sampling designs. At the community level, changes in the structure of assemblages were observed, with the replacement of sensitive species by tolerant ones (Archambault et al., 2001; Bishop et al., 2002; Littler and Murray, 1975). Polychaete assemblages seem to increase their abundances and species richness near sewage discharges, due to the opportunistic species from families like Capitellidae or Spionidae (Dauer and Conner, 1980; Elías et al., 2006). Crustaceans and bivalves filter-feeders (e.g. Balanidae, Mytilidae) have been reported to be more abundant near sewage discharges due to the higher amount of suspended solids (López-Gappa et al., 1993; Pinedo et al., 2007). However, amphipods appear to be very sensitive to environmental changes due to the presence of sewage discharges (Dauvin and Ruellet, 2007). A general decrease in abundance and the disappearing of sensitive species near sewage discharges is also known for gastropod assemblages (Atalah and Crowe, 2012; Terlizzi et al., 2005a). In our sampling areas, at the eulittoral, the abundance of suspension feeders (*Chthamalus montagui*) increased near sewage discharges. This feeding type seems to be favoured by the presence of sewage discharges, and the natural competition between barnacles and limpets (*Patella* spp.) becomes unbalanced. Previous studies have pointed out the tolerance of *C. montagui* (Borja et al., 2000) and have associated *Patella* spp. with high-good status in rocky shores (Díez et al., 2012). At the sublittoral fringe the community structure changed: sensitive species seem to prefer the reference areas, while tolerant species dominate this fringe near the sewage discharges.

Finally, the incorporation of secondary production in the biological assessment brought more information about the ecosystem health. The importance of secondary production to assess the anthropogenic effects has already been recognized for freshwater systems and estuaries (Whiles and Wallace, 1995; Woodcock and Huryn, 2007; Faupel et al., 2012; Dolbeth et al., 2011, 2012), but not yet for rocky shore habitats. Secondary production is a measure of ecosystem functioning and includes life history characteristics such as body mass, recruitment, age, life span, taxonomy and trophic status, and population biomass and density (Cusson and Bourget, 2005). In Peniche shores, the secondary production confirmed that the presence of sewage discharges benefit suspension feeders (*Mytilus galloprovincialis* and *C. montagui*) and reduced the presence of sensitive species (e.g. *Rissoa parva*).

On balance, the most consistent response to sewage discharges seems to be the replacement of sensitive species by tolerant ones as already observed by several authors (Archambault et al., 2001; Atalah and Crowe, 2012; Bishop et al., 2002; Littler and Murray, 1975; Terlizzi et al., 2005a). Even though, some feeding types appear to be favoured by the organic and nutrient enrichment. Nevertheless, the tolerance of each individual species to the changes in the environmental parameters may be the ultimate justification for the changes in the biological assemblages.

5.1.2.2 How to measure: destructive *versus* non-destructive sampling

There are two types of sampling: destructive and non-destructive. In the destructive sampling, all the organisms are removed from the quadrat. The individuals can be counted, and dry-weighted to estimate biomass. This method has the advantage of being the most accurate way to quantify organisms, including the smaller species. But, it has also some disadvantages: it is time consuming, needs expertise knowledge and disturbs the habitat. In the non-destructive sampling, such as visual census, the survey is carried out in the field, by estimation of the percentage cover or number of individuals found in the quadrat. Non-destructive sampling has the disadvantage of underestimating species with smaller dimensions, and does not allow biomass estimations (Hawkins and Jones, 1992).

In our study area located in Peniche, the eulittoral was sampled by the two methods: destructive sampling and visual census. Table 5.1 shows the differences in species richness, density and biomass using destructive and non-destructive sampling. As can be seen the number of species detected in the destructive sampling is higher, especially in the impacted area.

Table 5.1 Comparison of number of species, density and biomass values of eulittoral assemblages using destructive sampling and visual census. R1, R2 – reference areas; Imp – impacted area.

