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Influence of urbanisation on an intertidal ecosystem engineer: *Chthamalus* barnacles and their associated epifauna

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INFLUENCE OF URBANISATION ON AN INTERTIDAL ECOSYSTEM ENGINEER: *CHTHAMALUS* BARNACLES AND THEIR ASSOCIATED EPIFAUNA

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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ABSTRACT

Acorn barnacles are conspicuous ecosystem engineers occurring in the intertidal of many rocky shores worldwide. They harbour diverse epifaunal assemblages among the crevices of their living or empty tests. Due to their sessile lifestyle, barnacles cannot escape from potential environmental and anthropogenic pressures affecting the intertidal. Urbanisation of the coastal areas is the main anthropogenic disturbance contributing to the deterioration of the marine ecosystems worldwide. Heavy metal contamination is often associated with urbanisation due to the effluents coming from industrial plants and other urban sources. In this study, the influence of urbanisation in the structural complexity of *Chthamalus* barnacles and their associated epifauna was studied. Moreover, the effects of urbanisation on the spatial patterns of epifaunal distribution were also considered. Two different conditions (urban and extra-urban) were considered, where two different shores along North Portugal were sampled for each condition. Heavy metal content in the soft tissues of barnacles was used as proxy of urbanisation and the number of full and empty tests were used as proxy of barnacles' structural complexity. Comparing each condition, the parameters of structural complexity and the epifaunal abundance, taxa richness and Shannon-Wiener index were analysed by means of univariate tests. Differences at the spatial scale for the patterns of epifaunal distribution were also analysed by means of univariate tests between conditions. The whole epifaunal assemblage and the most prominent taxonomical groups were compared between conditions using multivariate tests.

A higher content of heavy metals was found in the urban shores. The univariate analysis for the barnacles' structural complexity did not show significant differences between conditions. Along the study area, diverse invertebrate assemblages associated with *Chthamalus* barnacles were found, with a total of 16,283 individuals belonging to 28 different taxa. Uni- and multivariate analysis did not reveal any significant differences in the epifaunal assemblages associated with barnacles between urban and extra-urban shores. However, univariate tests revealed significant differences on the spatial variability of epifauna between conditions at the scale of site. The results of this study indicated that the barnacles' structural complexity and their epifaunal assemblages are not significantly shaped by urbanisation. However, urbanisation significantly affects the spatial variability of the epifauna associated with *Chthamalus* barnacles.

Keywords: urbanisation, ecosystem engineer, epifaunal assemblage, *Chthamalus*, spatial variability, North Portugal.

RESUMO

As cracas são “engenheiros de ecossistema” muito abundantes ao longo do intertidal rochoso de muitas praias em todo o mundo. Estes organismos albergam comunidades epifaunais diversas nas aberturas e fendas entre as suas carapaças vivas ou vazias. Por serem organismos sésseis, as cracas não se conseguem evadir de potenciais pressões ambientais e antropogénicas que incidem no intertidal. A urbanização das áreas costeiras é a principal perturbação antropogénica a contribuir para a deterioração dos ecossitemas marinhos. A contaminação por metais pesados é muitas vezes associada ao desenvolvimento urbano devido aos efluentes provenientes de indústrias e outras fontes urbanas. Neste trabalho foi estudada a influência da urbanização na complexidade estrutural de cracas do género *Chthamalus*, bem como a epifauna associada a estes organismos. Para além disso, também foram estudados os efeitos da urbanização nos padrões espaciais de distribuição da epifauna. Foram consideradas duas condições: urbana e extra-urbana. Para cada condição foram amostradas duas praias no Norte de Portugal. O conteúdo em metais pesados nos tecidos moles das cracas foi usado como dimensão da urbanização e o número de carapaças cheias e vazias foi usado como medida da complexidade estrutural das cracas. De forma a comparar cada condição, usaram-se testes univariados para analisar os parâmetros de complexidade estrutural e a abundância, riqueza taxonómica e o índice de Shannon-Wiener relativos às comunidades epifaunais. Também foram feitos testes univariados para analisar as diferenças nos padrões de distribuição epifaunais à escala espacial entre as diferentes condições. De seguida recorrerram-se a testes multivariados para comparar, entre condições, as comunidades epifaunais inteiras e os grupos taxonómicos mais abundantes.

Os resultados revelaram um maior conteúdo de metais pesados nas praias urbanas. As análises univariadas relativas à complexidade estrutural das cracas não revelaram diferenças significativas entre condições. Ao longo da área de estudo foram registadas comunidades epifaunais diversas associadas às cracas *Chthamalus* spp. Foram encontrados 16.283 indivíduos pertencentes a 28 taxa diferentes. As análises uni- e multivariadas não revelaram diferenças significativas nas comunidades epifaunais entre as praias urbanas e extra-urbanas. No entanto, os testes univariados revelaram diferenças significativas na variabilidade espacial da epifauna entre condições, à escala de sítio. Os resultados deste estudo denotaram que a complexidade estrutural das cracas e as suas comunidades epifaunais não são moldadas pela urbanização. No entanto, a urbanização afeta significativamente a variabilidade espacial da epifauna associada às cracas.

Palavras-chave: urbanização, engenheiro de ecosistema, comunidade epifaunal, *Chthamalus*, variabilidade espacial, Norte de Portugal.

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1. INTRODUCTION

1.1 URBANISATION OF THE COASTAL AREAS

Throughout the history, the greatest civilizations have established their foundations along coastlines. Nowadays, the coastal areas are still the centres for the concentration of population, resources, infrastructures and economic activities (e.g. Kasanko et al., 2006; Mahknovsky, 2014) because the marine environment facilitates many human activities (e.g. fishing, industry, tourism, transportation) (Barragán and de Andrés, 2015; de Andrés et al., 2017). This phenomenon has been coined as 'coastalisation' (Bell et al., 2013; Salvati, 2014; Salvati and Forino, 2014; Serra et al., 2014; Mikhaylov et al., 2018). However, the establishment of population in the urbanised coastal areas has important ecological impacts. The most common disturbances associated with urbanisation of the coastline are the habitat destruction, the introduction of alien species, pollution and the contamination by marine debris (Bertocci et al., 2017a; Doxa et al., 2017). These disturbances may lead to the decline, up to extinction, of native species, landscape modification and biotic homogenization (Bertocci et al., 2017a). Moreover, larvae supply, recruitment, adult and juvenile mortality of marine organisms may also be affected by the urbanisation (Dias et al., 2017). In this way, urbanisation is nowadays considered one of the strongest, most widespread and rising anthropogenic disturbances affecting the ecosystems worldwide (see Barragán and de Andrés, 2015; Doxa et al., 2017). As urbanisation increases pressure on ecosystems this has also an effect in human well-being (UNEP, 2006). The value of the ecosystem services provided by the coastal ecosystems is more than a third of the total for the whole world, even though coastal areas occupy a very small percentage of the planet's surface (Barbier et al., 2011; Underwood and Chapman, 2013; Barragán and de Andrés, 2015). Therefore, the ability of these ecosystems to sustain important services may become compromised by the anthropogenically driven disturbances and stressors that impact their biodiversity and functioning (Vinagre et al., 2017) such as urbanisation. Coastal organic and inorganic pollution caused by urban and industrial discharges is considered a main driver of the degradation of these ecosystems (de Andrés et al., 2017), leading to marine biodiversity losses and community shifts (Johnston and Roberts, 2009; Scherner et al., 2013). Heavy metals are strongly related with anthropogenic activities and prolonged exposure to some elements such as cadmium and chromium might be dangerous to animal and human health (Álvaro et al., 2016).

In addition to the anthropogenic disturbances, naturally driven processes and physical disturbances also shape coastal ecosystems. The effects of those factors can be prominently observed in intertidal rocky shores (Underwood and Chapman, 2013). In these habitats, the biological processes of recruitment, predation, grazing, and competition are among the most important, influencing or maintaining patterns of local biodiversity (Underwood and Chapman, 2013). The stress caused by physical factors such as desiccation and wave action also influences faunal abundance and diversity (Underwood and Chapman, 2013). However, some organisms can induce the amelioration of the biotic and abiotic stress by creating, maintaining and/or modifying habitats.

1.2 ECOSYSTEM ENGINEERS

Ecosystem engineers are organisms that physically modify, create or maintain habitats for other species (Jones et al., 1994, 1997). Jones et al. (1994) defined ecosystem engineers as “organisms that directly or indirectly modulate the availability of resources (other than themselves) to other species, by causing physical state changes in biotic or abiotic materials”. Jones et al. (1997) emphasized that the physical ecosystem engineering consists in the “physical modification, maintenance, or creation of habitats”. These authors established the nomenclature for the research on this ecological topic by giving a name to a concept already known. Ecosystem engineer species can either create habitats through their morphological features or through their behaviour (Coleman and Williams, 2002). Thus two kinds of engineers can be distinguished: *autogenic engineers* – species which living and/or dead tissues change the environment – and *allogenic engineers* – species whose activity lead to the transformation of biotic or abiotic features of an habitat (Jones et al., 1994). Ecosystem engineering does not involve a direct trophic interaction, so when an organism directly provides another species with a resource, in the form of living or dead tissue, it is not considered engineering. However, this ecological process always co-occurs with trophic interactions, which sometimes makes it difficult to separate ecosystem engineering from other ecological processes. One important characteristic of the engineering process is its action scale, both in space and time. Ecosystem engineers may have implications across local, landscape-scale and biogeographic gradients (Crain and Bertness, 2006). Jones et al. (1994) enumerated six factors to quantify the scale the impact of engineers: “1. life time per capita activity of individual organisms; 2. population density; 3. the spatial distribution, both locally and regionally, of the population; 4. the length of time the population has been present at a site; 5. the durability of constructs, artifacts and impacts in the absence of the original engineer; 6. the number and types of resource flows that are modulated by the constructs and artifacts, and the number of other species dependent upon these flows.” (Jones et al., 1994).

Ecosystem engineers have important consequences in their surrounding environment and in their associated communities (Harley, 2006). One of the most important processes of the ecosystem engineering is that it usually increases the habitat complexity by providing living space with different structural components and by generating quantitative changes in the amount of living space (Sueiro et al., 2011; Mendez et al., 2015). A greater complexity will provide more resources, habitats and niches (Connor and McCoy, 2001). Moreover, biogenic habitats provided by ecosystem engineers alleviate the abiotic and biotic stress affecting the intertidal organisms by providing cool, moist and hydrodynamically benign micro-habitats (Harley, 2006). In this way, ecosystem engineers usually increase the abundance and richness of species (Harley, 2006). However, the ecological importance of the ecosystem engineering process is more pronounced in physically stressful environments.

1.3 CONTROVERSY AROUND THE ECOSYSTEM ENGINEERING CONCEPT

The terms ecosystem engineer and ecosystem engineering proposed by Jones et al. (1994) arose from the necessity of grouping and classifying the common properties that habitat-forming organisms have in common. As stated by Jones et al. (1997) the concept behind these terms was poorly studied and developed until then as opposed to the studies of trophic interactions among species, interspecific competition, species diversity and ecosystem fluxes. In fact, these designations established new grounds for ecological research but also left room for some conceptual discussions.

