Università Politecnica delle Marche

Department of Life and Environmental Sciences

PhD in Civil and Environmental Protection

BIODIVERSITY AND TRACE ELEMENTS

Community Assessments and Application of Benthic Organisms as Biomonitors of Environmental Pollution

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XXXIV Cycle

To my father.

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SUMMARY

Marine ecosystems are facing a huge loss of biodiversity worldwide, together with a widespread collapse of habitats and their functionality. Direct and indirect anthropogenic pressures (e.g., release of pollutants, overexploitation of marine resources, introduction of invasive species, climate change, etc.) are the main responsible of this phenomenon, while the lack of long-term monitoring programs creates an obstacle in the implementation of successful management plans and policies. Recognizing the situation, the European legislation (e.g., Biodiversity Strategy for 2030, Green Deal, Marine Strategy Framework Directive, and others) aims to increase the number of monitoring plans to create temporal baselines, fundamental to understand and measure possible shifts in biodiversity over time, providing stakeholders with precise information on the knowledge gaps in currently available biodiversity data. In addition, various European Directives (e.g., the Water Framework Directive and the Marine Strategy) suggest the application of benthic taxa as bioindicators of environmental pollution, since mainly edible species have been used so far, with pollutants law limits almost exclusively referred to the human consumption.

In this context, the aim of this PhD project was to assess the benthic communities of an Italian Archipelago aimed to become a future Marine Protected Area, to inform stakeholders and policy makers of the hydroid (Cnidaria: Hydrozoa) and sponges (Porifera) characterising the studied area. In addition, a systematic review was conducted on the application of benthic organisms, proposed as non-conventional bioindicators of trace elements (TEs) in coastal shallow waters. In fact, this project also deepens the application of Porifera as biomonitoring tools for mercury pollution, with a multi-level analysis (from the community to the individual), exploring the involvement of sponges' associated microbiota in the bioaccumulation processes, for their possible application in bioremediation scenario.

In the framework of the PADI FOUNDATION project "Marine biodiversity of Montecristo Island (Mediterranean Sea)", samplings were carried out by SCUBA diving during June 2019 and 2020 in Montecristo and Giglio, two islands included in the Tuscan Archipelago National Park (TANP) (Tyrrhenian Sea, Italy), to investigate hydroid and sponge assemblages in relation to a bathymetric gradient. This work provides the first baselines of these assemblages in the considered islands, adding new records for the area (40 hydroid species and 17 sponge species). In addition, it improves the knowledge on the ecology of the two taxa, giving new information on hydroids' epiphytic habits and on sponges' distribution of the entire Archipelago. These are the first studies summarising hydroid and sponge diversity of the area, and one of the few focussing entirely on

these organisms. Although a high species richness was encountered, future studies are needed to fully describe the actual diversity of a poorly studied area.

Following the suggestion of the EU legislation in identifying benthic organisms as bioindicators of environmental pollution, an extensive bibliographic research was conducted on Elsevier's Scopus database on six sessile taxa of filter- and suspension-feeders (sponges, cnidarians, bryozoans, polychaetes, cirripeds, and tunicates). A total of 225 documents were included in the study, with most of them carried out in the Temperate Northern Atlantic and on a few TEs (i.e., Cd, Cu, Pb and Zn), focussing their attention mainly on two groups, Cnidaria and Porifera. The literature analysis allowed to summarize the current knowledge on the application of these taxa as bioindicators in coastal shallow waters, and to identify their possible use also as bioremediation tools.

To this end, some of the sponge samples collected in the TANP for the community assessment were also used for further analysis on the total mercury content (THg). Among the analysed species, a high intra- and inter-variability have been recorded, with the species *Cliona viridis* showing the lowest concentration, and *Chondrosia reniformis* and *Sarcotragus spinosulus* the highest. From a literature research, other authors also highlighted these two species as better bioindicators than others, since they reflect the level of contamination of a location more accurately. For this reason, the following studies were conducted considering only on the Atlanto-Mediterranean sponge *C. reniformis*. From additional samples of *Chondrosia* collected in the Tuscan Archipelago, a different distribution of Hg was found inside the sponge itself. In fact, since *Chondrosia* is characterized by two body regions, an external cortex and an internal choanosome, the two parts were analysed separately, showing higher concentration in the choanosome, were the actual filtration takes place.

Another factor to consider in the bioaccumulation process was the possible involvement of the sponges' microbiota. Therefore, the associated prokaryotic community was investigated on samples of *C. reniformis* collected in Faro (Portugal), thanks to the financial support of an Assemble Plus grant received during fall of 2020. Although the THg content in the microbial cell pallets was lower than the one showed by the sponge fraction (devoid of the microbiota), a considerable amount of metal was detected, suggesting a possible implication of the microbiota not only in the secondary metabolism but also in the bioaccumulation of TEs. Additionally, the microbial community of the sponge was defined and explored, highlighting that ammonia-oxidizing organisms dominate the whole microbial consortium, thus proposing the ammonium oxidation/nitrification as a key metabolic pathway taking place within *C. reniformis*.

A collection of six manuscripts is included in this thesis. Five of them have been published on international journals (with Q2 ranking), while one paper is still in preparation. All these manuscripts contribute to the dissemination of knowledge in the marine taxonomic and ecological fields, giving new insights and laying the groundwork to stakeholders and policy makers in the implementation of new strategies and policies.

In addition, at the end of the thesis, I listed other products and publications not concerning the topic of this thesis but performed during the PhD period.

INTRODUCTION

BIODIVERSITY: ROLE, ASSESSMENT, AND SCIENTIFIC TRANSFER

Although a multitude of definitions have been given to the term *biodiversity* (Kaennel 1998), it was defined by the Convention on Biological Diversity as "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (SCBD 2010, page 15), indicating that the concept covers a wide range of different aspects, at different scales and in different fields of activity, from natural to human sciences (Kaennel 1998).

Biodiversity provides humans many ecosystem services and biological resources, delivered through subsistence agriculture, fishing and hunting, together with important social and economic benefits (Jaisankar et al. 2018; EC 2020). To keep using all the services and benefits supplied, biodiversity needs to be maintained: any disturbance in the delicate ecological balance kept by the multitude of organisms, habitats, and ecosystems, will lead to severe consequences, which may threaten the survival of many species (Jaisankar et al. 2018). However, in the last century, at a global level more than the 60% of ecosystem services have been deteriorated or already overused, several species have become extinct, and many others are threatened due to anthropogenic activities (SCBD 2010; Mace et al. 2012; Jaisankar et al. 2018).

Marine biodiversity is nowadays subjected to a huge loss worldwide, together with a widespread collapse of ecosystems and their functionality. Considering the 5,291 marine species included in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, the 25% are threatened (O'Hara et al. 2019). Nonetheless up to 23.2% of the assessed species fall into the Data Deficient category (IUCN 2021), making impossible to determine meaningful trends in the status of marine biodiversity, and creating problems in the drawing of new legislations and policies.

Another important issue for marine biodiversity is related to taxonomy (i.e., the science dedicated to discovering, describing, naming, and identifying organisms) which, in the last decades, is facing the so-called "taxonomic crisis"; this phenomenon is characterized by a lack of taxonomic experts, strongly concentrated in a few countries, and by insufficient funding for taxonomic works, a situation widely recognized in Europe (UNEP-MAP RAC/SPA 2010; Costello et al. 2012). Considering only the Mediterranean Sea, the state-of-knowledge is unbalanced towards a few metazoans, such as fish and a few vertebrates (i.e., echinoderms, crustaceans, and molluscs), while only a little is known about all other invertebrates, especially for small body size taxa (Costello et al. 2012), which are the main representatives in the marine environment.

Although the Mediterranean is considered the best investigated sea globally (Costello et al., 2012), the knowledge of marine and coastal biodiversity is not homogenous and has many lacunae: the sampling effort results insufficient, being focussed only on a few areas, with many biocenoses still under-sampled on a basin scale (UNEP-MAP RAC/SPA 2010). Thus, data is often patchy, lacking information on the many species, habitats, and communities especially on a regional scale (UNEP-MAP RAC/SPA 2010). Moreover, the rates of marine diversity loss due to continuous anthropogenic pressures (e.g., pollution, climate change, overexploitation of marine resources, introduction of invasive species, etc.), coupled with the low taxonomic effort, make urgent the need of collective action plans across European Union (EU) Member States aiming the monitoring, conservation, and recovery of degraded ecosystems at national and regional scales. Recognizing the situation, the EU legislation is moving to invest more and more in the protection and restoration of natural environments: the EU Biodiversity Strategy and the Marine Strategy Framework Directive (MSFD), together with the transition to a circular sustainable economy with the EU Green Deal, were developed around the so-called "ecosystem approach"[1] for the management of anthropogenic activities impacting the marine environment, aiming to integrate the concepts of environmental protection with those of sustainable use, in order to maintain or reach the Good Environmental Status (GES) along the coasts of all European countries (EC 2008, 2019, 2020).

To achieve these goals, EU Directives are intended to increase the number of monitoring plans to create temporal baselines, fundamental to understand and measure possible shifts in biodiversity over time, providing stakeholders with precise information on the knowledge gaps in currently available biodiversity data (Mihoub et al. 2017). The lack of historical long-term data is one of the major problems in understanding fluctuations of marine communities (McClenachan et al. 2012; Fortibuoni et al. 2017a). Most of long-term data series are related to fishery and plankton (e.g., Beaugrand et al. 2000; Coro et al. 2016; Fortibuoni et al. 2017a, 2017b; Zingone et al. 2019), and, despite these kinds of studies are becoming more and more frequent on benthic assemblages (Sandulli et al. 2021), datasets are still scarce (Clare et al. 2017). In fact, in the case of the benthic communities, most of monitoring plans started only within the last decades (Mihoub et al. 2017), even though most of the anthropogenic pressures have been operating over centuries, most

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[1] The "ecosystem approach" is defined as the strategy for the integrated management of land, water and living resources, promoting conservation and sustainable use in an equitable way (For further information see https://www.cbd.int/ecosystem/).

remarkably during the Industrial Revolution in the middle of the 19th century and the "Great acceleration" in the 1950s (Ostberg et al. 2015; Steffen et al. 2015). Thus, the identified mismatch between the later application of benthic biodiversity monitoring and the start of anthropogenic pressures limits any assessment of the full impacts on biodiversity (Mihoub et al. 2017). Nonetheless, monitoring programs, especially for long-term studies, must not be underestimated, as they undoubtedly help to collect important information and give a general picture of the status of an area, fundamental for the implementation of new restrictions or protected areas or to strengthen the existing network of MPAs.

In this spirit, the EU Biodiversity Strategy aims to protect at least the 30% of the European seas and to improve the health of existing protected areas (EC 2020). Therefore, the interaction between researchers and stakeholders represents a crucial tool to support the societal transition to sustainability (Figure 1) (Knaggård et al. 2019).

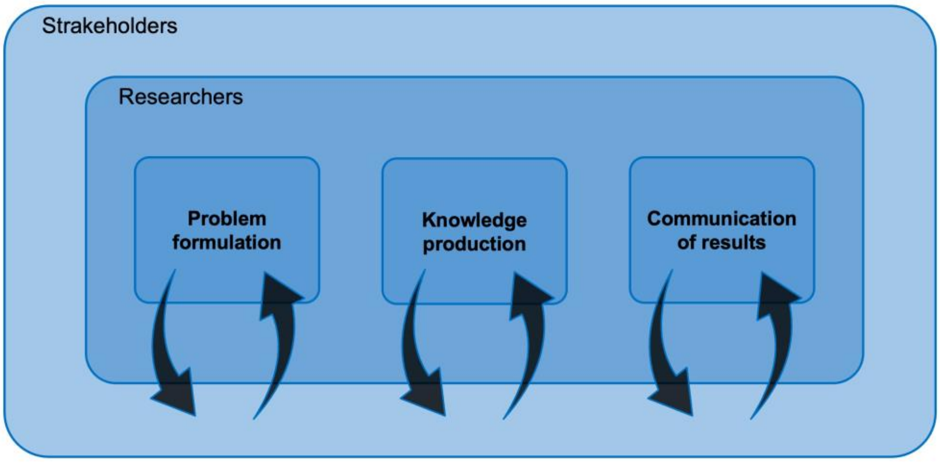

Figure 1 – The stakeholders interaction model (modified from: Knaggård et al. 2019).

The scientific knowledge not only has to be transferred from research institutions to stakeholders and decision makers, but research efforts need to be aligned with stakeholders' priorities, considering establishing an academic community-based partnership (Camden et al. 2019). This will lead to a strict collaboration between the two parties, working synergically toward a same objective (Figure 1) (Camden et al. 2019; Knaggård et al. 2019).

Stakeholders' engagement results of fundamental importance in the achievement of the main goals of the new EU Directives and in the protection of our seas, to identify priority conservation areas, and to define their level of protection, management strategies and connectivity, enhancing a species-based protection to safeguard ecosystems' diversity.

TRACE ELEMENTS: THE CASE OF MERCURY (HG)

Thousands of contaminants are released in the environment every year consequently to their production, use and disposal (Chiarelli and Roccheri 2014). They can be of natural (mainly from erosion and from volcanism) or of anthropogenic origin, from household use to manufacturing process (e.g., combustion of fossil fuels, leachate from the land, run-off from agricultural activities, mining and smelting operations, etc.) (Chiarelli and Roccheri 2014; Rebelo et al. 2014). During operational activities or abnormal circumstances, contaminants can be discharged and reach the marine coastal environment throughout different pathways (e.g., atmospheric depositions, rivers, industrial discharges, maritime traffic, or submarine groundwaters) affecting organisms and ecosystems (Tedetti et al. 2010; Rebelo et al. 2014; Senthil Rathi et al. 2021).

Trace elements (TEs) are undoubtedly among the most common contaminants in coastal waters (Ghani 2015), and they represent one of the most important issues for marine ecosystems, ensuring that some of these metals and their derived organic compounds are listed in the WDF as priority pollutants (EC 2000). TEs are under the heading of "heavy metals", even though a clear definition of this term is not yet available (Duffus 2002). They include all the transition elements (bloc d of the periodic table), the rare earth elements (lanthanides and actinides), all those elements presenting the typical characteristics of metals (bloc p), together with some metalloids (Ge, As, and Te) and nonmetals (Se) (Figure 2).

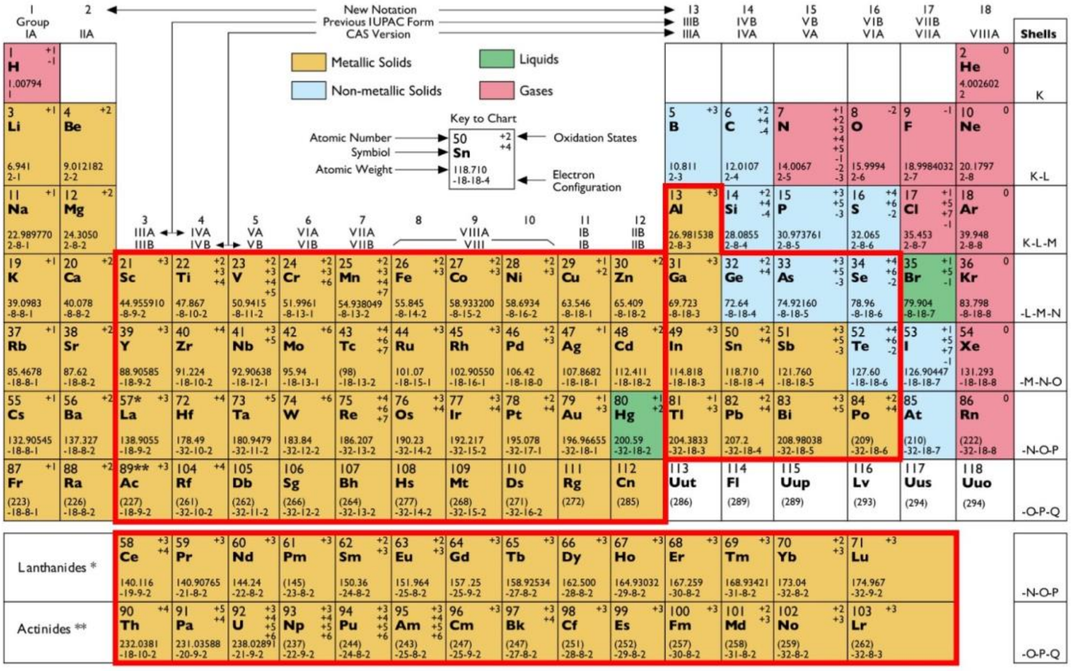

Figure 2 – Periodic tables of elements. Red squares indicate groups and elements included under the heading of "trace elements" (modified from: Dan Cojocari - Own work - Print It Here, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=34444082).

Some TEs are micronutrients (e.g., Fe, Cu, Co, Mn, and Zn), essential elements playing key roles in the functioning of enzyme systems. However, concentrations above specific thresholds can induce toxic effects in organisms, leading to physiological and metabolic alterations (Zoroddu et al. 2019). Others (e.g., Al, As, Ba, Bi, Cd, Pb, Hg, Ni, Pt, and Ag) are considered as nonessential elements, having a natural toxicity and no specific biological functions (EC 2000; Chang et al. 1996). Due to their characteristics, the hazard on wildlife and ecosystems represented by TEs is widely recognized. In fact, they: (1) do not degrade and have long half-lives, (2) can bioaccumulate in organisms and, in the aquatic environment, (3) can be converted by chemical and biological reactions in organic compounds, more toxic than their inorganic forms (Simkiss and Taylor 1995; Jakimska et al. 2011).

Owing to its relatively reduced area (2.3 x 10^6 km^2) and the fact that it is an almost enclosed basin, the Mediterranean Sea is particularly subjected to atmospheric and river inputs of TEs, whether anthropogenic or natural (e.g., Saharan dust events), causing high concentration of these contaminants in the surface waters (Elbaz-Poulichet et al. 2001; Migon 2005). Among TEs, mercury (Hg) is listed by the WDF as a priority pollutant (EC 2000), being recognized as a risk for the good chemical status for the aquatic environment. Mercury is, in fact, characterized by a high persistence in the environment – especially in its organic form of methylmercury (MeHg) –, bioaccumulation in organisms and biomagnification in the trophic chain (Ramalhosa et al. 2008; EEA 2018; Droghini et al. 2019). Most mercury emissions result from anthropogenic activities, such as primary Hg mining and processing, its use in industrial and artisanal processes, coal combustion and the management of mercury waste (EC 2017). In addition, the Mediterranean basin is considered as a mercuriferous area, since it is characterized by the presence of natural deposits of Hg along the coasts of many countries (e.g., Algeria, Italy, Spain, Slovenia, and others), containing about 65% of the world's cinnabar (HgS) deposits (Cossa and Martin 1991; Covelli et al. 2001).

TEs can be detected directly from water samples, but they are often monitored indirectly with the application of organisms as biomonitors (e.g., Pan et al. 2018; O'Callaghan et al. 2019; Conti et al. 2020; Costa et al. 2021; Girolametti et al. 2021; Singh and Gupta 2021), since they can be present in the water column but not in bioavailable form (Simkiss and Taylor 1995). Marine invertebrates can introduce and accumulate Hg in their tissues, uptaking it from solution or food. If associated with particles, Hg usually becomes available to organisms after ingestion and digestion in the alimentary tract. In addition, its concentration can largely vary between taxonomic levels, from phyla down to species of the same genus (Rainbow 1990, 2002).

The destructive potential represented by mercury is well known for humans. It is enough to mention the acute MeHg poisoning occurred in 1953 in Minamata (Japan), which caused chronic and, in many cases, lethal effects on the population (Ekino et al. 2007). Physiological effects of Hg exposure have also been recorded in many marine organisms. For example, signs of apoptosis and alteration of cells' movement were observed in various sponge species (Batel et al. 1993; Cebrian et al. 2007), mutagenicity, sterility and increase in the asexual reproduction in different cnidarians (Kasschau et al. 1980; Chotimah et al. 2014; Lucas and Horton, 2014), significant hemocyte mortality in oysters (Gagnaire et al. 2004), decrease of sperm density in polychaetes (Lockyer et al. 2019), reduction of the phenoloxidase activities and DNA damage in sea squirts and sea urchins respectively (Tujula et al. 2001; Schröder et al. 2005), and many others. Nonetheless, Hg law limits are defined for water, sediments, and various species of commercial interest (Table 1), being referred only to human health (EC 2002), and, for most marine invertebrates, thresholds are still not defined.

Table 1 – Mercury (Hg) law limits in different environmental matrices. MAC = Maximum Allowable Concentration.

Environmental compartment	Hg MAC	
Water	0.7 $\mu g\ L^{-1}$	Directive 2000/60/EC
Sediment	5 $\mu g\ Kg^{-1}$	Directive 2000/60/EC
Fishery products*, with the exception of the muscles of the following fish species:	0.5 mg Kg^{-1} w.w.	Directive 2001/466/EC
Acipenser species	1.0 mg Kg^{-1} w.w.	
Anarhichas lupus		
Aphanopus carbo		
Centroscymnes coelolepis		
Coryphaenoides rupestris		
Esox lucius		
Gempylus serpens		
Hippoglossus hippoglossus		
Istiophorus platypterus		
Katsuwonus pelamis		
Lepidocybium flavobrunneum		
Lepidopus caudatus		
Orcynopsis unicolor		
Ruvettus pretiosus		
Sarda sarda		
Sebastes marinus		
Sebastes mentella		
Sebastes viviparus		
Tricopterus minutes		
Xiphias gladius		
Raja spp.		
Mullus spp.		
Anguilla spp.		
Euthynnus spp.		
Hoplostethus spp.		
Lepidorhombus spp.		
Lophius spp.		
Makaira spp.		
Pagellus spp.		
Thunnus spp.		
All shark species		

*see the Directive 2001/22/EC for sampling method and analysis

The application of non-conventional taxa in the biomonitoring of contaminants have been deeply discussed in the literature (e.g., Rainbow 1995; Chiarelli and Roccheri 2014) and by various EU Directives (EC 2000, 2008), but they are still of little use for this purpose. Sponges are important suspension-feeders, able to filter a large volume of water per day (many thousands of litres kg^{-1} day^{-1}), together with sediment and food particles, unicellular algae, bacteria, fungi (Schmitt et al. 2007), and various contaminants, including TEs (Perez et al., 2005; Cebrian et al., 2006; Batista et al., 2014). Thanks to their characteristics, Mediterranean sponges have been already applied as bioindicators of TEs, but only a few studies investigated the presence of Hg and most of them have been carried out mainly on Dictyoceratida of the genus *Spongia*, collected along the coast of Marseille (France) (see Verdenal et al. 1990; Perez et al. 2004, 2005; Berthet et al. 2005).

The potentiality of this taxon is not only linked to its possible use as bioindicator, but also in its application in bioremediation scenarios (Santos-Gandelman et al. 2014a, 2014b). Many sponge species host complex communities of microorganisms (which can contribute up to 60% of the biomass) in their mesohyl matrix, acquired both from a vertical transmission through larvae, both horizontally from seawater, and composing the associated microbiota. These sponges take the name of “bacteriosponges” or “high microbial abundance” (HMA) sponges, in which the microbiota take part in the nutrient and metabolic cycles, and exchange different metabolites with their host (Schmitt et al. 2007). It has been demonstrated that the bacteria composing the microbiota can actively participate in the bioaccumulation processes by storing considerable amounts of TEs (Selvin et al. 2009; Santos-Gandelman et al. 2014a, 2014b; Keren et al. 2017). However, to the best of our knowledge, only two studies testing mercury resistance of microbial strains isolated from marine invertebrates have been carried out (Salvin et al. 2009; Santos-Gandelman et al. 2014b), and no information is currently available on the Hg content in the microbiota itself.

AIM OF THE STUDY

Periodic biodiversity assessments are of fundamental importance for describing possible changes in marine communities caused by natural and/or anthropogenic impacts. In fact, the application of different organisms as bioindicators of human impacts and pollution has increased in the last decades thanks to the implementation of the European legislation (e.g., Water Framework Directive, Marine Strategy, etc.).

From the analysis conducted on the current state-of-knowledge, different gaps were identified:

- A general lack of taxonomic baselines useful to understand possible impacts or important shifts in biodiversity over time at regional and/or national scales;
- An insufficient sampling effort, especially for taxa with small body size (e.g., Bryozoa, Cnidaria, Porifera, Tunicata and others), along the coasts of the Mediterranean basin;
- A lack of a general picture regarding the application of unconventional non-edible taxa as biomonitors of TEs pollution;
- A few information available on the Hg content in Mediterranean sponges, with studies frequently considering the same species and areas;
- No data on the Hg content in sponges' microbiota, coupled with a characterization of the entire microbial community to understand which are the main players in the bioaccumulation process.

In this context, this project aims to analyse benthic assemblages with a multidisciplinary approach (i.e., taxonomy, ecology, analytical chemistry, genetic) at different level (i.e., community, population, individual, microbial) to partially fulfil the listed knowledge gaps. Therefore, the main objectives of this study were to: (1) increase the knowledge on the benthic communities of an Italian Archipelago aimed to become a future MPA; (2) confirm the role of sponges as suitable bioindicators of TEs pollution; (3) investigate the possible involvement of sponges' microbiota in the bioaccumulation process to identify sponges not only as bioindicators but also as useful tools in a bioremediation scenario.

For this end, this PhD project:

- Provided new data on the zoobenthic communities of the Tuscan Archipelago National Park, useful to assess, with future monitoring, possible changes in the studied assemblages related to direct or indirect impacts (**Chapter 1 – paper 1 and 2**)

- Identified the sampling effort applied in the characterization of the sponge assemblages of the entire Tuscan Archipelago (**Chapter 1 – paper 2**)
- Provided a review on the application of unconventional non-edible taxa as biomonitors of TEs pollution (**Chapter 2 – paper 3**)
- Highlighted the role of sponges as attractive bioindicators of Hg pollution (**Chapter 2 – paper 4, 5**)
- Provided a first complete characterization of the microbial community of the common Atlanto-Mediterranean sponge *Chondrosia reniformis*, together with new insights on the Hg content in the sponge microbiota (**Chapter 3 – paper 6**).

RESULTS AND DISCUSSION

CHAPTER 1 – NEW INSIGHTS INTO THE MARINE BIODIVERSITY OF MONTECRISTO AND GIGLIO ISLANDS (TUSCAN ARCHIPELAGO, ITALY)

This Chapter includes two papers related to the study of hydroid (Cnidaria:Hydrozoa) and sponge (Porifera) assemblages, characterising the benthic communities of the Tuscan Archipelago National Park (TANP, Italy).

In the framework of the PADI FOUNDATION project “Marine biodiversity of Montecristo Island (Mediterranean Sea)”, sampling activities were carried out in SCUBA diving in two islands of the Archipelago, Montecristo and Giglio, at five depths, applying the visually-oriented sampling method. Collected organisms were identified at the lowest taxonomic level possible and stored in a reference collection deposited in the Zoology Laboratory at DiSVA (Department of Life and Environmental Sciences) of Università Politecnica delle Marche.

In paper 1, entitled “**The effect of substrate and depth on hydroid assemblages: a comparison between two islands of the Tuscan Archipelago (Tyrrhenian Sea)**”, we explored if the nature of the substrate, divided in living (i.e., macroalgae) and non-living (i.e., bare rock), could influence the hydroid assemblages of the studied islands, along a bathymetric gradient.

In paper 2, entitled “**Unravelling the sponge diversity of the Tuscan Archipelago National Park (Northern Tyrrhenian, Italy)**”, we assessed the current sponge biodiversity of the hard bottoms of the two studied islands. Considering the current knowledge on sponge communities of the TANP present in the literature, we also evaluated if the sampling effort applied in the Archipelago is sufficient to describe the sponge diversity of the area.

Our data improve the knowledge on the biodiversity of the zoobenthic communities of the Archipelago, adding new records for the area and creating useful taxonomic baselines. These data can also contribute to laying the groundwork for stakeholders in the establishment of the TANP as a future MPA, following the Italian legislation and in agreement with the Italian legislation and the EU Directives (MSDF, Biodiversity Strategy for 2030, etc.).

PAPER 1

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The effect of substrate and depth on hydroid assemblages: a comparison between two islands of the Tuscan Archipelago (Tyrrhenian Sea)

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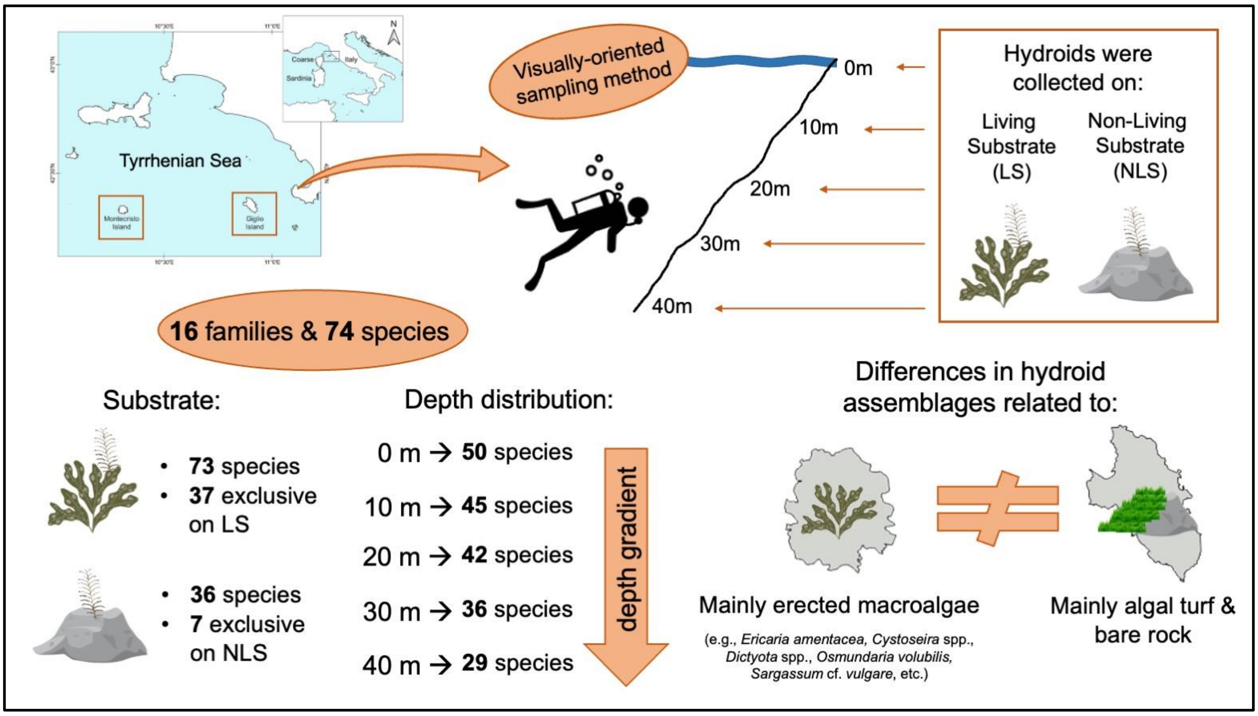

Supplementary material available at:

https://drive.google.com/drive/folders/1qaso9AXvCjVegtXTRbah-6vvidaTJ8RL?usp=sharing

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SENCKENBERG

ORIGINAL PAPER

The effect of substrate and depth on hydroid assemblages: a comparison between two islands of the Tuscan Archipelago (Tyrrhenian Sea)

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Abstract

Substrate and depth are known to be crucial factors in determining the spatial distribution of benthic communities. Herein, we explored if the nature of the substrate, living (i.e., macroalgae) and non-living (i.e., bare rock), may influence the hydroid assemblages of the Tuscany Archipelago (Italy), in relation to the bathymetric gradient. Samples were collected in June 2019 and 2020 in two islands, Montecristo and Giglio. Overall, 16 families (12 Leptothecata and 4 Anthoathecata) and 74 species were identified, with exclusive species observed for each substrate type and depth. The pattern of assemblages' distribution indicates an interaction between two factors (substrate and depth) also depending on the considered island. The DISTLM-forward analysis revealed that the percentage coverage of several algal taxa significantly explained a part of the observed variation in the hydroid assemblages. This study improves the knowledge of the zoobenthic communities of the Tuscan Archipelago National Park and points out the important role of the living substrate and its variation in the distribution patterns of the hydroids assemblages. In addition, following the Italian legislation and the EU Biodiversity Strategy for 2030, this paper contributes to improve the knowledge of the biodiversity of the Tuscan Archipelago providing a baseline for the biodiversity management of the forthcoming marine protected area.

Keywords Hydrozoa · Biodiversity · National Park · MPA · Western Mediterranean

Introduction

The loss of marine diversity and the continuous anthropogenic pressure make urgent to implement action plans for the conservation and recovery of degraded habitats and ecosystems (Mihoub et al. 2017; Jaisankar et al. 2018). The EU Biodiversity Strategy to 2030, together with the application of a sustainable economy announced by the EU Green Deal (EC 2019), aims to invest in nature protection and restoration, enlarging existing Natura 2000 areas and widening the network of marine protected areas (MPAs) (EC 2020). To achieve these goals, monitoring biodiversity of extant MPAs and areas designed for future protection is crucial to set updated taxonomic baselines that will help to identify long-term changes in marine communities (Mihoub et al. 2017; EC 2020).

Due to their sensitiveness to environmental changes, sessile benthic species and assemblages are widely recognized as effective bioindicators (Giangrande et al. 2005; Muxika et al. 2005; Ballesteros et al. 2007; Orfanidis et al. 2007;

Luigi Musco and Stefania Puce contributed equally to this work.

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Puce et al. 2009; Frontalini and Coccioni 2011; D'Archino and Piazzi 2021; Roveta et al. 2021), and their monitoring is recommended by European Directives, such as the Water Framework Directive (EC 2000) and the Marine Strategy Framework Directive (MSFD) (EC 2008), as a useful tool for assessing the health status of the marine environment. In fact, monitoring plans usually focus on benthic taxa abundance and diversity to get information on habitats and ecosystems (EC 2000; Haynes et al. 2014; Yilmaz et al. 2020).

Among benthic taxa, hydroids have also been considered suitable indicators for environmental monitoring (Topçu et al. 2018; Yilmaz et al. 2020), being sensitive to variation in environmental conditions (e.g., substrate type, water movement, light, salinity, sedimentation, exposure to air, temperature, and food availability) (Boero 1984; Boero and Fresi 1986; Di Camillo et al. 2008). It is thus not surprising that hydroid assemblages vary along bathymetric gradients. In fact, the vertical zonation of hydroid assemblages is a topic that has been investigated both on natural and artificial substrata and in several geographic areas (Boero and Fresi 1986; Chaplygina 2006; Di Camillo et al. 2008; Puce et al. 2009; González-Duarte et al. 2014). Light and water movement have been reported as the main factors determining this zonation, with water movement particularly relevant at shallower depth (Boero and Fresi 1986; González-Duarte et al. 2014). The vertical zonation of hydroid assemblages has been noticed even in a limited depth range (González-Duarte et al. 2014), and some hydroid species show a gradual change in their abundance with depth while other species are reported only in shallow or deep zones (Boero and Fresi 1986; Di Camillo et al 2008; González-Duarte et al. 2014).

Various studies (e.g., Canessa et al. 2019, 2020) showed that the structure and diversity of sessile zoobenthic assemblages are influenced not only by physico-chemical factors and biological interactions, but also by substrate lithology. Laboratory experiments also demonstrated that larvae of *Eudendrium glomeratum* Picard, 1952 primarily settle on carbonate substrates (marble) rather than on granitic ones (quartz) (Bavestrello et al. 2000). In addition, hydroids are known for their ability to grow as epibionts on other organisms such as macroalgae and other metazoans, e.g., Porifera, Cnidaria, Mollusca, Annelida, Bryozoa, Crustacea, Echinodermata, and Chordata (Calder 1991; Piraino et al. 1992; Morri and Bianchi 1999; Faucci and Boero 2000; Puce et al. 2008; Di Camillo et al. 2017; Monti et al. 2018). Many species- or genus-specificity have been recorded for epizoic hydroids; this is the case for example of *Hebella* Allman, 1888 and *Anthohebella* Boero, Bouillon & Kubota, 1997 species, which have been exclusively found on other hydroids, or of *Halocoryne epizoica* Hadzi, 1917, only recorded in association with bryozoans (Puce et al. 2008). However, hydroids living on macroalgae are considered typical guests, even though they are not host-specific and usually shared with other biogenic or hard substrates (Pinna et al. 2020). Moreover, as reported from several geographic areas, hydroid richness and abundance can be influenced by features of the living substrate they colonize, e.g., density, texture, and percentage coverage (Van Winkle et al. 2000; Dougherty and Russell 2005; Puce et al. 2005; Castellanos-Iglesias et al. 2018). It is thus expected that changes in abundance and diversity of these macrobenthic taxa may in turn affect the associated hydroid assemblages.

In the current work, we analyzed the hydroid assemblages of two Mediterranean islands belonging to an Italian Archipelago planned to become an MPA. We hypothesize that the features of the substrate may influence the species composition of hydroid assemblages of the considered islands, also in relation to the bathymetric gradient. Particularly, we herein analyzed and compared the hydroid assemblages associated to non-living substrates (i.e., hydroids growing directly on the hard bottom) and living substrates (i.e., hydroids growing on macroalgae) at various depths from 0 to 40 m.