	Destructive sampling	**Visual census**
Number of species	R1: 8 R2: 8 Imp: 23	R1: 6 R2: 6 Imp: 6
Density (ind/m^2±SE)	R1: 7823± 2482 R2: 8104± 1723 Imp: 62948± 8840	R1: 5475±1031 R2: 3822±528 Imp:5811±1128
Biomass (g/m^2±SE)	R1: 68,7± 14,9 R2: 90,3± 19,9 Imp: 148± 28,7	—

Also, the densities have much higher values, and the discrimination between impacted and reference areas is higher using the destructive sampling. The species that are not found at the eulittoral using visual census are small gastropods (*Littorina saxatilis*, *Skeneopsis planorbis*), bivalves (*Lasae adansoni*, *Modiolus modiolus*), crustaceans (*Campecopea hirsuta*, *Dynamene* spp., *Tanais dulongii*) and some rare polychaetes (*Perineris marionii* and *Eulalia viridis*). These species live in empty barnacle’s cases (Fish and Fish, 2011) and the estimation by visual census is difficult due to their small dimensions. Moreover, PERMANOVA only detected differences in the multivariate structure of eulittoral invertebrate assemblages when using destructive sampling. Therefore, it seems that destructive sampling is the most precise way of quantifying the community, and thus the most sensible method to detect changes in the biological patterns due to anthropogenic impacts. Nevertheless, the time spent to sort and identify all the specimens of all the samples, as well as the expertise knowledge necessary may not always be available.

How to choose the best sampling method? The decision will depend on several factors. What is the goal of the work? Are we interest in a rapid assessment or detail knowledge of the community? How much time and human resources do we have available?

For a detailed knowledge of the community responses it seems necessary to undertake a destructive sampling, with all species identified to the lowest taxonomic level. Similarly, to search for cost-effective indicators, a solid choice will be dependent of the amount of information available. After that first approach, in order to proceed to the monitoring of the area, the visual census may be the most appropriate method if, saving money and time, could be able to assess the ecological status of the biological communities. Therefore, beyond a detailed knowledge of the community, it is essential to find cost-effective indicators that allow an efficient management of the coastal areas.

5.1.2.3 Where to look: searching for a suitable indicator

Several methods or indices have already been developed to assess the ecological status of rocky shores being most of them based in macroalgae assemblages (Díez et al., 2012). Macroalgae communities usually respond with a decrease in the number of species and a reduction of stratification due to the disappearance of large perennial species, replaced by opportunistic ones (May, 1985, Soltan et al., 2001, Terlizzi et al., 2002). There are several quality indices using macroalgae communities, such as, the Ecological Evaluation Index (EEI) from Greece, the CARLIT from the Mediterranean, the Reduced Species List (RSL) or the Quality of Rocky Bottoms (CFR) from the Atlantic (Díez et al., 2012). The EEI method is based on the ecological status group (ESG) of macroalgae, and states that perennial species (ESG I) dominate in pristine habitats and opportunistic species (ESG II) prevail in impacted areas (Orfanidis et al., 2001). The CARLIT methodology integrates both cartographic data and information about the value of communities as indicators of water quality (Ballesteros et al., 2007). From pristine to impacted areas the communities changed from *Cystoseira*-dominated, to *Corallina*-dominated (intermediated state), to *Ulva*-dominated (Pinedo et al., 2007). The RSL is based on several features of the community structure like species richness, ecological status groups and percentages of chlorophyta, rhodophyta and opportunistic algae (Wells et al., 2007). The CFR takes into account the cover and species richness, as well the presence of opportunistic species (Juanes et al., 2008). Few have attempt to use intertidal macrofauna (Hiscock et al., 2005), and the existent metric is based on species richness and number of sensitive and tolerant species. More recently, the simultaneous use of both flora and fauna lead to the creation of RICQI: Rocky Intertidal Community Quality Index (Díez et al., 2012) based on indicator species abundances, morphologically complex algae cover, species richness and faunal cover. The usefulness of these indices is undeniable, but their application is generally geographically restricted, due to the changes in the community composition. Therefore, it is necessary an index able to respond to this changes in natural variability, and still sensible to detect the anthropogenic disturbances.

In this thesis, our goal was not the construction of an index, but a detailed knowledge of the responses of intertidal macrofauna, and the comparison between several geographical areas, in order to search for a common pattern that could be used to assess the ecological status of the rocky shores. In order to achieve this goal eulittoral assemblages from Atlantic (island and mainland) and Mediterranean shores were compared, searching for similar responses of those communities to the presence of sewage discharges. The choice of eulittoral assemblages has already been explained in chapter 4. From all the intertidal levels it seems the best option. At the littoral fringe the effects of sewage discharges were not detected, and also in sheltered shores this fringe can be extremely reduced. At the sublittoral fringe the algae composition differs across bioregions and can only be sampled at spring tides. Even in Portugal, the north is dominated by large brown algae (such as *Laminaria* spp., *Saccorhiza polyschides*) while the centre and south regions are distinguished by the presence of red algae turf composed by *Corallina* spp., *Plocamium cartilagineum*, among others (Boaventura et al., 2002a). Moreover, the faunal species are smaller, and live within the algae turfs, which could lead to difficulties in the species quantification by visual census. The eulittoral is accessible to sample, and the species present have widespread distributions and are easy to identify at high taxonomic resolution, which makes these assemblages potential cost-effective indicators of anthropogenic impacts.