Jones et al. (1994) listed a group of concepts related to ecosystem engineers. These authors compared their new terms with the concept of *keystone species*. These two concepts are not completely different from each other. Some ecosystem engineers can be considered keystone species but not every keystone species is capable of engineering. Keystone species can structure the communities in which they occur either by trophic effects or by engineering the physical structure of the ecosystem. Whenever there are direct trophic interactions between organisms the concept of ecosystem engineering cannot be applied, nonetheless the engineering activity of some species can lead to indirect effects in the food web. To distinguish these two concepts, Boogert et al. (2006) introduced the term of *key (ecosystem) engineers* to denote those keystone species that can exert their effects in their community by engineering processes. This term also came up to distinguish keystone species from important ecosystem engineers, since the latter is not merely a subset of the former as stated by Boogert et al. (2006). Important ecosystem engineers are not necessarily keystone species but their effects on the ecosystem functioning are very significant and can be quantified (e.g. Jones et al., 1994, 1997).

In a review by Reichman and Seabloom (2002a) on the engineering effects of pocket gophers (Geomyidae) the authors restricted the term “ecosystem engineer” to keystone species and ended up trivializing the concept assuming that all living organisms affect the physical environment in some way. These statements triggered off a discussion around this topic when a response was made by Wilby (2002). This author argued that the fact that all organisms affect their surrounding environment emphasizes the possible ubiquity of the ecosystem engineering phenomenon. However, the author recognized the fact that by focusing only in the ecosystem engineers rather than understanding the engineering processes the result can be an accumulation of “just-so stories” (Jones et al., 1994). To continue the discussion, Reichman and Seabloom (2002b) replied that the term “ecosystem engineering” was unfortunate because the concept of engineering implies a purposeful act. However, these authors assigned importance to the concept of ecosystem engineering rather than to the term itself. More recently, Jones et al. (2010) attempted to consolidate the concept and clarify the term “ecosystem engineering” after six years since they introduced it in the literature. These authors established an integrated framework in order to link four components of the engineering process: “1. An engineer causes structural change; 2. Structural change causes abiotic change; 3. Structural and abiotic change cause biotic change; 4. Structural, abiotic, and biotic change can feedback to the engineer.” (Jones et al., 2010).

Despite all the controversy and discussion around the term and concept of ecosystem engineering, the nomenclature have endured over the years (Wright and Jones, 2006). Furthermore, after the integration of ecosystem engineering in the framework provided by Jones et al. (2010) this ecological process gained more acceptance among the scientific community.

1.4 ANTHROPOGENIC DISTURBANCES AND THE EFFECTS ON THE SPATIAL PATTERNS

In many ecosystems, the community structure, the biodiversity and the functioning of the ecosystems are usually driven by ecosystem engineers. According to Thompson et al. (1996), biogenic habitats have higher abundance and diversity of many taxa and functional groups than nearby areas lacking these organisms. Therefore, disturbing these ecosystem engineers may disrupt the ecosystem where they live. The removal of an engineer species can result in a collapse of its engineering function. Nonetheless, an enhancement of an engineer's population beyond some threshold can dramatically modify important ecosystem attributes (Harley and O'Riley, 2011).

Patterns in faunal assemblages are shaped by physical (abiotic) and biological processes such as colonisation of different organisms from surrounding habitats, tidal height and wave exposure, different patterns of dispersal of organisms (Reimer, 1976a; Mrowicki et al., 2014; Bertocci et al., 2017b), effects of the topography of the substratum, grazing and patchy algal recruitment (Bertocci et al., 2011, 2017). These processes influence the recruitment, growth and mortality of organisms at a variety of spatial and temporal scales (Benedetti-Cecchi et al., 2000). The variability of the distribution is related with the variability of habitat quality or quantity. On topographically more complex habitats it is expected that the occurring species show greater variability on smaller scales than species occurring in more uniform habitats. In very heterogeneous and complex habitats habitat-related effects on the abundance of organisms vary more on smaller scales than in homogeneous habitats (Burrows et al., 2009). Small-scale variability depends on the irregularity of the coastline. A high variability is expected to occur in more indented and fragmented coasts made up of shores of varying soft and hard substrata (Burrows et al., 2009). According to Burrows et al. (2009), the analysis of spatial patterns is important to understand processes structuring biological communities. Understanding this natural variability of assemblages is important for implementing suitable monitoring programs and environmental impact studies (Li et al., 1997; Veiga et al., 2011, 2013). It is known that anthropogenic disturbances may alter spatial patterns of abundance, biodiversity and distribution of intertidal organisms (Bertocci et al., 2017b). Therefore, it is fundamental to quantify the assemblages' natural variability in order to identify relevant scales for investigating either natural processes or anthropogenic impacts (Veiga et al., 2013). Among coastal ecosystems, rocky intertidal shores usually constitute extremely diverse biotopes. These systems are configured by many ecosystem engineers, such as macroalgae (Rubal et al., 2018), mussels (Arribas et al., 2014), reef-building polychaetes (Dubois et al., 2006) and acorn barnacles (Barnes, 2000). There is a gap of knowledge about the effects triggered by human activities and their associated outcomes on barnacles' engineering traits. Therefore, studies dealing with the effects of urbanisation on aggregations of acorn barnacles would significantly contribute to fill this gap of knowledge.

1.5 ENGINEERING ROLE OF ACORN BARNACLES

Among the most conspicuous intertidal species that occur worldwide, acorn barnacles play important roles in shaping intertidal communities and the ecosystems they are settled in. Acorn barnacles are cirriped crustaceans in the suborder Balanomorpha (order Sessilia). All species of acorn barnacles live in marine habitats and most of the species are encountered in shallow waters, with 26% of the species inhabiting the intertidal zone, between high and low tides (Doyle et al., 1996). Acorn barnacles, as well as the other cirripedes, have complex life cycles in which they hatch as planktonic larvae and then go through a dramatic metamorphosis to become adults (Burrows et al., 1999). The last moults lead to the transformation of free-living stages to sessile suspension-feeder adults that attach to rocks or other available surfaces (Rainbow, 1984). The adults enclose themselves inside a calcareous shell (test) and feed of particles in suspension with the help of modified articulated legs called cirri (Rainbow, 1984; Riisgård and Larsen, 2010; Nishizaki and Carrington, 2014). The settled barnacles can constitute stands of solitary individuals or can establish stands of high densities, in which at very high densities they can form hummocks. According to Bertness et al. (1998) the hummocking conditions are a high-recruitment and a high-growth. The aggregation of individuals may reduce the effects of environmental stress in the barnacles (Bertness, 1989). Since the mature adults live fixed to the substrate, the reproduction between individuals is difficult. However, most of the species are hermaphroditic and each individual copulate with the surrounding individuals by extending its particularly long penis.

Acorn barnacles change their surrounding environment through their own structure and, therefore, they are considered to be autogenic ecosystem engineers (Dean, 1981; Barnes, 2000). As reviewed by this last author, either the living barnacles or their empty tests provide protection from stressful environmental conditions, serving as important refuges for a wide variety of organisms belonging to the microfauna (*e.g.* protozoans), meiofauna (*e.g.* small nematodes) and macrofauna (*e.g.* isopods, collembolans, small fishes). This large variety of organisms uses those microhabitats, such as the crevices among barnacles and the interior of empty barnacle tests, to settle and to avoid predation and desiccation (Reimer, 1976a; Raffaelli, 1978). These and other factors, including the alterations in light, temperature, wave action, sedimentation, and food availability, influence the abundance and distribution of the benthos associated with barnacles (McDougall, 1943; Branch, 1976; Creese, 1982; Underwood and McFadyen, 1983; Bros, 1987; Barnes, 2000; Harley, 2006; Sueiro et al., 2011). Barnacle matrices have a high topographical complexity (Thompson et al., 1996), with empty tests providing high-quality habitat and live barnacle cover providing medium-quality habitat (Harley and O'Riley, 2011). It is known that more complex and heterogeneous habitats support higher number of species by enhancing the habitat quality (Crooks, 2002; Veiga et al., 2014b; Mendez et al., 2015; Torres et al., 2015;).

In spite of the increasing knowledge about the ecosystem engineering process done by acorn barnacles, little has been done recently about the diversity of invertebrates that live among the crevices and tests of these engineers (see Harley, 2006; Sueiro et al., 2011; Mendez et al., 2015 for the most recent studies that explored the faunal assemblages associated with different species of acorn barnacles). Most of the data on this topic comes from the last decades of the twentieth century and this information was compiled by Barnes (2000). In its review, this author stated that organisms ranging from protozoans to small vertebrates could be found in association with barnacle shells. Inside the protozoan group, the ciliates (Ciliophora) were the most important group. The most relevant phyla of invertebrates could be enumerated as follows: Cnidaria, Platyhelminthes, Nemertea, Annelida, Sipuncula, Arthropoda and Mollusca. Furthermore, fishes in the group of blennies (order Perciformes, suborder Blennioidei) were also found. By supporting diverse epifaunal assemblages, the aggregations of species of acorn barnacles provide and maintain important ecosystem services to human populations (Leslie et al., 2005).

The living portion of the habitat-forming barnacles is not the important feature that contributes to the colonisation of faunal assemblages. It is the physical structure provided by barnacle shells that harbour these communities instead. This physical structure is also an important part in the interaction with other colonists, affecting their recruitment (Bros, 1987; Yakovis and Artemieva, 2017). In fact, the death of a barnacle does not imply major changes in the recruitment of other organisms since the walls of adult shells often remain intact for a period after the barnacle's death (Qian and Liu, 1990). However, this recruitment may be greatly influenced by the removal of the entire test (Bros, 1987).

1.6 *CHTHAMALUS* BARNACLES ACROSS THE WESTERN EUROPEAN COASTLINES

Barnacles are among the most abundant organisms in the intertidal zone of rocky shores around the world and they play key functions in their ecosystems as autogenic engineers, regardless of taxonomic differences (Crisp et al., 1981; Power et al., 2001; Martins et al., 2016). According to Range and Paula (2001), on the Atlantic coast of Europe there are five very common intertidal acorn barnacle species: *Semibalanus balanoides* (Linnaeus, 1767), *Austrominius modestus* (Darwin, 1854), *Chthamalus* *montagui* Southward, 1976, *Chthamalus stellatus* (Poli, 1791) and *Perforatus perforatus* (Bruguière, 1789). *C. stellatus* and *C. montagui* often occur together, creating conspicuous aggregations in the intertidal zone of European rocky shores. They have similar northern and eastern limits in their European distribution, occurring from N. Scotland to N. Africa, including the Mediterranean and Black Sea (Southward, 1976; Crisp et al., 1981). The distribution patterns at regional (100s km) and local (1-10s km) scales of the adult barnacles are influenced by abiotic and biotic factors (Rainbow, 1984; O'Riordan et al., 2010). Important abiotic factors include desiccation, temperature, winds and fetch (O'Riordan et al., 2010). Fetch, and thus wave exposure, is considered the main factor influencing the distribution of the two *Chthamalus* species, with *C. stellatus* favouring more wave exposed locations and *C. montagui* becoming more abundant in sheltered shores (Crisp et al., 1981). Barnacle reproduction, settlement and recruitment are the most important biotic factors determining the distribution patterns of these species (O'Riordan et al., 2010). However, these factors, together with competition (see Connell, 1961), predation and facilitation, exert a more significant influence at the microhabitat scales (Power et al., 2006; O'Riordan et al., 2010). Abiotic factors are also important in determining distribution patterns at small spatial scales. For instance, rock surface wetness has been studied as one of the parameters contributing to the distribution of *C. stellatus* and *C. montagui* on the intertidal habitats (Power et al., 2001).