Materials and methods

Study area

The Tuscan Archipelago National Park (TANP, Italy) is located in the Western Mediterranean Sea (Fig. 1a). The Archipelago includes seven main islands (Capraia, Elba, Giannutri, Giglio, Gorgona, Montecristo, and Pianosa) and other smaller islands and rocks between Tuscany and Corsica (IslePark 2021). In the current study, we focused on two islands: Montecristo and Giglio (Fig. 1b, c). The choice was due to the geological features of Montecristo and Giglio since they have a similar mineralogical composition. Both islands are made of granitic rocks (Alvisi et al. 1994; Innocenti et al. 1997), except for Promontorio del Franco in Giglio, which is composed of limestone (Alvisi et al. 1994: 25, Fig. 1, for the mineralogical composition of Giglio). Montecristo is an almost uninhabited, isolated offshore island (Innocenti et al. 1997), established as an Integral Nature Reserve in 1971, and it is presently a Special Protection Area (EEC 1979). Giglio hosts 1500 residents, spread out among three villages (Giglio Castello, Giglio Porto and Campese), with a high flow of tourists during summertime (IslePark 2021).

Sample collection and identification

Samplings were carried out by SCUBA diving June 2019 and June 2020 in Montecristo (TANP permission #00068010) and Giglio islands. Sixteen sites were sampled (eight sites per island, Fig. 1b, c; Online Resource 1). Samples of

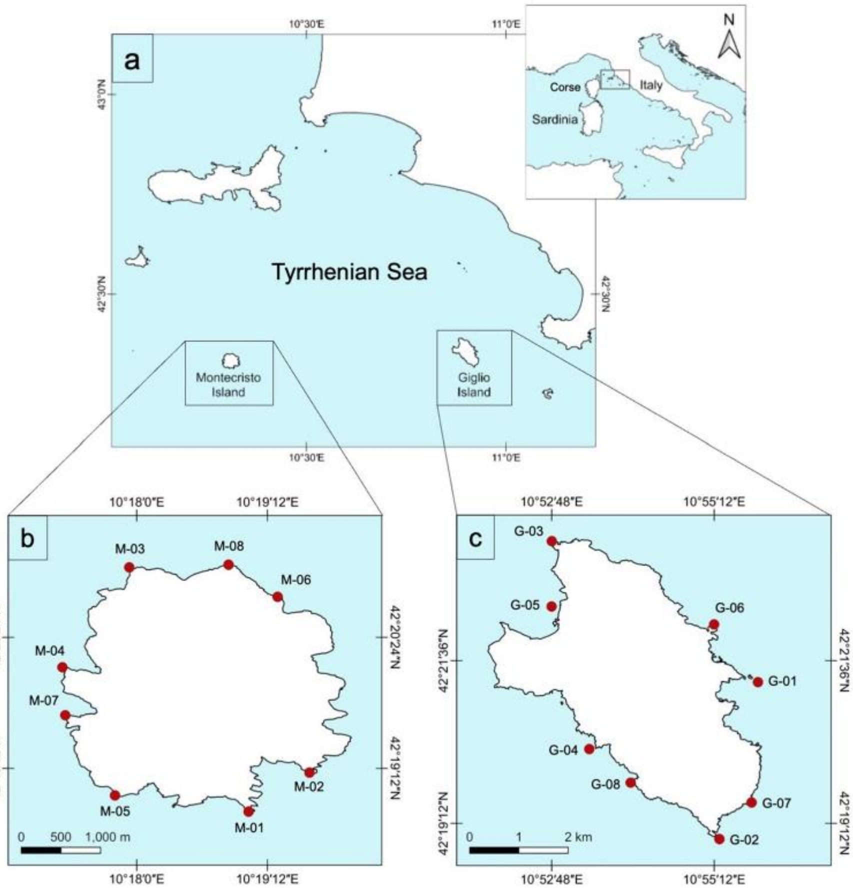

Fig. 1 GIS map showing the: **a** location of the Tuscan Archipelago National Park; **b** study sites at the Montecristo Island; **c** study sites at the Giglio Island (map drawn using the following data sources: Mediterranean coastline—polygon shapefile downloaded from the open source website European Environmental Agency, https://www.eea.europa.eu/data-and-maps/data/eea-coastline-for-analysis-1/gis-data/europe-coastline-shapefile; Tuscan Archipelago and Giglio and Montecristo islands—polygon shapefile downloaded from the open source website OpenToscana, http://dati.toscana.it/dataset?res_format=SHP)

hydroids were collected from hard-bottom assemblages at five depths, every 10 m from 0 to 40 m depth, and the collected material was fixed in ethanol 95%. At each depth, two scuba operators spent about 6 min, covering an area, chosen randomly, of about 20 m × 1 m (20 m^2) each and applying a visually oriented sampling. One operator focused on picking hydroid colonies directly from the hard bottom (non-living substrate, NLS), while the other scuba operator focused on collecting any living substrate (LS) (i.e., macroalgae and sessile metazoans). The visually oriented sampling method was selected since it is considered one of the most effective methods in the detection of the highest numbers of hydroid species in an area, allowing the use of a large sampling unit which more efficiently represents the hydroid diversity and microhabitat diversity in shallow coastal benthic habitats (Boero and Fresi 1986; Puce et al. 2009; Megina et al. 2013; Piraino et al. 2013).

The preserved specimens were examined with a Nikon Eclipse Ni compound microscope and identified at the lowest possible taxonomic level using temporary or permanent preparations on microscope slides (see Gibbons and Ryland 1989 for additional details). Samples and permanent slides are stored in a reference collection deposited in the Zoology Laboratory at DiSVA (Department of Life and Environmental Sciences), Polytechnic University of Marche. The status of each taxon was validated by WoRMS (Horton et al. 2021).

The visually oriented sampling data were expressed as presence/absence of the hydroids at each sampling time at each site.

Photo survey and analysis

Together with the collection of samples, during sampling in June 2019, a photographic survey was conducted at the same depths to analyze the macrophytic taxa characterizing the two islands.

Pictures were taken using a Canon G7X camera, at 34 cm distance from the substrate with the support of a 28 cm × 21 cm frame (0.0588 m^2).

Five pictures per each depth of each site were randomly taken for the characterization of the living substrate. From each photo, macroalgal species were identified at the lower taxonomic level possible, and the percentage coverage of each taxon was calculated using the ImageJ free software (Rasband 2012).

Experimental design and data analyses

The experimental design aiming at testing differences in hydroid assemblages between living and non-living substrates at varying depth included three crossed factors: island (fixed, 2 levels: Montecristo, Giglio), depth (fixed, 5 levels: 0, 10, 20, 30, 40), and substrate (fixed, 2 levels: NLS, LS). Data were analyzed with permutational multivariate analysis of variance (PERMANOVA) (Andeson 2001), using a similarity matrix of presence-absence data (based on Jaccard similarity), using 9999 permutations. Permutational analysis of multivariate dispersions (PERMDISP) was used to test the homogeneity of samples dispersion from their group centroids (Anderson et al. 2008). A posteriori pairwise comparisons were conducted in case of interaction among factors. The analysis of similarity percentages (SIMPER) (Clarke 1993) was used to determine species contributions to the dissimilarity in hydroid assemblages between substrates (NLS vs LS) among depths.

In order to identify the macrophytes explaining the variation of the hydroid assemblages associated to living substrates, non-parametric multiple regression analyses were performed using the DISTLM-forward procedure (distance-based multivariate analysis for a linear model using forward selection); the predictor variables included the percent coverage of macrophytes (cut-off 0.1% of total coverage).

All analyses were carried out using the software PRIMER 7.0.20 (Clarke and Gorley 2015) with the add-on PERMANOVA + (Anderson et al. 2008).

Results

Characterization of the leaving substrate (LS)

Based on the photographic sampling, 34 algal species have been identified (9 Chlorophyta, 11 Phaeophyceae, 1 Prasinodermatophyta, and 13 Rodophyta) (Table 1). In some cases, the identification at species level was not possible due to the small size or impossibility to observe with sufficient detail taxonomically important characters; in these cases, algae were categorized as "Green filamentous algae," "Red filamentous algae," "Crustose brown algae," "Crustose corallines," "Corticated algae," "Algal mat," and "Mixed turf" (Table 1).

At Giglio, the algal composition appeared homogeneous among depths, especially between 0 and 30 m depth (Table 1), being mainly characterized by algal mat and mixed turf; from 10 m depth, green filamentous algae and *Dictyota* spp. appeared abundant (Table 1). At 0 and 10 m depth, *Padina pavonica* and *Acetabularia acetabulum* appeared also moderately abundant, while at 20 and 30 m, *Sporochnus pedunculatus*, *Peyssonnelia* sp., and red filamentous algae were also observed (Table 1). In addition, crustose corallines and cf. *Stilophora tenella* (Table 1) were observed at 30 m depth. At 40 m depth, *Peyssonnelia* sp., crustose corallines, and green and red filamentous algae appeared dominant, together with a small coverage of algal mat and mixed turf (Table 1).

At the Montecristo Island, at 0 m depth, the most abundant macrophytes were crustose corallines, *Dictyota* spp., fucalean brown algae (mainly *Ericaria amantacea*, *Cystoseira* cf. *humilis*, and other *Cystoseira* species), together with the red algae *Gastroclonium clavatum* and mixed turf (Table 1). At 10 and 20 m depths, the main components of the macroalgal community were represented by crustose corallines, *Cystoseira* spp., mixed turf, and *Jania* spp. In addition, at 10 m, the presence of corticated algae, *Halopteris scoparia*, *Dictyota* spp., *Dictyopteris polypodioides*, and red filamentous algae was recorded, while at 20 m, *Sargassum* cf. *vulgare* *Peyssonnelia* sp. and green filamentous algae were also found (Table 1). The community at 30 m depth was mainly characterized by turf-forming algae, *Peyssonnelia* sp., crustose corallines, and *S.* cf. *vulgare*, together with green filamentous algae, cf. *Wrangelia penicillata*, and *S. pedunculatus* (Table 1). At 40 m depth, coralline algae, mainly including crustose corallines, *Jania* spp., and *Lithophyllum stictiforme*, appeared dominant, followed by *H. scoparia*, mixed turf, *Peyssonnelia* sp., the encrusting *Palmophyllum crissum*, and green filamentous algae (Table 1).

Composition and diversity of hydroid assemblages

In all the 16 sampling sites, 16 families (12 Leptothecata and 4 Anthoathecata) and 74 species were identified (Table 1, Online Resource 2). Within the 4 families of Anthoathecata, 12 species were found, 9 of which belonged to the genus *Eudendrium*, whereas among the 12 Leptothecata families, 62 species were identified, most of which belonged to the families Campanulariidae (16), Aglaopheniidae (10), and Haleciidae (9) (Table 2). In some cases (*Antennella* sp., *Campanularia* spp., Campanulariidae sp., *Halecium* spp., *Scandia* spp., Bouganvilliidae sp., Corynidae sp., and *Eudendrium* spp.), identification to species level was impossible due to the small amount of available material, poor material (i.e., specific diagnostical characters not recognizable), or the lack of structures essential for identification (e.g., reproductive structures) (Table 2).

A total of 36 species were found settling on NLS, of which 7 were exclusive of this substrate (*Aglaophenia kirchenpaueri*, *Eudendrium carneum*, *E. merulum*, *E. moulouyensis*, *E. racemosum*, *Halopteris catharina*, *Zygophylax*

Table 1 List of identified microphytes from the photo survey conducted during the sampling activity of June 2019

Taxa	Giglio Island					Montecristo Island				
	0 m	10 m	20 m	30 m	40 m	0 m	10 m	20 m	30 m	40 m
Chlorophyta										
Acetabularia acetabulum (Linnaeus) P.C.Silva 1952	2.09 ± 1.19	2.41 ± 2.09	0.49 ± 0.76	–	–	–	0.006 ± 0.023	0.017 ± 0.037	0.01 ± 0.03	–
Anadyomene stellata (Wulfen) C.Agardh 1823	0.08 ± 0.19	–	–	–	–	–	–	–	–	–
Caulerpa cylindracea Sonder 1845	–	0.77 ± 1.94	0.17 ± 0.27	0.12 ± 0.29	–	–	0.05 ± 0.15	0.44 ± 0.62	0.48 ± 0.89	–
Cladophora prolifera (Roth) Kützing 1843	–	–	0.43 ± 1.59	–	–	–	0.16 ± 0.69	01.48 ± 2.97	0.63 ± 2.07	0.09 ± 0.41
Codium bursa (Linnaeus) C.Agardh 1817	–	–	0.18 ± 0.80	–	–	–	–	–	–	–
Flabellia petiolata (Turra) Nizamuddin 1987	–	–	0.86 ± 1.62	0.65 ± 6.24	1.91 ± 3.16	–	1.08 ± 1.72	0.53 ± 1.04	0.98 ± 2.58	1.59 ± 1.75
Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux 1816	–	–	–	–	0.15 ± 0.31	–	–	–	0.09 ± 0.24	0.05 ± 0.14
Ulva sp.	–	–	–	–	–	0.16 ± 0.72	–	–	–	–
Valonia utricularis (Roth) C.Agardh 1823	–	–	–	–	–	–	–	–	0.015 ± 0.067	–
Green filamentous algae	1.02 ± 1.70	7.33 ± 9.05	34.33 ± 27.81	22.36 ± 20.72	11.56 ± 13.21	–	–	2.37 ± 5.55	4.88 ± 8.38	2.45 ± 5.06
Ochrophyta—Phaeophyceae										
Cystoseira cf. *humilis* Schousboe ex Kützing 1860	–	–	–	–	–	6.5 ± 15.1	–	–	–	–
Cystoseira spp.	–	–	–	–	–	2.19 ± 6.94	24.3 ± 29.3	18.86 ± 31.68	1.18 ± 5.25	–
Dictyota spp.	0.89 ± 1.78	3.23 ± 8.44	3.79 ± 6.58	7.46 ± 14.48	0.33 ± 1.21	27.29 ± 35.75	3.37 ± 7.26	0.99 ± 2.11	1.88 ± 2.81	0.36 ± 1.19
Dictyopteris polypodioides (A.P.De Candolle) J.V.Lamouroux 1809	–	–	0.08 ± 0.34	–	–	0.54 ± 1.81	2.34 ± 5.61	0.62 ± 1.48	0.82 ± 2.49	0.14 ± 0.61
Ericaria amentacea (C.Agardh) Molinari & Guiry 2020	–	–	–	–	–	8.55 ± 23.98	–	–	–	–
Halopteris scoparia (Linnaeus) Sauvageau 1904	–	0.77 ± 2.45	0.48 ± 1.46	–	–	–	3.64 ± 9.34	1.06 ± 3.28	–	11.45 ± 21.22
Padina pavonica (Linnaeus) Thivy 1960	7.39 ± 6.93	8.86 ± 5.84	0.64 ± 1.07	0.01 ± 0.04	–	–	1.9 ± 4.3	0.03 ± 0.09	0.03 ± 0.08	–
Sargassum cf. *vulgare* C.Agardh, nom. illeg. 1820	–	–	–	–	–	–	0.13 ± 0.56	6.53 ± 11.02	8.23 ± 15.07	–
Sporochnus pedunculatus (Hudson) C.Agardh 1817	–	–	2.59 ± 6.63	6.69 ± 12.15	–	–	–	–	2.28 ± 4.67	–
cf. *Stilophora tenella* (Esper) P.C.Silva 1996	–	–	–	3.39 ± 10.49	–	–	–	–	–	–
Taonia atomaria (Woodward) J.Agardh 1848	–	–	–	–	–	0.58 ± 1.93	–	–	–	–
Crustose brown algae	–	–	0.06 ± 0.18	–	0.54 ± 1.21	2.33 ± 7.95	0.07 ± 0.29	–	–	–

Table 1 (continued)

Taxa	Giglio Island					Montecristo Island				
	0 m	10 m	20 m	30 m	40 m	0 m	10 m	20 m	30 m	40 m
Prasinodermatophyta										
Palmophyllum crassum (Naccari) Rabenhorst 1868	–	–	0.24 ± 1.01	0.19 ± 0.47	0.36 ± 0.91	0.38 ± 1.52	0.18 ± 0.78	0.08 ± 0.36	–	2.94 ± 1.91
Rodophyta										
Amphiroa rigida J.V.Lamouroux 1816	–	0.12 ± 0.36	–	–	–	–	0.03 ± 0.11	–	–	–
cf. *Chrysymenia ventricosa* (J.V.Lamouroux) J.Agardh 1842	–	–	–	–	–	–	0.35 ± 1.57	–	–	–
Ellisolandia elongata (J.Ellis & Solander) K.R.Hind & G.W.Saunders 2013	–	–	–	–	–	1.07 ± 3.01	1.07 ± 2.85	–	–	–
Gastroclonium clavatum (Roth) Ardissone 1883	–	–	–	–	–	5.59 ± 9.88	–	–	–	–
Gloiocladia repens (C.Agardh) N.Sánchez & Rodríguez-Prieto 2007	–	–	–	–	–	–	–	–	–	0.11 ± 0.37
cf. *Hypnea musciformis* (Wulfen) J.V.Lamouroux 1813	–	–	–	–	–	–	1.85 ± 3.35	–	–	–
Jania spp.	0.15 ± 0.65	1.4 ± 3.1	2.18 ± 3.94	1.25 ± 1.52	–	0.4 ± 1.4	7.15 ± 20.26	3.75 ± 6.05	0.36 ± 0.84	9.22 ± 20.20
Laurencia cf. *obtusa* (Hudson) J.V.Lamouroux 1813	–	–	–	–	–	0.56 ± 1.41	–	–	–	–
Lithophyllum stictiforme (Areschoug) Hauck 1877	–	–	–	0.85 ± 2.82	0.41 ± 1.81	–	–	–	–	2.77 ± 5.92
Mesophyllum sp.	–	–	–	–	0.21 ± 0.94	–	–	–	0.39 ± 1.77	1.86 ± 4.04
Osmundaria volubilis (Linnaeus) R.E.Norris 1991	–	–	0.04 ± 0.18	0.38 ± 1.68	–	–	–	–	0.4 ± 1.7	0.62 ± 1.89
Peyssonnelia sp.	–	–	2.44 ± 7.59	5.39 ± 9.61	45.21 ± 31.56	–	0.81 ± 1.29	2.46 ± 3.83	14.01 ± 26.29	8.84 ± 13.40
cf. *Wrangelia penicillata* (C.Agardh) C.Agardh 1828	–	–	–	0.89 ± 3.98	1.03 ± 3.84	–	–	1.51 ± 2.85	2.68 ± 4.44	0.02 ± 0.09
Crustose corallines	–	–	1.79 ± 4.73	15.96 ± 23.24	15.74 ± 22.95	33.84 ± 27.92	21.14 ± 24.01	30.43 ± 26.66	12.04 ± 20.94	33.33 ± 28.69
Red filamentous algae	–	0.19 ± 0.83	7.13 ± 16.21	3.13 ± 12.25	7.05 ± 8.82	–	2.88 ± 6.20	1.4 ± 4.4	0.98 ± 3.20	0.52 ± 2.33
Corticated algae	–	–	–	–	0.53 ± 2.37	–	5.31 ± 8.99	0.16 ± 0.69	0.16 ± 0.69	0.92 ± 2.77
Algal mat	56.47 ± 34.13	53.13 ± 33.01	29.29 ± 27.81	15.74 ± 22.83	6.81 ± 17.37	–	–	–	–	–
Mixed turf	20.16 ± 36.48	19.13 ± 33.56	5.01 ± 11.05	16.65 ± 15.91	3.1 ± 7.3	10.61 ± 26.37	15.67 ± 17.08	20.21 ± 21.78	41.67 ± 36.63	10.63 ± 17.41

biarmata), while 73 species were recorded on LS, almost all epiphytes on macroalgae, with 37 exclusive species belonging to the families Aglaopheniidae (1), Campanulariidae (16), Haleciidae (7), Halopterididae (1), Hebellidae (1), Lafoeidae (4), Lovenellidae (1), Plumulariidae (1), Sertulariidae (3), Bouganvilliidae (1), and Cladocorynidae (1) (see Table 1 for species). However, only a few species were observed on other LS represented by sponges and hydroids: in fact, *Orthopyxis crenata*, *Clytia linearis*, and *E. racemosum* were observed on sponges, *Clytia hemisphaerica* and *C. noliformis* on *Eudendrium* spp., and *Filellum serratum* on *Z. biarmata* (Table 2).

Among the investigated depths, most of species were found at 0 m (50), followed by 10 (45) and 20 m (42), thus showing a general decreasing trend with depth (Online Resource 2). Exclusive species were recorded at all depths; in particular, 9 species were only found at 0 m (*Aglaophenia picardi*, *A. pluma*, *Clytia hummelincki*, Corynidae indet., *Eudendrium moulouyensis*, *Halopteris liechtensternii*, *Laomedea angulata*, *Orthopyxis integra*, *Polyplumaria flabellata*), 3 at 10 m (*Calyptospadix cerulea*, *Filellum serpens*, *Mitrocomium cirratum*), 2 at 20 m (*Gonothyraea loveni*, *Halecium liouvillei*), 4 at 30 m (*E. rameum*, *Halecium conicum*, *H. muricatum*, *Sertularella cubica*), and 1 at 40 m (*Z. biarmata*) (Online Resource 2).

Out of the 74 identified species, 45 were recorded at the Giglio Island and 63 at the Montecristo Island. One family (Zygophylacidae) and 9 species were found only at Giglio, while 4 families (Bouganvilliidae, Cladocorynidae, Corynidae, and Lovenellidae) and 30 species were uniquely observed in Montecristo (Table 1). The site with the highest number of species was M-08 (39), followed by M-05 (29), G-06 (25), and M-01, M-07, and G-08 (23) (Online Resource 2). Conversely, G-03 and G-01 were the two sites with the lowest number of species, 7 and 10 respectively (Online Resource 2). Considering the sampling depths, the number of species collected at Montecristo Island was higher than at Giglio Island, except for 20 m depth (Fig. 2). Most species were recorded at 0 and 10 m at Montecristo Island, while decreasing at greater depths (Fig. 2). At Giglio Island, the number of species was almost constant from 0 to 20 m depth, while at 30 m and 40 m was lower (Fig. 2). No hydroids were recorded at various sites at 30 and 40 m depths (Online Resource 2).

Except for species belonging to the families Aglaopheniidae, Eudendriidae, Halopterididae, Plumulariidae, Sertulariidae, and Zygophylacidae, which were found on NLS almost at all depths at Giglio Island, the representative species of the other identified families were mainly found on LS (Table 2). Moreover, at 40 m, species belonging to Halopterididae (*Antennella* spp.) were found as epiphytes on calcareous algae (Table 2). Alternatively, at Montecristo Island, almost all species were growing on macroalgae, especially at 0 m (41 species) and 10 m (35), while only a few were recorded on NLS, especially at 30 and 40 m (Table 2).

PERMANOVA revealed a complex scenario with assemblages varying between the two analyzed substrates depending on the depth and the considered island (Table 3). The pairwise comparison highlighted differences in the hydroid assemblages between NLS and LS at 0, 10, and 30 m at Montecristo and at 0, 10, and 40 m depth at Giglio (Table 4).

PERMDISP indicated significant dispersion in the analyzed data ($F_{19,140} = 4.4678$; $p = 0.0006$). The highest average dispersion was observed for LS samples at Montecristo at all depths, revealing a general higher beta diversity associated to this substrate in this island compared to the rest of the samples. At Giglio, the average dispersion appeared higher for NLS samples compared to LS ones. Considering the NLS, average dispersion was particularly high from 0 to 20 m depth at Giglio compared to Montecristo, while at 30 and 40 m depth, the average values appeared similar between the two islands (Table 5).

The SIMPER analysis revealed high dissimilarity in the assemblages associated to the two substrates at all depths with values ranging from 98.87% at 20 m to 96.26% at 0 m (Online Resource 3). Dissimilarities at 0 m were mainly related to *Clytia hemisphaerica* (7.96%), *Aglaophenia octodonta* (7.62%), and *Clytia linearis* (7.14%), at 10 m to *C. linearis* (14.08%) and C. *hemisphaerica* (7.47%) (Online Resource 3). Dissimilarities between NLS and LS at 20 and 30 m were mainly explained by *C. linearis* (10.24%), *Scandia* spp. (6.68%), *Filellum serratum* (5.69%), and *Antennella siliquosa* (12.36%), *C. linearis* (8.95%), and *Antennella secundaria* (7.91%) respectively (Online Resource 3), while at 40 m by *F. serratum* (12.50%), *A. secundaria* (11.83%), and *C. linearis* (9.19%). A large part of the species contributed to between-substrate dissimilarity at the analyzed depths being absent in one of the two (Online Resource 3).

The DISTLM sequential test revealed that, when considering the percent coverage of each algal taxon, altogether, they explained 87.5% of the observed variation in the hydroid assemblages. However, considering the algal taxa significantly related to the hydroid assemblages only (7 over 31), they explained 36.8% of the variation (Table 6). In particular, *Cystoseira* spp. ranked first among predictor variables explaining 7.7% of the observed variation, followed by *Gastroclonium clavatum* (5.6%), corticated algae (5.7%), *Cladophora prolifera* (5.1%), crustose brown algae (4.9%), crustose corallines (4.1%), and cf. *Hypnea musciformis* (3.7%).

Table 2 List of identified hydroids collected at five depths (0, 10, 20, 30, 40 m) on non-living substrates (NLS) and living substrates (LS) of Giglio and Montecristo islands

Taxa	Giglio Island															Montecristo Island														
	0 m			10 m			20 m			30 m			40 m			0 m			10 m			20 m			30 m			40 m		
	NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS	
		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT
Leptothecata																														
Aglaopheniidae																														
Aglaophenia acacia Allman, 1883																	X						X		X					
Aglaophenia elongata Meneghini, 1845																						X	X		X	X		X		
Aglaophenia harpago Schenck, 1965					X		X			X							X			X										
Aglaophenia kirchenpaueri (Heller, 1868)	X						X																							
Aglaophenia lophocarpa Allman, 1877																	X						X			X		X		
Aglaophenia octodonta Heller, 1868	X			X												X	X		X	X										
Aglaophenia picardi Svoboda, 1979																	X													
Aglaophenia pluma (Linnaeus, 1758)																X	X													
Aglaophenia tubiformis Marktanner-Turneretscher, 1890	X			X			X										X		X	X			X		X					
Aglaophenia tubulifera (Hincks, 1861) *				X													X			X										
Campanulariidae																														
Campanularia hincksii Alder, 1856 *																				X									X	
Campanularia spp.																				X										
Campanularia volubilis (Linnaeus, 1758) *																	X									X				
Campanulariidae sp.																	X						X							
Clytia gracilis (Sars, 1850) *		X						X									X													
Clytia hemisphaerica (Linnaeus, 1767)		X			X			X			X	X		X			X			X			X			X			X	
Clytia hummelincki (Leloup, 1935)																	X													
Clytia linearis (Thorneley, 1900)		X			X			X			X	X		X			X			X			X			X			X	
Clytia noliformis (McCrady, 1859) sensu Calder, 1991 *		X			X							X		X			X			X										
Clytia paulensis (Vanhöffen, 1910)		X			X			X																						

Table 2 (continued)

Taxa	Giglio Island															Montecristo Island														
	0 m			10 m			20 m			30 m			40 m			0 m			10 m			20 m			30 m			40 m		
	NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS	
		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT
Gonothyraea loveni (Allman, 1859) *								X																						
Laomedea angulata Hincks, 1861		X																												
Laomedea calceolifera (Hincks, 1871)																	X						X							
Laomedea flexuosa Alder, 1857 *																	X												X	
Obelia bidentata Clark, 1875																				X									X	
Obelia dichotoma (Linnaeus, 1758)					X			X															X			X			X	
Orthopyxis crenata (Hartlaub, 1901)									X								X			X	X					X				
Orthopyxis integra (MacGillivray, 1842)																	X													
Haleciidae																														
Halecium conicum Stechow, 1919 *																							X			X				
Halecium delicatulum Coughtrey, 1876 *				X	X		X	X									X			X			X							
Halecium liouvillei Billard, 1934 *																							X							
Halecium muricatum (Ellis & Solander, 1786) *																										X				
Halecium nanum Alder, 1859		X			X												X						X							
Halecium petrosum Stechow, 1919 *	X	X																		X						X				
Halecium pusillum Sars, 1856		X															X						X			X				
Halecium sessile Norman, 1867 *																							X							
Halecium spp.																													X	
Halecium tenellum Hincks, 1861 *																							X			X				
Halopterididae																														
Antennella ansini Peña Cantero & García Carrascosa, 2002 *							X							X			X		X	X			X							
Antennella secundaria (Gmelin, 1791) *				X						X				X									X			X		X	X	

Table 2 (continued)

Taxa	Giglio Island															Montecristo Island														
	0 m			10 m			20 m			30 m			40 m			0 m			10 m			20 m			30 m			40 m		
	NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS	
		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT
Antennella siliquosa (Hincks, 1877) *	X			X			X			X	X		X	X			X						X		X	X		X	X	
Antennella sp.														X																
Halopteris catharina (Johnston, 1833) *	X			X																										
Halopteris diaphana (Heller, 1868) *																	X			X					X	X				
Halopteris liechtensternii (Marktanner-Turneretscher, 1890) *	X			X													X													
Polyplumaria flabellata Sars, 1874 *																	X													
Hebellidae																														
Scandia gigas (Pieper, 1884) *																	X			X			X							
Scandia spp.					X			X			X						X			X			X			X				
Kirchenpaueriidae																														
Kirchenpaueria pinnata (Linnaeus, 1758)	X	X			X												X			X										
Lafoeidae																														
Acryptolaria conferta (Allman, 1877) *		X			X			X			X			X			X			X			X			X			X	
Filellum disaggregatum Peña Cantero, García-Carrascosa & Vervoort, 1998 *								X						X																
Filellum serpens (Hassall, 1848) *																				X										
Filellum serratum (Clarke, 1879) *					X			X			X			X	X					X										
Lovenellidae																														
Mitrocomium cirratum Haeckel, 1879 *																				X										
Phylactothecidae																														
Hydrodendron mirabile (Hincks, 1866) *								X					X	X						X										
Plumulariidae																														
Plumularia obliqua (Johnston, 1847)	X			X			X			X							X								X					
Plumularia pulchella Bale, 1882 *[b]																	X													

Table 2 (continued)

Taxa	Giglio Island															Montecristo Island														
	0 m			10 m			20 m			30 m			40 m			0 m			10 m			20 m			30 m			40 m		
	NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS	
		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT
Plumularia setacea (Linnaeus, 1758)				X																			X					X		
Sertulariidae																														
Dynamena disticha (Bosc, 1802)	X			X			X										X		X	X						X			X	
Salacia desmoides (Torrey, 1902) *	X			X						X										X			X						X	
Sertularella crassicaulis (Heller, 1868) *																	X									X			X	
Sertularella cubica García Aguirre & Gonzalez, 1980 *																										X				
Sertularella distans (Lamouroux, 1816) *				X			X										X			X										
Sertularella ellisii (Deshayes & Milne Edwards, 1836)	X			X			X										X			X			X		X	X				
Sertularella polyzonias (Linnaeus, 1758)														X												X				
Zygophylacidae																														
Zygophylax biarmata Billard, 1905 *													X																	
Anthoathecata																														
Bougainvilliidae																														
Bougainvilliidae sp.																				X										
Calyptospadix cerulea Clarke, 1882 *																				X										
Cladocorynidae																														
Cladocoryne floccosa Rotch, 1871 *																	X			X									X	
Corynidae																														
Corynidae sp.																	X													
Eudendriidae																														
Eudendrium armatum Tichomiroff, 1890	X			X			X			X				X						X					X					
Eudendrium capillare Alder, 1856							X									X	X			X						X				
Eudendrium carneum Clarke, 1882 *	X																											X		
Eudendrium glomeratum Picard, 1952																			X	X						X			X	

Table 2 (continued)

Taxa	Giglio Island															Montecristo Island														
	0 m			10 m			20 m			30 m			40 m			0 m			10 m			20 m			30 m			40 m		
	NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS		NLS	LS	
		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT		MA	OT
Eudendrium merulum Watson, 1985 *	X						X																							
Eudendrium moulouyensis Marques, Peña Cantero & Vervoort, 2000 *	X						X																							
Eudendrium racemosum (Cavolini, 1785)				X																X	X					X				
Eudendrium rameum (Pallas, 1766) *										X																				
Eudendrium ramosum (Linnaeus, 1758)	X			X																			X							
Eudendrium spp.	X													X			X			X						X		X		

MA, macroalgae; *OT*, other metazoans; *new records for the sampled area

Discussion

All species of hydroids collected in this study were already known for the Mediterranean Sea and are typical of rocky bottom assemblages (Bouillon et al. 2006; Morri et al. 2009; Puce et al. 2009; Yilmaz et al. 2020). Following the separation of the Italian seas into biogeographic areas, we compared our results with the hydroid species listed for Area 2 by Gravili et al. (2008), an area defined as "the coastline of Sardinia (and Corsica) and the north Tyrrhenian Sea from Piombino and including the Gulf of Gaeta, belonging to the northern section of the central-western area of the Mediterranean" (Relini 2008, page VI). In total, 32 species found in our study were already listed for Area 2 while 40 are new records (Table 1). Six of the species identified in our study (*Clytia hummelincki*, *Clytia linearis*, *Filellum serratum*, *Plumularia pulchella*, *Eudendrium carneum*, *Eudendrium merulum*) are considered non-indigenous species (NIS) listed by Gravili et al. (2013), with *C. hummelincki* and *C. linearis* classified as "invasive" and the other four species as "established" (Table 1). These new data contribute to fill a gap of knowledge about the hydroid distribution in the studied area, adding new records, most of which already reported and well-known in the surrounding areas (see Gravili et al. 2008).

From the analysis conducted by Iacono et al. (2013, Fig. 1, page 1711) on the surface currents of the Tyrrhenian Sea, it can be observed that water masses move from the southern part to the north, promoting larval dispersal toward the herein studied area (Cowen and Sponaugle 2009). Although in hydroids, and most cnidarians in general, the planula is considered only partly suitable for long-range dispersal (Boero and Bouillon 1987); longer distances can be reached by medusa stages, free-living fragments floating in the water column, or sessile stages settling on rafting objects (Aliani and Meloni 1999; Choong and Calder 2013). Therefore, the species composition of the studied area could be influenced by the assemblages present in the southern adjacent areas. A similar pattern was observed for the NIS recorded at the two islands. Apart from *P. pulchella*, for which there was no previous records in the surrounding areas, and *C. hummelincki* and *C. linearis*, which were already listed for the Area 2 (Gravili et al. 2008), the other NIS (*F. serratum*, *E. carneum*, and *E. merulum*) could have reached the islands via water currents. The two "invasive" NIS, *C. hummelincki* and *C. linearis*, originally described from the West Indies (Caribbean Sea, Atlantic Ocean) and New Britain (Papua New Guinea, Indo-Pacific) respectively, are now widespread in the Mediterranean (Gravili et al. 2013, Fig. 3 page 52 and Appendix 1 page 58) and easily found in many areas of the Italian seas (Gravili et al. 2008).