In this study, the effect of sewage discharges on the multivariate structure of eulittoral assemblages was only detected when the sampling was destructive. The differences between impacted and reference areas were not statically significant when the sampling was undertaken by visual census. The objective is to find a cost-effective indicator, so it appears that the eulittoral assemblage may not be the best option. On the other hand, the response of the dominant species seems more relevant in discriminating impacted and reference areas (Table 5.2).

Table 5.2 Asymmetric ANOVA testing for differences between impacted (Imp) and reference areas (Rs). Significant results are given in bold.

		Barnacles		*M. neritoides*		*Patella* spp.	
	Source	*F*	*P*	*F*	*P*	*F*	*P*
Atlantic (mainland)	Imp-v-Rs	0.276	0.605	0.192	0.660	11.052	**0.003**
	Ti×Imp-v-Rs	0.013	0.919	2.013	0.156	1.495	0.230
Mediterranean	Imp-v-Rs	0.890	0.508	2.558	0.114	5.865	**0.016**
	Ti×Imp-v-Rs	1.059	0.303	0.411	0.523	0.363	0.548
Atlantic Island	Imp-v-Rs	5.98	**0.0148**	5.60	**0.0248**	30.80	**0.0002**

Table 5.2 illustrates the responses of the dominant species, using visual census, in the three geographical areas. As can be seen limpets are the species that seem to consistently respond to the presence of sewage discharges. However, the response is not always the same (Fig. 5.1).

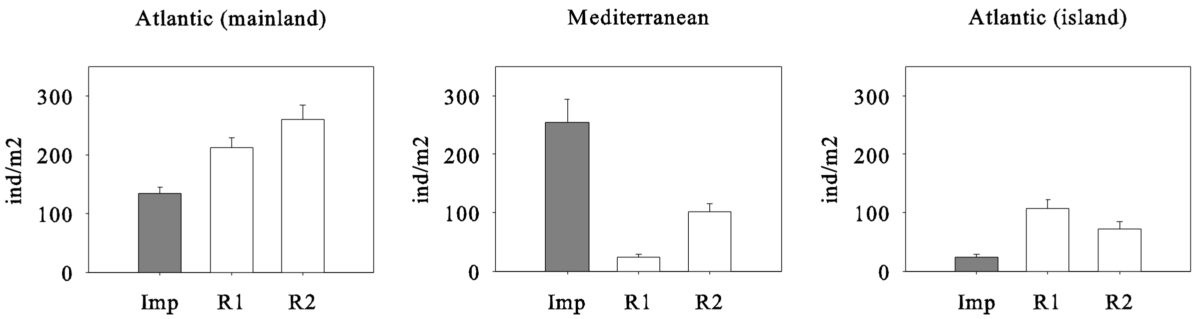


Figure 5.1 Density (ind/m^2±SE) of *Patella* spp. in Atlantic (mainland and island) and Mediterranean shores. Imp – impact; R1, R2 – reference areas.

The possible reasons for these different responses have already been discussed in chapter 4. The most probable reason is related to the fact that in the different regions was found distinct limpet species. In the Atlantic shores the dominant species were *Patella ulyssiponensis* and *P. aspera*. In the Atlantic island shores was only found *P. candei*. And, in the Mediterranean shores, the dominant species were *P. rustica* and *P. ulyssiponensis*. Such species may respond differently to sewage pollution. The tolerance or sensitiveness of each species could be the explanation for the patterns observed. Furthermore, and supporting the value of limpets as indicators of sewage pollution, is the potential of *Patella* spp. in detecting trace element contamination.
This thesis suggests that specific taxa, and especially limpets of the genus *Patella*, may be more effective in evaluating the environmental status of coastal systems in relation to main human impacts, such as sewage discharges, without the need of surveying the whole community which would be more costly and time-consuming. Nevertheless, further research will be necessary to ascertain their potential utilization as suitable environmental indicators over large spatial scales.