As autogenic ecosystem engineers chthamalid barnacles harbour epifaunal assemblages among test crevices and in the interior of empty tests. However, little is known about the diversity of organisms compounding these assemblages. *Chthamalus* barnacles are widely distributed along the Iberian Peninsula coastline and there is no information about their associated faunal assemblages in the literature. Since these barnacle species are conspicuous and of great ecological importance (Martins et al., 2016), it is relevant to understand which species of organisms use the engineered space as habitat. Exploring the biodiversity harboured by *Chthamalus* barnacles is important to understand the potential ecosystem services provided by these engineers and to identify the effects of anthropogenic disturbances on the structure of these intertidal communities.

2. AIMS AND HYPOTHESES

The first aim of this study was to test the effects of urbanisation on the abundance and structural complexity (percentage of empty and full tests) of *Chthamalus* barnacles along North Portugal rocky shores. The first hypothesis was:

(i) If barnacles are differently exposed to contaminants in urban and extra-urban shores, then the structural complexity of barnacles would be significantly different between shores of different conditions because urbanisation tends to increase the mortality of barnacles.

The second aim of the present study was to test if the faunal assemblages associated with barnacles were significantly different between shores of different conditions. The second hypothesis was:

(ii) If barnacles and their associated fauna are differently exposed to contaminants in urban and extra-urban shores, then epifaunal assemblages would be significantly different between shores of different conditions because there are species more tolerant than others to contaminants.

At last, differences on the spatial patterns of variability of the epifauna associated with barnacles were tested between urban and extra-urban shores. The third hypothesis was:

(iii) The spatial patterns of distribution of the epifauna on barnacles might be different in urban and extra-urban shores because physical and biological factors alter the patterns of distribution of species.

3. MATERIAL AND METHODS

3.1 STUDY AREA

This study encompassed four rocky shores along the north of Portugal (**Fig. 1**). The shores were classified based on their condition of urbanisation: urban shores and extra-urban shores. The latter included Moledo (41°50'30.12"N; 8°52'27.44"W) and Vila Praia de Âncora (41°49'14.37"N; 8°52'26.41"W), both in the Viana do Castelo district and 2.3 km apart from each other. These two shores are located in small urbanised areas with low levels of anthropogenic pressures. The considered urban shores were Cabo do Mundo (41°13'0.23"N; 8°43'0.01"W) and Leça da Palmeira, (41°12'17.78"N; 8°43'0.63"W) both in the Porto district and 1.3 km apart from each other. They are located in heavily urbanised areas with the presence of industrial plants. Due to their location, they are subject to greater anthropogenic pressures such as trampling and effluents discharge. The extra-urban shores were located 70 km north from the urban shores.

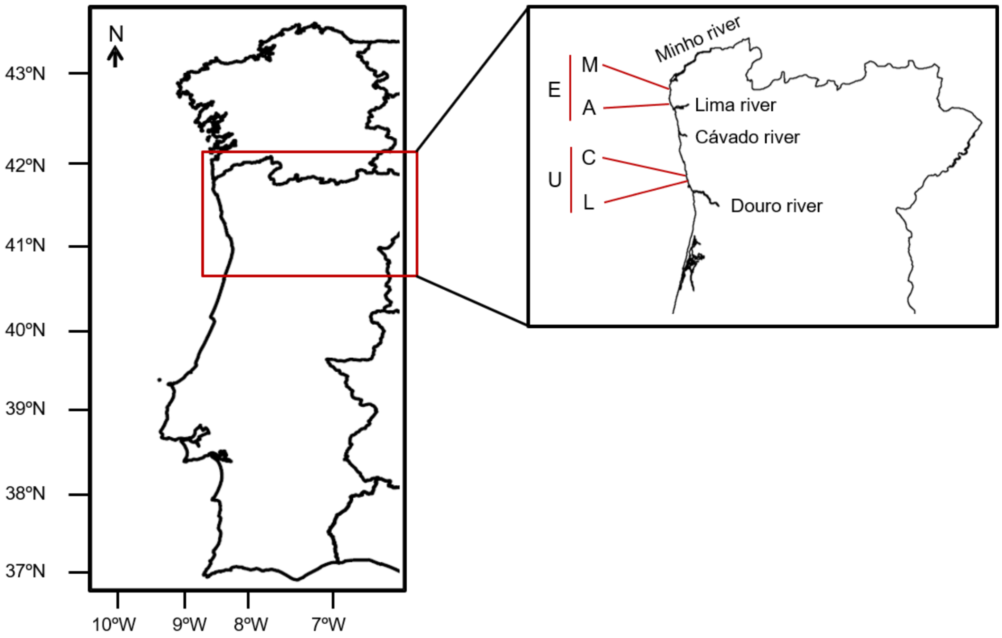

Fig. 1. Location of the four studied shores along the North Portugal coast: Moledo (M) and Vila Praia de Âncora (A) as the extra-urban shores (E) and Cabo do Mundo (C) and Leça da Palmeira (L) as the selected urban shores (U).

The environmental and physical features of the studied shores were comparable among them (Dias et al., 2002; Bertocci et al., 2017a). The wave regime along the study area is dominated by swells from the NW (73%) with those from the W contributing 16%. Most storms occur during winter (October-March) and on average the Portuguese coast is exposed to three storms a year. This area exhibits a semi-diurnal tidal regime in which the tidal wave propagates from south to north. The maximum tidal range is about 4 m and the most common tide is about 2 m (Dias et al., 2002). There are no significant geomorphological variations across the study area. The studied shores have a gentle slope and are mostly composed of a mixture of granite greywacke and schist (Rubal et al., 2013).

3.2 SAMPLING AND SAMPLE PROCESSING

Sampling was done in April 2017. Samples were collected in the high intertidal level of the four studied rocky shores during the low tide. At each shore, two different sites, separated by 10s of meters, were randomly chosen. In each site, six quadrats 10 x 10 cm of acorn barnacles were haphazardly placed on the exposed bedrock. The enclosed barnacles were photographed (see **Fig. S1**) and posteriorly removed with a scrapper. The removed barnacles were placed in flasks, properly labelled to study the associated fauna. Moreover, in each sampling site, one flask with barnacles was collected to analyse the metal content and assess the contamination levels of the studied shores that was used as proxy of urbanisation. The samples for the metals were stored in previously decontaminated flasks and transported in refrigerated containers to the laboratory within 8 h. The samples were stored in a freezer until posterior processing.

At the laboratory, the samples to study the fauna were washed in tap water to remove the associated epifauna. Water was then sieved to retain the macrofaunal invertebrates on a 500-µm mesh. The retained fauna was preserved in formalin (4%) with Rose Bengal for later sorting, counting and identification at the species level (whenever possible). The species nomenclature was confirmed at WoRMS, World Register of Marine Species database (http://www.marinespecies.org/) (accessed on October 2018). To analyse the metal content, the entire body (soft tissue) of *Chthamalus* individuals was dissected and separated from their shell plates (as in Reis et al., 2012). The concentration of the heavy metals chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and cadmium (Cd) were measured in an atomic absorption spectrometer (see Reis et al., 2009 for description of the method). Concerning the quadrat photographs, the number of full and empty *Chthamalus* barnacle (**Fig. 2**) tests were counted in each sample as a proxy of habitat complexity. Empty tests were considered more complex because these structures have more surface area that can be exploited by other organisms. Thus, it was assumed that the death of barnacles, and further emptying of their tests, increases the complexity of the habitat engineered by these organisms.

Fig. 2. Detail from a sampled quadrat of barnacles showing two empty tests among several full tests.

3.3 DATA ANALYSES

To compare the concentrations of heavy metals between urban and extra-urban shores bar graphs were plotted using the mean concentrations for each analysed metal.

Species accumulation curves (also known as collector's curves) were used to extrapolate species richness as function of the sampling effort. In order to estimate species richness from samples, non-parametric methods (Chao1 and Chao2) were considered. Chao1 is an estimator of the true number of species in an assemblage based on the number of rare species in the sample (Colwell and Coddington, 1994). This estimator involves calculating the number of 'singletons' (number of species represented by only a single individual in a sample) and the number of 'doubletons' (number of species represented by exactly two individuals in that sample). Chao2 method is based on species presence/absence and involves calculating the number of 'unique' species (number of species that occur in only one sample) and the number of species that occur in exactly two samples (Colwell and Coddington, 1994).

The abundance of full tests, the percentage of empty tests and the total abundance of tests were used as proxies for the structural complexity of barnacles. After processing the samples and the identification of the fauna, *analyses of variance* (ANOVA) were done to test for significant differences between shore conditions in complexity of barnacles, the abundance (N), diversity (Shannon-Wiener index, H') and taxa richness (S) of epifauna associated with barnacles. ANOVA analyses were based in a design with three factors: *condition*, fixed with 2 levels; *shore*, random and nested in *condition*, with 2 levels; *site*, random and nested in *condition* and *shore*, with 2 levels and 5 replicates.

Permutational analysis of variance (PERMANOVA) based on Bray-Curtis similarity matrix of no transformed abundance data was used to test for significant differences between shore conditions in the multivariate epifaunal assemblage structure (Anderson et al., 2008). Therefore, it was done for each major group (Mollusca, Arthropoda and Nematoda) the same analyses to understand the influence of each group on the statistical results. These tests were done considering the same statistical design described above for ANOVA. When the number of unique permutations was less than 30, Monte-Carlo *P*-values were considered (Anderson et al., 2008). Moreover, to visualise multivariate patterns in mollusc, arthropod and nematode assemblages between conditions, non-metric multidimensional scaling (nMDS) was used as an ordination method.

In order to understand if the structure of the community associated with barnacles had a significantly different spatial variability in shores with different conditions, ANOVA analyses were done considering two factors: *condition*, fixed with 2 levels; *scale*, fixed with 3 levels and 2 replicates. The factor condition encompassed the two studied conditions: urban and extra-urban. The factor scale included the scales of shore, site and replicate. SNK (Student-Newman-Keuls) post-hoc tests were considered whenever significant differences ($P < 0.05$) were found. Multivariate analyses were carried out using PRIMER v.6+PERMANOVA package. For univariate tests GMAV5 was used.

4. RESULTS

4.1 HEAVY METAL ANALYSES

Metal concentrations obtained in *Chthamalus* spp. were higher in urban than extra-urban shores, except for cadmium (**Fig. 3**). Considering the four studied shores the range for each metal was 0.24-0.77 mg Cr kg^{-1}, 4.03-5.98 mg Mn kg^{-1}, 161-231 mg Fe kg^{-1}, 1.5-2.9 mg Cu kg^{-1}, 159-202 mg Zn kg^{-1} and 0.55-1.65 mg Cd kg^{-1}. Consequently, the mean concentrations of the analysed metals followed the increasing distribution Cr < Cd < Cu < Mn < Zn < Fe. Comparing each shore, Cabo do Mundo showed the highest concentrations for chromium (0.77 mg kg^{-1}), manganese (5.98 mg kg^{-1}), iron (231 mg kg^{-1}) and copper (2.9 mg kg^{-1}). Leça da Palmeira showed the highest concentration for zinc (202 mg kg^{-1}) and Moledo showed the highest concentration for cadmium (1.65 mg kg^{-1}).