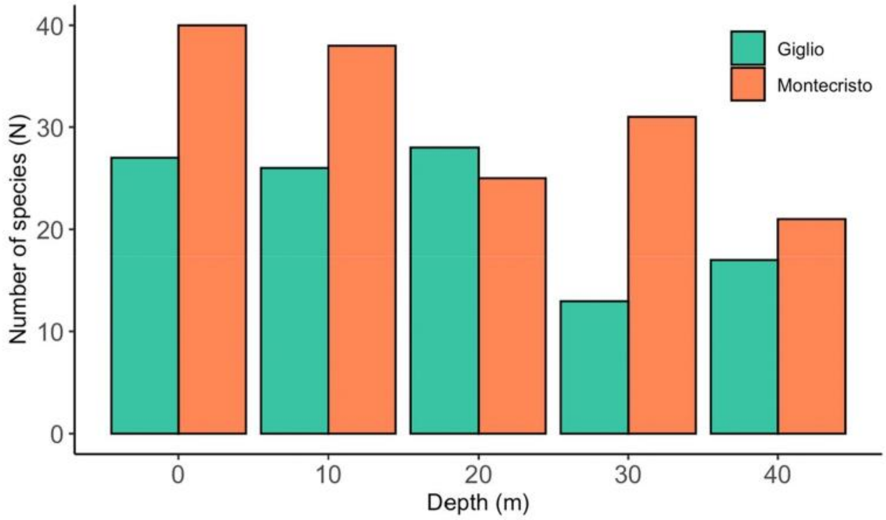

Fig. 2 Number of species per sampled depth per island

Table 3 Results of the three-way PERMANOVA

Source	df	SS	MS	Pseudo-*F*	*P*(perm)	Up	*P*(MC)	Estimate	Sq.root
Island (Is)	1	4643	4643	1.9736	**0.0138**	9885	**0.0165**	28.631	5.3508
Substrate (Su)	1	19504	19504	8.2907	**0.0001**	9889	**0.0001**	214.39	14.642
Depth (De)	4	17631	4407.7	1.8736	**0.0002**	9812	**0.0004**	64.225	8.014
Is × Su	1	20352	20352	8.6515	**0.0001**	9900	**0.0001**	450	21.213
De × Is	4	12726	3181.5	1.3524	**0.0348**	9811	**0.0405**	51.813	7.1982
De × Su	4	11048	2762	1.1741	0.1395	9801	0.1561	25.594	5.059
Is × De × Su	4	15381	3845.2	1.6345	**0.0014**	9772	**0.0035**	186.59	13.66
Residuals	140	329350	2352.5					2352.5	48.502
Total	159	430630							

Analysis of hydroid assemblage variation considering islands, depths, and the nature of the substrate. *df*, degrees of freedom; *SS*, sum of squares; *MS*, mean squares; *Pseudo-F*, *F*-ratio; *P(perm)*, probability; *Up*, unique perms; *P(MC)*, probability with the Monte Carlo method; *Sq.root*, square root. Significant *p*-values ($p < 0.05$) are given in bold

Table 4 Results of the a posteriori pairwise comparisons within depths and islands depending on the nature of the substrate (LS vs NLS)

Groups	Montecristo				Giglio			
	t	*P*(perm)	Uniqueperms	*P*(MC)	*t*	*P*(perm)	Up	P(MC)
0 LS vs NLS	1.6251	**0.0294**	924	**0.0347**	1.9527	**0.0001**	3971	**0.0009**
10 LS vs NLS	2.5151	**0.0002**	693	**0.0001**	1.7318	**0.0003**	3111	**0.0066**
20 LS vs NLS	1.3881	**0.0384**	56	0.0962	1.0969	0.2017	367	0.2924
30 LS vs NLS	1.7929	**0.0043**	1627	**0.0099**	1.2626	0.1326	66	0.1605
40 LS vs NLS	1.0161	0.4006	491	0.3997	1.7744	**0.003**	1061	**0.0107**

t, *t*-test; *P(perm)*, probability; *Up*, unique perms; *P(MC)*, probability with the Monte Carlo method. Significant *p*-values ($p < 0.05$) are given in bold

Among the 74 identified species, *Acryptolaria conferta*, *Aglaophenia octodonta*, *Antennella secundaria*, *Antennella siliquosa*, *C. hemisphaerica*, *C. linearis*, *Dynamena disticha*, and *Scandia* spp. were the most common among sites and sampling depths (see Online Resource 2). In fact, these species have been recognized in the literature as very frequent and abundant hydroid components in the benthic communities of Atlanto-Mediterranean rocky bottoms (Boero and Fresi 1986; Svoboda and Cornelius 1991; Bouillon et al. 2004, 2006; Boero et al. 2005; Moura et al. 2012; Gravili et al. 2013).

Benthic suspension feeders can present annual patterns dynamics characterized by an increase in activity and in

Table 5 Results of the PERMDISP analyses at the different depths (0, 10, 20, 30, 40 m) in the analyzed islands both on non-living substrate (NLS) and living substrate (LS)

	Group	NLS			LS		
		Size	Average	SE	Size	Average	SE
Montecristo	0	8	35.926	7.0859	8	53.706	1.2836
	10	8	26.573	6.2897	8	54.883	0.85059
	20	8	20.192	8.6538	8	51.132	3.2192
	30	8	38.755	4.1872	8	53.324	1.274
	40	8	43.983	3.5641	8	47.642	3.2447
Giglio	0	8	51.78	1.7115	8	43.947	1.4184
	10	8	52.137	2.0887	8	41.505	3.5471
	20	8	45.502	5.3154	8	42.976	4.5444
	30	8	38.456	4.8309	8	34.232	6.1119
	40	8	45.147	3.0863	8	37.132	4.4565

SE, standard error

secondary production during spring and summer or by a variation in the abundance and species composition of assemblages (Coma et al. 2000; Bavestrello et al. 2006). In many seas worldwide (e.g., Calder 1990; Migotto et al. 2001; Cunha and Jacobucci 2010; Petrova et al. 2011; Ajala-Batista et al. 2020) and, specifically, in the Mediterranean, most of benthic hydroid species exhibit a seasonal pattern, causing continuous changes in the composition of hydroid populations between winter and summer (Boero 1984; Coma et al. 2000; Bavestrello et al. 2006). Water temperature is the main driver of these variations, mostly influencing the bathymetric range between 5 and 20 m depth, since in the intertidal zone, only a few specialized species are present (Boero 1984; Bavestrello et al. 2006). However, also light, salinity, and sedimentation can play an important role in hydroids' seasonality (Bavestrello et al. 2006). Since the checklist of Gravili et al. (2008) gives information on hydroids regardless seasonality, possible comparison of the species found in the same season of our sampling (June) was done with investigations conducted in adjacent areas. The only documents found for the southern Tyrrhenian Sea were related to deep assemblages (Bo et al. 2011, 2012), marine caves (Boero 1985), or they were conducted in a different season (Boero and Fresi 1986; Piraino and Morri 1990). While from the northern areas, e.g., northern Tyrrhenian and Ligurian seas, only one paper could be considered, that is, Puce et al. (2009), which analyzed the changes in the hydroid assemblages' composition from the Portofino Promontory between 1980 and 2004. Since the community was found very different between the studied years, only data from 2004 were considered for the comparison. In total, 22 species belonging to the families Aglaopheniidae (3 species), Campanulariidae (7 species), Haleciidae (1 species), Halopterididae (1 species), Hebelliidae (1 species), Lafoeidae (1 species), Sertulariidae (3 species), and Eudendriidae (5 species) were common between the two datasets in June. Basing on the seasonality given by Bouillon et al. (2004), among the 22 common species, 14 (*Aglaophenia kirchenpaueri*, *A. octodonta*, *Aglaophenia picardi*, *A. secundaria*, *Campanularia hincksii*, *C. hemisphaerica*, *C. linearis*, *D. disticha*, *Eudendrium capillare*, *E. ramosum*, *Halecium delicatum*, *Obelia dichotoma*, *Orthopyxis integra*, and *Sertularella polyzonias*) can be considered perennial. Moreover, the seasonality recorded in various species probably evolved to avoid the competition for substrate (Bavestrello et al. 2006). In fact, during the spring/summer period, hydroids are usually influenced by the presence of large algae and by the seasonal turnover of the frond: during the winter season, large hydroid colonies (e.g., species belonging to the genus *Eudendrium*) can be found growing directly on bare rock frequently forming facies, while in the spring/summer, are usually found as epiphytes (Boero 1984). This is in line with our observation conducted in June, where most of species were found as epiphytes on a flourishing macroalgal community (see Table 1).

The role of living organisms (i.e., habitat-forming species) in influencing the local diversity has been largely investigated in the literature (Rossi et al. 2017; Piazzi et al. 2018). Our results showed how hydroid assemblages deeply differ in terms of identity and number of species between living substrate, in our case represented by macroalgae, and non-living substrate, as bare rock, in agreement with previous studies (Pinna et al. 2020). In fact, macroalgae can be seen as a continuous available space for the settlement of opportunistic hydroid species (i.e., small size, extensive hydrorhizal growth, seasonality) (Coma et al. 1992; Cunha and Jacobucci 2010). This is the case of species belonging to some hydrozoan families—e.g., Campanulariidae, Haleciidae, and Lafoeidae—which, in the current study, have been exclusively found on living substrates, accordingly to other works (e.g., Coma et al. 1992; Faucci and Boero 2000). On the other hand, species identified as exclusive representatives of the non-living substrate are all considered in the literature as k-strategists (i.e., large colony size, loss of

Table 6 Results of DISTLM-forward analysis

Variable	SS (trace)	Pseudo-*F*	*P*	Prop	Cumul
Cystoseira spp.	7609.20	3.15	**0.001**	0.077	0.077
Gastroclonium clavatum	5569.50	2.39	**0.012**	0.056	0.133
Corticated algae	5613.60	2.51	**0.011**	0.057	0.189
Cladophora prolifera	5105.00	2.37	**0.018**	0.051	0.241
Dictyota spp.	4398.80	2.11	0.054	0.044	0.285
Mesophyllum sp.	3755.30	1.84	0.091	0.038	0.323
Dictyopteris polypodioides	3635.10	1.83	0.117	0.037	0.359
Ellisolandia elongata	3481.10	1.79	0.173	0.035	0.394
Crustose brown algae	4854.80	2.64	**0.011**	0.049	0.443
Crustose corallines	4099.70	2.32	**0.019**	0.041	0.485
cf. *Hypnea musciformis*	3641.90	2.15	**0.048**	0.037	0.521
Palmophyllum crassum	2861.50	1.73	0.122	0.029	0.550
Cystoseira cf. *humilis*	2800.20	1.74	0.129	0.028	0.578
Peyssonnelia sp.	2322.30	1.47	0.176	0.023	0.602
cf. *Wrangelia penicillata*	1932.10	1.23	0.275	0.019	0.621
Jania spp.	1806.20	1.16	0.333	0.018	0.639
Osmundaria volubilis	1979.20	1.29	0.270	0.020	0.659
cf. *Stilophora tenella*	1735.40	1.14	0.341	0.017	0.677
Lithophyllum stictiforme	1743.20	1.15	0.342	0.018	0.694
Ericaria amentacea	1404.50	0.92	0.443	0.014	0.709
Sargassum cf. *vulgare*	1406.20	0.92	0.469	0.014	0.723
Padina pavonica	1387.70	0.90	0.481	0.014	0.737
Acetabularia acetabulum	1789.90	1.18	0.316	0.018	0.755
Algal mat	1868.50	1.25	0.282	0.019	0.773
Caulerpa cylindracea	1581.00	1.06	0.378	0.016	0.789
Mixed turf	2114.20	1.46	0.191	0.021	0.811
Sporochnus pedunculatus	1277.40	0.88	0.483	0.013	0.824
Red filamentous algae	944.70	0.63	0.726	0.010	0.833
Flabellia petiolata	1172.30	0.76	0.575	0.012	0.845
Green filamentous algae	1311.30	0.84	0.535	0.013	0.858
Halopteris scoparia	1669.90	1.08	0.380	0.017	0.875

Variables: percent coverage of each algal taxa (5 cut-off 0.1% of total cover). *SS*, sum of squares; *Pseudo-F*, F-ratio; *P*, probability; *Prop.*, proportion of explained variation; *Cumul.*, cumulative proportion of explained variation. Variables significantly contributing to explain variation of hydroid assemblages ($p < 0.05$) are in bold

medusa stage, long-term settlement, often perennial), such as species representative of the families Aglaopheniidae, Halopterididae, and Eudendriidae (Cornelius 1990; Leclère et al. 2009). As suggested by various authors (Riedl 1966; Boero 1984; Puce et al. 2008), the epibiotic habit of hydroids can be seen as an adaptation to face the competition for the space when the non-living substrate is already occupied by other organisms, since they compete poorly with other benthic sessile species (Faucci and Boero 2000). The relationship between hydroids and other organisms can range from a simple epibiosis to a mutualistic symbiosis; the association of hydroids with other metazoans, such as sponges, cnidarians, molluscs, and bryozoans, is generally highly specific and is frequently characterized by morphological and behavioral adaptations (Puce et al. 2008). Alternatively, hydroids living on algae do not show any particular host-specificity, but they can be found both on biogenic and/or non-biogenic substrates, even artificial ones (Boero 1981; Morri and Bianchi 1999; Faucci and Boero 2000; Megina et al. 2016; Pinna et al. 2020), and algal selectivity is known only for a few species (Morri and Bianchi 1999), such as *Zanclea alba*, *Pelagiana trichodesmiae*, and *Fraseroscyphus sinuosus* (Puce et al. 2008). Even though in this study most of identified species were recorded exclusively as epiphytes (Table 1), these hydroids are considered substrate generalists and are also typical inhabitants of hard rocky bottom substrates (Boero and Fresi 1986; Faucci and Boero 2000; Puce et al. 2009). Nonetheless, the high complexity of the algal coverage encountered in the analyzed islands possibly explains the observed pattern. In fact, the dominant erected and corticated algae (see Table 1) represent a complex and heterogeneous substrate suitable for many hydroid species (Boero and Fresi 1986; Faucci and Boero 2000; De Oliveira and Marques 2007). Verily, among the significant variables identified by the DISTLM analysis, most of them (*Cystoseira* spp., corticated algae, *Cladophora prolifera*, crustose corallines, and cf. *Hypnea musciformis*) are known to be frequently colonized by hydroids (De Oliveira and Marques 2007; Calder 2019), while no records have been reported for *Gastroclonium clavatum* and crustose brown algae. The role of *Cystoseira* spp. as top algal predictor in the variation of the hydroid assemblage is an interesting result in agreement with the well-known environmental significance of these macroalgae. Fucalean brown algae belonging to this genus (and the closely related genera *Ericaria* and *Gongolaria*) are among the most important ecosystem engineers in the shallow sublittoral zone of Mediterranean rocky shores; their canopies are well-known repositories of a great biological diversity (Cebrian et al. 2021; Tamburello et al. 2021). Due to their high sensitivity to all forms of anthropogenic disturbance, they are typically associated with pristine environments and are used as indicators of high environmental quality (Ballesteros et al. 2007; Orfanidis et al. 2021). It is therefore not surprising to find a higher abundance of these seaweeds in Montecristo, which among the two islands is the one characterized by lower impacts.

Our results are also in line with the observations made in other geographical areas (e.g., Apulian Sea (Italy), São Sebastião Channel (Brazil), South Water Cut (Belize), Taiwan) where most or all recorded species belonged to the order Leptothecata (Kaehler and Hughes 1992; Fraschetti et al. 2002; De Oliveira and Marques 2011; Tseng et al. 2014). In fact, the lower frequency of Anthoathecata as epiphytes was already explained by Riedl (1966) and Faucci and Boero (2000), who highlighted the fact that this group results more sensitive to the mechanical stress caused by strong water movement, due to the lack of rigid thecae.

The evident difference between NLS and LS in terms of species composition and number of hydroids is also observed in the formal statistical analyses. The average dissimilarity is high at all considered depths and similar among them. These results can be explained by the fact that the flourishing macroalgal community observed can be colonized by a multitude of hydroid species (Morri and Bianchi 1999; Faucci and Boero 2000; De Oliveira and Marques 2007, 2011), and that different portion of an algal thallus can host different assemblages (Fraschetti et al. 2006). An important contribution in the within group dissimilarity at the various depths was given by species exclusively found on one of the two considered substrates (see Online Resource 3).

In general, significant differences between the two substrates tend to characterize the analyzed area albeit at some depth this pattern is not clearly observed. This might be due to unchecked environmental factors related to depth. In fact, as underlined by many authors, hydroid assemblages are usually characterized by a vertical zonation, mainly related to light and water movement (Boero and Fresi 1986; Chaplygina 2006; Di Camillo et al. 2008; Puce et al. 2009; González-Duarte et al. 2014). Water movement has been recognized as an important factor, especially in shallow waters, inducing deep morphological and size adaptations of the colonies (i.e., small size, perisarc annulations), and changes in their orientation (Boero 1984; Gili and Hughes 1995). Light is another environmental factor varying with depth (both in terms of amount and spectral composition), affecting the spatial distribution of hydroids (Boero 1984; Gili and Hughes 1995). In fact, large hydroids are usually less abundant in shallow and well-lit zones where algae are the predominant organisms on the substrate (Gili and Hughes 1995). Various hydroids, such as some *Aglaophenia* species, *Eudendrium moulouyensis*, and *Halecium nanum*, also found in this study, host associated zooxanthellae, limiting their bathymetric distribution (Boero 1984; Gili and Hughes 1995; Di Camillo et al. 2008). As pointed out by González-Duarte et al. (2014) for Chafarinas Islands, some species occur in a narrow bathymetric range. This pattern is evident also in our study, as showed by statistical analysis (Table 3), and with the exclusive presence of some hydroids at specific depths: in fact, 9 species were limited to the shallower depth (0 m), while from 1 to 4 species were exclusively distributed at 10, 20, 30, and 40 m depth.

In addition, the analyzed pattern of hydroid assemblages indicates an interaction between the factors substrate and depth, also depending on the island considered, and focusing on islands' features might help to better interpret the results. The differences recorded between the hydrozoan assemblages of Montecristo and Giglio can be mainly referred to differences in the substrate on which hydroids settle. The high average dispersion recorded on LS at the Montecristo Island can be considered a proxy of beta diversity probably related to the natural variability of this pristine and protected island especially if associated to its species richness, and high diverse substrates (García Charton et al. 2000), in this case represented by a well-developed macroalgal community (see Table 1). In fact, Montecristo resulted to be mainly characterized by erected macroalgae especially at shallower depths (e.g., *Ericaria amentacea*, *Cystoseira* spp., *Dictyota* spp., *Acetabularia acetabulum*, *Flabellia petiolata*, cf. *Hypnea musciformis*, *Osmundaria volubilis*, *Padina pavonica*, and *Sargassum* cf. *vulgare*), which have been demonstrated to host a variety of hydroid species (Morri and Bianchi 1999; Faucci and Boero 2000; Fraschetti et al. 2006; De Oliveira and Marques 2007, 2011; Calder 2019). On the other hand, the high average dispersion recorded on the non-living substrate of Giglio, especially at the shallower depths

(0–20 m), as well as the low number of species found compared to Montecristo (see Online Resource 2), may be an indication of high fragmentation in assemblages. In fact, a more homogenous macroalgal community was observed in Giglio's sites, where most of the substrate was covered by algal mat and/or by undefined turf-forming algae (see Table 1), or where only bare rock was present.

In conclusion, this work is the first focusing on the hydroids of the TANP, providing additional insights on the Tuscan Archipelago benthic communities, and it represents a major advancement compared to the fragmentary and scattered information previously available, based mostly on investigations not specifically targeting hydroids. Since many hydrozoan species present seasonal cycles (Bavestrello et al. 2006; Bouillon et al. 2006), we stress the need of further studies extended to other seasons to have a better knowledge in terms of temporal variation and to create a baseline useful to assess future changes due to Mediterranean warming (Puce et al. 2009). Finally, our data may contribute to laying the groundwork for the policymakers in the establishment of the TANP as a future MPA, following the Italian legislation (L. 979/82 art.31 and L. 394/91 art.36) and in agreement with the EU Biodiversity Strategy to 2030 (EC 2020).

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Declarations

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PAPER 2

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Unravelling the sponge diversity of the Tuscan Archipelago National Park (Tyrrhenian Sea, Italy)

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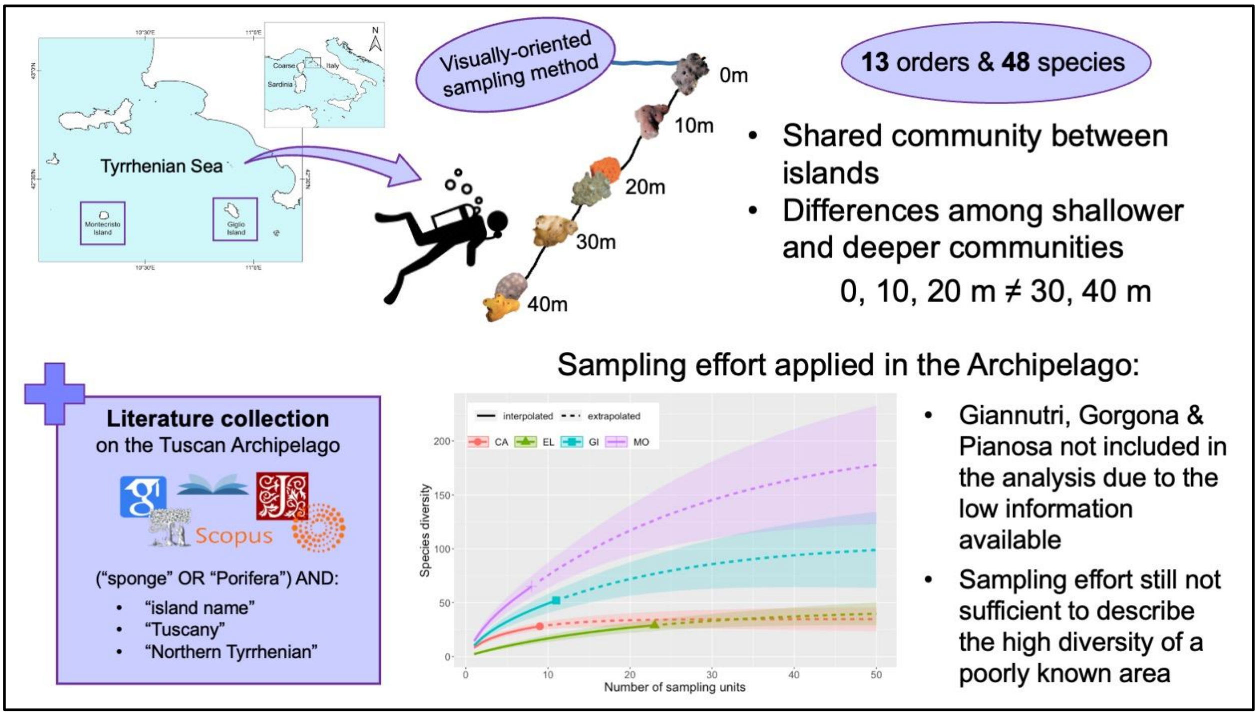

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Unravelling the sponge diversity of the Tuscan Archipelago National Park (Tyrrhenian Sea, Italy)

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Abstract
Porifera are considered key components of benthic communities, being typical inhabitants of both shallow and deep waters, from the mesolittoral to the bathyal zone. Although sponges are one of the most common organisms in Western Mediterranean ecosystems, in many areas the knowledge on this taxon is still very limited. In the current study, we aim to analyse and compare the sponge communities characterising the hard bottoms of two islands, Montecristo and Giglio, belonging to the Tuscan Archipelago National Park (TANP, Italy), in relation to a bathymetric gradient. A total of 340 samples were collected, 13 orders and 48 species (46 Demospongiae and 2 Calcarea) identified, with *Chondrosia reniformis* representing the most recorded species, being found at all sites at almost all depths. Depth was the only significant factor in the observed pattern, while the sponge community is shared between the two Islands. In addition, our data were analysed together with data available in the literature on sponges of the Tuscan Archipelago, to assess the sampling effort. Among all the Islands of the Archipelago, Giglio and Montecristo showed the higher mean species diversity. Nonetheless, the sampling effort resulted not sufficient for each Island and future studies targeting Porifera are needed. Our work is the first summarising the sponge diversity of the entire Tuscan Archipelago, and one of the few focussing entirely on the sponge fauna. These results provide new important information on the area of the TANP, aimed to become a future Marine Protected Area, adding new records, and highlighting the high diversity of a poorly known area.

Keywords: *Porifera, sampling effort, western mediterranean, MPA*

Introduction

Porifera are considered key components in many benthic ecosystems (Bell 2008), supporting rich biodiversity levels and, in many cases, representing a secondary substrate for a specialized associated fauna (Ilan et al. 1994; Koukouras et al. 1996; Bo et al. 2012). They are crucial ecosystem engineers in many habitats worldwide, but they can also act as bioeroders, especially in the coralligenous and coral reefs, behaving as intermediate disturbers and representing the main driving force in the turn-over of bioconstructions (Van Soest et al. 2012; Maldonado et al. 2017). Being suspension-feeders, sponges are a significant trophic link between the benthos and the water column, deeply impacting the benthic-pelagic coupling of inorganic nutrients (e.g., dissolved carbon, various nitrogen compounds and silicates) through a complex combination of metabolic processes and the elaboration of biogenic silica (Maldonado et al. 2012).

Due to the fundamental functional role covered by sponges in marine ecosystems (Bell 2008; Maldonado et al. 2012, 2017), several species are under the protection of the international legislation. For example, for the Mediterranean Sea, a total of 15 sponge species are listed in the Annexes of the Bern and Barcelona Conventions, as well as many are included in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, being

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*Correspondence: T. Marrocco, Department of Life and Environmental Sciences, Università Politecnica Delle Marche, Ancona, Italy. Email: t.marrocco@pm.univpm.it considered severely endangered or threatened (IUCN 2021). The importance of sponge protection and conservation is also related to the economic value associated with their seawater filtration rates, estimated by Pham et al. (2019) as nearly double the market value of the fish catch.

The simple body organization and the high plasticity, together with the symbiotic association with a complex community of microorganisms, allowed sponges to adapt to different environmental conditions (Schmitt et al. 2007; Van Soest et al. 2012). Sponges are typical inhabitants of both shallow and deep waters, from the mesolittoral to the circalittoral zones, including crucial habitats as *Posidonia* meadows, coral reefs, marine caves and coralligenous assemblages, and in the bathyal zone, where they can create the so-called "sponge grounds" (Corriero et al. 2000; Bo et al. 2012; Calcinai et al. 2015; Padiglia et al. 2018; Hawkes et al. 2019; Pawlik & McMurray 2020). Most of sponge species generally present a wide bathymetric distribution (Van Soest et al. 2012), in some cases making difficult the identification of a vertical zonation at a narrow depth range. However, various factors can contribute to the bathymetric distribution of Porifera, such as light (especially for species hosting symbionts, e.g., *Cliona viridis* and *Petrosia (Petrosia) ficiformis*), sedimentation rates, nutrients availability, water movements and predation (Wilkinson & Evans 1989; Rosell & Uriz 1991; Arillo et al. 1993).

Porifera represents one of the most common organisms of the Mediterranean benthic communities, for which 785 valid species have been listed by now (de Voogd et al. 2022). Although among the seven Mediterranean ecoregions (Spalding et al. 2007), the Western Mediterranean shows the highest number of sponge species (422) (de Voogd et al. 2022), the sampling effort results insufficient, being focussed only on a few areas, with many biocenoses still under-sampled (UNEP-MAP RAC/SPA 2010). In fact, different areas are interested by various studies targeting sponges, as for example the Portofino Marine Protected Area (MPA), from where almost 300 species have been described (Cerrano et al. 2001; Bertolino et al. 2013; Calcinai et al. 2015), while many other areas are still less investigated.

Aim of the current study is to analyse and compare the sponge communities characterising the hard bottoms of two Islands belonging to the Tuscan Archipelago (Italy), planned to become a future MPA. In particular, we explored if the sponge species composition changes in relation to a bathymetric gradient, from 0 to 40 m. In addition, our data were evaluated with reference to data available in the literature on sponges of the Tuscan Archipelago, to determine if the sampling effort applied is sufficient to describe the diversity of the area.

Materials and methods

Sample's collection and identification

Samplings were carried out by SCUBA diving during June 2019 and 2020 in Montecristo and Giglio, two Islands belonging to the Tuscan Archipelago National Park (TANP, Italy) (permission #00068010) (Figure 1). Montecristo is an Integral Nature Reserve since 1971, under the surveillance of the Italian Carabinieri Corps and Coast Guard, and all maritime activities are strictly forbidden (Angeletti et al. 2010; Bo et al. 2014). Conversely, Giglio hosts 1,500 residents and three villages (Campese, Giglio Castello and Giglio Porto), and its adjacent waters do not have any protection (IslePark 2021). The two Islands present a similar mineralogical composition, being mainly composed by granitic rocks (Alvisi et al. 1994; Innocenti et al. 1997).

A total of 14 sites were sampled (Table S1) and samples of Porifera were collected on rocky bottom assemblages at five depths: 0.5, 10, 20, 30 and 40 m. At each depth, two scuba operators spent about 6 minutes, covering an area of 20 m × 1 m (20 m^2) each and applying a visually oriented sampling method to collect the highest number of species of the area (Calcinai et al. 2011). For each sponge sample, a small portion was collected, thus allowing the regeneration of the specimen. The visually oriented sampling data were expressed as the presence/absence of sponges at each sampling time at each site.

The collected material was fixed in ethanol 95%. Specimens were processed following the standard methods for slides' preparations (Rützler 1978), and when possible, identified at species level, using a Nikon Eclipse Ni compound microscope. The slides used for the identification were stored in a reference collection deposited at the Zoology Laboratory at DiSVA (Department of Life and Environmental Sciences), Polytechnic University of Marche (Ancona, Italy). The taxonomic identification was conducted using "Systema Porifera" (Hooper & Van Soest 2002), "Fauna d'Italia" (Pansini et al. 2011), "Proposal for a revised classification of the Demospongiae (Porifera)" (Morrow & Cárdenas 2015) and the World Porifera Database (de Voogd et al. 2022).

Literature collection

A comparison with the previous literature on the area of the TANP has been carried out. The

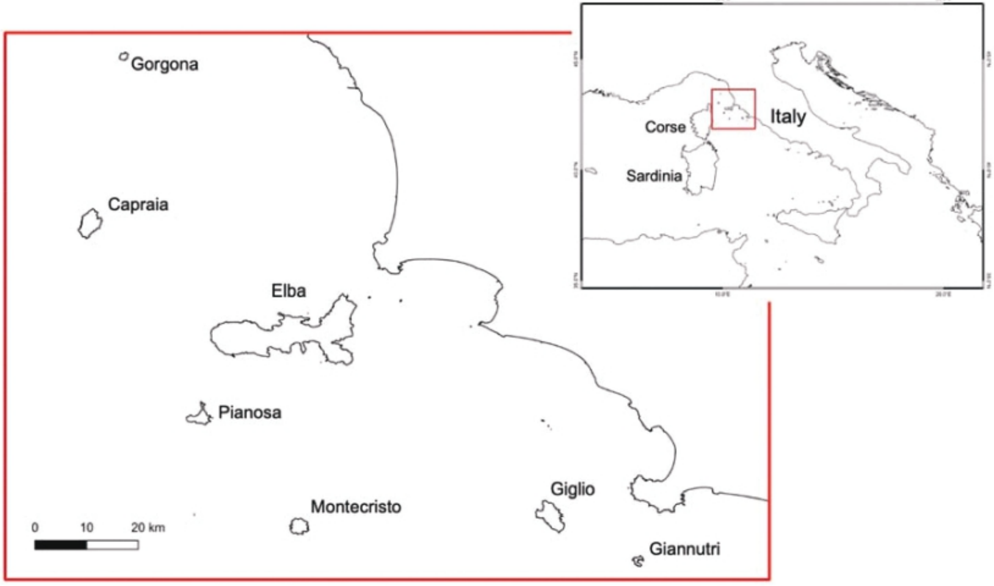

Figure 1. Map of the localization of the Tuscan Archipelago National Park, and of the seven Islands included.

literature research was conducted following the rules given by Pautasso (2013) for literature review. Since the considered area is limited, the present compilation is the result of an extensive bibliographic research on different databases (e.g., Google Scholar, JSTOR search, Scopus, Web of Science, Biodiversity Heritage Library), entering different keywords (“sponge” OR “Porifera”) AND (“Island name” OR “Tuscany” OR “Northern Tyrrhenian Sea”). An additional manual research was conducted on the references of the found documents, to include the possible literature on the area that could have been missed with the online search. The literature considered in this work includes journal articles and grey literature, as congress proceedings and technical reports. We did not include the paper of Roveta et al. (2020) by choice, since specimens used in that study were also considered in the present analysis. In addition, we checked for duplicate records and standardized the nomenclature using the World Porifera Database (de Voogd et al. 2022).

Experimental design and statistical analyses

The experimental design aiming at testing differences in the sponge communities at varying depth included two crossed factors: Island (fixed, 2 levels: Montecristo, Giglio) and depth (fixed, 5 levels: 0.5, 10, 20, 30, 40). Using a similarity matrix of presence-absence data (based on Jaccard similarity), data were analysed with Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001), with 9999 permutations. Permutational analysis of multivariate dispersions (PERMDISP) was used to test the homogeneity of samples dispersion from their group centroids (Anderson et al. 2008). *A posteriori* pairwise comparisons were conducted in case of significancy in the PERMANOVA. The analysis of Similarity Percentages (SIMPER) (Clarke 1993) was used to determine species contributions to the variability in sponge assemblages within Islands and depths.

Occurrences of sponge species obtained from the literature (considering documents in which the sampled Island(s) was specified) and our data were included in a presence/absence matrix, which was used as a similarity matrix. Dissimilarities among Islands was calculated using the Jaccard semimetric distance to perform a hierarchical clustering using the R package *Vegan* (Oksanen et al. 2020). The same dataset was used to estimate the sampling effort applied at each Island. A rarefaction curve per Island was built using the *iNEXT* R package (Chao et al. 2014), based on sampling-unit-incidence data (presence/absence), and the Hill number was measured in relation to the species richness (q = 0) of each Island. To standardize our data, we outrun a similarity matrix per Island, acquiring site and sponge species collected in every study toward the definition of the sampling unit and the species diversity respectively. If a document did not specify in which sampling site species were found, all sponge species were considered as found in a single site. In the analysis, if a species was indicated as sp. or spp. in different studies, they were grouped together to minimize bias.

Results

Considering all sites, a total of 340 samples were collected, with 13 orders and 48 species (46 Demospongiae and 2 Calcarea) of Porifera identified (Table I). A table containing further information (Island, site code, latitude, longitude, depth, and the list of all the identified species) is reported in the supplementary material as Table S2. Most of species belong to the orders of Poecilosclerida (14), Dictyoceratida (9) and Haplosclerida (8), and to the genera *Hymedesmia* (8) and *Haliclona* (5) (Table I). Due to the small amount of material available, in few cases (*Crella* sp., *Dictyonella* sp., *Fasciospogia* sp., *Ircinia* sp., *Sycon* spp., Calcarea indet., Haplosclerida indet.) identification to species level was prevented (Table I).

Considering the investigated depths, the total number of taxa showed a general increase with depth, with 17 species at 0.5 m, 18 at 10 m, 24 at 20 and 30 m, and 27 at 40 m (Table S2). Some species were found at only one depth, in particular three species were only recorded at 0.5 m (*Cacospongia mollior, Fasciospongia cavernosa, Ircinia* sp.), 2 at 10 m (*Halisarca dujardini, Penares helleri*), 4 at 20 m (*Acanthella acuta, Callyspongia subcornea, Fasciospongia* sp., *Spongia (Spongia) officinalis*), 2 at 30 m (*Aplysina cavernicola, Crella* sp.), and 6 at 40 m (*Dictyonella* sp., *Halichondria (Halichondria) contorta, Hymeniacidon perlevis, Haliclona (Reniera) mediterranea, Hymedesmia (Hymedesmia) consanguinea, Hymedesmia (Soestella) arenata*) (Table S2).

Overall, *Chondrosia reniformis* was the most widely distributed species, being found at all sites and at almost all depths (Table S2). Other common species were *Cliona viridis, Haliclona (Halichoclona) fulva, Hemimycale columella* and *Hymedesmia (Hymedesmia) baculifera* (Table S2).

Four species listed in Table I (*Aplysina aerophoba, A. cavernicola, Sarcotragus foetidus, Spongia (Spongia) officinalis*) are included in the Annexes II and III of the SPA/BD Protocol of Barcelona Convention, while three species (*Agelas oroides, A. cavernicola, Hymedesmia (Hymedesmia) rissoi*) are endemic to the Mediterranean Sea (Table I).

Among the 48 species, 40 were recorded at Montecristo and 34 at Giglio (Table I). Two orders (Agelasida and Chondrillida) and 14 species were recorded only at the Montecristo Island, while one order (Bubarida) and 8 species were uniquely found at Giglio. The site with the highest number of species was M-07 (23), followed by M-05 (15), G-01 and G-05 (14), while G-02 (5), M-01 and M-03 (7) were the sites with the lowest number of taxa recorded (Table S2). Considering the sampled depths at each Island, the number of species is almost constant within depths (Table S2).

PERMANOVA showed statistical differences only within depths (Table II), and the pairwise comparison highlighted differences in the sponge species composition between the shallower depths (0.5, 10 and 20 m) and the deeper ones (30 and 40 m) (Table III). However, the PERMDISP indicated a similar average dispersion within depths (Table IV), making the dispersion not significant ($F_{4,65} = 2.1736$; $p = 0.1269$).

The SIMPER analysis revealed that the similarity within Islands was given only by a few species. The average similarity is higher at Giglio (21.39%) with the contribution of *Chondrosia reniformis* (44.48%) and *Hemimycale columella* (30.04%), while the lower similarity at Montecristo (18.20%) was given by *C. reniformis* (40.50%) and *Cliona viridis* (20.23%), together with *Haliclona (Halichoclona) fulva* (8.87%) and *Hymedesmia (Hymedesmia) baculifera* (7.41%). Dissimilarities were calculated within depthsin particular, the ones which showed a significant difference in the pairwise comparison (see Table III). The highest average dissimilarity in species composition was between 10 and 40 m depth (92.31%) and the lowest between 10 and 30 m depth (86.05%) (Table S3). Dissimilarities between 0.5 and 30 m (89.31%) were mainly given by *H. columella* (10.74%), *C. reniformis* (9.90%) and *C. viridis* (8.36%), between 0.5 and 40 m (90.18%) by *C. viridis* (11.37%), *H. columella* (9.82%) and *C. reniformis* (8.71%) (Table S3). Species responsible for dissimilarity between 10 and 30 m and between 10 and 40 m depth were mainly explained by *C. reniformis* (13.87%), *C. viridis* (8.80%), *H. (H.) baculifera* (8.72%), and *C. reniformis* (13.96%), *C. viridis* (12.11%), *H. (H.) fulva* (8.53%), respectively (Table S3). While between 20 and 40 m depth (91.87%), the average dissimilarity was mostly related to *C. viridis* (12.50%), *C. reniformis* (9.43%) and *H. (H.) fulva* (8.08%) (Table S3).