5.2 Final remarks and future perspectives

The urgency to find efficient indices and indicators to prevent further deterioration of coastal areas is one of the hot topics in today's scientific publication. However, a detailed knowledge of communities' response to anthropogenic impacts is essential to sustain those indices. This thesis contributes with important information to understand the response of intertidal macroinvertebrate assemblages to sewage pollution. The identification of all species to the lowest taxonomic level possible, and the sampling along all vertical levels, allowed a comprehensive knowledge of the effects of sewage discharges on macrofauna assemblages from rocky shores. Furthermore, a first attempt to evaluate and compare the responses of rocky shore macrofauna assemblages to sewage pollution in different geographical areas was carried out. Eulittoral assemblages from Atlantic and Mediterranean shores were studied, and the results seem to suggest that limpets of the genus *Patella* may be effective in evaluating the environmental status of coastal systems in relation to human impacts, such as sewage discharges.

The knowledge acquire with this thesis brings new questions that should be address in future research. It would be interesting to promote further studies on areas where the scientific debate is still open, such as “destructive vs. non-destructive sampling” or “indicator taxon vs. whole community” approaches. Furthermore, due to our results, it seems important to continue the effort to find efficient indices or indicators that will assist coastal area management. The potential utilization of limpets of the genus *Patella* as indicators of anthropogenic impacts should be followed. For that, the representativeness must be increase with further studies in different geographical and ecological scenarios. Moreover, it is essential to reach a scientific consensus and standardize methodologies and integrate activities both nationally and internationally. Another challenge is to bring the scientific knowledge to technicians and politicians, especially because marine biodiversity is not yet a priority to governments, and even for the general public.

Finally, in the Atlantic island shores, an ecological opportunity has raised to study the recovery potential of eulittoral assemblages. Although the reference conditions had not yet been reached after 17 months, a promising recovery of the assemblages near the decommissioned outfall was evident, especially of limpet and barnacle species. The resilience of these important habitats may be an incentive to the improvement of the sewage treatment plants. As a consequence, the priority should be to prevent further deterioration of marine habitats, in order to continue to benefit from all the products, services al well-being that coastal areas provide.

“The marine environment is a precious heritage that must be protected, preserved and, where practicable, restored with the ultimate aim of maintaining biodiversity and providing diverse and dynamic oceans and seas which are clean, healthy and productive.” (Directive 2008/56/EC)

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Appendix

Appendix A. Feeding guilds, life span and weight-to-energy conversion factors (from Brey 2001) for each species used for the secondary production estimates

Species	Feeding guild	Life span (yr)	Conversion factor
Barleeia unifasciata	herbivore	2	23.04
Melarhaphe neritoides	herbivore	4	23.04
Patella spp.	herbivore	5	23.04
Tricolia pullus	herbivore	1	23.04
Rissoa parva	herbivore	1	23.04
Runcina coronata	herbivore	1	23.99
Skeneopsis planorbis	herbivore	1	23.04
Lasae adansoni	suspension feeder	3	22.79
Modiolus modiolus	suspension feeder	5	22.79
Musculus costulatus	suspension feeder	3	22.79
Mytilus galloprovincialis	suspension feeder	8	23.26
Hyale perieri	herbivore	0.8	22.74
Hyale pontica	herbivore	0.8	22.74
Parajassa pelagica	suspension feeder	0.8	22.74
Chthamalus montagui	suspension feeder	2	22.74
Pirimela denticulata	omnivore	7	22.26
Idotea pelagica	omnivore	1.5	22.74
Campecopea hirsuta	herbivore	1.5	22.74
Campecopea lusitanica	herbivore	1.5	22.74
Dynamene spp.	herbivore	1.5	22.74
Ischyromene lacazei	herbivore	1.5	22.74
Tanais dulongii	omnivore	1.5	22.57
Capitellidae	deposit feeder	0.8	23.33
Lumbriconereis impatiens	carnivore	3	23.33
Perineris cultrifera	omnivore	3	23.33
Platynereis dumerilli	omnivore	2	23.33
Orbiniidae	deposit feeder	4	23.33
Pholoe inornata	omnivore	4	23.33
Eulalia viridis	carnivore	3	23.33
Sabellaria alveolata	suspension feeder	3	23.33
Syllis amica	carnivore	1.5	23.33
Syllis gerlachi	carnivore	1.5	23.33
Syllis prolifera	carnivore	1.5	23.33
Nematoda	omnivore	2	23.33
Nemertina	carnivore	2	23.33
Oligochaeta	deposit feeder/herbivore	2	23.33
Acanthochitona crinita	herbivore	3	23.33
Acanthochitona fascicularis	herbivore	3	23.33
Echinoidea	omnivore	5	20.53
Ophiuridea	omnivore	3	21.75
Larvae	omnivore	0.1	23.81