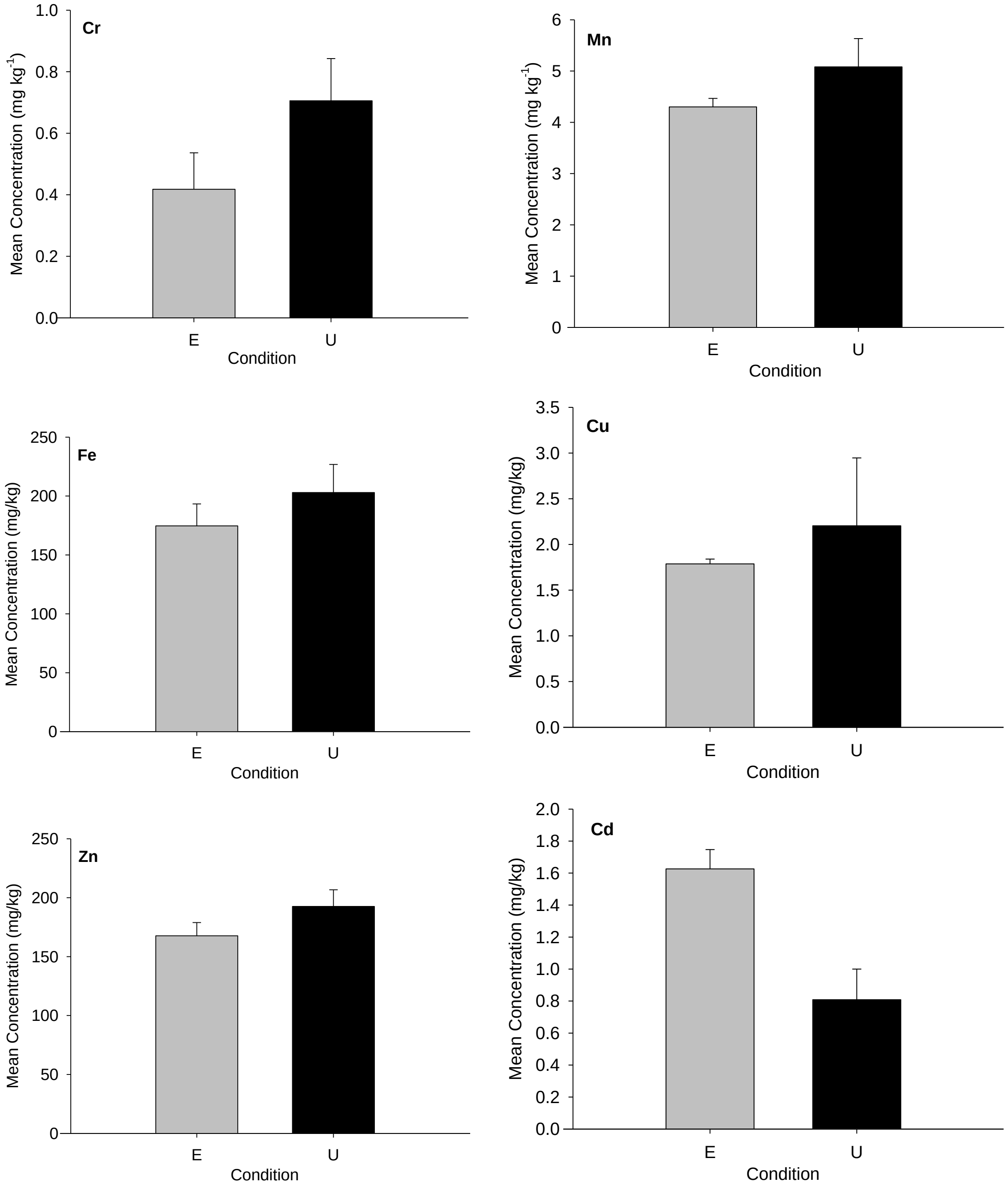

Fig. 3. Mean concentrations (mg/kg) (+ standard error) of heavy metals in the soft tissues of *Chthamalus* barnacles sampled from the extra-urban (E) and urban (U) shores.

4.2 BARNACLES’ STRUCTURAL COMPLEXITY

In every quadrat of barnacles, the number of empty tests was always considerably lower than the number of full tests, with the percentage of full tests above 90% of the total tests in every quadrat (**Fig. 4**). The urban shores showed a slightly bigger mean percentage of empty tests, with 3.74 % in Cabo do Mundo and 3.85 % in Leça da Palmeira. In Vila Praia de Âncora it was found a mean of 3.27 % and in Moledo 3.61 % of empty tests. Cabo do Mundo and Vila Praia de Âncora were the shores in which the total of tests (full and empty) was higher (**Fig. 5**). In Cabo do Mundo, a mean of 1214 tests were found and in Vila Praia de Âncora a mean of 1175 tests were found. The total number of tests counted in Moledo and Leça da Palmeira was only slightly lower, with a mean of 1103 tests found in Moledo and a mean of 1048 tests found in Leça da Palmeira (**Fig. 5**).

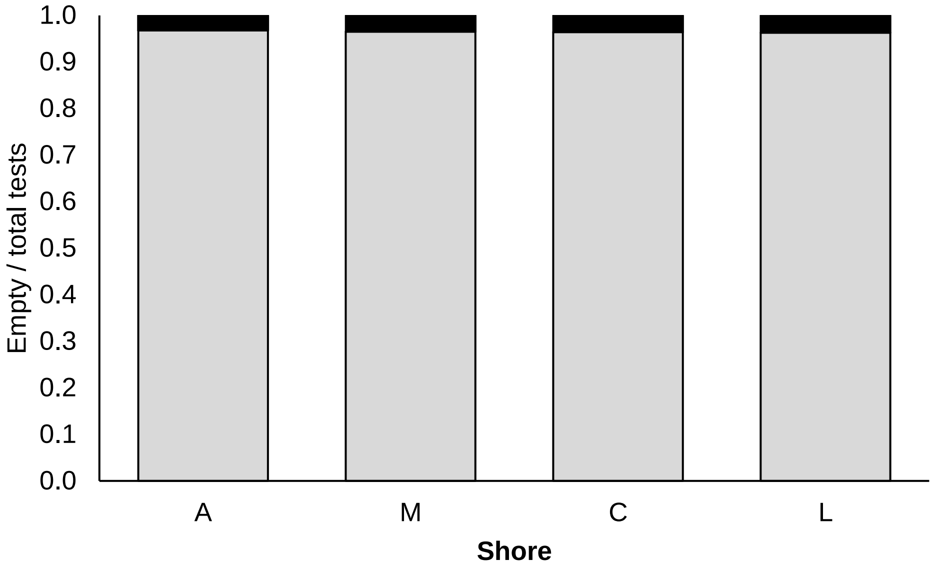

Fig. 4. Mean percentage of empty tests in relation to the mean abundance of barnacle tests in each shore. Grey bars: mean percentage of the full tests; Black bars: mean percentage of the empty tests; A: Vila Praia de Âncora; M: Moledo; C: Cabo do Mundo; L: Leça da Palmeira.

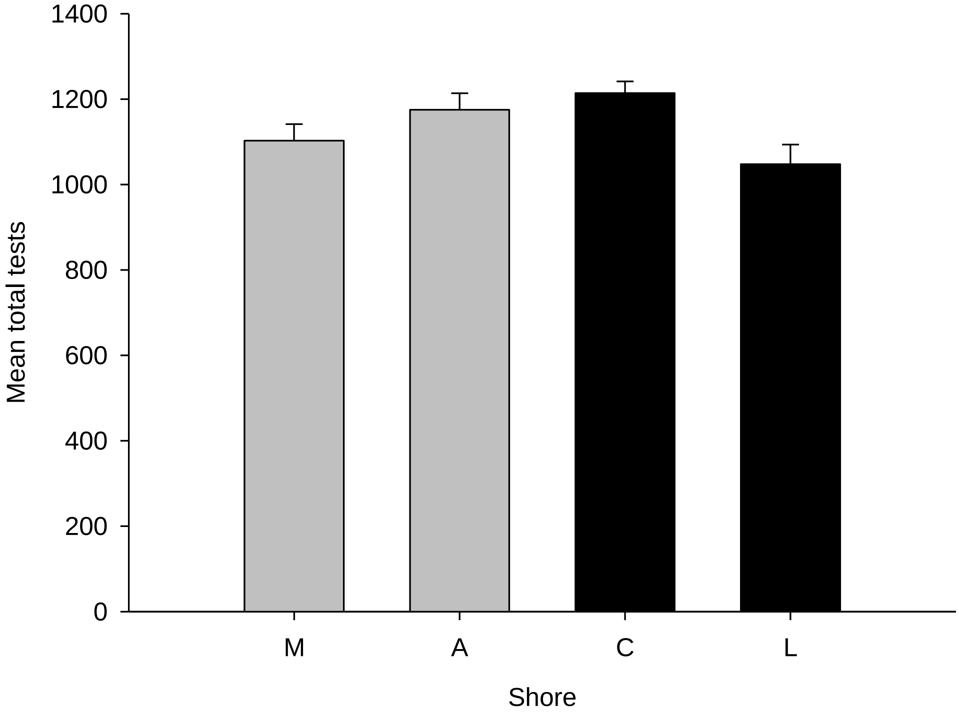

Fig. 5. Mean total number of tests (+ standard error) in each studied shore. Grey bars: extra-urban shores; Black bars: urban shores; M: Moledo; A: Vila Praia de Âncora; C: Cabo do Mundo; L: Leça da Palmeira.

ANOVA tests revealed that different measures of structural complexity of barnacles did not show significant differences between conditions nor shores. Among sites, significant variability was found for the number of full tests and total number of tests (*P* = 0.003, *P* = 0.030, respectively) (**Table 1**).

Table 1. Results of ANOVA tests for complexity of barnacles. Co: condition; Sh: shore; Si: site; *: P < 0.05.

Source of variation	**df**	**Full tests**		**% Empty tests**		**Total tests**	
		MS	*F*	MS	*F*	MS	*F*
Co	1	1322.500	0.02	1.285	3.81	640.000	0.01
Sh (Co)	2	78286.100	2.30	0.337	0.19	8216.500	2.27
Si (Co x Sh)	4	33968.900	3.17*	1.811	2.17	36160.050	3.06*
Residual	32	10718.475		0.833		11805.763	
Total	39						

4.3 EPIFAUNAL ASSEMBLAGES

A total of 16,283 individuals were sorted and assigned to 28 different taxa (**Table S1**). In urban shores, 8,413 individuals belonging to 18 taxa were found, against the 7,870 individuals belonging to 27 taxa found in extra-urban shores (see **Fig. 6** for some representative species). Thus, in urban shores more individuals were counted but in extra-urban shores more species were recorded.