From all the analysed literature, including a total of 73 documents (Tables S4 and S5), 84 Demospongiae, 8 Calcarea and 4 Homoscleromorpha have been listed (Table V), with the highest number of species recorded at Montecristo (34), followed by Capraia (30), Elba and Giglio (29), while the other Islands showed only a few species (13, 4 and 8 at Giannutri, Gorgona and Pianosa respectively) (Table V; Figure 2). Most of the listed species belong to the orders Poecilosclerida (24), Dictyoceratida (16) and Haplosclerida (10) (Table V). Table V also includes

Table I. List of Porifera taxa identified for Montecristo and Giglio Islands. E = endemic Mediterranean species; M* = new records for the Montecristo Island; G* = new records for the Giglio Island; P = species included in the Annexes II and III of SPA/BD of Barcelona Convention.

Porifera	Status	Montecristo Island	Giglio Island
Demospongiae			
Agelasida			
Agelas oroides (Schmidt, 1864)	E, M*	X	
Axinellida			
Axinella damicornis (Esper, 1794)	M*	X	X
Bubarida			
Acanthella acuta Schmidt, 1862			X
Dictyonella sp.	G*		X
Chondrillida			
Halisarca dujardinii Johnston, 1842	M*	X	
Chondrosiida			
Chondrosia reniformis Nardo, 1847	M*	X	X
Clionaida			
Cliona viridis (Schmidt, 1862)		X	X
Spirastrella cunctatrix Schmidt, 1868	M*	X	X
Dictyoceratida			
Cacospongia mollior Schmidt, 1862		X	
Fasciospongia cavernosa (Schmidt, 1862)	M*	X	
Fasciospongia sp.	M*	X	
Ircinia retidermata Pulitzer-Finali & Pronzato, 1981	M*	X	
Ircinia sp.		X	
Ircinia variabilis (Schmidt, 1862)	G*	X	X
Sarcotragus foetidus Schmidt, 1862	P, M*	X	
Sarcotragus spinosulus Schmidt, 1862	G*	X	X
Spongia (Spongia) officinalis Linnaeus, 1759	P, M*	X	
Haplosclerida			
Callyspongia subcornea (Griessinger, 1971)	G*		X
Haliclona (Halichoclona) fulva (Topsent, 1893)	M*	X	X
Haliclona (Reniera) mediterranea Griessinger, 1971	G*		X
Haliclona (Soestella) arenata (Griessinger, 1971)	M*	X	
Haliclona (Soestella) mucosa (Griessinger, 1971)	G*, M*	X	X
Haliclona (Soestella) valliculata (Griessinger, 1971)	G*, M*	X	X
Haplosclerida indet.		X	
Petrosia (Petrosia) ficiformis (Poiret, 1789)	M*	X	X
Poecilosclerida			
Crambe crambe (Schmidt, 1862)	M*	X	X
Crella sp.	G*		X
Crella (Crella) elegans (Schmidt, 1862)	G*, M*	X	X
Hemimycale columella (Bowerbank, 1874)	G*, M*	X	X
Hemimycale mediterranea Uriz, Garate & Agell, 2017	G*, M*	X	X
Hymedesmia (Hymedesmia) baculifera (Topsent, 1901)	G*, M*	X	X
Hymedesmia (Hymedesmia) consanguinea Lundbeck, 1910	G*		X
Hymedesmia (Hymedesmia) mollis Lundbeck, 1910	G*, M*	X	X
Hymedesmia (Hymedesmia) pansa Bowerbank, 1882		X	X
Hymedesmia (Hymedesmia) peachii Bowerbank, 1882	G*, M*	X	X
Hymedesmia (Hymedesmia) rissoi Topsent, 1936	E, G*, M*	X	X
Hymedesmia (Hymedesmia) versicolor (Topsent, 1893)	G*, M*	X	X
Hymedesmia (Stylopus) coriacea (Fristedt, 1885)	G*	X	X
Phorbas tenacior (Topsent, 1925)	M*	X	X
Suberitida			
Aaptos aaptos (Schmidt, 1864)	M*	X	
Halichondria (Halichondria) contorta (Sarà, 1961)	G*		X
Hymeniacidon perlevis (Montagu, 1814)	M*	X	
Tetractinellida			
Penares euastrum (Schmidt, 1868)	G*, M*	X	X
Penares helleri (Schmidt, 1864)	G*		X
Verongiida			
Aplysina aerophoba (Nardo, 1833)	P	X	X

(*Continued*)

Table I. (Continued).

Porifera	Status	Montecristo Island	Giglio Island
Aplysina cavernicola (Vacelet, 1959)	E, P, M*	X	
Calcarea			
Calcarea indet.		X	X
Leucosolenida			
Sycon spp.	M*	X	X
TOT Porifera x Island		40	34

Table II. Results of the two-way PERMANOVA. Analysis of sponge community variation considering Islands and depths. df = degrees of freedom; SS = sum of squares; MS = mean squares; Pseudo-F = F-ratio; P (perm) = probability; Up = unique perms.

Source	df	SS	MS	Pseudo-F	P(perm)	Up
Depth (De)	4	22,270	5567.4	2.0658	**0.0002**	9845
Island (Is)	1	3203.3	3203.3	1.1886	0.2562	9915
De x Is	4	12,783	3195.7	1.1858	0.1587	9842
Residuals	60	161,700	2695			
Total	69	199,960				

Table III. Results of the a posteriori pairwise comparisons within depths. t = t-test; P (perm) = probability; Up = unique perms. Significant p-values ($p < 0.05$) are given in bold.

Groups	t	P(perm)	Up
0.5–10	1.3258	0.0633	9935
0.5–20	1.1911	0.1387	9936
0.5–30	1.5495	**0.0011**	9908
0.5–40	1.8474	**0.0001**	9916
10–20	0.94994	0.5252	9911
10–30	1.5374	**0.0048**	9917
10–40	2.1225	**0.0001**	9917
20–30	1.0521	0.343	9911
20–40	1.4679	**0.0102**	9912
30–40	0.90663	0.6386	9923

Table IV. Results of the PERMDISP analyses at the different depths (0.5, 10, 20, 30, 40 m). SE = standard error.

Group	Size	Average	SE
0.5	14	49.242	1.9882
10	14	44.381	2.8533
20	14	51.301	2.3144
30	14	52.091	1.6693
40	14	51.507	1.6711

six species (*Aplysina aerophoba, A. cavernicola, Axinella polypoides, Sarcotragus fasciculatus, Spongia (Spongia) officinalis, Tethya aurantium*) reported in the Annexes II and III of the SPA/BD Protocol of Barcelona Convention. In addition, four species (*A. oroides, A. cavernicola, Delectona ciconiae, Eurypon vescicularis*) are endemic to the Mediterranean Sea (Table V).

Comparing the species extrapolated from the literature with the ones identified in this study, we found that 24 species were shared between the two datasets and 17 were new records for the area of the Tuscan Archipelago (Tables I and V). Considering Montecristo and Giglio separately, 9 and 10 species, respectively, were already recorded, while 30 and 21, respectively, were new records for the two Islands (Tables I and V).

In Table S4 we included all documents providing species listed in Table V, with all details (Porifera – with changes in species names –, authors, title, publication year, DOI/reference, Island, document type, application field). Species (35) reported in documents with no information on the sampling area/Island, that cannot be listed in Table V and thus used for statistical analysis, are also included in Table S4. Moreover, we would have added to the list other two endemic species (*Clathria (Clathria) toxistricta, Haliclona (Rhizoniera) sarai*) and four protected ones (*Geodia cydonium, Hippospongia communis, Sarcotragus foetidus, Tethya citrina*) (Table S4). Since many documents (26) did not specify the taxon collected but referred to sponges with the general terms “Porifera” or “sponge(s)”, these studies were not considered for statistical analysis but included in the Supplementary Material as Table S5, together with all document’s information (authors, title, publication year, DOI/reference, Island, document type, application field). Due to the low number of species and sampling units listed for the Islands of Giannutri, Gorgona and Pianosa (Tables V and S4), the cluster analysis and the extrapolation curves were not statistically reliable and thus, these Islands were excluded. However, for the sake of completeness, a cluster analysis, with the corresponding levels of dissimilarity, and the rarefaction curves considering all Islands are presented in the Supplementary Material as Figures S1 and S2.

Considering the joined dataset, obtained gathering data from this study and the scientific literature, the cluster analysis indicated that Montecristo and Giglio formed a group at a dissimilarity level of 0.64, while Capraia resulted related to the previous group

Table V. List of Porifera taxa extrapolated from the literature collection. E = endemic Mediterranean species; P = species included in the Annexes II and III of SPA/BD of Barcelona Convention. CA = Capraia; EL = Elba; GN = Giannutri; GI = Giglio; GO = Gorgona; MO = Montecristo; PI = Pianosa.

Porifera	Status	CA	EL	GN	GI	GO	MO	PI
Demospongiae								
Agelasida								
Agelas oroides (Schmidt, 1864)	E	X	X	X	X			X
Axinellida								
Axinella damicornis (Esper, 1794)		X	X		X			
Axinella polypoides Schmidt, 1862	P		X		X			
Axinella verrucosa (Esper, 1794)			X		X			
Eurypon lacazei (Topsent, 1891)					X			
Eurypon vescicularis Sarà & Siribelli, 1960	E				X			
Raspaciona aculeata (Johnston, 1842)					X			
Bubarida								
Acanthella acuta Schmidt, 1862		X	X	X	X			
Dictyonella incisa (Schmidt, 1880)		X						X
Chondrosiida								
Chondrosia reniformis Nardo, 1847		X	X	X	X			
Chondrosia sp.			X					
Chondrillida								
Chondrilla nucula Schmidt, 1862			X					
Clionaida								
Cliona celata Grant, 1826		X						
Cliona lobata Hancock, 1849							X	
Cliona sp.		X						
Cliona viridis (Schmidt, 1862)		X			X	X	X	X
Spirastrella cunctatrix Schmidt, 1868		X		X	X			
Spirastrella sp.			X					
Dendroceratida								
Aplysilla rosea (Barrois, 1876)							X	
Dictyoceratida								
Cacospongia mollior Schmidt, 1862					X		X	
Cacospongia sp.			X					
Dysidea avara (Schmidt, 1862)			X					X
Dysidea fragilis (Montagu, 1814)							X	
Dysidea incrustans (Schmidt, 1862)							X	
Fasciospongia cavernosa (Schmidt, 1862)				X				
Hippospongia communis (Lamarck, 1814)	P	X						
Ircinia dendroides (Schmidt, 1862)		X						
Ircinia spp.		X	X					
Ircinia variabilis (Schmidt, 1862)		X		X			X	
Pleraplysilla spinifera (Schulze, 1879)				X			X	
Sarcotragus fasciculatus (Pallas, 1766)	P	X	X					
Sarcotragus spinosulus Schmidt, 1862		X		X			X	
Scalarispongia scalaris (Schmidt, 1862)			X				X	
Spongia (Spongia) officinalis Linnaeus, 1759	P	X		X				X
Spongia (Spongia) virgultosa (Schmidt, 1868)		X					X	
Haplosclerida								
Chalinula limbata (Montagu, 1814)		X						
Haliclona sp.					X			
Haliclona (Gellius) angulata (Bowerbank, 1866)					X			
Haliclona (Halichoclona) fulva (Topsent, 1893)					X			
Haliclona (Reniera) sp. Schmidt, 1862					X			
Haliclona (Soestella) mucosa (Griessinger, 1971)		X						
Haliclona (Soestella) valliculata (Griessinger, 1971)			X					
Haplosclerida							X	
Petrosia (Petrosia) ficiformis (Poiret, 1789)		X	X		X	X		
Siphonodictyon coralliirubri (Calcinai, Cerrano & Bavestrello, 2007)			X					

(*Continued*)

Table V. (Continued).

Porifera	Status	CA	EL	GN	GI	GO	MO	PI
Poecilosclerida								
Antho (Plocamia) sarasiri Costa, Pansini & Bertolino, 2019			X					
Batzella inops (Topsent, 1891)					X		X	
Clathria (Microciona) sp.					X		X	
Crambe crambe (Schmidt, 1862)		X	X	X	X			
Crella (Pytheas) sigmata Topsent, 1925		X						
Crella (Yvesia) sp. (cited as *Yvesia* sp.)							X	
Forcepia (Leptolabis) luciensis (Topsent, 1888)							X	
Hamigera hamigera (Schmidt, 1862)			X					
Hemimycale columella (Bowerbank, 1874)		X						
Hemimycale sp.			X					
Hymedesmia spp. Bowerbank, 1864							X	
Hymedesmia (Hymedesmia) pansa Bowerbank, 1882							X	
Hymedesmia (Hymedesmia) paupertas (Bowerbank, 1866)							X	
Hymedesmia (Stylopus) coriacea (Fristedt, 1885)		X					X	
Hymedesmia (Stylopus) sp. (cited as *Stylopus* sp.)							X	
Lissodendoryx sp.		X						
Mycale (Aegogropila) retifera Topsent, 1924							X	
Mycale (Aegogropila) tunicata (Schmidt, 1862)							X	
Myxilla (Myxilla) incrustans (Johnston, 1842)		X			X			
Phorbas dives (Topsent, 1891)					X			
Phorbas fictitius (Bowerbank, 1866)		X			X			
Phorbas sp.							X	
Phorbas tenacior (Topsent, 1925)		X		X	X			
Tedania (Tedania) anhelans (Vio in Olivi, 1792)		X						
Suberitida								
Halichondria sp.							X	
Hymeniacidon perlevis (Montagu, 1814)		X						
Terpios gelatinosus (Bowerbank, 1866)							X	
Tethyida								
Tethya aurantium (Pallas, 1766)	P		X					
Tethya meloni Corriero, Gadaleta & Bavestrello, 2015			X					
Timea fasciata Topsent, 1934							X	
Tetractinellida								
Delectona ciconiae Bavestrello, Calcinai & Sarà, 1996	E		X					
Dercitus (Stoeba) plicatus (Schmidt, 1868)					X			
Penares euastrum (Schmidt, 1868)								X
Verongiida								
Aplysina aerophoba (Nardo, 1833)	P		X		X		X	
Aplysina cavernicola (Vacelet, 1959)	E, P		X		X			
Aplysina sp.				X				
Calcarea								
Clathrinida								
Ascandra contorta (Bowerbank, 1866)							X	
Clathrina clathrus (Schmidt, 1864)			X		X			
Leucetta solida (Schmidt, 1862)			X					
Leucosolenida								
Leucandra aspera (Schmidt, 1862)							X	
Sycon ciliatum (Fabricius, 1780)							X	
*Sycon elegans (*Bowerbank, 1845)							X	
Sycon raphanus Schmidt, 1862					X			
Sycon sp. Risso, 1827			X					
Homoscleromorpha								
Homosclerophorida								
Corticium candelabrum Schmidt, 1862					X		X	
Oscarella lobularis (Schmidt, 1862)				X		X		X
Plakina monolopha Schulze, 1880							X	
Plakortis simplex Schulze, 1880						X		X

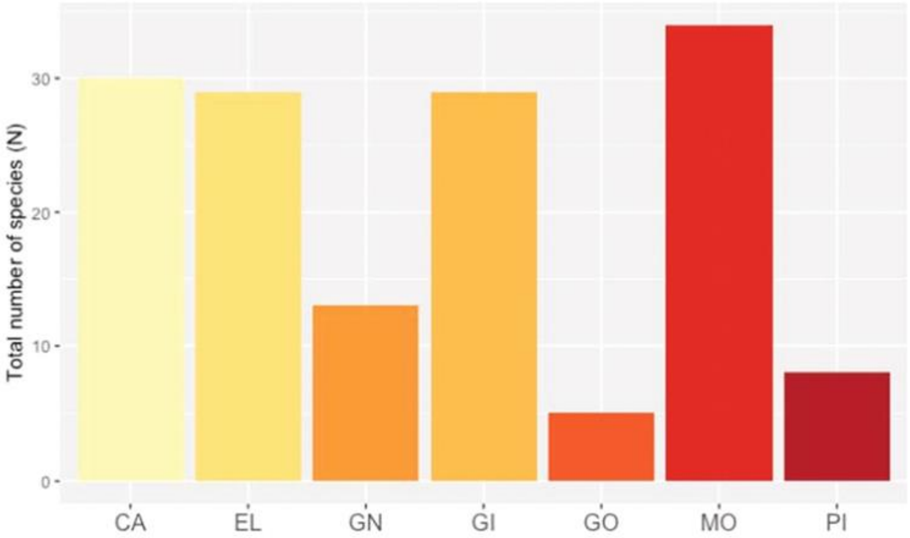

Figure 2. Total number of sponge species (N) per Island. CA = Capraia; EL = Elba; GN = Giannutri; GI = Giglio; GO = Gorgona; MO = Montecristo; PI = Pianosa.

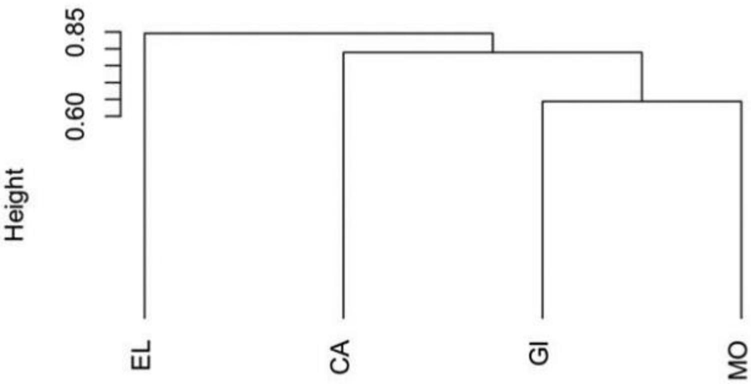

Figure 3. Dendrogram showing dissimilarity among Islands, based on their sponge fauna. CA = Capraia; EL = Elba; GI = Giglio; MO = Montecristo.

at a level of 0.79 (Figure 3). The Elba Island was the less related Island with the above group, with a dissimilarity of 0.84 (Figure 3).

Although the number of documents increased significantly in time (Table S4), the extrapolation curves indicate that the sampling effort applied by now to the area is not sufficient (Figure 4). The curves demonstrated that the Montecristo Island is expected to have the highest species diversity, even though it was the Island with the lower number of sampling units, contrary to Capraia where, with almost the same number of sampling units, the number of estimated species was less than the half (Figure 4). In general, Giglio and Montecristo showed a higher mean species diversity than the other two Islands, while Capraia and Elba presented an almost total overlapping between the 95% confidence intervals and the average diversity (Figure 4).

Discussion

The species of Porifera identified in the current study are all well-known and typical of Italian and Mediterranean coastal habitats (Hooper & Van Soest 2002; Pansini & Longo 2008). Species recognized as the most frequent (*Chondrosia reniformis, Cliona viridis, Haliclona (Halichoclona) fulva, Hemimycale columella* and *Hymedesmia (Hymedesmia) baculifera*) are also described in the literature as among the most common species of shallow waters (Carballo 1994; Ben

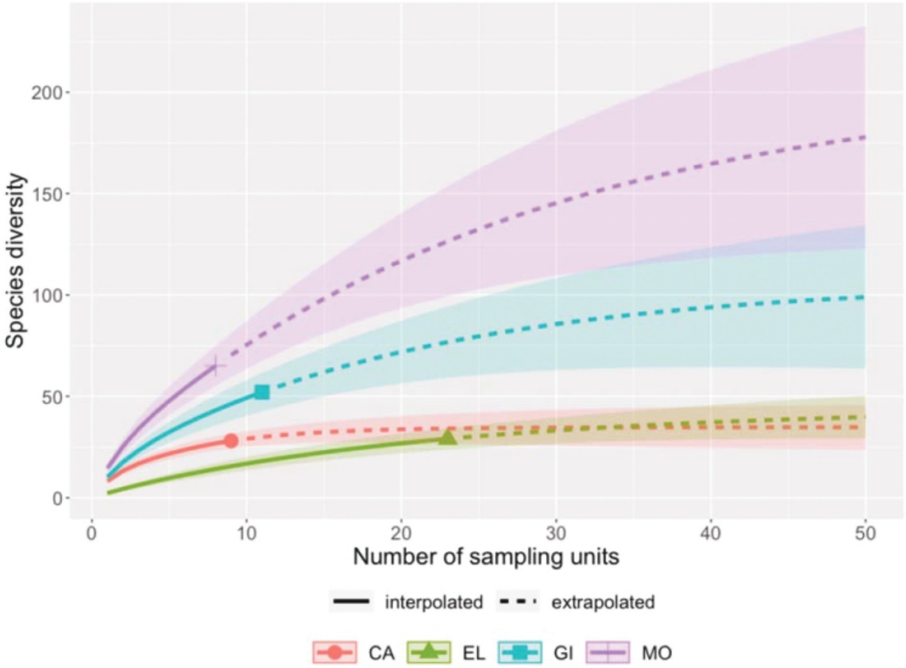

Figure 4. Sampling-unit-based rarefaction (solid line segment) and extrapolation (dotted line segments) curves with 95% confidence intervals (shaded areas) for sponge data. CA = Capraia; EL = Elba; GI = Giglio; MO = Montecristo.

Mustapha et al. 2002; Hooper & Van Soest 2002; Bertolino et al. 2013).

Depth has been previously highlighted to be fundamental in structuring marine benthic assemblages (Samaai et al. 2010). Even though sponges can be characterised by a wide bathymetric range, and a same species can be found from the surface to the bathyal waters (Van Soest et al. 2012), differences in the species composition can be also identified in a narrow vertical range. It is thus not surprising that our analysis showed significant differences in the sponge community composition among the investigated depths. In particular, differences were recorded between the shallower depths (0.5, 10 and 20 m) and the deeper ones (30 and 40 m). In fact, in all sampled sites, around 30 m depth, the shift from the littoral community, mainly characterized by algae, to a pre-coralligenous or coralligenous habitat was observed (Roveta C., Pulido Mantas T., Pica D. personal observation). The 30 and 40 m corresponded to the depths where most of species were recorded, and where typical sponge species characterizing coralligenous assemblages (e.g., *Aplysina* spp., *Axinella* spp., *Cliona viridis, Crambe crambe, Haliclona* spp., *Hemimycale columella, Hymedesmia* spp., *Penares euastrum, Phorbas tenacior*) (Bertolino et al. 2013; Longo et al. 2018) were the most common (Table S2) and the main responsible for the dissimilarities within the shallower and the deeper populations (Table S3). A similar pattern was already observed in the Tuscan Archipelago by Balduzzi et al. (1995), specifically at Giglio and Capraia Islands, and by Ferdeghini et al. (2000), at the Giannutri Island.

The mineralogic composition of the substrate is known to influence larvae recruitment and, therefore, species composition and distribution (Bavestrello et al. 2000; Canessa et al. 2019, 2020). For example, Canessa et al. (2020) highlighted how coralligenous assemblages can differ between limestone and granite, and that several sponge species, as *Sarcotragus foetidus* or the ones belonging to the genus *Axinella*, are more abundant on granitic substrates. This is in line with our observations and the results of the statistical analysis, which did not highlight significant differences in the species composition of the two Islands. In fact, Montecristo and Giglio share the nature of the substrate, being both granitic (SiO_2) (Alvisi et al. 1994; Innocenti et al. 1997), except for Promontorio del Franco in Giglio (an area not considered in this study), mainly composed of limestone (Alvisi et al. 1994, page 25, fig. 1). Together with the mineralogical composition of the substrate, current is another factor that can play an important role in structuring the sponge community composition of the considered Islands. Even though sponge larvae tend to settle nearby the parental organism due to their limited swimming capacity (Van Soest et al. 2012), water movements can promote larval dispersion (Mariani et al. 2006). Tyrrhenian Sea surface currents move from its southern part to the northern one and, once coastal waters encounter the promontory of Monte Argentario, they are drifted from the coast to the offshore (Iacono et al. 2013, p. 1711, fig. 1). Therefore, it is possible that larvae (and/or propagules) released by sponges at the Giglio Island can be transported to Montecristo, promoting a similar community.

Among the sessile fauna of the Tuscan Archipelago, Porifera represents one of the most common groups (Benedetti-Cecchi et al. 2003). However, information on sponges inhabiting the area is scant, fragmentary, and sparse in the literature, and many of the observations derive from sampling not targeting sponges in a systematic way. In fact, species listed for Montecristo derived just from two documents, that are Corriero and Pronzato (1987) and Taviani et al. (2009) – the last one providing only one species (Table S4) –, and the present work. For the other Islands, only a few documents were taxonomical papers, targeting sponges, while most of them were mainly ecological (see Table S4 for references). For Elba, which was the Island with the higher number of documents recorded (36), a considerable amount were biotechnological studies, focussing their attention on the extraction of natural compounds from a few target species and their microbial communities (e.g., *Aplysina* spp., *Axinella* spp., *Chondrosia reniformis, Hamigera hamigera, Petrosia (Petrosia) ficiformis, Sarcotragus fasciculatus*) (see Table S4 for references). In addition, regarding Giannutri, Gorgona and Pianosa, only few documents were found per Island, providing a low number of species, and not allowing us to include them for further analyses.

The cluster analysis suggested a more similar species composition at Giglio and Montecristo, together with Capraia, while Elba resulted as the less related. Once again, the mineralogical composition of the various Islands can partially explain the results obtained with the analysis. Capraia, Giglio and Montecristo are mainly composed of SiO_2, with values ranging from 58% to 73%, being Giglio and Montecristo granitic and Capraia volcanic (Innocenti et al. 1997; Westerman et al. 2003; Gasparon et al. 2009), while Elba presents a high mineralogical diversity, being both carbonatic (e.g., limestone) and granitic (Bortolotti et al. 2001). Therefore, the different geomorphology characterizing the analysed Islands can play a role in the similarity of their sponge populations.

In general, many problems were encountered during the literature analysis carried out in the current study: (1) sampled Island(s) and site(s) information were present in the Materials and Methods, but records were not georeferenced; (2) the sampled Island(s) was specified, but not the study site(s) (see Table S4); (3) sampled Island(s) and site(s) were given, but insufficient taxonomical information were provided (different species grouped in relation to their growth form, as "encrusting Porifera", "massive Porifera", etc., or merely listed as "Porifera") (see Tables S4 and S5). As already pointed out for other phyla, studies providing the reader with geographical information of the sampling area(s) and site(s), together with georeferenced records, not only improve the data quality, but also facilitate the literature analysis for other researchers (e.g., timesaving in data compilation) (Ajala-Batista et al. 2020). The lack of taxonomical information, especially in ecological papers, avoid the gathering of datasets regarding pattern of species richness and endemism, and do not allow a better understanding of organisms' natural history (Ajala-Batista et al. 2020). However, even though we found different high-quality in grey literature studies, crucial for improving our dataset, more complete information would have allowed us to add more species in Table V, and to have more sampling units in the extrapolation of the species diversity, thus leading to a more accurate prediction.

The lack of communication among disciplines has been highlighted not only in ecological papers, which were mainly or exclusively focussed on the application of indexes to evaluate the ecological status of a habitat, without considering any taxonomical aspect (see Tables S4 and S5 for references), but also in pure taxonomical works. Most of them did not present any ecological information (see Table S4 for references), leading to a reduction in value and attractiveness, with all the useful information on species distribution and diversity often lost (Di Camillo et al. 2018).

Although sponges have key ecological and economical roles (Padiglia et al. 2018; Pham et al. 2019), the sampling effort applied on this group in the Tuscan Archipelago is still not sufficient. Due to this underestimation, non-recorded species are probably present within the area, especially for the cryptic nature of many sponges, as underlined by the work of Bertolino et al. (2013) and Calcinai et al. (2015). Thus, sampling of sponges at different depths and on different substrates (e.g., rocky shores, port structures, *Posidonia* meadows, marine caves, and other living organisms, such as macroalgae and other benthic metazoans) should be carried out to increase the likelihood of their encounter. The increasing of the sampling effort will be crucial for the Tuscan Archipelago National Park, planned to become a future MPA (L. 979/82 art.31 and L. 394/91 art.36), and the Montecristo Island, a Natural Integral Reserve and previously identified as a reference site for ecological quality assessment in the Western Mediterranean (Turicchia et al. 2018), especially considering the presence of the endemic and protected species (SPA/BD) and accordingly to the new European legislation (EC 2020).

To conclude, our work is the first summarising the sponge diversity of the entire Tuscan Archipelago, and one of the few totally focussing on the sponge fauna. These results provide new important information on the area of the TANP, adding new records and highlighting the high diversity of a poorly known area.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

All data are provided within the main text and the supplementary material.

Supplementary material

Supplemental data for this article can be accessed here

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CHAPTER 2 – TEs AND MARINE BENTHIC ORGANISMS AS BIOINDICATORS

In the current Chapter, three papers on the application of unconventional marine sessile taxa as bioindicators of TEs pollution are included.

Paper 3, entitled "**Biomonitoring of Heavy Metals: The Unexplored Role of Marine Sessile Taxa**", is a systematic review conducted using Elsevier's Scopus database on the role of different filter- and suspension-feeders (sponges, cnidarians, bryozoans, polychaetes, cirripeds, and tunicates) as bioindicators in coastal shallow waters. The available literature about this topic was analysed to summarize the current knowledge and to identify possible applications of these taxa in a bioremediation scenario.

In paper 4 "**Hg Levels in Marine Porifera of Montecristo and Giglio Islands (Tuscan Archipelago, Italy)**", the suitability as bioindicators of Hg pollution were tested on various sponge species collected in the Tuscan Archipelago. A high variability was encountered in the Hg contents, underlining a species-specificity of metal concentrations for Porifera, and allowing to identify a few species as better bioindicators for Hg pollution.

In paper 5, entitled "**Distribution of mercury inside the Mediterranean sponge *Chondrosia reniformis*: a study case from the Tuscan Archipelago National Park (Tyrrhenian Sea)**", the attention was focussed on the species *C. reniformis*, a common Mediterranean Demospongiae. The Hg content and the bioconcentration factor were calculated, suggesting the role of *Chondrosia* as an attractive bioindicator of toxic metal pollution in coastal waters, and pointing out the usefulness of using organisms in the monitoring programs of coastal areas, as proposed by various European marine policies.

PAPER 3

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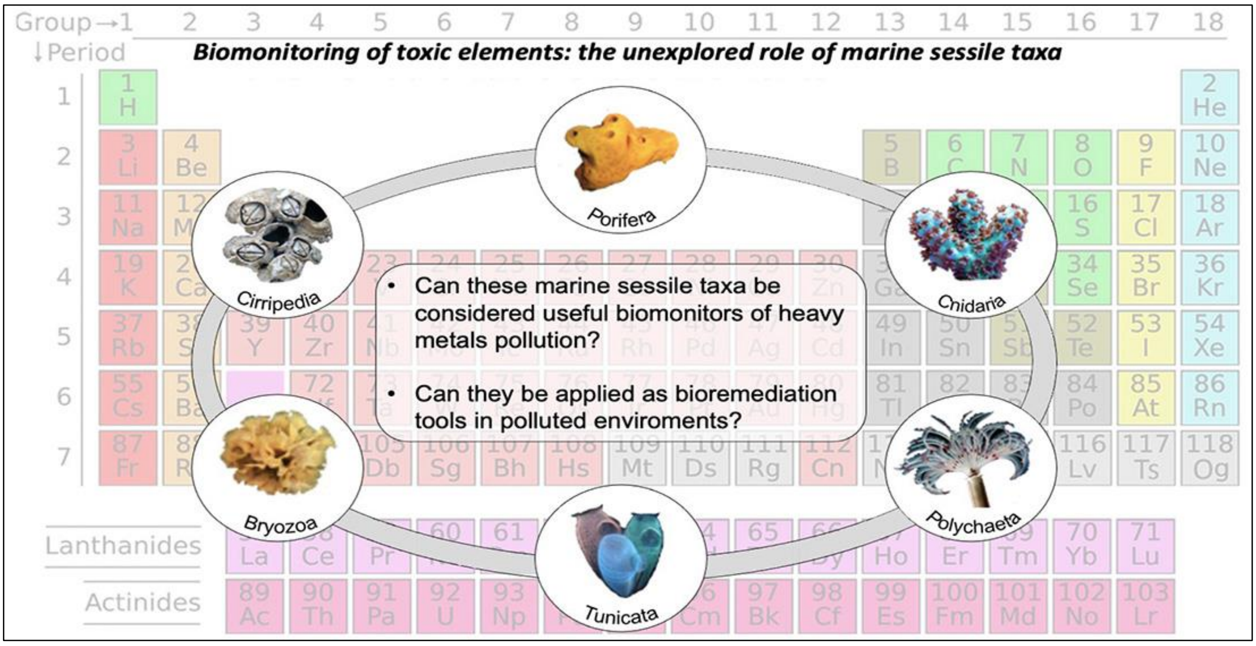
Biomonitoring of Heavy Metals: The Unexplored Role of Marine Sessile Taxa

Roveta Camilla, Annibaldi Anna, Afghan Afghan, Calcinai Barbara, Di Camillo Cristina Gioia, Gregorin Chiara, Illuminati Silvia, Pulido Mantas Torcuato, Truzzi Cristina, Puce Stefania



- Can these marine sessile taxa be considered useful biomonitors of heavy metals pollution?
- Can they be applied as bioremediation tools in polluted enviroments?

Supplementary material available at:

https://drive.google.com/drive/folders/1qaso9AXvCjVegtXTRbah-6vvidaTJ8RL?usp=sharing

applied sciences

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Review

Biomonitoring of Heavy Metals: The Unexplored Role of Marine Sessile Taxa

Camilla Roveta [1], Anna Annibaldi [1,*], Afghan Afghan [1], Barbara Calcinai [1], Cristina Gioia Di Camillo [1], Chiara Gregorin [1,2], Silvia Illuminati [1,*], Torcuato Pulido Mantas [1], Cristina Truzzi [1] and Stefania Puce [1]

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Abstract: Coastal areas are known to receive significant anthropogenic inputs, mainly deriving from metropolitan areas, industries, and activities related to tourism. Among these inputs, some trace elements are listed as priority pollutants in the European Water Framework Directive, due to their ability to bioaccumulate in organisms. Many studies have been conducted on heavy metals (HMs) accumulation and on their possible effects on different edible marine species. While the most studied sessile organisms are bivalves, in the current review, we focus our attention on other sessile taxa (sponges, cnidarians, bryozoans, polychaetes, cirripeds, and tunicates), proposed as bioindicators in coastal shallow waters. Although their potential as bioindicator tools has been repeatedly highlighted in the literature, these organisms are still poorly investigated and considered for monitoring. In this context, we analyze the available literature about this topic, in order to summarize the current knowledge and identify possible applications of these organisms in a bioremediation scenario.

Keywords: zoobenthos; sentinel species; suspension feeders; water pollutants; bioaccumulation; heavy metals

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1. Introduction

Marine coastal areas are among the most exploited and vulnerable ecosystems, constantly subjected to multiple anthropogenic pressures [1,2], due to the intensification of metropolitan areas, tourism, and industrial and agricultural activities [3,4]. These human-induced perturbations cause in many cases the release of various contaminants, which can be transported in coastal waters through different pathways, as atmospheric depositions, rivers, industrial discharges, maritime traffic, or submarine groundwaters [3], representing important hazards for the ecosystem's health [1]. These contaminants principally include chemicals (e.g., heavy metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, etc.) and fecal contaminants (e.g., *Escherichia coli*, total coliforms, and enterococci) [3]. Therefore, understanding the fate and distribution of contaminants in the sea and their origins is crucial to assess the ecological and chemical status of water and organisms and to implement management plans [2,5].

Heavy metals (HMs) are a problematic issue for marine ecosystems, ensuring that some of these elements and their derived compounds are listed in the European Water Framework Directive (WFD 2000/60/EC) as priority pollutants. Heavy metals are naturally occurring elements that can be found throughout the earth's crust. Nevertheless, anthropogenic activities (e.g., mining and smelting operations, industrial production and use, metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, and sediment resuspension) are primarily responsible for the environmental contamination and human exposure to these contaminants [6].

To date, a clear definition of the term “heavy metals” is not yet available. The term has been widely used in chemistry but no authority such as the International Union of Pure and Applied Chemistry (IUPAC) has ever defined it [7]. Even if its use is strongly discouraged, the term is increasingly used in the scientific literature especially in articles pertaining to multidisciplinary environmental issues [8]. On a chemical basis, the group of HMs includes all the transition elements (bloc d of the periodic table), the rare earth elements (the series of lanthanides and the series of actinides, including La and Ac themselves), and all the elements presenting the typical characteristics of metals (bloc p), together with some metalloids (Ge, As, and Te) and some nonmetals (Se) [7,9].

Some HMs (e.g., Fe, Cu, Co, Mn, and Zn) are essential elements for organisms, playing key roles in the functioning of enzyme systems. However, high concentrations induce detrimental effects in organisms and in the environment. Other metals (e.g., Al, As, Ba, Bi, Cd, Pb, Hg, Ni, Pt, and Ag) have no specific biological functions and are considered as nonessential metals [10].

In the marine environment, heavy metals can be found in various chemo-physical forms (metal ions, hydrated ions, charged metal complexes, uncharged inorganic complexes, and organometallic complexes), each of them presenting different bioavailability, toxicity, bioaccumulation, mobility, and biodegradation rates [11]. All aquatic organisms can introduce and/or accumulate HMs in their tissues, taking them up from water or food, and the concentration of these elements can vary between taxonomic levels, from phyla down to species of the same genus [12]. Many studies have been carried out on the bioaccumulation and the effects of the different elements on marine organisms, but mainly on edible species (as bivalves, cephalopods, decapod crustaceans, and fish) e.g., [13,14]. However, other sessile organisms, e.g., polychaetes, tunicates, sponges, and barnacles, can be considered as suitable bioindicators for their physical and physiological characteristics [15,16], and they have been recommended by many authors, e.g., [17–22], by the WFD and by the Marine Strategy Framework Directive (MSFD) [23]. Moreover, these organisms can represent an excellent functional tool since they do not only show detectable concentrations of trace elements [18,24] but the presence of these chemicals in their tissues can also stimulate different physiological responses [25]. Therefore, they can be considered as useful biomonitors of HMs contamination, providing important information on the ecological and chemical status of an area.

The main aim of this systematic review is a spatiotemporal analysis of the literature on the bioaccumulation of HMs at global scale on six sessile marine taxa (Porifera, Cnidaria, Bryozoa, Polychaeta, Cirripedia, and Tunicata), considered as nonconventional biomonitors for chemicals pollution. The current work will provide a schematic summary of the current knowledge on the relationship between the selected taxa and HMs. It will identify possible applications of these organisms as bioremediation tools in contaminated environments, also accordingly to the European MSFD within the achievement of the Good Environmental Status (GES) (MSFD 2008/56/EC).

2. Materials and Methods

The present compilation is the result of an extensive bibliographic research on Elsevier’s Scopus database (www.scopus.com), entering different keywords, “heavy metal” AND “taxa” and “heavy metal” AND “common name of the organism” (Figure 1), in the option “Article title, Abstract, Keywords,” in all years until the cut-off date of 20 June 2020. The literature considered in this work includes journal articles and grey literature, as congress proceedings. Systematic reviews are not included by choice. All documents found with the aforementioned queries have been screened by reading titles and abstracts, excluding those not matching our criteria (Figure 1), and only the ones eligible for this study have been considered for the analysis. Duplicates, e.g., same documents found using different keywords, were counted as one. Since the Mediterranean province showed the highest number of documents on the topic, we conducted an additional manual research on the references reported in the found articles, in order to include possible documents on the area that could have been missed with the online search (Figure 1). A flow chart of the searching strategy and the eligibility process is given as Figure 1.

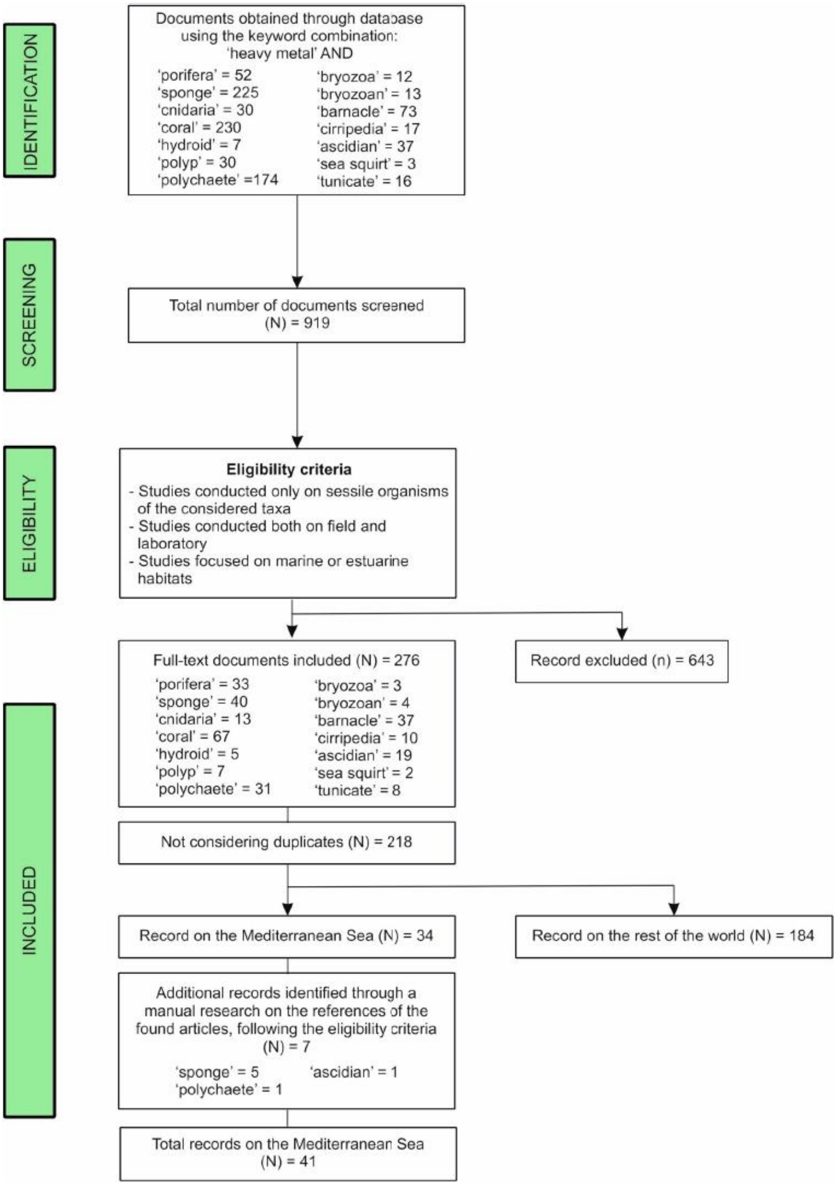

Figure 1. Flow chart illustrating the steps for obtaining documents about the study of heavy metals (HMs) on organisms from Scopus search engine and from the manual research.

Studies from the web-based and manual researches have been analyzed based on the taxa considered (Porifera, Cnidaria, Bryozoa, Polychaeta, Cirripedia, Tunicata), temporal distribution, marine realm of the sampling site(s), habitat (sea, estuarine), analyzed body part (cells and tissues, entire specimen, microbiome, skeleton), science field (biomonitoring, microbiology, physiology), HM(s), work setup (field, laboratory). Moreover, HMs considered in the documents have been divided in two categories, essential (Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, V, and Zn) and nonessential (Ag, Al, As, Bi, Cd, Ce, Cs, Eu, Ga, Hf, Hg, La, Lu, Nb, Pb, Sb, Sc, Sn, Te, Th, Ti, U, Y, and Zr) elements following [26–28].

Documents related to the Mediterranean Sea province have been analyzed on the basis of the considered taxa (Porifera, Cnidaria, Polychaeta, Cirripedia, and Tunicata), temporal distribution, ecoregion of the sampling site(s), sampling depth(s), HM(s), considered order(s) per taxa. In all the analyses, every document could be included in one or more categories.

The analysis reported in Figure 2B was carried out following the bioregionalization of coastal and shelf areas proposed by Spalding et al. [29]. While, for the creation of the map of documents distribution in the Mediterranean Sea, geographic coordinates were used. In studies showing only a map of the sampling point(s), geographic coordinates were estimated from it. In the absence of a map, coordinates of the sampling point(s) were estimated using the information across the text, if available. When the given coordinates ended up in land, they were adjusted to the nearest coastal points.

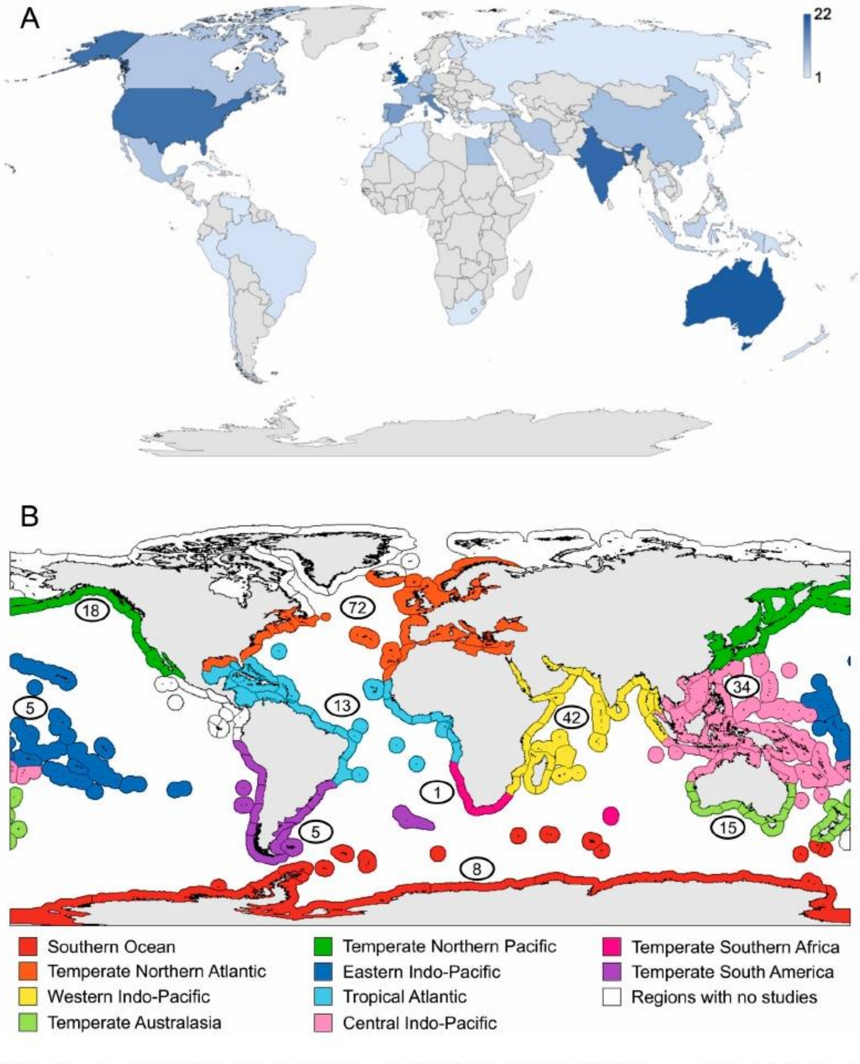

Figure 2. Map showing the number of documents per: (**A**) first authors' affiliation country and (**B**) marine realm.

A list of works divided per taxa with all document's details (publication year, authors, title, DOI/Reference, source, first author affiliation country, realm of the sampling site, habitat, basic physicochemical parameters of water, taxa, application field, analyzed body part, data validation, HM(s), and work setup) is reported in the Supplementary Material as Table S1. The Supplementary Material also included a detailed list of documents (taxa, authors, publication year, marine ecoregion of the sampling site(s), latitude, longitude, depth, application field, order of the analyzed species, analyzed species, HM(s), concentration, and unit) on the Mediterranean Sea province as Table S2. In the case of HMs, concentrations were not reported along the text, they were estimated from graphs. Concentration units, if different, were standardized to make concentrations comparable between documents.

3. Results

3.1. Literature Analysis at Global Scale

A total of 919 documents (Figure 1), including duplicates, have been screened and analyzed by reading the title and abstract, using the aforementioned keywords. Those not following our eligibility criteria were removed (643), leading to 276 documents, spread among the six considered taxa (Figure 1). After excluding duplicates, the final number of analyzed works was 218 (Figure 1; Table S1).

Considering all the literature found, most of the works have been published from researches with an affiliation in the United Kingdom (22), Australia (21), India (19), the United States (18), and Italy (15) (Figure 2A). While in many eastern and northern European countries and various Asian and most of the African countries, only a few studies have been published (Figure 2A). Australia and the United Kingdom were the only two, out of 44 countries, where all six taxa were studied (Table S1). Regarding the sampling area, most of the studies were carried out with organisms collected in the Temperate Northern Atlantic (32.0%) and the Western (18.7%) and Central (15.1%) Indo-Pacific marine realms (Figure 2B). Inside the Temperate Northern Atlantic realm, the Mediterranean Sea province showed the highest number of documents (34). All the other studies are scattered among the other marine realms, with only one work in the Temperate Southern Africa and a total absence of studies in the Tropical Eastern Pacific and the Arctic (Figure 2B). Up to the 12.8% of the total documents have not been taken into account for the creation of Figure 2B in as much as 12 works did not give any information about the sampling site(s), 12 considered marine organisms breed in aquarium, and 5 regarded organisms collected on hydrothermal vents located in areas not included in the division proposed by Spalding et al. [29] (Table S1). Most of the studies included species collected in the marine environment (92.8%), and only an 8.0% focused on estuarine organisms (Table S1).

The heatmap presented as Figure 3A showed that the first document, found using the query "heavy metal" AND "ascidian," was published before 1970 (1956, see Table S1 for citation). After this publication, for all taxa except Bryozoans, for which the first publication is registered in the decade 1991–2000 (1992, see Table S1 for citation), no works could be found until 1971–1980 (Figure 3A). In general, there is an upward trend in the number of documents through time, for almost all the taxa, while for Cirripedia, it is possible to identify a peak in 1991–2000 and a decrease in documents in the following decades (Figure 3A). The taxa of Cnidaria shows the highest number of works in almost all the decades, with the 37.0% of the publications, resulting the most studied group for HMs, followed by Porifera (18.5%), a group presenting a great increase in documents starting from 2001 (Figure 3A).

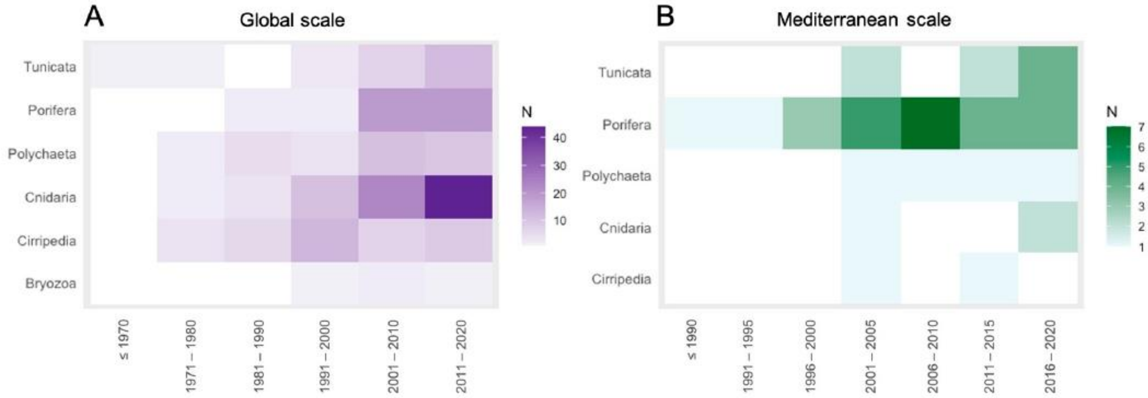

Figure 3. Number of documents per: (**A**) decade, at global-scale and (**B**) 5-year period, at Mediterranean-scale.

From the literature collection, it was evident that most of the documents analyzed both essential and nonessential elements (72.0%), in particular Cd (146), Cu (172), Pb (110), and Zn (142), while the 19.1% analyzed only essential elements and the 8.9% only nonessential ones (Table S1).

Organisms belonging to the six considered taxa have been mainly collected for biomonitoring analysis (Figure 4A), aiming to measure HMs concentrations in organisms and tissues. In particular, a significant proportion of documents in each taxon, for their analysis, processed the entire specimen, followed by the skeleton, especially in the Cnidaria group (Figure 4B). In fact, among the 43 documents on skeletal analysis, 36 examined the one of cnidarians (Table S1). Figure 4A showed also a total absence of physiological studies on Bryozoa, while only 2 have been conducted on Cirripedia (see Table S1 for citations). The remaining documents are spread among the other four taxa (Figure 4A). Works on microbiology represent a small component (15 documents) of the total, most of them (7) on Porifera (Figure 4A). A comparable situation can be observed for studies on cells and tissues and the microbiome, which have not been taken into account very frequently (Figure 4B).

The 59.1% of documents processed samples after their collection on the field (Figure 5A), especially for Cirripedia and Cnidaria (Figure 5B), while the 36.0% conducted laboratory experiments (Figure 5A). Among all taxa, organisms mainly belonging to the Cnidaria group have been used for this aim (Figure 5B). Only a small percentage (4.9%) of documents regarded studies in which collected organisms have been processed immediately to analyze a possible bioaccumulation of HMs and they have been bred to conduct laboratory experiments on the possible physiological effects of elements (Figure 5A). Apart from Cirripedia and Cnidaria, all the other taxa showed studies conducted with this work set up (Figure 5B).

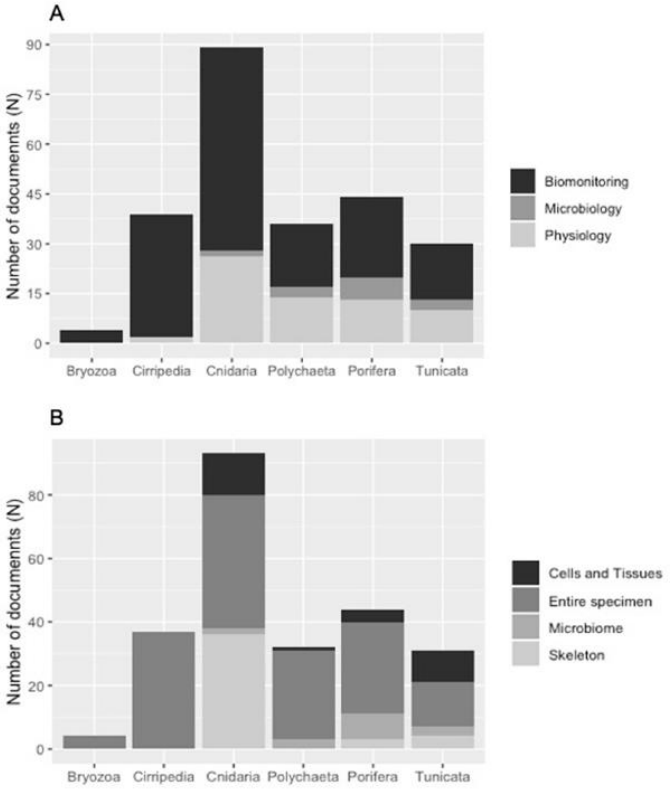

Figure 4. Number of documents per: (**A**) science field and (**B**) analyzed body part.

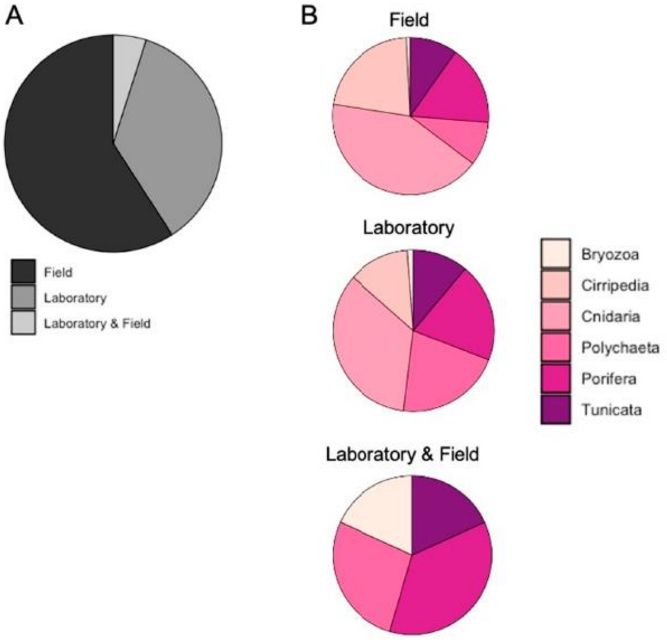

Figure 5. Percentage of documents per: (**A**) work setup and (**B**) taxa in each work setup.

3.2. Literature Analysis Focused on the Mediterranean Sea Province

The literature analysis on documents found within the Elsevier's Scopus database led to the record of 34 works on organisms collected in the Mediterranean Sea province. The manual research carried out on the references reported in these works led to the addition of seven documents. The total 41 documents (Figure 1) regarded 5 of the 6 considered taxa: Porifera (24), Cnidaria (3), Polychaeta (4), Cirripedia (2), and Tunicata (8) (Table S2).

Among all documents, only one work regarding Porifera was published before 1991 (1990, see reference in Table S1) (Figure 3B). The temporal distribution of documents showed how Porifera were considered in every 5-year period, with a peak in 2006–2010 (Figure 3B). Polychaeta were included in a constant number of works from 2001 to 2005, while for Tunicata, there is an increment of documents during time (Figure 3B). On the other hand, the low number of works on Cnidaria and Cirripedia is scattered through the different 5-year periods, from 2001 to 2005 till today (Figure 3B).

Inside the basin, most of the studies collected organisms in the Western Mediterranean ecoregion (22), mainly along the French and Spanish coasts (Figure 6; Table S2), followed by the Adriatic Sea (7), the Ionian Sea (6), the Levantine Sea (4), and the Aegean Sea (2) (Table S2). Figure 6 shows that most of the Porifera have been collected in the Western Mediterranean, while almost all studies on Tunicata and all on Cirripedia are referred to organisms collected in the Levantine and the Aegean Sea. Two-third of documents, moreover, gave information on the sampling depth (Figure 7). Most of the organisms were collected between 0 and 5 m depth, while Porifera is the only taxon collected at all the considered depth ranges (Figure 7).

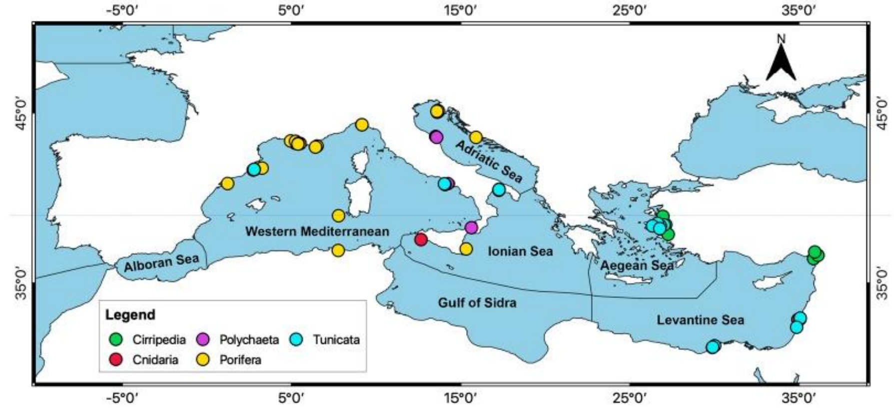

Figure 6. Map of the distribution of documents per taxa in each ecoregion of the Mediterranean Sea.

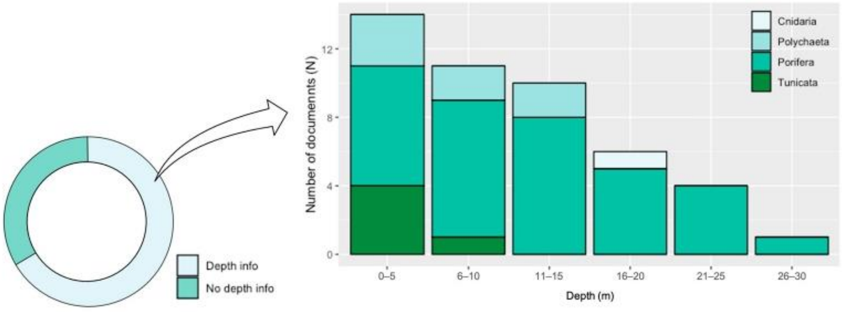

Figure 7. Donut chart of the percentage of documents giving information of the sampled depth(s) (**left**), and number of documents per taxa per depth range (**right**).

Most of the documents studied the role of organisms as bioindicators (28) especially of Cu (28), Pb (23), and Cd (20) contamination. Fourteen works explored the main effects of Cd (10) and Cu (9) on the collected species (Table S2), while one paper highlighted the tolerance of the microbiota extracted from polychaetes to Cd, Cu, and Zn (see Table S1 for citation).

Since Porifera resulted to be the most studied taxa, we considered this group for further considerations. Species studied belong to 11 orders of sponges (Table S2), and Dictyoceratida is undoubtedly the one to which most of works on biomonitoring are related. These organisms showed Cd concentrations ranging between 0.2 and 1.60 mg·kg-1 d.w., Cu between 22 and 300 mg·kg-1 d.w., and Pb between 0.1 and 90 mg·kg-1 d.w. (Table S2). Dictyoceratida has not been investigated for possible effects given by the presence of trace elements, but orders as Poecilosclerida and Scopalinida were considered more suitable for physiological studies and mainly on Cd and Cu (Table S2).

4. Discussion

At present, the sessile taxa considered in this study (Porifera, Cnidaria, Bryozoa, Polychaeta, Cirripedia, and Tunicata) are still little used as biomonitors compared with other groups, despite the fact that their potential application has been highlighted repeatedly during time [12,23], with an increasing annual trend in the last years. For example, searching on Scopus database using the keywords "heavy metal" AND "bivalve" or "heavy metal" AND "fish," without conducting the screening, 1148 and 6800 documents were obtained, respectively, more than all the works screened in our research, conducted using many keywords. In fact, the vast majority of the studies on the topic referred to organisms representing food resources for human consumption, which do not have to exceed specific levels, established by Community legislation or other relevant standards [23,30]. Another factor to take into account is that the considered taxa are not of the same economic importance as bivalves or fish, even though the economic role covered by some of them (e.g., sponges) is more and more often highlighted by the scientific community [31]. Nonetheless, looking at the physical and physiological characteristics a bioindicator should present (see Butler et al. [15] and Haug et al. [16]), these groups fit in many of the listed features. In fact: (i) with the exception of some cnidarians and polychaetes, they are exclusively sessile; (ii) most of them are active filter-feeders; (iii) they present a global distribution, therefore they can be collected and used for this purpose all around the globe, even at the Poles; (iv) these taxa comprehend modular organisms [32], therefore the collection of small portions does not affect their survival and they can rapidly regenerate; (v) some of them present also a gregarious behavior [33–37], allowing to sample just a part of a population and to observe possible changes on long-term monitoring.

From our review, it is clear that, at a global scale, studies are not equally distributed among marine regions [29], with most studies focused the Mediterranean Sea province [29], probably due to its nature of being a semienclosed basin [38], and thus more subjected to anthropogenic stress and pollution [39], as the one caused by HMs (see Table 1 from Danovaro [40], for quantities released annually, and Table 1 from Tovar-Sánchez et al. [41], for the concentration in the Mediterranean water). The basin, moreover, is subjected to the European legislation (e.g., MSFD 2008/56/EC), which suggests biological indicators (e.g., sponges and cnidarians) as potential tools to monitor the environmental status of coastal areas. Therefore, researchers could be more addressed to use unconventional organisms in their biomonitoring studies. However, also in the Mediterranean basin, there is a bias in the studies distribution, since research activities mainly focused on the Western Mediterranean ecoregion (22) [29], while the others are interested only by a low number of studies. This condition should be explained with the affiliation of the researchers studying the topic, which are mostly present in countries facing the Western Mediterranean ecoregion. Moreover, most of studies are limited to the most accessible shallow waters, mainly investigated by scuba diving.

Another strong bias highlighted by the present work is the difference in the number of documents per taxon, most of them concerning Cnidaria (37%) and Porifera (18.5%). Considered cnidarians are especially from tropical areas (Table S1), in which studies principally investigated the concentrations of HMs particularly in the hard skeleton of many scleractinian corals. Their skeleton is, in fact, characterized by the presence of growth bands, used in the determination of coral's age [42,43], and as a proxy of ocean responses to environmental factors, as temperature and salinity [44,45], and also contaminants, such as heavy metals [46,47], hydrocarbons [48], and pesticides [49]. This peculiarity makes the coral skeleton an important biomonitoring tool for marine pollution, allowing to analyze and detect possible variations in the concentrations of HMs over different time periods. However, together with scleractinians, other groups of cnidarians, e.g., octocorals and stylasterids, showed the presence of growth bands in their skeletons [50,51], making these taxa additional organisms suitable for biomonitoring studies.

Regarding the other well-studied group, Porifera have been mainly collected in the Temperate Northern Atlantic realm [29]. Especially interesting are the few studies analyzing the role of their associated bacteria [52–58]. Many sponges, in fact, are characterized by the presence of complex communities of microorganisms composing the associated microbiota, being named "bacteriosponges" or "high microbial abundance sponges" [59]. Since the microbiota can contribute up to 40% of sponge biomass and cover a fundamental role in their secondary metabolism [60,61], it has been suggested that it can actively participate in the bioaccumulation process. Many bacteria isolated from sponges, e.g., *Fasciospongia cavernosa*, have been found to be resistant to different antibiotics and pollutants, including Persistent Organic Pollutants (POPs) and various heavy metals, as Cu, Pb, Co, Cd, Zn, Ni, and Hg together with their organic compounds [55]. Different researches showed how also the bacteria associated with polychaetes and ascidians can be important in the sequestration of HMs from water [62–67]; therefore, increasing study of the effective role of the microbiota is necessary to fully understand the dynamics of the process. Besides, we want to stress the importance of works on the possible negative physiological effects of HMs in organisms (e.g., apoptosis of sponge tissues, bleaching in corals, decreasing of sperm density in polychaetes, etc.) [68–70], but also the "positive" consequences that HMs can trigger, e.g., in the moon jellyfish *Aurelia* spp., which polyps were observed to increase their asexual reproduction if exposed to Ag and Hg [4,71]. However, what we call a "positive" response can have severe environmental consequences. Referring to the example of *Aurelia* spp., an increase in the asexual reproduction can be followed by increase in polyp strobilation and ephyra release and, thus, intense jellyfish blooms [72], with multiple ecological and socio-economic consequences on human health, marine trophic chains, fishery, and aquaculture [73,74]. Moreover, an increase in this kind of studies will help to identify different physiological traits known as "biomarkers." Biomarkers are biological responses of suitable sentinel species to pollutants, which can act as early-warning tools able to detect pollutant-induced stress syndromes in organisms before the occurrence of severe habitat and communities' alterations [75].

From our review emerged clearly that the most studied HMs have been Cd, Cu, Pb, and Zn, both due to their natural toxicity (Cd and Pb) (WFD 2000/60/EC) and both for being used by organisms as micronutrients (Cu and Zn). In fact, essential elements can be considered HMs since they can become toxic, for an organism, above specific thresholds [28], as it was observed, e.g., in *Aurelia aurita* polyps [71] or in the sponge *Crambe crambe* [76]. Many groups of invertebrates are known to synthetize metallothioneins or metallothionein-like proteins, including the taxa considered in this review [77–80], except for Cnidaria and Bryozoa, for which there is still no information available [81–83]. Metallothioneins are cysteine-rich metal-binding proteins, which can sequester metals, being involved in metal resistance and in the detoxification processes [82]. If the detoxification mechanism is not overwhelmed, organisms can increase body metal concentrations without showing toxic effects or signs of suffering. However, if in the environment metal concentrations are elevated, the mechanism overwhelms and metals can bind to sensitive intracellular targets, inducing toxicity [84]. Therefore, to understand the thresholds not to be overcome, for both essential and nonessential elements, results fundamental to set general concentration limits focused on organism wellness, since by now, the actual low limits for HMs concern only edible species (Directive 2002/32/EC) and many are referred to human health [30].

Another important issue emerging from our bibliographic analysis was the lack of a standardization of presented data. First of all, almost half of the studies (generally written in the last century) did not show any information about the quality assurance (Certified Reference Material (CRM), recovery of standard addition, etc.) and, even though the 54% presented quality information, they were provided in an unclear way (no table of data and explanation of the reference material) or inappropriate material has been used (Table S1). In addition, only about the 15% analyzed the physicochemical parameters of water (e.g., pH, conductivity, alkalinity, organic matter, etc.) (Table S1), factors which could influence the speciation of HMs in the marine environment [85] and, thus, modify their bioavailability [11]. Different concentration orders and units are also given, making difficult the data management and the intercomparison among studies. Most of documents expressed data in dry weight (d.w.) while some others expressed results in wet weight (w.w.). If data presented in w.w. do not also provide the water concentration in samples, they are not comparable with those expressed in d.w. These issues arouse the attention of the scientific community since the 1970s [86], and the suggestion for the creation of "quality assurance" protocols for data comparison was given repeatedly in past decades [75,87]. Examples are the MED POL Mediterranean Sea Biomonitoring Programme [88] and the Pollution Effect Network (PEN) [75]. However, these activities did not pursue until today, while the intercomparison problem persists.

5. Conclusions and Future Perspectives

The results of our review showed a growing interest by the scientific community in the identification of sessile taxa as nonconventional bioindicators of HMs. The increasing interest is mainly linked to the higher awareness of researchers to metals contamination in the marine environment and to the international legislations, which are more and more focused on the role of these taxa as fundamental monitors of environmental pollution. Different studies suggested that some sessile organisms can be used also in the bioremediation processes, thanks to their characteristics and the part played by the associated microbiota (bacteria and fungi). This is particularly true for sponges, cnidarians, polychaetes, bivalves, and tunicates [60,64,89–91]. The application of vagile organisms as bioremediation and biomonitoring tools (as fish, cephalopods, or decapods crustaceans) results instead more difficult, since they are not sessile and cannot detect the actual values of a single location. In addition, even if the present work focused only on marine species, some of the considered taxa (Porifera, Cnidaria, and Bryozoa) also includes freshwater species. Therefore, these organisms can be applied as useful tools also near inland farms, industries, and plantations, as it is already done for freshwater bivalves [92].

Nonetheless, researchers should also focus their attention in identifying model organisms, which proved to be more suitable as bioindicators or bioremediation tools compared to other species of the same taxa.

These research activities cannot exclude the consultations of specialized taxonomists, since (1) species belonging to the same genus can bioaccumulate different concentration of HMs [12] or can present different responses if subjected to the same metal (see Figure 1 from Viarengo et al. [75] for the different physiological responses of *Mytilus galloprovincialis*); (2) some species considered by the literature revised for this study (e.g., *Geodia cynodium*, *Spongia (Spongia) lamella*, and *S. (S.) officinalis*) (see Tables S1 and S2 for citations) are included in the Annex II of the SPA/BIO Protocol of Barcelona Convention and, therefore, subjected to protection, due to their status of "threatened" species [93].

Our work highlighted many research gaps, but two aspects need special attention. As future perspectives, we recommend increasing sampling and collection efforts and widening the geographic and bathymetric distributions of the studies not only in the

Mediterranean Sea but also in the other marine realms, provinces and ecoregions, especially in the less investigated ones, integrating the results with the studies currently available. Moreover, data standardization is still a big issue in the field, therefore, we suggest facing the problem again, reopening old programs (e.g., MED POL and PEN) or crating new ones with new possible protocols to standardize methods, validation, analysis, and data presentation.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/2/580/s1, Table S1: List of works divided per taxa with all document's details, Table S2: List of documents on the Mediterranean Sea province.