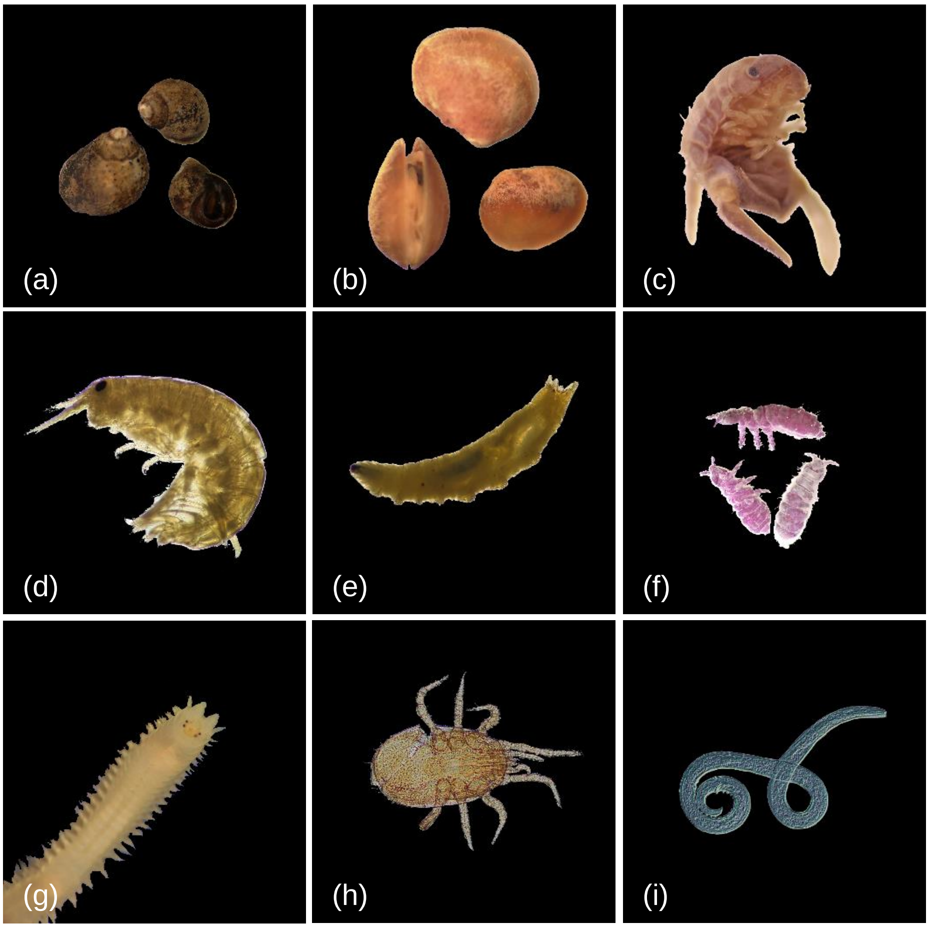

Fig. 6. Representative species of the several taxa found in association with *Chthamalus* barnacles: *Melarhaphe neritoides* (Linnaeus, 1758) (a), *Lasaea rubra* (Montagu, 1803) (b), *Campecopea hirsuta* (Montagu, 1804) (c), *Apohyale prevostii* (H. Milne Edwards, 1830) (d), Dolichopodidae sp. (e), Collembola sp. (f), Nereidae sp. (g), *Hydrogamasus littoralis* (Canestrini & Canestrini, 1881) (h), Ethmolaimidae sp. (i). Images (a) – (g) were captured under a stereo microscope (Zeiss Stemi 305) and images (h) and (i) were captured on an optical microscope (Leica DM2500 LED).

Mollusca was the most abundant phylum, with 12,816 individuals counted. *Melarhaphe neritoides* was the most abundant species, with 11,201 individuals sorted. Besides *M. neritoides*, two more species of gastropods were recorded: *Littorina saxatilis* (30 individuals) and *Patella* sp. (2 individuals). However, the abundance of these two species was residual in comparison with the abundance of the bivalves *Lasaea rubra* (1,235 individuals) and *Mytilus galloprovincialis* (348 individuals). Molluscs were slightly more abundant in extra urban shores, except for *L. saxatilis* and *Patella* sp, which were only recorded for extra-urban shores. The phylum Arthropoda constituted the second most abundant taxon, with 2,227 individuals counted. From the subphylum Crustacea, *Campecopea hirsuta* was the most abundant species (1,559 individuals). The other crustaceans were far less abundant and only recorded in extra-urban shores. From the subphylum Hexapoda, collembolans (Collembola sp.) were the most abundant organisms (410 individuals), followed by Chironomidae spp. larvae (157 individuals). With 991 individuals counted, Nematoda was the third most abundant taxon being also the most diverse, with 7 taxa recorded. The abundance of nematodes in urban shores (315 individuals) was about half the abundance of nematodes in extra-urban shores (676 individuals). This difference was also observed for mites (Acari), with 21 individuals in urban shores and 48 individuals in extra-urban shores. Mites were also a diverse group among the epifaunal assemblages, with 4 taxa in urban and extra-urban shores.

The total number of taxa estimated by Chao1 (30 ± 3) and Chao2 (44 ± 17) were higher than those observed (**Fig. 7**).

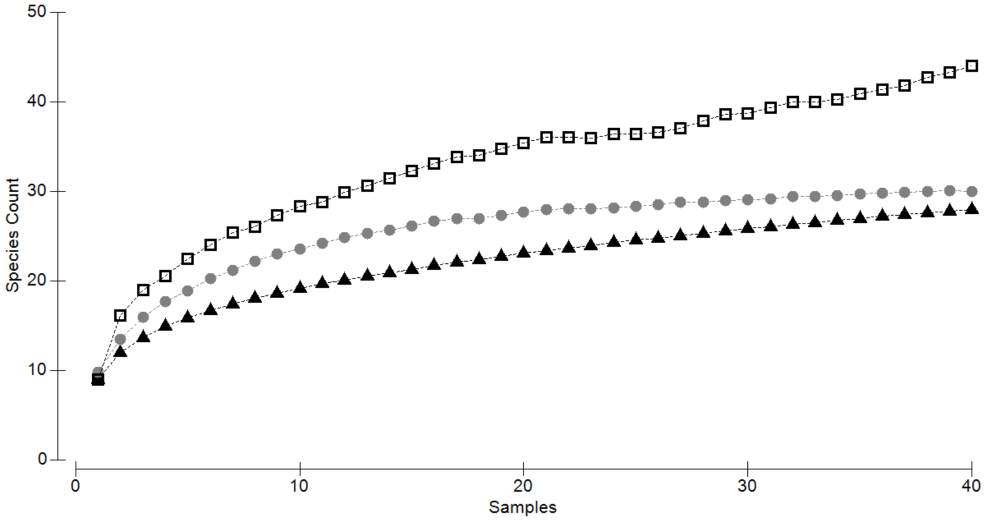

Fig. 7. Species accumulation curves. Curves obtained for the observed taxonomic richness (triangle), Chao 1 (circles) and Chao 2 (quadrats).

Regarding the univariate variables (N, S and H'), Vila Praia de Âncora and Leça da Palmeira were the shores with higher abundance (N) (**Fig. 8**). The shores with a higher taxa richness (S) and Shannon-Wiener index (H') were Vila Praia de Âncora and Cabo do Mundo (**Fig. 8**). However, ANOVA analyses did not reveal significant differences between conditions in the total abundance, the taxa richness nor the Shannon-Wiener index (**Table 2**). Significant variability was found between sites for N (*P* = 0.017), shores for S (*P* = 0.028) and sites for H’ (*P* = 0.010) (**Table 2**).

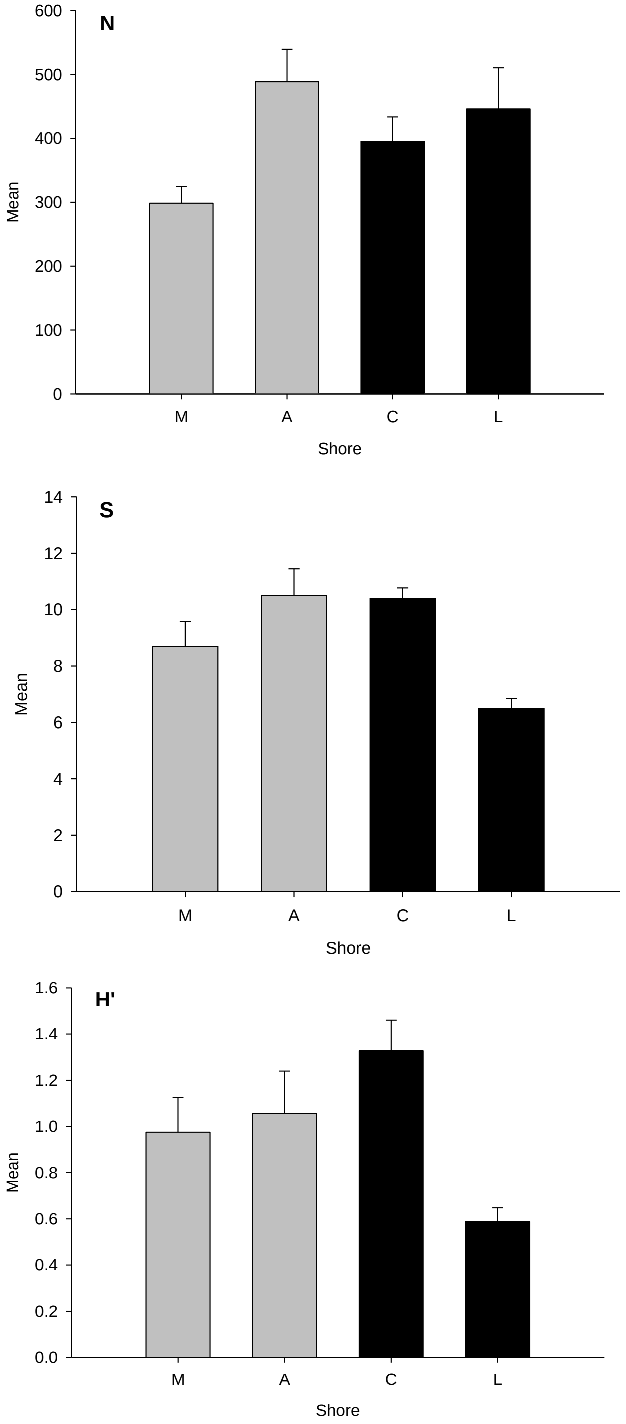

Fig. 8. Means (+ standard error) for abundance (N), taxa richness (S) and diversity (H’) of epifauna associated with *Chthamalus* barnacles in extra-urban (grey) and urban (black) shores. M: Moledo; A: Vila Praia de Âncora; C: Cabo do Mundo; L: Leça da Palmeira.

Table 2. Results of ANOVA tests for the abundance (N), taxa richness (S) and diversity (H’) of epifauna. Co: condition; Sh: shore; Si: site; *: P < 0.05; **: P < 0.001.

Source of variation	**df**	**N**		**S**		**H’**	
		MS	*F*	MS	*F*	MS	*F*
Co	1	7371.225	0.08	13.23	0.29	0.03	0.02
Sh (Co)	2	96625.63	1.57	46.13	9.87*	1.39	2.39
Si (Co x Sh)	4	61354.78	3.55*	4.68	0.97	0.58	4.01**
Residual	32	17279.79		4.84		0.14	
Total	39						

The multivariate analysis using PERMANOVA did not reveal significant differences between conditions for the structure of the epifaunal assemblages associated with *Chthamalus* barnacles (*P* = 0.323) (**Table 3**). For shores and sites, PERMANOVA revealed significant variability (*P* = 0.015, *P* = 0.002, respectively) (**Table 3**).

Table 3. Results of PERMANOVA analysis for the structure of epifaunal multivariate assemblage. Due to the low number of permutations for the factor Co, Monte Carlo P-values were considered. Co: condition; Sh: shore; Si: site; *: P < 0.05.

Source of variation	**df**	**Faunal assemblages**		
		MS	Pseudo-*F*	Perms
Co	1	4649.8	1.44	3
Sh (Co)	2	3232.5	3.43*	300
Si (Sh(Co))	4	941.54	2.78*	997
Residual	32	338.83		
Total	11			

The PERMANOVA tests for the most abundant groups of invertebrates (Mollusca, Arthropoda and Nematoda) did not revealed significant differences between conditions: *P* = 0.377 for Mollusca; *P* = 0.554 for Arthropoda; *P* = 0.466 for Nematoda. The mollusc and arthropod assemblages only revealed significant differences between sites (*P* = 0.001 and *P* = 0.002, respectively). For nematodes, the multivariate analyses revealed significant differences between shores (*P* = 0.005) and between sites (*P* = 0.003). The nMDS for the nematode assemblages elucidated that Leça da Palmeira was the shore that most contributed for the differences revealed in the PERMANOVA analysis (**Fig. 9**).

The nMDS for molluscs and arthropods did not reveal a clear contribution of any scale (see **Fig. S2** for molluscs and **Fig. S3** for arthropods).