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PAPER 4

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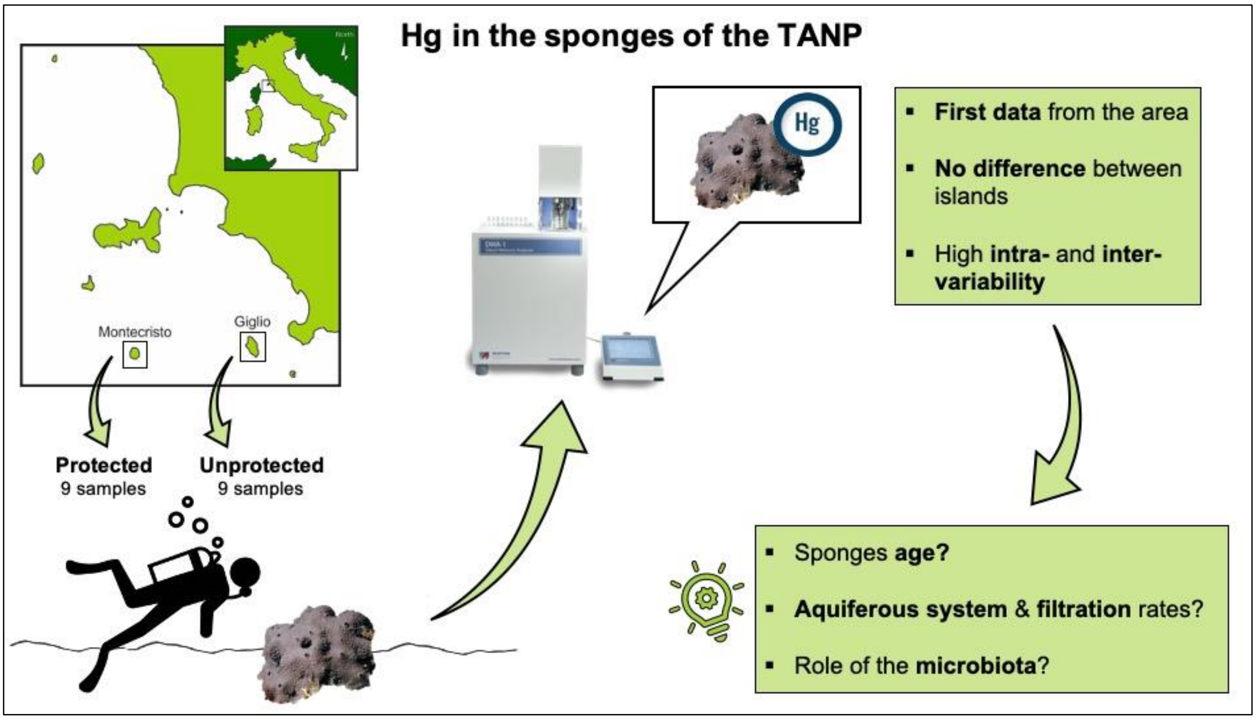

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Article

Hg Levels in Marine Porifera of Montecristo and Giglio Islands (Tuscan Archipelago, Italy)

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Abstract: Porifera are filter-feeding organisms known to bioaccumulate different contaminants in their tissues. The presence of mercury (Hg) has been reported in different Mediterranean species, mainly collected in the southern coast of France. In the present study, mercury concentrations in the tissue of the sponges of Montecristo and Giglio, two islands of Tuscany Archipelago National Park (TANP), are presented for the first time. Analyses of total mercury content were performed by Direct Mercury Analyzer. Statistical differences have been reported in the Hg concentrations of species collected in both islands, but they do not appear related to the anthropic impacts of the islands. Among the collected species, a high intra- and inter-variability have been recorded, with *Cliona viridis* showing the lowest concentration (0.0167–0.033 mg·kg^{-1} dry weight), and *Chondrosia reniformis* and *Sarcotragus spinosulus* the highest (0.57 ± 0.15 and 0.64 ± 0.01 mg·kg^{-1} dry weight, respectively). The variability of Hg measured did not allow us to identify sponges as bioindicators of toxic elements. Anyway, these results improve knowledge on the ecosystem of the TANP, underlining the species-specificity of metal concentrations for Porifera, and providing additional data to address the main input of the Marine Strategy guidelines to protect coasts, seas and oceans.

Keywords: toxic element; Mediterranean Sea; sponges; biomonitoring

1. Introduction

Mercury (Hg) is considered to be one of the most toxic heavy metals due to its persistence in the environment, bioaccumulation in organisms and biomagnification in the trophic chain [1,2]. Atmospheric inputs of Hg have tripled during the last 150 years, being two-thirds of its actual concentration from anthropogenic sources [2,3]. Traces of mercury have been found in all the compartments of the ecosphere (atmosphere, hydrosphere, lithosphere and biosphere) [4–6]. In aquatic environments, it is transformed by chemical and biological reactions in organomercury compounds, as methylmercury (MeHg), the most toxic mercury species, which can be bioaccumulated more than other trace elements along the trophic chain [6]. Mercury can have many different effects on a wide range of organisms, both vertebrates, e.g., References [7–9], and invertebrates, e.g., References [10–12]. For all these reasons, Hg is listed in the European Water Framework Directive (WFD 2000/60/EC) as a priority substance representing a risk for the good chemical status for the aquatic environment, with a Maximum Allowable Concentration (MAC) of 0.07 μg·L^{-1}.

The presence of mercury in the Mediterranean Sea has been documented since the 1970s [13,14]. The principal mercury input (94%) was recognized in rivers' dischargements, while only 5.5% was related to direct industrial wastewater and 0.5% to domestic sewage [15]. The Mediterranean area presents a high number of natural deposits of mercury distributed along the coasts of many countries, containing about 65% of the world's cinnabar (HgS) deposits [15,16]. Particularly, in Tuscany (Italy), the levels of Hg in different environmental matrices are derived from both a natural contribution of the mineralization and the pollution caused by the huge exploitation of the area of Mount Amiata [17]. In fact, Mount Amiata, located in the south of Tuscany, is part of the geologic anomaly of the Mediterranean basin and it is characterized by a large cinnabar deposit [16,18]. Moreover, it is well known from ARPAT (acronym for Regional Agency for Environmental Protection of Tuscany) and ISPRA (acronym for Higher Institute for Environmental Protection and Research) technical reports, and scientific papers [19], the presence of mercury in the waters and organisms of the Tuscany coast and island. From 2012 to 2017, high concentrations of mercury have been reported in water, sediment and biota (as *Posidonia oceanica*, *Mytilus galloprovincialis* and different fish species) in the Tuscan Archipelago islands, e.g., References [20–24].

Many studies have been conducted worldwide on the bioaccumulation and the effects of mercury and its organic compounds in edible species, such as bivalves, cephalopods, decapods crustaceans and fish [6,25–27]. Although, other filter-feeding organisms, such as polychaetes, tunicates, sponges and barnacles, have been proposed as bioindicators in shallow waters [28], but these taxa are still little used for this purpose. Especially, sponges satisfy all the characteristics listed in References [29,30] for a suitable bioindicator and have been recommended by many authors, e.g., References [31,32], and by the WFD as possible monitors for heavy metals. Being filter-feeders, sponges can filter a large amount of water, and they can collect and accumulate many different contaminants (such as hydrocarbons, organochlorinated compounds, heavy metals, etc.) in their tissues, which are present in the water column both in the soluble and particulate phases [10,28]. Moreover, the level of the bioaccumulation in their tissues is a function of the contaminants' concentration in the water [33]. The presence of heavy metals in sponges can affect their physiology and survival [28,34,35]. Only a few authors have investigated the presence of Hg in sponges and most of the studies have been conducted on samples, mainly on the genus *Spongia,* collected in the coast of Marseille [10,36–38].

The current paper presents, for the first time, total mercury content in the tissue of the sponges of Montecristo and Giglio, two islands of Tuscany Archipelago National Park (TANP). The study addresses two hypotheses: (1) sponges collected show detectable total mercury contents, and (2) there are any differences in Hg concentrations between the sponge species collected in both studied islands. Our results point out that sponges have detectable Hg concentrations, showing a high inter- and intra-specific variability. This variability could also be responsible of the lack of trend in the Hg concentrations between the specimens collected from the more anthropic Giglio and the Integral Reserve of Montecristo. Anyway, the results obtained with statistical analyses give new important insights for the area on the differences among Hg concentration in sponges.

2. Materials and Methods

2.1. Study Area

The study was carried out in June 2019 in Montecristo and Giglio islands (Tuscany, Italy) (Figure 1).

The Montecristo Island (42.3317° N, 10.3083° E) (Figure 1B) is an uninhabited and isolated island, sub-circular in shape, fourth in size after Elba, Giglio and Capraia islands, and completely mountainous, reaching a height of 645 m above sea-level with an almost constant slope of 25° [39–42]. Its history and geography distinguish the island from the others: during the Quaternary period, Montecristo remained in contact with the Tuscany's littoral for a shorter period than the other islands, ending up located 63 km from the mainland, on the limit of the continental shelf, closer to Corsica than to the mainland [39].

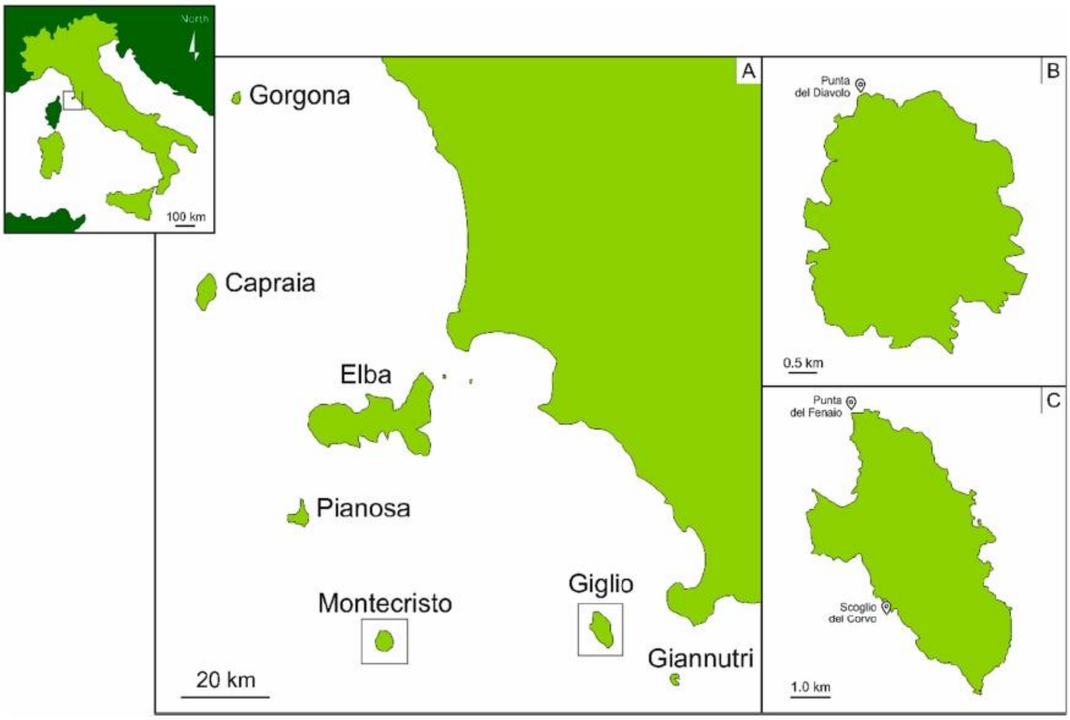

Figure 1. Map of the Tuscan Archipelago National Park (TANP) (**A**), Montecristo Island (**B**) and Giglio Island (**C**). The sampling sites are indicated in the islands' maps.

In 1971, Montecristo was established as an Integral Nature Reserve, and in 1988, was declared a biogenetic nature reserve by the Council of Europe. Moreover, the island has the status of Special Protection Area (SPA, Directive 79/409/EEC). The land and the adjacent waters, up to 1 km offshore, are controlled by the Coast Guard and the Carabinieri Corps [41,43]. Bathing, diving, fishing, mooring and circumnavigating are forbidden, while landing, berthing and scientific activities are allowed, only under specific conditions and with the permission of the Territorial Office for Biodiversity of the Carabinieri Corps of Follonica [41]. Due to the high protection which Montecristo has undergone, the island is considered one of the most pristine and best-preserved sites of the Mediterranean Sea, being previously identified as a reference site for the ecological quality assessment of the western Mediterranean benthic assemblages on rocky bottoms [44,45].

The Giglio Island (42.3603° N, 10.9229° E) (Figure 1C), being the second in size after Elba, is included in the southern group of the Archipelago, only 14 km from the Italian coast [42,46]. It is an oval-shaped island, characterized by a mountain chain along the north–south axis, and it reaches a height of 496 m above the sea-level. Contrary to Montecristo, the island hosts 1500 residents, spread out among the villages of Giglio Castello, Giglio Porto and Campese [42]. Only 40% of the island is included inside the protected area, while adjacent waters and many terrestrial areas do not have any protection (DPR 22 July 1996) [47]. The areas included in the legislation of the TANP are divided into four zones: (1) zone A of strict reserve, including five rocks (Cappa, Corvo, Mezzo Franco, Pietra Bona, and Le Scole), (2) zone B of general reserve, (3) zone C of general protection and (4) zone D of socio-economic promotion [42]. In general, Giglio is considered to have undergone anthropogenic pressures [47], due to the high flow of tourists during Summer [42], and to be more exposed to potential stress deriving from the mainland.

2.2. Samples Collection and Identification

Surveys were conducted by SCUBA diving and eighteen sponge samples were collected between 5 and 40 m, nine on the hard bottom assemblages of Punta del Diavolo (42°21′02.46″ N; 10°17′55.86″ E) in the Montecristo Island (TANP permission #00068010) (Figure 1B), and nine between Punta del Fenaio (42°23′21.54″ N; 10°52′48.18″ E) and Scoglio del Corvo (42°20′17.76″ N; 10°53′21.36″ E) in the Giglio Island (Figure 1C). The low number of samples for this preliminary study is strictly subordinated to protect and respect the island ecosystem, which needs to be protected even in the case of scientific research.

Samples were immediately frozen on dry ice, and then stored at −20 °C until analysis.

Among the samples, fourteen species of Demospongiae have been identified: *Agelas oroides* (Schmidt, 1864), *Axinella damicornis* (Esper, 1794), *Cliona viridis* (Schmidt, 1862), *Haliclona* (*Halichoclona*) *fulva* (Topsent, 1893), *Haliclona* (*Soestella*) *mucosa* (Griessinger, 1971), *Penares euastrum* (Schmidt, 1868) and *Sarcotragus spinosulus* Schmidt, 1862 in Montecristo, and *Chondrosia reniformis* Nardo, 1847, *C. viridis*, *Crambe crambe* (Schmidt, 1862), *H.* (*H.*) *fulva*, *Hemimycale columella* (Bowerbank, 1874), *Hymedesmia* (*Hymedesmia*) *baculifera* (Topsent, 1901) and *Petrosia* (*Petrosia*) *ficiformis* (Poiret, 1789) in Giglio. For the species *P. euastrum* and *C. reniformis*, three samples each were collected; therefore, we denominated them with the name of the species followed by the number 1, 2 or 3 between brackets.

2.3. Samples Treatment and Mercury Analysis

A clean room laboratory ISO 14644-1 Class 6, with areas at ISO Class 5 under laminar flow, was used for all laboratory activities. After the identification, samples were weighted (laboratory analytical balance, AT261 Mettler Toledo Greifensee, Switzerland, readability 0.01 mg, repeatability standard deviation (SD) = 0.015 mg) and cleaned with ultrapure water (A10 Milli-Q system, Merk Millipore, Bedford, MA, USA). The acid-cleaning procedures, used for all the laboratory materials, were performed as described in References [48,49].

After cutting samples into small pieces, sponges were lyophilized (Edwards EF4 modulyo, Crawley, Sussex, England), minced, homogenized and divided in aliquots of about 0.02 g each. Analyses of total mercury content (THg) were performed by Direct Mercury Analyzer (DMA-1 Milestone, Sorisole (BG), Italy), as described in Reference [50]. Briefly, the total mercury content was quantified by thermal decomposition amalgamation atomic absorption spectrometry at 253.7 nm. The calibration curve method was used for the quantification of Hg content. All measurements were replicated at least 4 times.

2.4. Accuracy

Quality assurance and quality control were assessed by processing blank samples and certified reference material (dogfish muscle DORM-2, NRCC; Ottawa, ON, Canada). The experimental values obtained for Hg in blanks are negligible compared with the metal content in sponge tissue (<1%). For DORM-2 analysis (n = 8), Hg content (4.58 ± 0.10 mg·kg^{-1}) is in agreement with the certified value (4.43 ± 0.05 mg·kg^{-1}) and no statistically significant differences were observed (*p*-value > 0.05, Student's T test, STATGRAPHICS 18 Centurion, 2018).

2.5. Data Analyses

Data are expressed as arithmetic mean ± standard deviation (SD) of the performed replications. Statistical analyses of differences within organisms were performed using the analysis of variance (one-way ANOVA) after testing the homogeneity of the variance with Levene's test [51]. In case of heteroscedasticity, we applied the non-parametric Kruskal–Wallis analysis of variance. Depending on the resulting statistics, post-hoc comparison was eventually performed with the Bonferroni correction, always considering a significant level of 0.05. All graphs and statistical analyses were performed using STATGRAPHICS (STATGRAPHICS Centurion 2018, Statgraphics Technologies Inc., The Plains, VA, USA).

3. Results

THg, expressed on a dry weight (dw) basis, in sponges collected in the area of Montecristo and Giglio islands, are reported in Figure 2A,B, respectively.

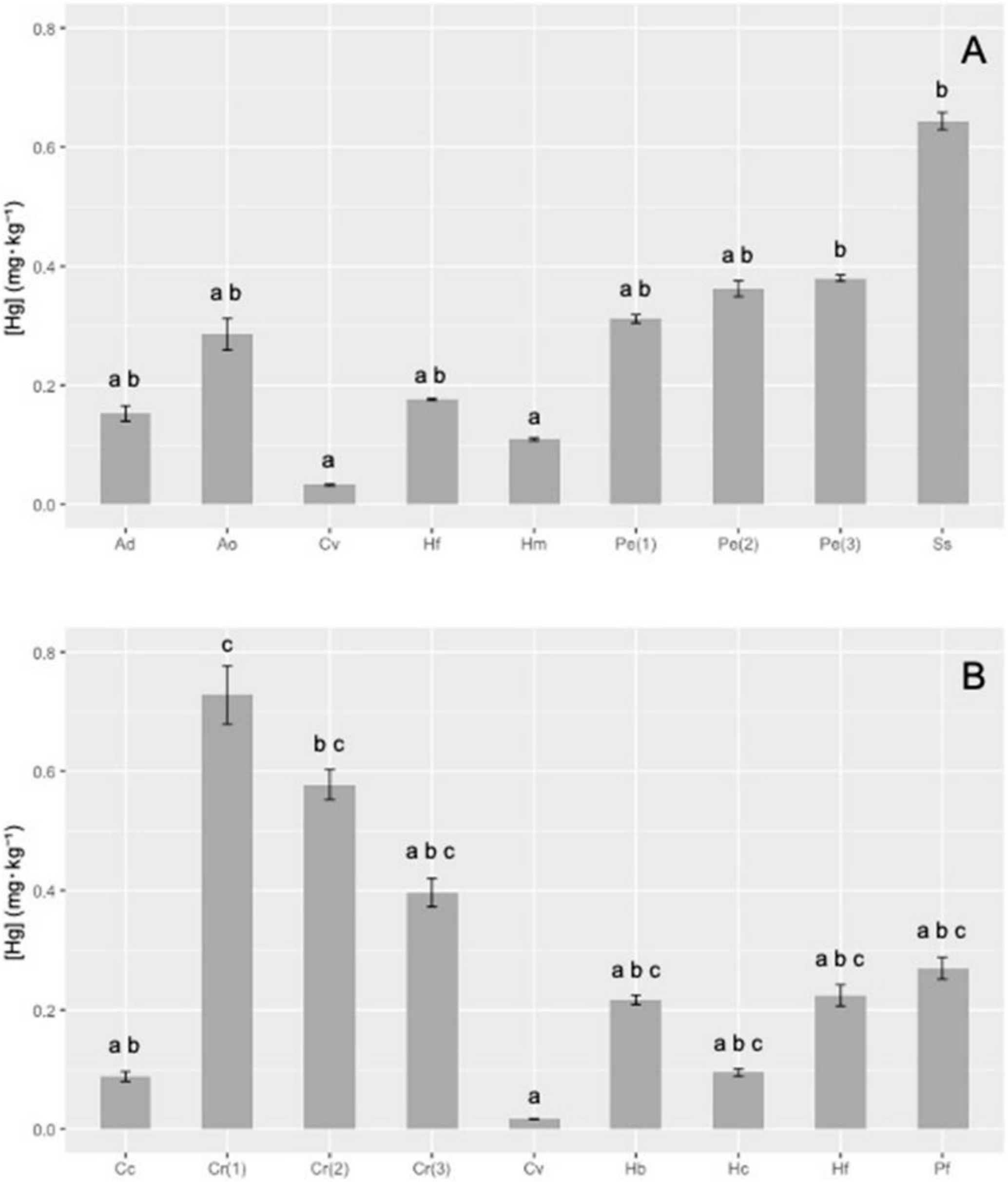

Figure 2. Mercury levels (± standard deviation (SD)) in the sponges collected in Montecristo (**A**) and Giglio (**B**) islands. In each graph, different letters (a, b, c) indicate statistically significant differences among sponges (Kruskal–Wallis test, $p < 0.05$). Ad = *Axinella damicornis;* Ao = *Agelas oroides;* Cv = *Cliona viridis;* Hf = *Haliclona* (*Halichoclona*) *fulva;* Hm = *Haliclona* (*Soestella*) *mucosa;* Pe = *Penares euastrum;* Ss = *Sarcotragus spinosulus;* Cc = *Crambe crambe;* Cr = *Chondrosia reniformis;* Hb = *Hymedesmia* (*Hymedesmia*) *baculifera;* Hc = *Hemimycale columella;* Pf = *Petrosia* (*Petrosia*) *ficiformis.*

Total mercury showed high variability in sponges coming from the marine area of Montecristo island, ranging from 0.033 to 0.64 mg·kg^{-1}, with the minimum content in *Cliona viridis* (0.033 ± 0.001 mg·kg^{-1}) and the maximum (20-fold higher) in *Sarcotragus spinosulus* (0.64 ± 0.01 mg·kg^{-1}). Values ranging from about 0.11 to 0.29 mg·kg^{-1} were recorded in *Agelas oroides, Axinella damicornis, Haliclona (Halichoclona)* *fulva* and *H. (Soestella) mucosa*, whereas slightly higher values were found for the three samples of *Penares euastrum* (1), (2) and (3) (0.35 ± 0.03 mg·kg^{-1}), that showed similar concentration among them. Statistical analysis of the data showed a significant difference (Kruskal–Wallis test, $p < 0.05$) between *C. viridis* vs. *P. euastrum* (3) and *S. spinosulus*, *H. (S.) mucosa* vs. *P. euastrum* (3) and *S. spinosulus* (Figure 2A).

A high variability of THg content was also found in sponges collected in the marine area of Giglio Island: total Hg ranged from 0.017 to 0.73 mg·kg^{-1} dw. Even in this case, the minimum content was measured in *C. viridis* (0.0167 ± 0.0003 mg·kg^{-1}), which showed values from 10 to 70 times lower than other species. On the other hand, the three samples of *Chondrosia reniformis* (1), (2) and (3) showed the highest concentrations (ranging from 0.39 to 0.73 mg·kg^{-1}). In the other sponges, the THg content ranged from ≈0.1 (*Crambe crambe, Hemimycale columella*) to about 0.22 mg·kg^{-1} in *H. (H.) fulva, Hymedesmia baculifera* and *Petrosia (P.) ficiformis.* The Kruskal–Wallis test highlighted significant differences ($p < 0.05$) between *C. reniformis* (1) vs. *C. crambe*, *C. viridis* vs. *C. reniformis* (1) and *C. reniformis* (3) (Figure 2B).

Since samples of *C. viridis* and *H. (H.) fulva* were found in both islands, differences in the THg content between the two islands have been investigated, to compare the possible influence of different sites. *C. viridis* showed a higher THg in Montecristo island (Kruskal–Wallis test, $p < 0.05$), while the concentration of mercury in *H. (H.) fulva* was higher in Giglio (Kruskal–Wallis test, $p < 0.05$).

4. Discussion

Samples show the presence of mercury both in Montecristo and Giglio. Although statistical differences in the Hg concentrations have been detected in the sponge species (*Cliona viridis* and *Haliclona (H.) fulva*) collected in both islands of the Tuscan Archipelago, the lack of a trend excludes a possible influence of the site. In fact, even though since 1971 Montecristo has been an Integral Nature Reserve considered a pristine area not interested by mechanic impacts, such as anchoring and diving disturbances, fishing, etc., its water can still be affected by other different impacts, such as climate change and alien species, e.g., References [52,53], and the presence of different pollutants [20–23] due to water circulation [54].

Currently, no law limit is defined for Hg in sponges and few data are available on Hg concentration in this phylum, most of which regard species and genera different from those collected in this study, e.g., References [28,55,56]. THg values of *Sarcotragus spinosulus,* measured by us, are comparable with the concentrations found in other species belonging to the subclass Keratosa, characterized by a skeleton of spongin fibers [57] such as *Scalarispongia scalaris* (Schmidt, 1862) (cited as *Cacospongia scalaris*) [37], *Spongia (Spongia) lamella* (Schulze, 1879) (cited as *S. agaricina*) [36,37], *S. (S.) nitens* (Schmidt, 1862) (cited as *S. nitens*) [36] and *S. (S.) officinalis* (Linnaeus, 1759) (cited as *S. officinalis*) [10,36–38] (Table 1). THg values of *Chondrosia reniformis* recorded in specimens from Montecristo and Giglio are similar to the ones recorded in Reference [37] for the same species collected in different localities near Marseille (Table 1). On the other hand, results obtained for *Agelas oroides* and *Cliona viridis* in Reference [37] are higher than our THg values for both species (Table 1). Perez et al. [37] collected samples of *Cliona viridis* in polluted and non-polluted areas, and he did not find any differences in the Hg concentrations. Therefore, also the difference reported between our specimen and the one of Reference [37] could be related not only to the species-specificity but also to the individual specificity of sponges, with a high intra- and inter-specific variability [58,59].

Although it is known that some heavy metals (e.g., copper, lead and vanadium) have effects on sponges, increasing their fission frequency, inducing changes in cellular aggregation and reducing growth and filtration rates [34,35], no information is available for Hg. However, some studies showed, from laboratory experiments, that mercury can cause death, inhibition of gemmule formation and malformation in gemmoscleres in the freshwater sponge *Ephydatia fluviatilis* (Linnaeus, 1759) (0.001 to 1.000 mg·kg^{-1}) [60], and it can arrest movement of single sponge cells of *Scopalina lophyropoda*

(Schmidt, 1862) (1 and 5 μg·kg^{-1}), which tended to be rounded without pseudopodia [61], while a high concentration of MeHg (0.6 mg·kg^{-1}) induces apoptosis in tissue of *Geodia cydonium* (Linnaeus, 1767) [62].

Table 1. Selection of literature data for mercury concentrations in sponges.

Species	Location	Methodology	Hg (mg·kg^{-1}) dw	References
Agelas oroides	Montecristo Island, Tuscany (Italy)	Mean ± SD	0.29 ± 0.03	This study
	Coutiou, Marseille (France)	Mean ± SD	1.7 ± 0.2	[37]
Chondrosia reniformis	Giglio Island, Tuscany (Italy)	Mean ± SD	0.73 ± 0.05 0.58 ± 0.02 0.39 ± 0.02	This study
	Coutiou, Marseille (France)	Mean ± SD	0.8 ± 0.1	[37]
Cliona viridis	Montecristo Island, Tuscany (Italy)	Mean ± SD	0.033 ± 0.001	This study
	Giglio Island, Tuscany (Italy)		0.0167 ± 0.0003	
	Coutiou, Marseille (France)	Mean ± SD	0.3 ± 0.4	[37]
Sarcotragus spinosulus	Montecristo Island, Tuscany (Italy)	Mean ± SD	0.64 ± 0.01	This study
Cacospongia scalaris	Coutiou, Marseille (France)	Mean ± SD	0.8 ± 0.0	[37]
Spongia (Spongia) lamella	Marseille and Saint Tropez (France)	Max	0.52	[36]
	Coutiou, Marseille (France)	Mean ± SD	0.3 ± 0.0	[37]
Spongia (Spongia) nitens	Marseille and Saint Tropez (France)	Max	0.52	[36]
Spongia (Spongia) officinalis	Marseille and Saint Tropez (France)	Max	0.52	[36]
	Coutiou, Marseille (France)	Mean ± SD	0.9 ± 0.1	[37]
	Coutiou, Marseille (France)	Mean ± SD	0.8 ± 0.1	[38]
	Maire, Marseille (France)		0.5 ± 0.1	
	Plane, Marseille (France)		0.9 ± 0.3	
	Jarre, Marseille (France)		0.6 ± 0.1	
	Riou, Marseille (France)		0.5 ± 0.1	
	Veyron, Marseille (France)		0.6 ± 0.1	
	Lavera, Marseille (France)		0.5 ± 0.1	
	Niolon, Marseille (France)		1.1 ± 0.5	
	Port-Cros, Saint Tropez (France)		0.6 ± 0.4	
	Coutiou, Marseille (France)	Mean ± SD	0.4 ± 0.1	[10]
	Port-Cros, Saint Tropez (France)		0.3 ± 0.1	

The highest THg values were found in *Chondrosia reniformis* and *Sarcotragus spinosulus*, collected in Giglio and Montecristo islands, respectively. These two species share the absence of a mineral skeleton, showing *C. reniformis*, a dispersal fibrillary collagen, and *S. spinosulus*, an organic skeleton made of spongin [57]. Three papers on Antarctic and Mediterranean species [59,63,64] showed that spicules accumulate only a small part of heavy metals compared to the sponge tissues; while in *Spongia* spp., the skeletal spongin fibers can trap and consequently concentrate metals, such as Fe, Pb, Cr, Zn and V [37,65]. These studies suggest that a collagenous skeleton instead of a mineral one can bioaccumulate a higher concentration of heavy metals. On the other hand, the mercury concentrations recorded for *C. viridis*, which is the only boring species and symbiotic with zooxanthellae [66], were the lowest among all the species collected. It is possible that the association with zooxanthellae influences the accumulation capacity of the sponge, as also pointed out in Reference [37]. It has been demonstrated that *C. viridis* can vary its filtration rates depending on the photosynthetic activities of its zooxanthellae [67], and this could partially explain why our samples present low concentrations of THg in their tissues.

The aforementioned intra- and inter-specific variability suggested by our observations and other authors [37,38] (Table 1) appear to not always be in agreement with the distance from the main source of pollutants. This can be a result of physiological and skeletal differences among sponge species. For example, the diversity of morphology of the aquiferous system could influence the size range of particles that can be filtered as well as the filtration rates [37]. Moreover, sponges are long-living organisms, with variable rate of growth, and it is very difficult to age each individual [36], therefore it is possible that low concentrations of heavy metals found in one organism could be related to its young age. In different sponge species, *Clathria (Clathria) prolifera* (Ellis and Solander, 1786) (cited as *Microciona prolifera*) [68], *Suberites domuncula* (Olivi, 1792) [69] and *Spongia (Spongia) officinalis* [10], authors found the presence of metallothioneins (MT) and metallothionein-like proteins (MTLPs), which are known to be used in the sequestration of metals in some invertebrates [70,71], and a positive correlation between MTLP concentrations and some heavy metals' (Cu, Zn and Hg) concentrations has been established [10].

Moreover, sponges can host complex communities of microorganisms, including bacteria, cyanobacteria and fungi, in their tissues [72]. The relationships between sponges and their microbiota can be defined as a symbiosis, in which microorganism communities can take part in the metabolic cycles and exchange different metabolites with their host [73]. It has been demonstrated that many bacteria, which can contribute up to 40% of the sponge biomass, are resistant to different antibiotics and pollutants, including Persistent Organic Pollutants (POPs) and heavy metals, such as Cu, Pb, Co, Cd, Zn, Ni, Hg and their organic compounds, e.g., References [72,74–76]. Moreover, some studies asserted that sponges and the associated bacteria can potentially be applied in the bioremediation of aquatic environments contaminated by mercury [74,75]. Therefore, the microbiota also seems to play an important role in the bioaccumulation of heavy metals in sponge tissues, suggesting the necessity of deepening the investigation towards this aspect.

5. Conclusions

In conclusion, our results are the first data about total Hg concentration in sponges from the TANP and suggest that these metazoa could accumulate toxic elements in coastal waters. On the other hand, the high variability of concentrations in THg measured in all specimens in both islands did not allow us to identify sponges as bioindicators of toxic elements. However, further studies with a higher number of sponge samples are needed to understand in which compartment (skeleton, tissue or microbiota) they accumulate the heaviest metals, analyzing the main effects on the histology and physiology of this group. Anyway, these results improve the knowledge on the ecosystem of the TANP, pointing out the species/individual-specificity of metal concentrations for Porifera and the key role of the organic skeleton, tissue and microbiota. Moreover, these data provide additional environmental information on the Tuscany Archipelago to address the main input of international guidelines on the Marine Strategy to protect and clean up coasts, seas and oceans.

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PAPER 5

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Distribution of mercury inside the Mediterranean sponge *Chondrosia reniformis*: A study case from the Tuscan Archipelago National Park (Tyrrhenian Sea)

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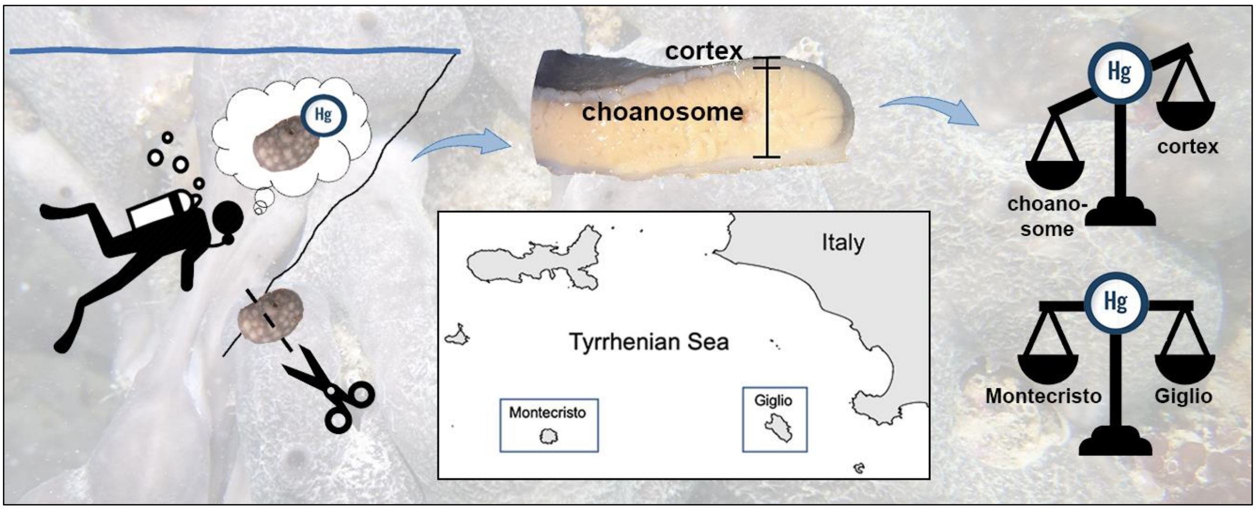

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Short Communication

Distribution of mercury inside the Mediterranean sponge *Chondrosia reniformis*: A study case from the Tuscan Archipelago National Park (Tyrrhenian Sea)

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ABSTRACT

Trace elements are ubiquitous substances in the marine environment, representing a problematic hazard for organisms since many of them lack any biological function. The main aim of the study was to investigate the variation of the total mercury content (THg) in the Mediterranean sponge *Chondrosia reniformis* collected in two islands of the Tuscan Archipelago, Italy. The two islands did not show harmful levels of THg (seawater and sediment) and no intraspecific variability was detected in THg values and bioconcentration factors of collected samples. Conversely, THg values recorded in the choanosome of *C. reniformis* were significantly higher than in the cortex, pointing out a possible involvement of the sponge microbiota in the bioaccumulation process. Our study suggests that *C. reniformis* could be an attractive bioindicator of toxic elements pollution in coastal waters, highlighting the potential use of these organisms in the monitoring programs of coastal areas, as suggested by various European marine policies.

1. Introduction

Trace elements are ubiquitous substances in the marine environment (Wurl and Obbard, 2004), which can be released by different anthropogenic activities (e.g., sewage effluents, industrial production, atmospheric deposition, fuel combustion, mining, and smelting, among others) (Jakimska et al., 2011; Roveta et al., 2021). Essential elements (e.g., Fe, Cu, Co, Mn, and Zn) are involved in the functioning of enzyme systems, however, over specific thresholds, they can induce detrimental effects in the organisms and environment. Other elements (e.g., Al, As, Ba, Bi, Cd, Pb, Hg, Ni, Pt, and Ag) have no biological functions and are considered as non-essential metals (Roveta et al., 2021), representing a problematic hazard due to their characteristics. In fact, non-essential metals: (a) do not degrade and have long half-lives, (b) can bioaccumulate in organisms and, in some cases, biomagnify in the trophic chain, and (c) can be converted by chemical and biological reactions in different organic compounds (Simkiss and Taylor, 1995; Jakimska et al., 2011; Roveta et al., 2021).

Trace elements can be detected directly from water samples, but they are more often monitored indirectly by the use of different organisms as biomonitors (e.g., Pan et al., 2018; O'Callaghan et al., 2019; Conti et al., 2020; Costa et al., 2021; Girolametti et al., 2021; Roveta et al., 2021; Singh and Gupta, 2021), in which the level of the bioaccumulation is a function of the metals' concentration in the water (Rainbow and Phillips, 1993; Truzzi et al., 2008). Sponges are sedentary filter-feeding organisms, able to filter a large volume of water (many thousands of liters kg^{-1} day^{-1}), including food particles, detritus, bacteria, fungi (Schmitt et al., 2007), and many different contaminants, as trace elements (Perez et al., 2005; Cebrian et al., 2006; Batista et al., 2014).

Chondrosia reniformis Nardo, 1847 is a Demospongiae, presenting an Atlanto-Mediterranean distribution (Lazoski et al., 2001), and being highly abundant on the shaded walls of the rocky bottom assemblages between 0- and 50-m depth (Wilkinson and Vacelet, 1979). Many of its characteristics (e.g., different physiological responses to changes in environmental conditions, incorporation of silica, production of collagen and other secondary metabolites, etc.) (Wilkinson and Vacelet, 1979; Bavestrello et al., 1995; Bavestrello et al., 1998a; Pozzolini et al., 2012, 2015, 2016, 2018) have made this species interesting for the scientific community. *C. reniformis* has also been identified in the literature as a suitable bioindicator of trace elements pollution (Perez et al.,

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2004; Cebrian et al., 2006; Cebrian et al., 2007), showing all the fundamental characteristics requested for an ideal biomonitor as described by Rainbow and Phillips (1993). In fact, *C. reniformis* is abundant in both polluted and pristine areas, and it presents higher concentrations than other species, accurately reflecting local levels of contamination (Perez et al., 2004).

Although sponges play a key ecological role and are common components of the zoobenthic communities (Padiglia et al., 2018), these organisms have only recently been started to be used, especially in the Mediterranean Sea, as biomonitoring tools for toxic elements pollution (Roveta et al., 2021). In this context, the current study investigates the variation of the total mercury content (THg) in the sponge *C. reniformis* in two islands from an Italian Archipelago, an area naturally characterized by the presence of Hg due to the large exploitation of a cinnabar deposit in the Mount Amiata (Fig. 1) (Roveta et al., 2020).

2. Materials and methods

Sampling was conducted in June 2020 by SCUBA diving at Giglio and Montecristo, islands belonging to the Tuscan Archipelago National Park (TANP) (Fig. 1). Since Montecristo has the status of Integral Nature Reserve and of Special Protection Area (79/409/EEC), a low number of samples and replicates per site have been collected to reduce the impacts on the island ecosystems. A total of eight sites were sampled (TANP permission #00068010), four in each island (Table S1; Fig. 1B, C), and two samples of *Chondrosia reniformis* (Fig. 2A) were collected per site between 0.5 and 40 m depth.

Five additional samples of *C. reniformis* have been collected at Giglio to investigate a possible variation between the two regions in which the sponge body is divided, the external cortex and the internal choanosome (Fig. 2B) (Bavestrello et al., 1998a, 1998b).

The collected sponge samples were of similar size (7.0 ± 1.2 cm), thus possibly of the same age, to minimize the potential differences in THg values related to the sponges' age (Cebrian et al., 2007; Orani et al., 2020). At each study site, one sample of seawater was collected by hand in decontaminated 1 L HDPE bottles (Illuminati et al., 2019), and three surface sediment samples (0–5 cm) were collected using PVC tube corers of 10 cm diameter and 70 cm length (Droghini et al., 2019).

Samples were frozen immediately after the collection, and then stored at −20 °C until analysis. Samples were cut into small pieces, using a ceramic knife, previously decontaminated (see Illuminati et al., 2016 for decontamination procedure), then lyophilized (Edwards EF4 modulyo, Crawley, Sussex, England), minced, homogenized, and divided in aliquots of about 0.02 g each. In samples where the THg content was analyzed in the two body regions, the cortex and choanosome were separated, and each body part was subjected to the aforementioned procedure. THg content was measured using a direct mercury analyzer (DMA-1) as reported in Roveta et al. (2020).

Seawater samples were filtered through 0.45 μm mixed esters of cellulose filters, diluted with ultrapure grade HCl 2% (*v/v*), and then analyzed with an AFS Titan 8220 spectrofluorometer (Fulltech Instruments, Rome, Italy). Argon 5.0 (99.999% purity) was used as a gas carrier and ultrapure grade HCl 5% (v/v) was used as the sample carrier. $NaBH_4$ 0.05% in NaOH 0.4% (m/v) was used as reductant agent to produce Hg hydrides. Instrumental parameters are reported in Table S2. Conversely, the experimental procedure described in Droghini et al. (2019) was followed for sediment samples treatment and analysis.

All measurements (sponges, seawater, sediments) were replicated at least 4 times. The calibration curve method was used to quantify THg contents. The analytical accuracy is routinely checked using the appropriate Certified Reference Materials (CRMs); in this study DORM-2 and MESS-2 (National Research Council Canada, Ottawa, Canada). Details are reported in Table S3 of the supplementary material.

For the THg content in sponge and sediment samples, data were expressed in mg kg^{-1} dry weight (dw), while seawater data were expressed in ng L^{-1}. To assess the capability of *C. reniformis* to bioconcentrate and bioaccumulate Hg, the bioaccumulation factor (BAF) and the bioconcentration factor (BCF) were calculated using the formulae: $BAF = THg_{sponge}/THg_{water}$ and $BCF = THg_{sponge}/THg_{sediment}$, after the appropriate conversion of the measurement units, according to the most cited literature (Orani et al., 2020 and references within).

Normality of the data was tested with a Shapiro-Wilk's test and the homogeneity of the variance with a Levene's test. A two-way analysis of variance (two-way ANOVA) with the islands (two levels) and sites (four levels) as factors was performed to test differences in the THg content, BAF and BCF among sponge samples. Differences in the THg content

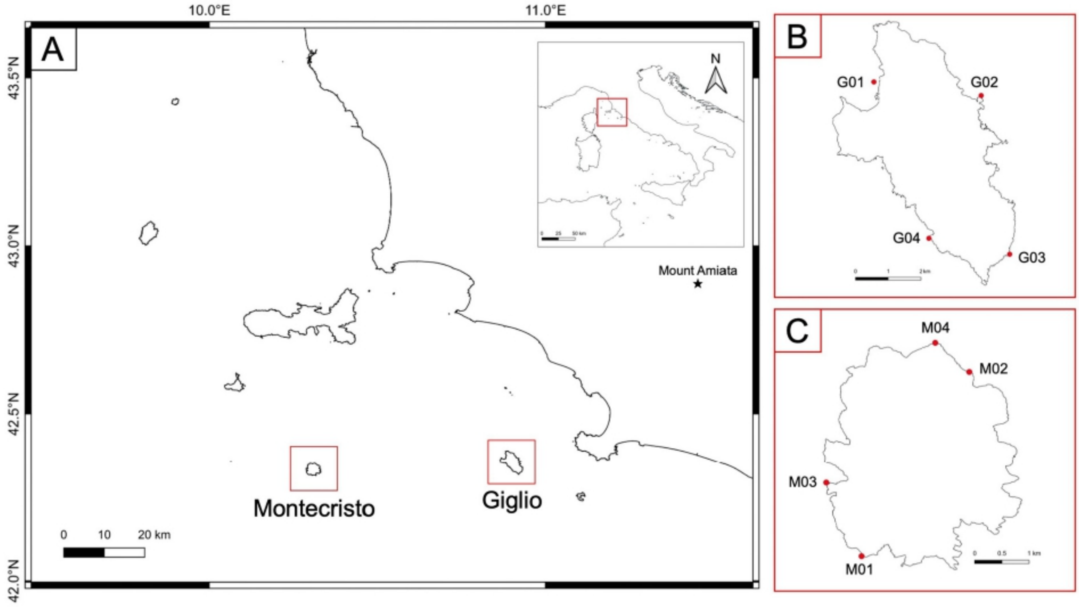

Fig. 1. Map of (A) the Tuscan Archipelago, and of the sites sampled at (B) Giglio and (C) Montecristo islands. For the alphanumeric code of sites see Table S1.

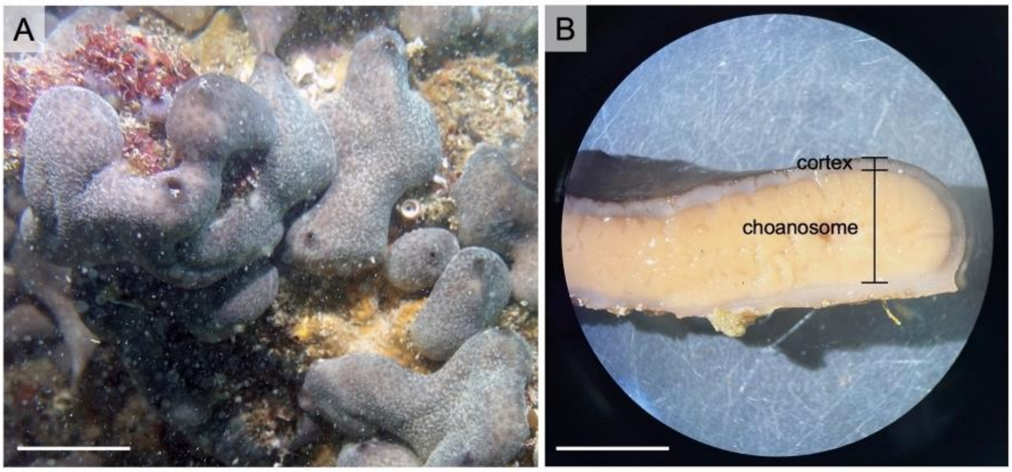

Fig. 2. (A) *Chondrosia reniformis* on the hard bottoms of the Giglio Island. (B) View of the inside of a *C. reniformis* sample, divided into two body regions, the external cortex and the internal choanosome. Scale bars: 2 cm.

between body regions of *C. reniformis* were analyzed by a two-way ANOVA with body regions (two levels) and samples (five levels) as factors. When a treatment factor was significant, the differences among levels were determined using the Tuckey's test. Data on the THg content in water and sediments were analyzed by a one-way ANOVA. In case of non-normality or heteroscedasticity, we applied the non-parametric Kruskal-Wallis analysis of variance. If statistically significant differences were found, the Dunn's post-hoc comparison with the Bonferroni correction of the *p*-value was performed. All statistical analyses were done with a 95% confidence level. All graphs and statistical analyses were performed using the free software R version 4.0.4 (R Core Team).

3. Results and discussion

THg concentrations found in our samples showed comparable values: in fact, at Giglio, THg overall concentration was 0.51 ± 0.03 mg kg^{-1} dw (ranging from 0.48 to 0.54 mg kg^{-1} dw), while at Montecristo, it was 0.53 ± 0.04 mg kg^{-1} dw (ranging from 0.48 to 0.57 mg kg^{-1} dw) (Table 1; Fig. 3A).

The two-way ANOVA did not highlight significant differences in the THg of sponge samples nor among sites nor between islands ($p > 0.05$, Table 2A). Similar THg values were detected in the same species collected at the Giglio Island by Roveta et al. (2020) (0.6 ± 0.1 mg kg^{-1} d.w.), and a similar outcome was also observed for copper by Cebrian et al. (2006) in the Blanes littoral (Spain). These observations could suggest a possible low intraspecific variability of some trace elements in *Chondrosia. reniformis* in uncontaminated sites. On the other hand, mercury levels obtained from our samples were lower than the ones recorded in the polluted site at Cortiou (Marseille, France) (0.8 ± 0.1 mg kg^{-1} d.w.) by Perez et al. (2004).

Table 1
THg levels in sponge ($n = 2$ per site), sediment ($n = 3$ per site), and water ($n = 1$ per site) samples. Bioaccumulation and bioconcentration factors (BAF and BCF, respectively) are also given. All measurements per sample of sponge, seawater and sediment were replicated at least 4 times.

Site code	Sponge (mg kg^{-1} dw)	Sediment (mg kg^{-1} dw)	BCF	Water (ng L^{-1})	BAF ($x10^4$)
G01	0.50 ± 0.07		109 ± 18		1.4 ± 0.2
G02	0.48 ± 0.06		103 ± 15		1.3 ± 0.2
G03	0.54 ± 0.08		117 ± 21		1.4 ± 0.3
G04	0.50 ± 0.04	0.0046 ± 0.0004	108 ± 13	37 ± 4	1.3 ± 0.2
M01	0.53 ± 0.04		104 ± 14		1.4 ± 0.3
M02	0.54 ± 0.06		105 ± 16		1.4 ± 0.3
M03	0.57 ± 0.01		113 ± 12		1.5 ± 0.3
M04	0.48 ± 0.08	0.0051 ± 0.0005	94 ± 18	16 ± 3	1.3 ± 0.3

Our results showed that THg levels in the seawater samples were higher at Giglio (37 ± 4 ng L^{-1}) than at the Montecristo Island (16 ± 3 ng L^{-1}) (Table 1) and similar to THg levels found in 2019, which were 36 ng L^{-1} and 21 ng L^{-1} at Giglio and Montecristo, respectively (Roveta et al., unpublished data). The THg levels resulted significantly different between islands (Dunn's test, $p < 0.05$), and far below the Maximum Allowable Concentration of 0.07 μg L^{-1} established by the European Directive 2008/105/EC (EC, 2008a), thus, satisfying the Descriptor 8 of the Marine Strategy Framework Directive (EC, 2008b), indicating a Good Environmental Status (GES) of the waters surrounding the southern islands of the Tuscan Archipelago. Due to the relevant differences encountered in the water samples between islands, also BAF values also resulted statistically different among islands ($p < 0.05$, Table 2B), with higher values in Montecristo than in Giglio (Table 1; 2B). However, THg values of the seawater were obtained from a single sampling in a specific period of the year and not from periodic monitoring. Trace elements concentration in the seawater is known to vary along the year (Annibaldi et al., 2009) and is also related to the sea-weather conditions, thus to a change in physico-chemicals parameters of the seawater itself (e.g., turbidity, pH, conductivity, alkalinity, organic matter etc.) and to the effect of sediment re-suspension (Domingos et al., 2015). A more useful and reliable way to evaluate trace elements accumulation in marine organisms is represented by the BCF, which is calculated as the ratio between the chemical concentrations measured in the organism and the one measured in local sediments (Orani et al., 2020), and usually used as a record of contamination for environmental studies. For this matrix the annual variation of trace elements due to the seasonality is nearly negligible and so more suitable for our study (Allafta and Opp, 2020). The mean values of THg in sediments collected at Giglio and Montecristo were very similar (Dunn's test, $p > 0.05$), being respectively 0.0046 ± 0.0004 mg kg^{-1} and 0.0051 ± 0.0005 mg kg^{-1} (Table 1), validating our hypothesis on the use of this matrix. Considering the current maximum allowable level of 0.3 mg kg^{-1} of Hg in sediments set by the Environmental Protection Agency

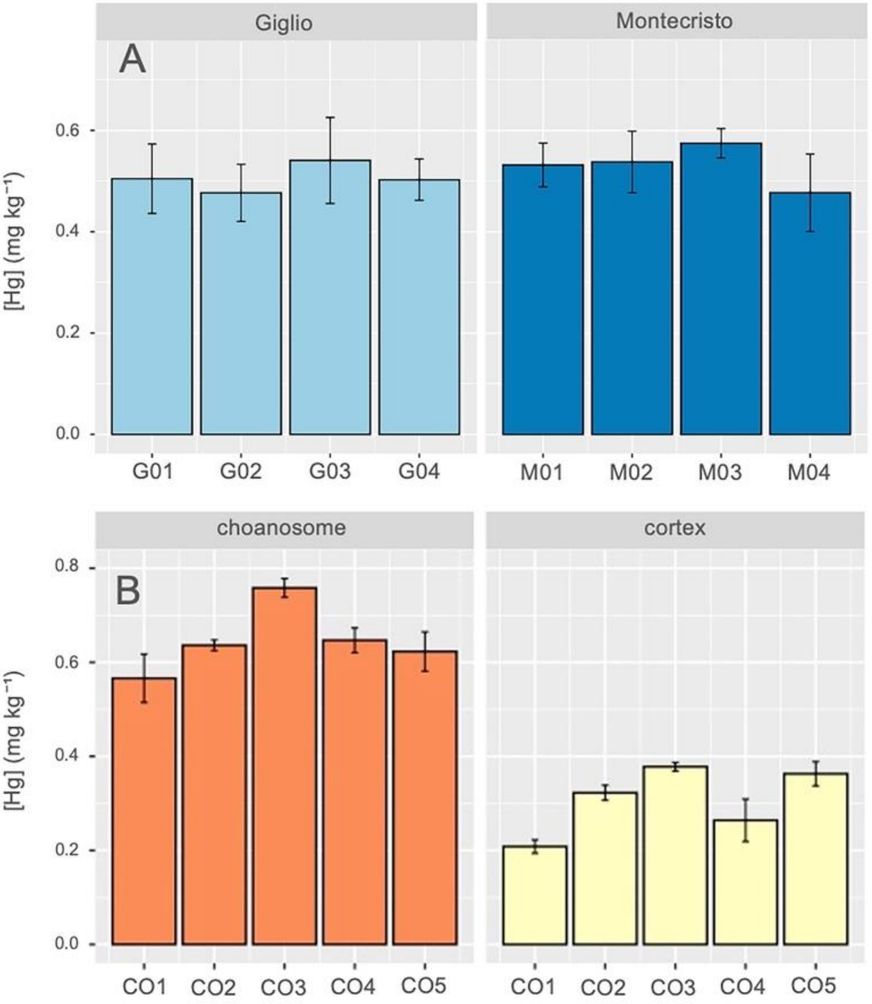

Fig. 3. THg content in (A) samples collected at the different study sites at Giglio and Montecristo islands, and in (B) the two body regions of *Chondrosia reniformis.*

(EPA, 2006), the values obtained in this study can be considered as typical for uncontaminated areas. In all samples, the BCF was higher than 1 (Table 1), meaning that *C. reniformis* actively bioaccumulates mercury, which has also been observed for other species by Orani et al. (2020). No significant differences were found within islands or sites ($p > 0.05$, Table 2C). However, to effectively verify the suitability of this sponge as biomonitor for mercury pollution an experiment under controlled conditions and in polluted environments should be carried out.

Since *C. reniformis* is characterized by two distinct regions (a) an external cortical zone (ectosome or cortex), composed by flattened pinacocytes and interwoven collagenous fibers densely packed, and (b) an internal one (choanosome), containing the choanocyte chambers (Bavestrello et al., 1998a, 1998b), we hypothesized a different bioconcentration of Hg between the two body regions. Statistical analysis highlighted how THg values differed markedly between cortex and choanosome ($p < 0.05$, Table 2D), with higher values in the latter (Fig. 3B). Illuminati et al. (2016) also investigated a possible variation in the concentration of Cd, Pb and Cu between the oscula and other areas of the sponges *Sphaerotylus antarcticus, Kirkpatrickia coulmani, Haliclona* sp. and *Petrosia ficiformis*, but no differences were found. The peculiarity observed in *C. reniformis* can be related to the presence of the choanocyte chambers in the choanosome (Bavestrello et al., 1998a, 1998b), in which the water filtration takes place (Schmitt et al., 2007). Another explanation can be related to a different distribution of the microbiota between the two body parts. Previous studies conducted by Manz et al. (2000) showed a compartmentalization of the microbial community in *C. reniformis*, with *Desulfovibrionaceae* (rod-shaped bacteria) colonizing the channel system of the sponge cortex, densely surrounded by coccoid *Bacteria* cells within the sponge mesohyl (extracellular matrix). In a so called "bacteriosponge" such as *C. reniformis*, the microbiota can reach 10^{8}–10^{10} cells g^{-1} of sponge wet weight and it was observed to actively participate in the bioaccumulation process, demonstrating resistance to different antibiotics and many potential toxic elements (e.g., Cu, Pb, Co, Cd, Zn, Ni and Hg, together with their organic compounds) (Selvin et al., 2009; Santos-Gandelman et al., 2014). Moreover, also in different sedentary polychaete species a differential bioaccumulation of various trace elements between their branchial crown and body was observed (Giangrande et al., 2017), while in ascidians belonging to the order Phlebobranchia, a different bioaccumulation of vanadium was found related to differences in the abundance of some strains belonging to their microbial community isolated from their branchial sac, intestine, and intestinal lumen (Ueki et al., 2019). Future research is needed to expand the current knowledge on the microbial community (not only in

Table 2
Two-way analysis of variance (ANOVA) to test differences in the (A) THg concentrations, (B) bioaccumulation factor (BAF) and (C) bioconcentration factor (BCF) among samples of *Chondrosia reniformis*, and in the (D) THg values between regions of *C. reniformis* body. Significant values ($p < 0.05$) are in bold. Df = degrees of freedom; SS = sum of squares; MS = mean squares; F = Pseudo-F statistic; p = probability.

A		Df	SS	MS	F	p
	Island	1	0.0399919	0.0399919	3.05	0.08839
	Site	3	0.0696316	0.0232105	1.77	0.1683
	Island x Site	3	0.0155976	0.00519921	0.3966	0.7562
	Within	40	0.524413	0.0131103		
	Total	47	0.625407			

B		Df	SS	MS	F	p
	Island	1	6.80E+09	6.80E+09	336.1	**>0.001**[a]
	Site	3	1.13E+09	3.76E+08	18.61	**>0.001**
	Island x Site	3	6.26E+08	2.09E+08	10.32	**>0.001**
	Within	40	8.09E+08	2.02E+07		
	Total	47	7.79E+09			

C		Df	SS	MS	F	p
	Island	1	416.657	416.657	0.6984	0.4083
	Site	3	2530.26	843.421	1.414	0.2529
	Island x Site	3	556.369	185.456	0.3109	0.8174
	Within	40	23,862.3	596.558		
	Total	47	26,926.5			

D		Df	SS	MS	F	p
	Body region	1	0.861331	0.861331	974.6	**>0.001**[b]
	Sample	4	0.102572	0.025643	29.01	**>0.001**
	Body region x Sample	4	0.0162851	0.00407127	4.606	**0.00846**
	Within	20	0.0176764	0.00088382		
	Total	29	0.997865			

[a] Tuckey's test: Montecristo > Giglio.

[b] Tuckey test: choanosome > cortex.

sponges), and to better understand the actual role played by the microbiota in the bioaccumulation, especially for a potential application of sessile benthic filter-feeding organisms (e.g., sponges, bivalves, polychaetes, ascidians) as bioremediation tools in contaminated environments.

4. Conclusions

This study is the first to highlight a differentiated bioaccumulation in different body parts of the sponge *Chondrosia reniformis*. Our results highlighted no intraspecific variability of THg levels in samples deriving from two natural areas, suggesting the role of *C. reniformis* as an attractive bioindicator of pollutants in coastal waters. Nonetheless, further studies are needed for a better understanding of the bioaccumulation process in this sponge species, for example considering additional areas and locations with different pollution levels, seasonality, and the involvement of the microbiota in the bioaccumulation mechanism. Finally, the current work helps to improve the knowledge about the ecology of *C. reniformis* and of the ecosystems characterizing the Tuscan Archipelago, aimed to become a future Marine Protected Area, and highlights the potential use of organisms in the monitoring programs of coastal areas, as suggested by various marine policies (e.g., Water Framework Directive, Marine Strategy Framework Directive).

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefania Puce reports financial support was provided by PADI Foundation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seares.2022.102206.

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CHAPTER 3 – TES AND THE ROLE OF SPONGES ASSOCIATED MICROBIOTA IN THE BIOACCUMULATION PROCESS

This last Chapter includes one papers in which the role of sponges' microbiota in the bioaccumulation of Hg is explored.

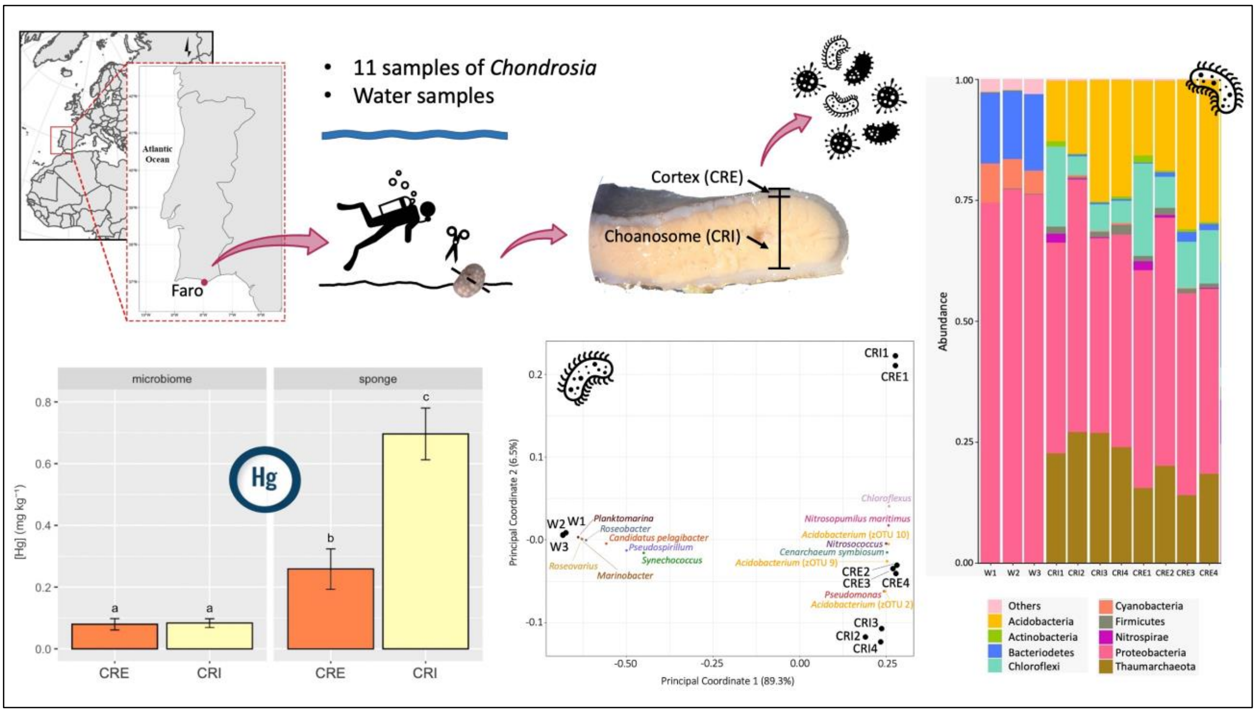
Paper 6 is entitled "**The Atlanto-Mediterranean sponge *Chondrosia reniformis* and its microbiota actively bioaccumulate mercury (Hg)**" and it is the only paper still in preparation for submission. This study was conducted in the framework of the ASSEMBLE Plus project "Some sponges like heavy meals: the role of the microbiota in trace metals bioaccumulation", and 11 samples of *Chondrosia reniformis* were collected by SCUBA diving at Faro (Portugal, Atlantic Ocean). This is the first detailed description of the prokaryotic communities characterizing *C. reniformis* and the first study conducted on the Hg content in sponges' microbiota, highlighting that the microbiome effectively bioaccumulate Hg and suggesting an involvement of the microbiota in the bioaccumulation processes.

PAPER 6

IN PREPARATION

The Atlanto-Mediterranean sponge *Chondrosia reniformis* and its microbiota actively bioaccumulate mercury (Hg)

Roveta Camilla, Fernandes Couceiro Joana, Calcinai Barbara, Girolametti Federico, Puce Stefania, Annibaldi Anna, Costa Rodrigo



Supplementary material available at:

https://drive.google.com/drive/folders/1qaso9AXvCjVegtXTRbah-6vvidaTJ8RL?usp=sharing

The Atlanto-Mediterranean sponge *Chondrosia reniformis* and its microbiota actively bioaccumulate mercury (Hg)

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Abstract

Microbial communities inhabiting sponges are known to take part in many metabolic pathways, including important nutrient cycling pathways, suggesting a possible implication also in the bioaccumulation of trace elements. Here, we estimated the total mercury content (THg) in the external cortex and internal choanosome characterizing the body of the bacteriosponge *Chondrosia reniformis* and in the corresponding microbial cell pellets. Furthermore, we identified the microorganisms composing the microbiome extracted from each body region using next-generation sequencing of 16S rRNA genes. Among the 11 phyla detected, the Bacteria domain dominated the microbial community, and no significant differences in the prokaryotic community composition of the two regions were recorded. Three lineages, the ammonium-oxidizing archaea *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*, and the ammonium-oxidizing bacterium *Nitrosococcus* sp., co-dominate the microbiome, suggesting ammonium oxidation/nitrification as a key metabolic pathway in the studied sponge. The microbiota also showed detectable concentrations of Hg, even though with lower levels than the sponge fraction, but with comparable values between the two regions of *C. reniformis'* body. Considering the fundamental role played by the microbiota in sponge metabolism and the evidence that it can actively participate in the bioaccumulation of metals, sponges should be considered not only as bioindicators of environmental pollution, as suggested by the European legislation (e.g., the Water Framework Directive and the Marine Strategy), but also as useful tools in the bioremediation in metal polluted environments.

Keywords: Porifera, metagenomics, microbiomes, 16S rRNA gene, trace elements, biomonitors

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Introduction

Microbes form complex relationships with most organisms, ranging from humans to invertebrates to plants (Thomas et al., 2016), with highly specialized reciprocal interactions, generally resulting in mutual benefits for both parties (Nyholm and McFall-Ngai, 2004; Schmitt et al., 2007). However, the diversity and highly dynamic nature of microbiomes make the understanding of the evolutionary and ecological drivers of symbiont composition challenging, as well as the possible roles played by the host-associated microbiota, which often result from multiple and interconnecting metabolic processes (Thomas et al., 2016).

Various marine invertebrates, including corals, worms, clams, mussels, sponges, and sea-squirts, are characterized by the presence of complex communities of microorganisms composing the associated microbiota (Thomson et al., 2021), generally hosted in their tissues, where nutrient cycling and host-microbe metabolite exchange takes place (Schmitt et al., 2007).

Marine sponges are sedentary filter-feeding organisms, whose microbiota can be acquired both horizontally from the seawater and vertically through larvae (de Oliveira et al., 2020). In many species, the sponge microbiota can contribute up to the 60% of host's biomass. Such sponges are usually referred to as "bacteriosponges" or "high microbial abundance" (HMA) sponges, in contrast to "low microbial abundance" (LMA) sponges, which possess microbial densities similar to those of the surrounding seawater (Vacelet and Donadey, 1977; Schmitt et al., 2007; Gloeckner et al., 2014).

Sponges are reservoirs of microbial genetic diversity, and the often-reported taxonomic diversification among so-far uncultivated symbionts of sponges is suggestive of metabolic novelties (Hardoim et al., 2014). Metabarcoding studies based on 16SrRNA gene amplicon sequencing showed how sponges not only present interspecific variability in the structure of their associated microbial communities, but also intraspecific variability between individuals of the same species collected in different localities (Hardoim et al., 2012). These patterns can be related to many factors, such as host-derived nutrients, chemical-physical parameters of the water (e.g., pH) and host characteristics (e.g., immune response), determining the composition and structure of symbiont communities over time and space (Thomas et al., 2016). However, only 1% to 14% of the total sponge bacterial community has been estimated to be cultivatable (Hardoim et al., 2014). Moreover, culturable symbionts of marine sponges usually correspond to low abundance ("rare") populations within this complex microbial consortium (Hardoim et al. 2014; Karimi et al., 2019) limiting our knowledge of the functional roles played by most of the sponge symbionts which remain uncultivated (Karimi et al., 2019).

Sponges can filter large volumes of water (many thousands of litres kg^{-1} day^{-1}), together with food particles and many different compounds, which are present in the water column both as dissolved and particulate organic matter (Berthet et al., 2005; Selvin et al., 2009; Batista et al., 2014). Among these compounds, contaminants (as hydrocarbons, organochlorinated compounds, trace elements, etc.) are largely collected. In fact, various studies demonstrated that sponges could present detectable concentrations of trace elements (TEs), and bioaccumulate these pollutants in their mesohyl (e.g., Perez et al., 2004, 2005; Cebrian et al., 2006; Batista et al., 2014). Thus, sponges are considered suitable bioindicators of environmental pollution, and they have been recommended by various European Directives, such as the Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC) as possible monitors of TEs contamination. In addition, since the microbiota plays a fundamental role in sponge metabolism, it has been suggested it can actively participate in the bioaccumulation process, and many bacteria have been found to be resistant to various antibiotics and pollutants, including Persistent Organic Pollutants (POPs) and TEs (e.g., Cd, Co, Cu, Hg, Ni, Pb, Zn, and their organic compounds) (e.g., Selvin et al., 2009; Versluis et al., 2016; Rodriguez Jimenez et al., 2021). For these reasons, sponges and their associated bacteria have been proposed as valid bioremediation tools (Santos-Gandelman et al., 2014a, 2014b).

Chondrosia reniformis Nardo, 1847 is a typical cushion-shaped, Atlanto-Mediterranean demosponge usually living in the shallow coastal waters (0-50 m) (Wilkinson and Vacelet, 1979; Lazoski et al., 2001). The sponge possesses profuse and dense endoskeleton made of collagen fibres, which confers the organism a high body plasticity (Bonasoro et al., 2001). The sponge's structure consists of two distinct body regions: an external cortical zone, named ectosome or cortex, composed by flattened pinacocytes and densely packed interwoven collagenous fibers; and an internal zone, named choanosome, which contains the choanocyte chambers (Bavestrello et al., 1998a, b). Lacking a siliceous skeleton, *Chondrosia* reinforces its body incorporating large amounts of foreign material: the cortex incorporates only siliceous particles, while the choanosome allow the attachment of every kind of mineral (Bavestrello et al., 1998a).

C. reniformis is known to be the source of several secondary metabolites, and therefore is commonly reared for in situ or aquaria studies. In fact, the species represents an important source of collagen and other important molecules, such as chondrosin, a newly discovered protein with anti-tumoral activity (Pozzolini et al., 2012, 2015, 2016, 2018; Scarfì et al., 2020). Moreover, *C. reniformis* has been already applied for biomonitoring studies and identified as a suitable bioindicator of TEs pollution, since it has shown higher concentrations than other sponge species, reflecting more accurately the level of contamination of a specific location (Perez et al., 2004; Cebrian et al., 2007).

In the present investigation we explored a possible implication of the microbiota in the bioaccumulation of mercury (Hg) in the common Atlanto-Mediterranean marine bacteriosponge *C. reniformis*, collected in Faro (Portugal, Atlantic Ocean). To this end, we estimated the total mercury content (THg) in the two body regions characterizing the sponge (external cortex and internal choanosome) and in the corresponding microbial cell pellets. In addition, we identified the microorganisms composing the microbiome extracted from each region using next-generation sequencing of 16S rRNA genes. The identification of the role played by sponges and microorganisms in the TEs bioaccumulation can contribute to the knowledge of the possible applications of sponges in a bioremediation scenario.

Materials and Methods

Samples collection and processing

Eleven samples of *Chondrosia reniformis* were collected into sterile bags by SCUBA diving around 18 m depth at offshore Faro beach, Algarve, Portugal ("Pedra da Greta": 36°58'47.2" N, 07°59'20.8" E), in June 2021. Samples were collected at least 3 m apart from each other, to avoid clones, and placed in 3L plastic bags (type Ziploc®) filled with surrounding seawater. The collected sponge samples were of similar size to avoid possible differences in sponge age, since different THg levels could be related to this factor (Cebrian et al., 2007; Orani et al., 2020; Roveta et al., 2022). Together with sponges, four samples of seawater (3 L each) were also collected in separate Ziploc plastic bags, 1.5 m above the bottom. All samples were transported to the Environmental Sample Processing Facility of the Centre of Marine Sciences (Faro, Portugal) in a cool box within 1.5 h post sampling and processed immediately.

Since *Chondrosia* is characterized by two body regions, the external cortex and the internal choanosome (Bavestrello et al., 1998a, b), samples were labelled with the following codes: CRI (*C. reniformis* internal, referring to the choanosome), CRE (*C. reniformis* external, referring to the cortex), and W (seawater), followed by sequential numbers.

Sponge samples were cleaned of rock reseals, washed with sterile seawater to remove planktonic or loosely associated microorganisms, following Thomas et al. (2016). The two regions of each *Chondrosia* sample were separated using a decontaminated knife (see Illuminati et al., 2016 for the decontamination procedure), and from each region, 10 g were weighted for Hg content analyses, and microbial cell pellets were obtained from sponge samples using a differential centrifugation protocol as described by Hardoim et al. (2014). Firstly, due to the hardness of the sponge given by collagenous fibres, each region of *C. reniformis* was mixed with a blender in sterile Ca^{2+} and Mg^{2+} free artificial seawater (CMFASW: 27 g L^{-1} NaCl, 1 g L^{-1} NaSO4, 0.8 g L^{-1} KCl and 0.18 g L^{-1} NaHCO3, 1 g of sponge per 4 ml CMFASW w/v) and then homogenised using a sterile mortar and pestle. Both the homogenate and the sponge fraction were centrifuged for 2 min at 500 g. The supernatants of the homogenates were then transferred into new centrifuge tubes and subjected to a final centrifugation step for 30 min at 10 000 g. The applied protocol allows to obtain a pellet enriched in microbial cells with negligible contamination of sponge cells, as estimated using shotgun metagenome sequenced (less than 2% of host-derived reads in the microbial cell pellets) (Karimi et al., 2017). In addition, 2.5 g of four *C. reniformis* samples were subjected to the protocol described above for the retrieval of microbial cell pellets, which were then used for total community microbial DNA extraction for the characterization of the prokaryotic communities associated with the species (see below).

Part of the water collected (three samples of 3 L each) was filtered using 0.22 μM nitrocellulose membrane filters (Millipore, Billerica, MA, USA; 47 mm) using a vacuum pump, to collect the water microbiota on top of the filters.

The obtained material was then stored at -80°C until further analysis.

Assessments of bacterial community diversity and composition in Chondrosia reniformis *and seawater*

The microbial pellets from sponge and the filters used to collect the seawater microbiome were subjected to total community DNA extraction using the Power Soil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol and as detailed in Costa et al. (2013). Before the extraction, water filters were aseptically cut into small pieces. Each sample was then transferred to a lysing matrix tube provided by the kit and subjected to bead-beating three times for 1 min (30.0 frequency) with a Mixer Mill MM 400 to improve submersion and contact between cells and lysing buffer. Metagenomic DNA yields and integrity were examined under ultraviolet (UV) light after standard agarose gel electrophoresis procedures, while the concentration of the DNA samples was quantified using a NanoDrop spectrophotometer (ThermoFischer Scientific).