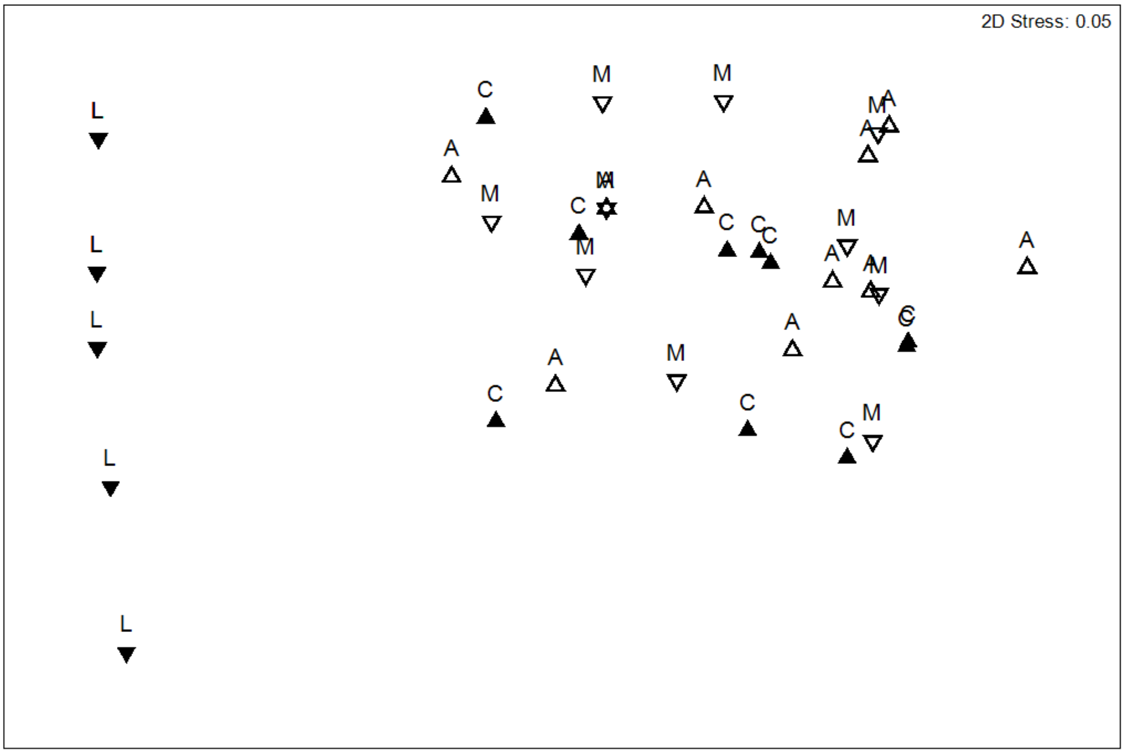

Fig. 9. nMDS for the sampling sites of each studied shore only considering the nematode assemblages. Extra-urban shores (empty triangles): Moledo (M) and Vila Praia de Âncora (A); Urban shores (full triangles): Cabo do Mundo (C) and Leça da Palmeira (L).

4.4 SPATIAL PATTERNS OF DISTRIBUTION

Univariate analysis for the spatial variation patterns on the structure of epifaunal assemblages revealed a significant interaction between the factors condition and scale (*P* = 0.032) (**Table 4**). Since this interaction was significant, the SNK post-hoc test was done to compare variances at each spatial scale (shore, site and replicate) between conditions (extra-urban and urban). The test revealed that the variances were larger for the extra-urban condition at the scale of site (variance of 537 for extra-urban and variance of 178 for urban condition) and replicate (variance of 424 for extra-urban and variance of 383 for urban condition), but only at the scale of site this difference was statistically significant (**Fig. 10**). At the scale of shore, the variance was larger for urban condition (variance of 67 for extra-urban and variance of 232 for urban condition) (**Fig. 10**).

Table 4. Results of ANOVA test for the spatial variability of the multivariate structure of the epifaunal community associated with barnacles. Co: condition; Sc: scale; *: P < 0.05.

Source of variation	**df**	**Spatial patterns**	
		MS	*F*
Co	1	18486.75	1.71
Sc	2	73453.52	6.81*
Co x Sc	2	69624.78	6.45*
Residual	6	10787.38	
Total	11		

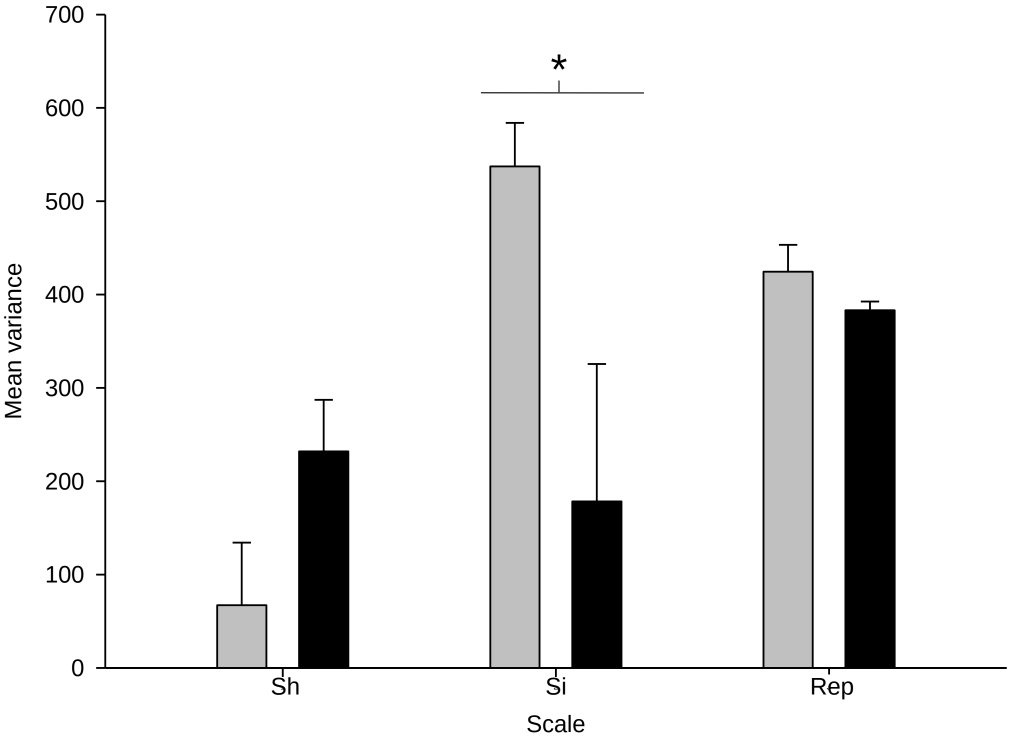

Fig. 10. Mean variances (+ standard error) for each level of the factor scale for the interaction between condition and scale calculated by means of ANOVA. Sh: shore; Si: site; Rep: replicate; *: *P* < 0.05; Grey bars: extra-urban shores; Black bars: urban shores.

5. DISCUSSION

All over the world, the increasing urbanisation is a major pressure in coastal areas. Nowadays, it is challenging to find a shore that is located far enough from urbanised centres so that there is no influence in that environment. Among industrialized countries the challenge becomes almost impossible. In Continental Portugal, there are some specific coastal regions that are far away from heavily populated cities and industries.

The studied shores Vila Praia de Âncora and Moledo are located in the Viana do Castelo district, which has a population density significantly lower than the estimates for Porto (visit https://www.pordata.pt/DB/Municipios/Ambiente+de+Consulta/Tabela). Within the Porto district, the other two studied shores – Cabo do Mundo and Leça da Palmeira – are flanked by urban centres and important industrial plants that dispose effluents in the vicinity of the rocky intertidal. For this study, the heavy metal content on living tissue of *Chthamalus* barnacles was considered as proxy for the impact of urbanisation and urban shores revealed overall higher concentrations than extra-urban shores. This was supported by other studies that considered the selected urban and extra-urban shores as disturbed and reference locations, respectively. (Reis et al., 2012, 2014; Rubal et al., 2014). Reis et al. (2012) studied for the first time the use of *Chthamalus montagui* as a biomonitor species in the Portuguese coast. These authors concluded that this species may be used for monitoring metal bioavailabilities in seawater of the NW coast of Portugal. Accordingly with that study, it was revealed that zinc, iron and manganese were the metals with the highest concentrations in the barnacle living tissue (Reis et al., 2012). Those results supported the results obtained in the present study. However, the mean concentrations were much lower in the present study than in Reis et al. (2012) for both urban/disturbed and extra-urban/reference shores. It was expected that zinc would be the metal with the highest concentration because several studies had tried to explain the high values of zinc that is accumulated in many species of barnacles (Pullen and Rainbow, 1991; Masala et al., 2004; Dionísio et al., 2013). Cadmium and chromium were the metals with lower concentrations found in both studies. However, in the present study the mean concentration of cadmium in urban shores was lower than the value for extra-urban shores. These results may point out to a subtle contamination in those shores. Though the mean concentration of cadmium in extra-urban shores in foodstuff according to the European Community Commission Regulation No 629/2008 was higher, it did not reach the maximum cadmium concentration allowed of 2 July 2008 (2.50 mg kg^{-1}, dry wt.) (EU, 2008).

The barnacle species *C. stellatus* was first tested than *C. montagui* as a biomonitor organism (Stenner and Nickless, 1975). These species have the characteristics that enable them to be considered good biomonitoring organisms: they are sessile, very abundant in rocky shores, available in all seasons, easy to sample and capable of accumulating contaminants above environment levels (Stenner and Nickless, 1975; Barbaro et al., 1978). However, in the present study the results did not reveal a clear picture of the contamination state of both conditions. Most likely, further studies will have to consider other levels than the high level of the rocky intertidal. In the mid intertidal of the studied shores, *Chthamalus* barnacles and *Mytilus galloprovincialis* mussels dominate the substrate and are more prone to contamination (Rubal et al., 2014).

For this study, three main aims were established. The first aim was related to the barnacles' structural complexity and how this could be influenced by urbanisation effects. The results revealed that urbanisation did not have a significant effect on the number of full tests, the percentage of empty tests or the total number of tests. These results may be due to that levels of contamination may not be enough to increase the mortality of barnacles. Among the heavy metals considered for this study, zinc, cadmium and chromium are the most toxic metals for aquatic organisms (Bryan, 1971). However, the minimum concentration, which will kill organisms is very dependent both on the metal and on the organism, showing different species different susceptibilities to heavy metals (Bryan, 1971). Some acorn barnacle species can bioaccumulate high concentrations of heavy metals in their soft tissues and have mechanisms of detoxification and bioreduction (see the review by Reis et al., 2011). Besides anthropogenic sources, natural mortality and predation are responsible for increasing the number of empty tests of barnacles. Intertidal invertebrates such as dogwhelks (*Nucella* spp.), nemertean worms and larval dipterans are responsible for the consumption of barnacles, leaving empty tests (Haven, 1971; Hawkins and Hartnoll, 1983; Harley and O'Riley, 2011). Sea stars and crabs also feed on barnacles but they typically remove the whole barnacle tests (Harley and O'Riley, 2011).

Results of the present study did not corroborate the first hypothesis stated. Subtle pollution affecting the shores can produce a variety of recognizable effects without killing an organism. These effects include morphological changes, inhibitory effects and behaviour changes (Bryan, 1971). However, in this study it was only considered the number of empty barnacle tests. Therefore, future studies should explore sub-lethal effects on *C. montagui* and *C. stellatus* in urban and extra-urban shores.

There are few studies exploring the diversity of macrofauna associated with *Chthamalus* barnacles (Marine Biological Association, 1957; Martinez de Murguia and Seed, 1987). However, many studies explored the epifaunal communities associated with other barnacle species. In the present study, the number of taxa estimated by Chao1 (30 ± 3) and Chao2 (44 ± 17) were higher than those observed (28). These results suggest that *Chthamalus* barnacles harbour highly diverse communities of invertebrates. In the present study, molluscs were the most abundant group of invertebrates. This was also observed by Reimer (1976a, 1976b) that studied the communities of invertebrates associated with the acorn barnacle *Tetraclita stalactifera panamensis*. Among molluscs, gastropods constituted the most abundant class occurring among *Chthamalus*, followed by bivalves. The littorinid (Littorinidae) *Melarhaphe neritoides* represented almost 69% of the total invertebrate abundance. Many studies revealed that the littorinids develop a complex interaction with barnacles and that juveniles or small adults may shelter between the crevices of living or dead barnacles, as well as the empty tests of dead ones (Raffaelli, 1978; Underwood and McFadyen, 1983; Martinez de Murguia and Seed, 1987; Qian and Liu, 1990; McQuaid, 1996). Martinez de Murguia and Seed (1987) recorded among *C. montagui* and *Semibalanus balanoides* the same littorinids that were found in the present study: *M. neritoides* (as *Littorina neritoides*, using the old nomenclature) and *Littorina saxatilis*. When studying *C. stellatus* and *S. balanoides*, Lysaght (1941) observed that *M. neritoides* was only abundant where barnacles were present since they provided shelter during metamorphosis of littorinid larvae. The research by Emson and Faller-Fritsch (1976) supported that the juveniles of *M. neritoides* and other littorinids may be restricted to empty barnacle tests. In the present study, about 10% of the total invertebrate fauna was represented by bivalves, of which 8% was represented by *Lasaea rubra* and the other 2% was represented by *Mytilus galloprovincialis*. Martinez de Murguia and Seed (1987) also recorded the presence of *Lasaea rubra* and a *Mytilus* mussel species among *Chthamalus* barnacles. Moreover, *Lasaea rubra* was found together with *M. neritoides* in the interstices and empty tests of *C. stellatus* (Marine Biological Association, 1957). On New England coasts, *Mytilus* mussels were observed to be dependent, at least partially, on the irregular habitat provided by barnacles (Menge, 1976). There, the young individuals find protection and the areas where barnacles are absent there is virtually no recruitment (Menge, 1976; Lively and Raimondi, 1987).

Following the molluscs, crustaceans were the second most abundant group of invertebrates associated with *Chthamalus* barnacles. They represented about 10% of the total invertebrate fauna. However, this group revealed a small diversity of taxa, with the isopod *Campecopea hirsuta* representing more than 9.9% of the crustacean portion.

The presence of *C. hirsuta* among *Chthamalus* was reported by Wieser (1963) and Martinez de Murguia and Seed (1987). On *S. balanoides* the presence of *C. hirsuta* was recorded by Raffaelli (1978), and this author suggested that due to their larger size these isopods were the most important in terms of biomass. In the present study, it was observed that these isopods, when inside a barnacle, were tightly curled up, as observed by Raffaelli (1978) and drawn by Wieser (1963). These authors suggested that this posture allowed those crustaceans to reduce desiccation. In addition to this species of isopod, only one species of amphipod was found. *Hyale nilssoni* was recorded but its abundance represented less than 0.1% of the total invertebrate fauna. Among *C. montagui* and *S. balanoides* Martinez de Murguia and Seed (1987) also recorded the presence of this amphipod. When studying the epifauna associated with *Amphibalanus improvisus*, Zakutsky (1965) found 4 species of amphipods (*Erichthonius difformis*, *Stenothoe monoculoides*, *Gammarus locusta*, *Jassa ocia*). Some of these species bred inside of the barnacle tests (Zakutsky, 1965). However, in the present study there was no evidence that the individuals of *H. nilssoni* used *Chthamalus* barnacles to breed. In this study, only the mobile fauna associated with barnacles was counted. Nonetheless, barnacle recruits were observed growing between barnacle crevices and inside their empty shells. These observations were also recorded by Knight-Jones and Moyse (1961) for *S. balanoides* and Moyse and Hui (1981), who referred that the inside of the dead tests was apparently preferable for recruitment.

As in Mendez et al. (2015), molluscs and crustaceans were the dominant epifaunal taxa in *Chthamalus* barnacles. Besides these organisms, other conspicuous groups were found. Hexapods, such as collembolans (Collembola) and chironomid dipteran larvae (Chironomidae), seem to be a very common group of invertebrates occurring among chthamalid barnacles (Gorvett, 1958; Raffaelli, 1978; Martinez de Murguia and Seed, 1987). Chironomid midges probably constitute the most numerous group of marine insects. In the present study, some pupae of these midges were also recorded, thus it can be suggested that chironomids lay their eggs among barnacles to protect larvae from physical stress and from predators. Raffaelli (1978) and Martinez de Murguia and Seed (1987) observed that dipteran pupae and larvae suffer high mortality in the absence of protection provided by the crevices between barnacles and their empty tests. Some of these larvae are used as indicators of water pollution (Cheng, 1979), but there are no studies using these organisms as bioindicators in association with *Chthamalus* barnacles.

Martinez de Murguia and Seed (1987) found mites (Acari) as the most abundant group of organisms associated with *C. montagui* and *S. balanoides*. These authors revealed that the most surprising feature of the associated epifauna was the high diversity and abundance of these arthropods. Twelve species were recorded of which 7 were terrestrial and this high diversity is probably related to the high diversity of microscopic epifloral species found along the rough-textured shell plates of barnacles (Bénard, 1961; Stubbings, 1975; Fredrickson et al., 2016). Together with the accumulated detrital material, the associated epiflora provides the major source of energy input into the epifaunal communities. Gorvett (1958) found 5 species of mites when studying the barnacles *S. balanoides* and *C. stellatus* and observed that those organisms were the most numerous invertebrates of the studied communities. In the present study, 4 taxa of mites were found in both studied regions, but their abundance only represented 0.41 % of the total fauna. If the meiofauna was considered for this study, the abundance of mites would be considerably higher. Only one of the four taxa was exclusively marine (Halacaridae sp.), while the others were both marine and terrestrial (Erythraeidae sp., Ameronothridae sp., *Hydrogamasus littoralis*). Halacarid mites obtain the oxygen directly from the water, thus these organisms are more tolerant to the tidal inundations and can occur in the outer surfaces of the barnacles' crevices (Pugh and King, 1985). The non halacarid mites Erythraeidae sp. and *H. littoralis* are usually confined to sheltered conditions such as the inner zone of the barnacles' crevices (Pugh and King, 1985). During high tides, air is trapped in barnacle crevices and inside empty shells and this allows those species to survive until the next low tide (Baker, 1982). From the non halacarid mites observed in this study, Ameronothridae sp. is the most highly adapted to the littoral zone. It tolerates prolonged submersion, obtaining oxygen from sea water in a similar way to halacarid mites and consequently, may occur in the outer surfaces of the barnacles' crevices (Pugh and King, 1985).

A minor group that was found among the studied barnacles was Polycladida, known as polyclad flatworms. The presence of these organisms was residual but it has been observed that some species play an important ecological role as predators of barnacles (Rzhepishevskij, 1979; Hirata, 1987). Other studies just observed that these invertebrates use barnacle tests for shelter and as a protective environment for the development of their encapsulated eggs (McDougall, 1943; Skerman, 1960). An interesting finding in the present study was the great abundance (991 individuals) and diversity (7 taxa) of nematodes (Nematoda). Moreover, the nematode assemblages revealed significant variability between shores and sites, being Leça da Palmeira the shore that more contributed to these differences. Gorvett (1958) only recorded 4 nematodes and did not identify them to lower taxonomic levels. These invertebrates imply a special effort in their identification since they need to be mounted in microscope slides and the specific characters are difficult to observe clearly. Another limitation associated with the identification of nematodes is the lack of identification guides and Iberian Faunas, thus in the present study it was only possible to identify the individuals to the family level. This study added information to the knowledge of marine nematodes from North Portugal. Further research should explore the diversity of nematodes along the Iberian Peninsula considering the role of *Chthamalus* barnacles in harbouring nematodes among their tests.

The second aim of this study was to test if the structure of epifaunal assemblages associated with barnacles was influenced by urbanisation. The univariate variables (N, S, H’) did not reveal significant differences between conditions (urban and extra-urban). However, significant differences were found between sites for N and H’ and between shores for S. Therefore, these analyses detected the natural spatial variability in the epifaunal assemblages. These metrics are not always clear in quantifying the effects of anthropogenic disturbances. Moreover, these indices are often affected by sampling effort (Clarke and Warwick, 2001; Rubal et al., 2009; Bedini and Piazzi, 2012; O’Connor, 2013). In this study, the use of diversity indices such as the Shannon-Wiener does not seem adequate to detect the effects of subtle levels of anthropogenic contamination. Therefore, the alpha diversity of the studied assemblages appeared not to be correlated with the level of disturbance in concordance with Bedini and Piazzi (2012). Rubal et al. (2014), suggested that the absence of significant differences in the diversity indices could be due to the substitution of sensitive species by analogous numbers of tolerant ones. These authors also suggested that it could be because moderate pollution only changes the abundance of a few sensitive and tolerant species while the abundance of most dominant species remains unaltered. In the present study, when the identity and the abundance of all the epifaunal species were considered, the multivariate analysis did not reveal significant differences between conditions. Consequently, this study did not find evidence that the structure of the epifaunal assemblages is affected by urbanisation. In other studies, it was revealed that urbanisation-related disturbances could cause effects in marine invertebrate assemblages depending on the scale of analysis (Bertocci et al., 2017a). However, there is a gap of knowledge for the acorn barnacles and the urbanisation effects on their associated fauna. In the Iberian Peninsula, studies dealing with the epifauna associated with *Chthamalus* barnacles in urban and extra-urban shores are absent. Thus, the present study was a pioneering research, and in the future other concomitant urbanisation-related disturbances should be considered. Stressors that have not yet been studied across the Iberian Peninsula for chthamalid barnacles are trampling, removal of individuals, contamination by organic compounds, among others.

As with the first hypothesis, the second hypothesis of this study was also not corroborated by the results obtained. There was no evidence that the subtle levels of contamination affected the structure of epifaunal assemblages associated with barnacles.

It is known that natural assemblages are complex and intrinsically variable in space and time (Veiga et al., 2013). Anthropogenic impacts may alter this natural variability at different scales and it is imperative to identify relevant scales for investigating these disturbances on the ecological systems (Veiga et al., 2013). Therefore, the third aim of this study was to test if the urbanisation influences the spatial variability of the barnacles' epifaunal distribution. Spatial scale is a very central factor in a way that the ecological processes occurring in the ecosystems depend on the scale at which the variables are measured. It was revealed that the spatial patterns of the assemblages associated with *Chthamalus* barnacles were influenced by the interaction of shore condition and scale. Significant differences on the spatial variability of epifaunal assemblages between urban and extra-urban shores were revealed at the scale of site. These results might indicate that processes responsible for spatial variability (i.e. environmental or biological factors) are different between shore conditions. Many studies dealing with intertidal macro- and meiofauna concluded that the different patterns of distribution at different spatial scales were due to single or interacting abiotic or biotic factors (Bertocci et al., 2011; Veiga et al., 2011, 2014a; Bertocci et al., 2017a). These spatial patterns are shaped by the balance between intrinsic (e.g. behaviour and population dynamics) and extrinsic factors (e.g. responses to environment and other species) that influence spatial variability (Burrows et al., 2009). It is known that population's density is more variable at some spatial scales than at others (Veiga et al., 2013). The variables N and H' also detected the variability between sites, suggesting that the assemblages are naturally variable. Moreover, the multivariate analysis for the whole epifaunal assemblages also revealed significant differences between sites. By analysing three spatial scales, the present study has a strong power of spatial replication and in a more replicated study, it is more difficult to obtain statistical differences because the results are more accurate and approximate to what really happens in the nature by considering natural spatial variability. The post-hoc tests revealed that extra-urban shores presented a higher variability than urban shores. In extra-urban shores, the scale of sites revealed the larger variability, followed by replicates and shores. In urban shores the scale of sites revealed the lower variability. Moreover, spatial patterns do not necessarily remain constant over time and temporal scale must be considered before drawing any general conclusion (Fraschetti et al., 2005). The relevance of mid- to large-scale variability differ between habitats and among taxa (Fraschetti et al., 2005). Results of this study did not find significant differences between extra and urban shores on the variability at the larger scale analysed. It has been questioned to what extent does small-scale variability contribute to large-scale patterns of variation (Fraschetti et al., 2005). These authors suggested that small-scale variability can scale up to generate large-scale patterns. They also referred that the absence of variability at larger scales may be related to the environmental stress affecting the studied shores. Nonetheless, in the present study the larger analysed scale was probably not wide enough so that significant variability could be detected. The third hypothesis was supported by the results obtained in this study because the patterns of variability of the epifaunal assemblages at the scale of site were significantly different between shore condition.

6. CONCLUSION

Despite the high levels of urbanisation surrounding the studied urban shores the analyses only revealed subtle levels of contamination. The concentrations of heavy metals were not high enough to affect the structural complexity of *Chthamalus* aggregations. Between urban and extra-urban shores there were no significant differences in the number of empty and full barnacle tests. The subtle contamination was also not enough to affect the structure of the epifaunal assemblages associated with the barnacles. The most interesting results obtained from this study were the significant differences in the spatial patterns of the epifaunal assemblages. The interaction between scale and shore condition was significant, and site was the scale that contributed to these differences. Extra-urban shores revealed a higher variability than urban shores at the scale of sites. No significant differences were detected for the univariate faunal variables (N, S, H'). However, these metrics are not always clear in quantifying the effects of anthropogenic disturbances and are often affected by sampling effort. Further research should consider other contaminants and larger scales to detect more variability in the spatial patterns of distribution of the studied assemblages.

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SUPPLEMENTARY DATA

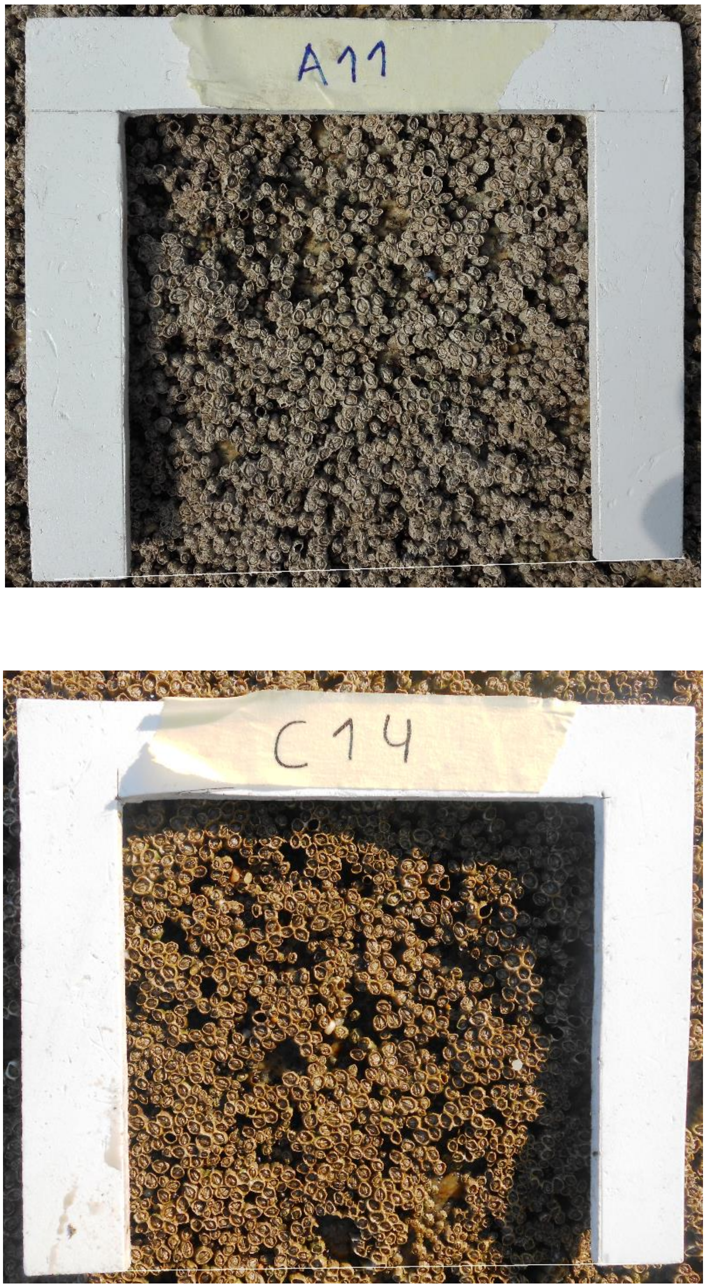

Fig. S1. Examples of photos used to count the empty and full barnacle tests.

Table S1. Total abundance of individual taxa associated with *Chthamalus* barnacles in urban and extra-urban shores.

Taxa	**Condition**	
	Urban	Extra-urban
Littorina saxatilis	4	26
Melarhaphe neritoides	5883	5318
Patella sp.	0	2
Mytilus galloprovincialis	252	96
Lasaea rubra	744	491
Hyale nilssoni	0	11
Campecopea hirsuta	690	869
Jaera forsmani	0	1
Calanoida sp.	0	1
Chironomidae indet.	90	67
Dolichopodidae sp.	6	11
Diptera larvae	0	2
Collembola sp.	266	144
Erythraeidae sp.	12	41
Halacaridae sp.	1	2
Ameronothridae sp.	6	1
Hydrogamasus littoralis	2	3
Pseudoscorpionida sp.	0	1
Nereidae sp.	0	2
Oligochaeta sp.	142	101
Nematoda sp. 1	27	3
Nematoda sp. 2	1	0
Oxystominidae sp.	2	1
Oncholaimidae sp. 1	219	369
Oncholaimidae sp. 2	0	5
Oncholaimidae sp. 3	0	2
Ethmolaimidae sp.	66	296
Polycladida indet.	0	4

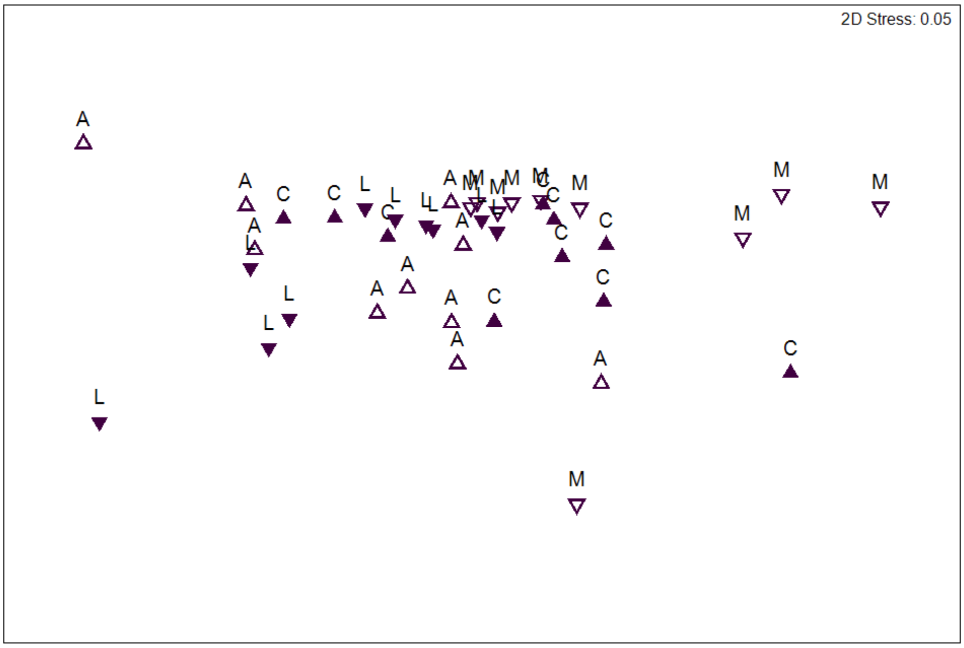

Fig. S2. nMDS for the sampling sites of each studied shore only considering the mollusc assemblages. Extra-urban shores (empty triangles): Moledo (M) and Vila Praia de Âncora (A); Urban shores (full triangles): Cabo do Mundo (C) and Leça da Palmeira (L).

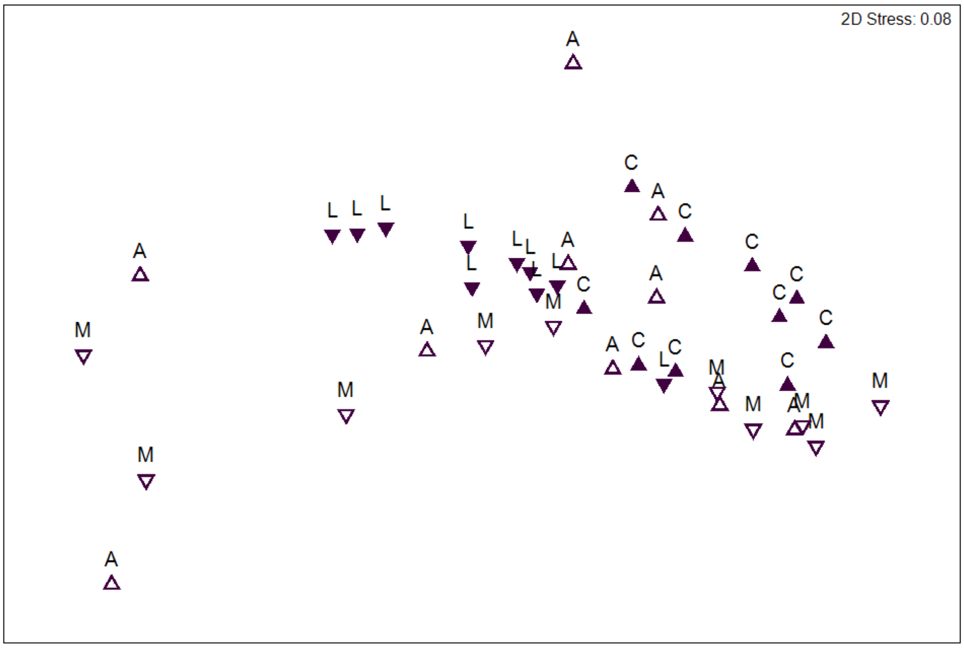

Fig. S3. nMDS for the sampling sites of each studied shore only considering the arthropod assemblages. Extra-urban shores (empty triangles): Moledo (M) and Vila Praia de Âncora (A); Urban shores (full triangles): Cabo do Mundo (C) and Leça da Palmeira (L).