Taxonomic profiling of bacterial communities was performed via high-throughput sequencing of 16S rRNA gene amplicons obtained by PCR from the metagenomic DNA samples. PCR amplification and sequencing of 16S rRNA gene amplicons was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). The primers used were the updated Earth Microbiome Project (EMP) primers 515F (5'-GTG YCA GCM GCC GCG GTAA-3') (Parada et al., 2016) and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') (Apprill et al., 2015), with barcodes on the forward primer. Firstly, a 30 to 35-cycle PCR was used to amplify the V4 hypervariable region (515 to 806) of the 16S rRNA gene, using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). The conditions for the PCR were as follows: a first step of 5 min at 95°C, followed by 30 to 35 cycles of 30 s at 95°C, 40 s at 53°C and 1 min at 72°C, after which there was one final elongation step of 10 min at 72°C. PCR products were checked in a 2% agarose gel under UV light to determine whether the amplification was successful. After amplification, the samples were multiplexed using unique dual indices, pooled together and later purified using calibrated Ampure XP beads. Afterwards, the pooled and purified PCR products were used to prepare an Illumina DNA library, and sequencing was performed on a MiSeq platform following the manufacturer's guidelines. During sequencing, an average 20,000 paired-end sequences, per sample, were generated.

Total mercury content analysis

Sponge samples were lyophilized (Edwards EF4 modulyo, Crawley, Sussex, England), minced, homogenized, and divided in aliquots of about 0.02 g each. The microbial pellet was centrifuged before the analysis at 1000 rpm for 1 minute to remove water residuals. Hg analyses were performed at the Department of Life and Environmental Sciences of the Polytechnic University of Marche, using a direct mercury analyser (DMA-1) as reported in Roveta et al. (2020).

Both filtered and non-filtered seawater were diluted with ultrapure grade HCl 2% (v/v), and then analysed with a AFS Titan 8220 spectrofluorometer (Fulltech Instruments, Rome, Italy). Argon 5.0 (99.999% purity) was used as a gas carrier and ultrapure grade HCl 5% (v/v) was used as the sample carrier. NaBH4 0.05 % in NaOH 0.4 % (m/v) was used as reductant agent to produce Hg hydrides. Instrumental parameters are reported in Table S1 of the supplementary material.

All measurements were replicated at least 4 times. The calibration curve method was used to quantify THg content. The analytical accuracy is routinely checked using the appropriate Certified Reference Materials (CRMs); in this study DORM-2 and MESS-2 (National Research Council Canada, Ottawa, Canada). Details are reported in Table S2.

Data treatment and analysis

Bacterial community diversity and composition. Reads obtained from the water and sponge samples were subjected to quality processing following the analysis pipeline of MR DNA (MR DNA, Shallowater, TX, USA). In total, 313,846 raw 16S rRNA (V4 region) gene sequence reads were obtained. Firstly, sequences were depleted of primers, and sequences below 150bp or with ambiguous base calls were removed. Then, sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. After denoising and dereplicating the sequences, zero-radius Operational Taxonomic Units (zOTUs), or Amplicon Sequence Variants, were generated and taxonomically classified using BLASTn against a curated database derived from NCBI (www.ncbi.nlm.nih.gov). Briefly, 16S rRNA gene reads possessing 100% nucleotide sequence homology are categorized into the same zOTU. Prior to downstream analyses, zOTUs classified as Eukaryota, chloroplasts or mitochondria were removed. The final analytical dataset comprised 303,732 16S rRNA gene reads sorted into 448 prokaryotic zOTUs, 17 belonging to the domain Archaea and 431 to the domain Bacteria (Table S3).

For alpha-diversity analyses, the dataset was normalised by rarefaction to 23,477 reads per sample (corresponding to sample W3 which had the lowest number of reads), and the rarefied dataset was then used for the determination of observed species richness, estimated species richness (Chao1 index) and diversity (Shannon's index). Rarefaction and calculations were carried out using the R package *phyloseq* (McMurdie and Holmes, 2013). To test for possible differences in alpha-diversity measures, a statistical analysis was performed using one-way ANOVA. Prior to analysis, the normality of the data was tested with a Shapiro-Wilk's test and the homogeneity of the variance with a Levene's test. In case of statistically significant differences, the Tukey's test was performed. Statistical tests were performed using R packages *car, stats* and *rstatix* (Fox and Weisberg, 2019; Kassambara, 2021; R Core Team, 2021).

To assess the prokaryotic community composition of each sample at phylum, class and genus levels, relative abundance data (percentages, non-rarefied) were used (Table S3). To improve the readability of taxonomy barplots, low abundance taxa (taxa below 1% relative abundance for the order plot and below 0.5% for the phylum and genus plots) were merged in a category called "Others". Plots were created using R packages *phyloseq*, *plyr* and *ggplot2* (Wickham, 2011; McMurdie and Holmes, 2013; Wickham, 2016). One-way ANOVAs followed by Tukey's post-hoc tests were used to test for differences in the relative abundance of the most dominant phyla across sample categories.

The prokaryotic community structure was also investigated by means of multivariate analysis. zOTU data (non-rarefied) were Hellinger-transformed and Bray-Curtis dissimilarities calculated from the Hellinger-transformed OTU-data table using the *vegan* package for R (Oksanen et al., 2020). The resulting distance matrix was then used as input in a Principal Coordinates Analyseis (PCoA) of the samples based on their zOTU profiles. To complement the result given by the PCoA, an analysis of Similarity Percentages (SIMPER) was carried out with the free software PAST (version 4.02) to rank the zOTUs contributing the most to community dissimilarities among sample groups. SIMPER was performed considering all sample groups together and the resulting top 15 zOTUs were plotted as "species data" on the PCoA graph. To investigate whether there was a significant difference between sample groups, data were analysed with Permutational Analysis of Variance (PERMANOVA) (Anderson 2001), using a Bray-Curtis dissimilarity matrix, with 9999 permutations. A posteriori pairwise comparisons were conducted in case of significant differences. Permutational analysis of multivariate dispersions (PERMDISP) was used to test the homogeneity of samples dispersion from their group centroids (Anderson et al., 2008). These analyses were carried out using PRIMER 7.0.20 (Clarke and Gorley, 2015) with the add-on PERMANOVA+ (Anderson et al., 2008).

Total mercury content. For the THg content in sponge samples and associated microbiota, data were expressed in mg kg^{-1} dry weight (d.w.), while seawater data (filtered and unfiltered) were expressed in ng L^{-1}. To assess the capability of both sponge fraction and microbiota to bioconcentrate Hg, the bioconcentration factor (BAF) was calculated using the formula: $BAF_{sponge} = THg_{sponge}/THg_{filtered\ water}$ and $BAF_{microbiota} = THg_{microbiota}/THg_{filtered\ water}$, after the appropriate conversion of the measurement units.

To test for possible differences in the THg content between filtered and unfiltered seawater samples, a one-tiled Student t test was carried out. Alternatively, one-way ANOVA (with Tuckey's post-hoc) was performed to test for differences in THg content (among samples, sponge fraction and microbial pellet) and BAF values, as described above for alpha-diversity measures. In case of non-normality or heteroscedasticity, non-parametric Kruskal-Wallis analysis of variance was applied and, if statistically significant differences were found, the Dunn's post-hoc comparison was performed. Statistical tests were performed using R packages car, stats and rstatix (Fox and Weisberg, 2019; Kassambara, 2021; R Core Team, 2021).

Results

Alpha-diversity analyses

Alpha-diversity (species richness and Shannon's diversity) in seawater and *Chondrosia reniformis* was explored using the rarefied (size-normalised) dataset. The observed number of zOTUs was significantly higher in seawater compared to CRE and CRI samples (Tukey's test, $p<0.05$) (Figure 1A). The estimated species richness (Chao1) was slightly higher than the observed richness in all samples, except for samples W3, CRI1 and CRE1 in which the two values were almost overlapping (Figure 1A). No differences among sample groups were found for Chao1 (ANOVA, $p=0.06829$), even though the seawater showed the higher values of estimated richness (Figure 1A). The

Shannon diversity indices were significantly higher for seawater than in CRI and CRE samples (Tuckey's test, p<0.05) (Figure 1B).

In general, between the two body regions of *Chondrosia*, CRI samples exhibited higher observed and Chao1 richness, and a lower Shannon Diversity compared to CRE samples (Figure 1), but no statistical differences were recorded.

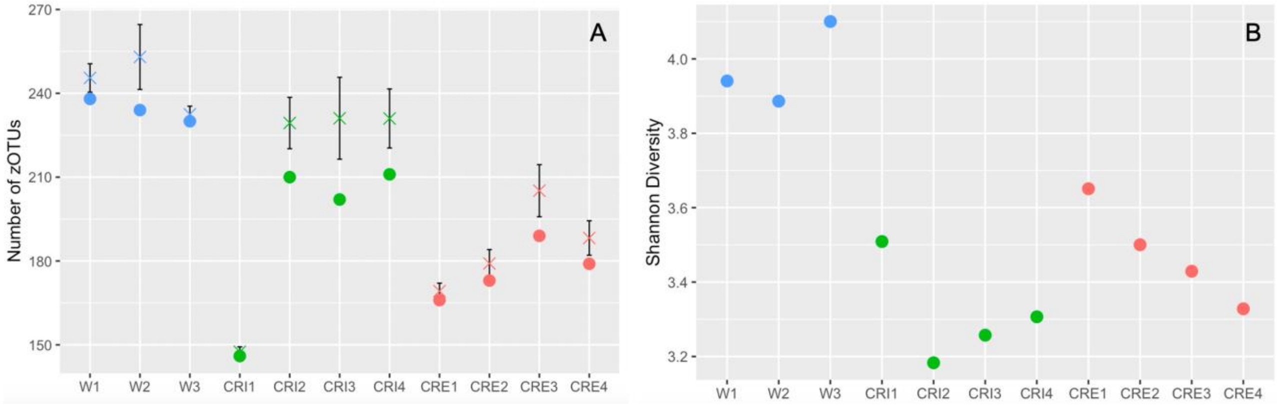

Figure 1 – Alpha diversity measures calculated on the rarefied dataset. (A) Observed (•) and Chao1 estimated (╳) zOTU richness per sample. (B) zOTU diversity per sample estimated with the Shannon diversity index. W = water; CRI = *Chondrosia reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Prokaryotic community composition

Proteobacteria, Thaumarcheota, Acidobacteria, Bacteroidetes, Chloroflexi (in this order) were the dominant phyla in the entire dataset (Figure 2A). Proteobacteria was the most abundant phylum in the three sample categories, with its relative abundance changing significantly between categories (Tuckey's test, p<0.05) (Figure 2A). For seawater samples, the relative abundance was slightly higher, accounting for about the 75% of the dataset, compared to the CRI and CRE samples, where Proteobacteria represented about 50% of the microbial community (Figure 2A). Another phylum with a significative relative abundance in water samples was Bacteroidetes, almost reaching 20%, while lower values (~7%) were represented by Cyanobacteria. Apart from Proteobacteria, the *Chondrosia* prokaryotic community was characterized by a high relative abundance of Thaumarchaeota, an archaeal phylum representing about the 20-30% of the microbiota, and of Acidobacteria, ranging from a 15 to 40% of the reads in CRE and CRI datasets (Figure 2A). Well represented was also the phylum Chloroflexi, with relative abundances around 10%, while lower relative abundances were observed for Actinobacteria, Bacteroidetes, Firmicutes and Nitrospirae (< 5%) (Figure 2A).

At the order-level, a drastic shift in taxonomic composition could be observed (Figure 2B). The overall community composition was most diversified in *Chondrosia*, with the highest relative abundance reached by the archaeal order Cenarchaeales, and by the bacterial orders

Acidobacteriales and Pseudomonales, each contributing with ~25% of the total reads to the dataset (Figure 2B). Other orders, such as Alteromonadales, Chloroflexales, Chromatiales, Nitrosopumilales and Rhodobacterales, also showed considerable relative abundances in CRE and CRI samples (Figure 2B). The order Rhodobacterales was dominant in all seawater samples, representing about the 45% of the dataset, while a lower contribution was given by Alteromonadales, Cellvibrionales, Flavobacteriales, Oceanospirillales, Pelagibacterales and Synechoccocales (Figure 2B).

Genus-level taxonomic composition differed sharply between seawater and *Chondrosia* (Figure 2C). Seawater samples were mostly dominated by *Roseobacter*, contributing with ~30% of the total reads in dataset, followed by *Flavobacterium*, *Marinobacter*, candidatus *Pelagibacter*, *Planktomarina*, *Roseovarius* and *Synechococcus,* contributing between 6 and 12% of the total reads in the dataset (Figure 2C), while CRE and CRI samples were dominated by *Pseudomonas*, *Cenarcheum* and *Acidobacterium*, each contributing with ~20% to the dataset, followed by *Chloroflexus*, *Nitrosococcus* and *Nitrosopumilus* (Figure 2C).

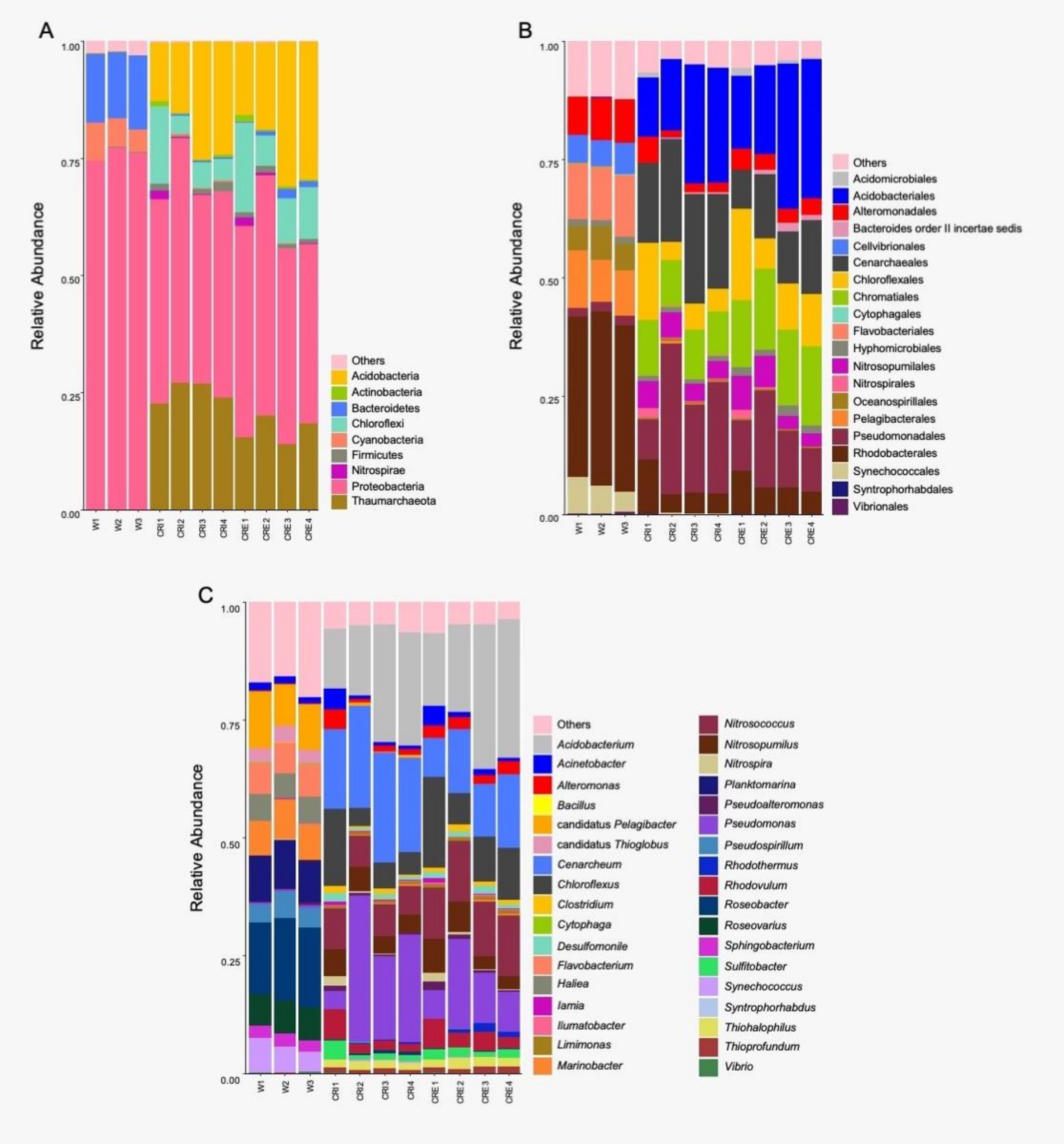

Figure 2 – Relative abundance of zOTUs of the prokaryotic community non-rarefied dataset at (A) phylum-, (B) order- and (C) genus-level for each sample of water and *Chondrosia reniformis* layers. Phyla and genera below 0.5%, and order below 1% of relative abundance were joined under the category "Others". W = water; CRI = *C. reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Beta-diversity analysis

The PCoA revealed a clear separation of seawater and *Chondrosia* samples (Figure 3). Except for CRI1 and CRE1, which form a complete separate group from the other samples, CRI and CRE samples formed two single and separate clusters (Figure 3). Nonetheless, their vicinity suggested that, in respect to their zOTU composition, these samples were similar.

In this ordination plot, the fifteen most differentiating zOTUs were also displayed, showing their effect and contribution to the dissimilarity between samples. For instance, zOTUs classified as

Marinobacter, candidatus *Pelagibacter, Planktomarina, Roseobacter, Roseovirus, Pseudospirillum* and *Synechococcus* were associated with water samples (Figure 3). Associated to *Chondrosia* samples were zOTUs affiliated with the Archaea species *Cenarchaeum symbiosum* and *Nitrosopomilus maritimus*, the bacterial genera *Chloroflexus, Nitrosococcus, Pseudomonas*, and zOTUs classified as *Acidobacterium* (Figure 3).

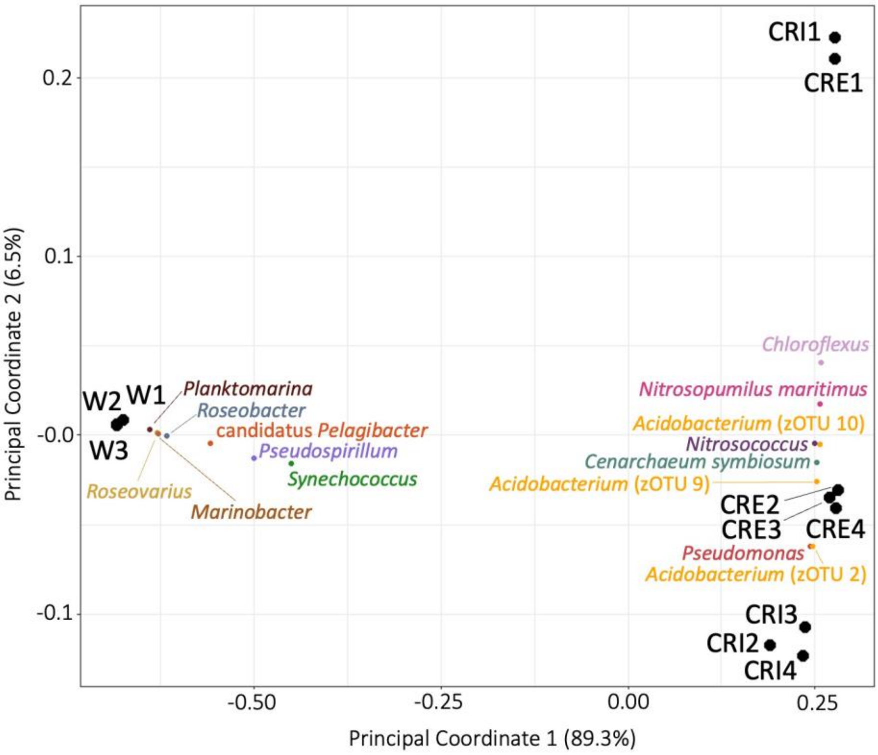

Figure 3 – Principal Coordinates Analysis (PCoA) of prokaryotic communities of the sponge *Chondrosia reniformis* layers and water samples. Community ordination was based on a Bray-Curtis distance matrix calculated from Hellinger-transformed zOTU abundance data. The 15 zOTUs with higher contribution to the dissimilarity between sample groups (SIMPER test) are plotted on the ordination diagram. The position of the zOTUs is a projection of their respective abundances in the space of the two principal components. The proximity of the zOTUs to the sample groups is due to higher abundance of zOTUs in those sample groups. Samples are represented by black dots and zOTUs are marked as coloured dots. Percentual values on each axis correspond to the amount of variance explained by that axis. W = water; CRI = *C. reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

PERMANOVA showed statistical differences within samples' groups (Table 1), and the pairwise comparison highlighted significant differences in the prokaryotic species composition between the seawater samples and both regions of *C. reniformis'* body (Table 2). However, the PERMDISP test indicated a similar average dispersion within samples' groups (Table 3), and thus differences in dispersion between groups were not significant ($F_{2,8} = 3.4322$; $p = 0.3295$).

Table 1 – Results of the one-way PERMANOVA. Analysis of microbial community variation within samples' groups. df = degrees of freedom; SS = sum of squares; MS = mean squares; Pseudo-F = F-ratio; P (perm) = probability; Up = unique perms. Significant p-values ($p < 0.05$) are given in bold.

Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Up**
Samples	2	19010	9504.8	38.087	**0.0027**	9697
Residuals	8	1996.4	249.55			
Total	10	21006				

Table 2 – Results of the a posteriori pairwise comparisons within samples' groups. t = t-test; P (perm) = probability; Up = unique perms. Significant p-values ($p < 0.05$) are given in bold. W = seawater; CRI = *Chondrosia reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Groups	**t**	**P(perm)**	**Unique perms**
W, CRI	7.5039	**0.0279**	9936
W, CRE	9.5549	**0.0288**	9908
CRI, CRE	1.0115	0.4563	9916

Table 3 – Results of the a posteriori pairwise comparisons within samples' groups. t = t-test; P (perm) = probability; Up = unique perms. Significant p-values ($p < 0.05$) are given in bold. W = seawater; CRI = *Chondrosia reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Group	**Size**	**Average**	**SE**
W	3	5.7312	0.41009
CRI	4	15.894	3.4514
CRE	4	12.988	2.3918

Total mercury content

Our results are presented as content of mercury in the seawater and the analysed sponge species. THg levels in the unfiltered seawater samples were slightly higher (3.9 ± 1.9 ng L^{-1}) than in the filtered one (3.3 ± 1.4 ng L^{-1}) (Table 4). The Student *t* test did not highlight any significant differences between them ($p>0.05$). Nonetheless, to calculate BAFs, the THg value obtained with the filtered seawater was used. The mean THg value recorded in the filters with the microbiome extracted from the seawater was nearly negligible, measuring 0.0034 ± 0.0008 mg kg^{-1} d.w. (Table 4).

Table 4 – THg levels in sponges, microbiota, and seawater samples. The bioaccumulation factor (BAF) is also given. CRI = *Chondrosia reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Samples code	**Sponge (mg kg^{-1} d.w.)**	**Microbiome (mg kg^{-1} d.w.)**	**Filtered seawater (ng kg^{-1})**	**Water microbiome (mg kg^{-1} d.w.)**	**Unfiltered seawater (ng kg^{-1})**	**BAF* (x10^{5}) in sponges**	**BAF* (x10^{5}) in microbiota**
CR-C	0.70 ± 0.08 (0.51 – 0.82)	0.008 ± 0.001 (0.06 – 0.12)	3.3 ± 1.4	0.0034 ± 0.0008	3.9 ± 1.9	2.1 ± 0.3 (1.6 – 2.5)	0.25 ± 0.04 (0.19 – 0.37)
CR-E	0.26 ± 0.07 (0.15 – 0.41)	0.079 ± 0.002 (0.068 – 0.12)				0.8 ± 0.2 (0.5 – 1.2)	0.25 ± 0.05 (0.19 – 0.35)

*calculated using filtered seawater

Considering *C. reniformis*, significant differences in the THg values were found between the two body regions (one-way ANOVA, $p<0.05$), with higher values in CRI (0.70 ± 0.08 mg kg^{-1} d.w., ranging from 0.51 to 0.82 mg kg^{-1} d.w.) than in CRE (0.26 ± 0.07 mg kg^{-1} d.w., ranging from 0.15 to 0.41 mg kg^{-1} d.w.) (Table 4; Figure 4). The opposite was observed in the microbial pellet, with the mean THg concentration in the microbiome extracted from CRI ranging from 0.06 to 0.12 mg kg^{-1} d.w. (0.08 ± 0.01 mg kg^{-1} d.w.) and in the one extracted from CRE from 0.068 to 0.12 mg kg^{-1} d.w. (0.079 ± 0.02 mg kg^{-1} d.w.), being extremely similar (one-way ANOVA, $p>0.05$) (Table 4; Figure 4).

Clear differences were recorded between THg values of the sponge fraction and the extracted microbiome within both body regions (Kruskal-Wallis, $p<0.05$), with higher THg values in the sponge compared to the microbial pellet (Dunn's test, $p<0.05$) (Figure 4), and a ratio of 8:1 and 3:1 for choanosome and cortex, respectively.

Similarly, for BAF values significant differences were found between body regions for the sponge fraction (one-way ANOVA, $p<0.05$), with higher values in the choanosome than in the cortex (Table 4), while no differences were recorded between the microbial pallet of the two regions (Kruskal-Wallis, $p>0.05$). Furthermore, BAF values were also different between the sponge fraction and the extracted microbiota in both choanosome and cortex (Kruskal-Wallis, $p<0.05$), with higher values in the sponge.

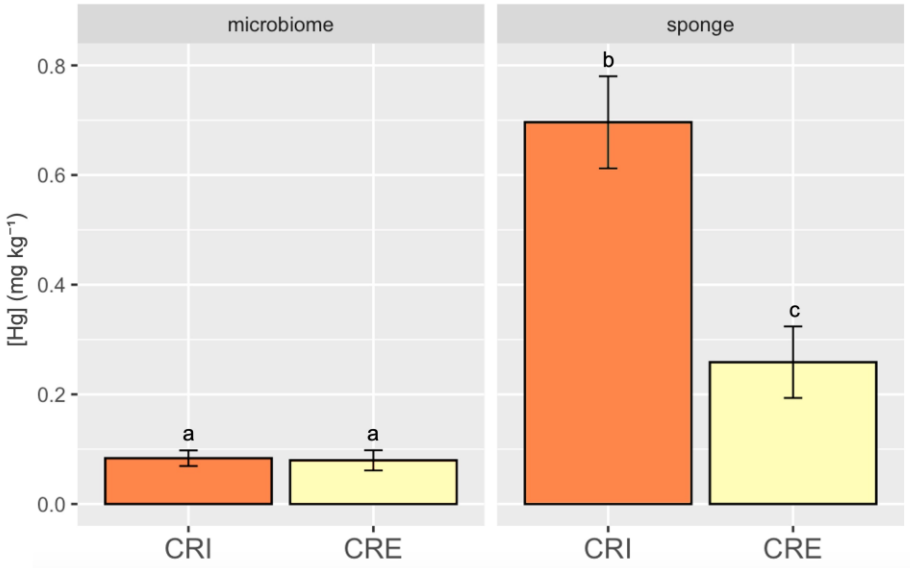

Figure 4 – Mean THg content (± standard deviation) in the microbiome and sponge devoid of microbial cells in the two body regions of *Chondrosia reniformis*. The different letters (a, b, c) indicate statistically significant differences (Dunn's test, $p<0.05$; Tukey's test, $p<0.05$). CRI = *C. reniformis* internal choanosome; CRE = *C. reniformis* external cortex.

Discussion

The current study represents the first detailed structural description of the prokaryotic communities of the two body regions characterizing *Chondrosia reniformis*, the internal choanosome and the external cortex. Among the 11 phyla detected, those belonging to the domain Bacteria dominated the microbial community, mainly represented by the phyla Proteobacteria, Acidobacteria, Bacteroidetes and Chloroflexi, while two phyla belonging to the domain Archaea, Euryarchaeota and Thaumarcheota, were also recorded. These observations are in line with previous studies on Atlanto-Mediterranean sponge microbes (e.g., Schmitt et al., 2007, 2012; Hardoim et al., 2012, 2014; Karimi et al., 2017; Slaby et al., 2017) and species from other geographic locations (e.g., Schmitt et al., 2007; Zarzycki et al. 2009; Schmitt et al., 2012; Webster et al., 2013; O'Connor-Sánchez et al., 2014). Particularly, the community profiles described here deepen our understanding of prokaryotic community assembly in *C. reniformis* owing to the higher resolution of the molecular approach employed (e.g., next-generation sequencing) in comparison with earlier studies relying on PCR-DGGE and clone libraries (Ribes et al., 2012) or fluorescence in situ hybridization (FISH) surveys (Manz et al. 2000). For instance, we revealed three lineages likely contributing to ammonium oxidation within *C. reniformis*: the ammonium-oxidizing archaea (AOA) *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*, and the ammonium-oxidizing bacterium (AOB) *Nitrosococcus* sp., while Ribes et al. (2012) found one AOB lineage of uncultivated Gammaproteobacteria based on cloning-and-sequencing of AmoA genes from sponge metagenomic DNA. In addition, using widefield deconvolution epifluorescence microscopy combined with FISH, Manz et al. (2000) showed a compartmentalization of the microbial community in *C. reniformis*, with Desulfovibrionaceae (rod-shaped bacteria) colonizing the channel system of the sponge cortex, densely surrounded by coccoid bacterial cells within the sponge mesohyl (extracellular matrix). Although we did not analyse the spatial arrangement of the associated microbiota, the family Desulfovibrionaceae was not recorded in our samples, neither in the choanosome nor in the cortex, instead a great diversity of bacterial species was found in both body regions, thus not only confined to the mesohyl. Since it is known that intraspecific variability between individuals of the same species collected in different localities can be detected (e.g., Hardoim et al., 2012; O'Connor-Sánchez et al., 2014; Griffiths et al., 2019; Easson et al., 2020), the differences in microbial community composition found in comparison with previous studies could be related to the fact that samples were collected at different sites and marine provinces, namely Montgrí Coast (Mediterranean Sea) in Ribes et al. (2012), and Faro (Atlantic Ocean) in this study, while no information on the sampling location was given by Manz et al. (2000). Moreover, some, if not most, of the differences observed among the three studies are likely related to the techniques and approaches employed: FISH in Manz et al. (2000), targeted approach on Desulfovibrionaceae; PCR-DGGE and clone libraries in Ribes et al., (2012), targeted approach on ammonium-oxidizing bacteria and archaea; next-generation, Illumina sequencing of "total" archaeal and bacterial 16S rRNA genes in this study.

C. reniformis is characterised by two body regions, the cortex and the choanosome, with a distinct texture and physical-chemical architecture: the first is composed by flattened pinacocytes and densely packed interwoven collagenous fibers, it is highly selective and able to incorporate only siliceous particles, while the latter contains the choanocyte chambers, allowing the entrance and attachment of every kind of material (Bavestrello et al., 1995, 1998a, b). Considering these differences, we thus hypothesized a distinct microbial community composition between the cortex and the choanosome. Surprisingly, the results obtained in this study reject the hypothesis, and no significant differences were recorded in the prokaryotic community structure between the two body regions of *Chondrosia,* as supported by statistical analysis. In this regard, both the PCoA and alpha-diversity analyses showed that *C. reniformis* sample 1 diverges from the other samples, but in light of the PERMDISP results, it cannot be seen as an outlier. This observation confirms the already observed variability that is possible to encounter between specimens of the same sponge species (e.g., Schmitt et al., 2007, 2012; O'Connor-Sánchez et al., 2014; Slaby et al., 2017) and it is likely that the extent of variation in microbiome composition between specimen 1 and the remainder of the specimens analysed contributes for the lack of statistical difference between the internal and the external region reported in this study.

Notably, sponges presenting a clear and strict separation between body regions (e.g., *Geodia* spp*.,* *Stelletta* spp., *Chondrosia* spp. and others) have been seldomly studied and, to our knowledge, the microbial community has never been analysed separately. In this context, the current study represents an important contribution to a better understanding of this issue.

The *C. reniformis* prokaryotic community was highly distinct from the one characterising the seawater. PCoA revealed that the difference between *C. reniformis* and seawater prokaryotic communities is caused by changes in both relative abundance and presence/absence of several bacterial and archaeal species, involving the enrichment, in *C. reniformis*, of archaeal ammonium oxidizers and of so-far uncultivated bacterial phylotypes in the Acidobacteria and Chloroflexi phyla. This is consistent with the community composition usually reported for bacteriosponges in the Atlanto-Mediterranean zone (e.g., Hardoim et al., 2014; Hardoim and Costa, 2014). Nevertheless, the dominance of archaeal species, namely *N. maritimus* and *C. symbiosum* (composing up to the 7 and 25% of the dataset of both body regions, respectively), in the total prokaryotic community is not frequently reported in studies of the marine sponge microbiome, and thus can be considered a distinguishing feature of the *C. reniformis* symbiotic consortium. This aspect was also clear by the alpha-diversity measures that were higher in the seawater than in the sponge, suggesting a selection of the symbiotic community by the sponge itself, as observed also in other sponge species and marine invertebrates such as corals (e.g., Karimi et al., 2017; Keller-Costa et al., 2017). Moreover, compared to the seawater, *Chondrosia* samples showed a higher individual-to-individual variation in their prokaryotic community structure and composition, a pattern also showed by other sponges, such as *Ircinia variabilis* (Schmidt, 1862) (Hardoim et al., 2012, 2014), *Ircinia campana* (Lamarck, 1814) (Griffiths et al., 2019) or *Luffariella variabilis* (Poléjaeff, 1884) (Webster et al., 2013), all recognized as HMA sponges. A lack of variability was instead recorded in *Cliothosa delitrix* (Pang, 1973) (cited as *Cliona delitrix*), a typical characteristic of low-microbial-abundance (LMA) sponges (Easson et al., 2020), since they reflect the more stable microbiota of the seawater, both in numbers and in phylogenetic composition (Schmitt et al., 2007).

Because *C. reniformis* is an HMA sponge (Ribes et al., 2012), its microbiota can reach densities of 10^8 – 10^{10} cells g^{-1} of sponge wet weight, which presumably are mainly acquired by vertical transmission through larvae (Lévi and Lévi, 1976). This microbiota can actively participate in the metabolic cycles of their host, also contributing with the biosynthesis of a multitude of inhibitory compounds, being thus considered as sources of novel metabolites with high biotechnological potential (Schmitt et al., 2007). PCoA and SIMPER highlighted the most characteristic taxa of *C. reniformis*, also belonging to the most representative genera, all recognized as important contributors in various pathways of sponges' secondary metabolism (Feng and Li, 2019). Among them, *C. symbiosum* and *N. maritimus* are archaeal species, which are typical components of the microbiome of many sponges such as *Aplysina aerophoba* (Nardo, 1833), *Axinella* sp., *Cliona celata* Grant, 1826, *Dragmacidon mexicanum* (de Laubenfels, 1935) (cited as *Axinella mexicana*), *Tethya aurantium* (Pallas, 1766) (cited as *Tethya aurantia*), *Theonella swinhoei* Gray, 1868, *Xestospongia* sp. (Preston et al., 1996; Hallam et al., 2006; Feng et al., 2016), but never identified in *C. reniformis* before. These Thaumarcheota species are well known to be involved in the first step of nitrification within the nitrogen cycle, that is, ammonia oxidation. The fact that these ammonium-oxidizing archaea rank among the most dominant symbionts of *C. reniformis*, along with the ammonium-oxidizing bacterium *Nitrosococcus*, strongly suggests ammonia oxidation as a key metabolic pathway dictating the transformation of N within this sponge species.

Similarly, important roles in carbon cycling may be played by *Chloroflexus* and *Nitrosococcus* spp. detected in our study, taxa well represented also in *C. reniformis* samples analysed by Ribes et al. (2012). Bacteria of the phylum Chloroflexi (in particular *SAR202*) are abundant and widespread in HMA sponges (Busch et al., 2020), and are usually involved in the 3-hydroxypropionate cycle for autotrophic CO_2 fixation (Zarzycki et al., 2009), but they can also participate in the central energy pathway, amino acid and fatty acid metabolism, and respiration, as observed in the sponge *A. aerophoba* (Bayer et al., 2018). On the other hand, *Nitrosococcus* is a Gram-negative ammonia-oxidizing bacterium (Chain et al., 2003), with the ability to oxidize methane to CO_2 in the absence of ammonium or nitrite (Jones and Morita, 1983) and involved in fundamental pathways, including the tricarboxylic acid cycle, and the Embden-Meyerhof-Parnass and pentose phosphate pathways (Klotz et al., 2006).

Another example of uncultivated bacterial phylum enriched in *C. reiniformis* as observed by Ribes et al. (2012) and this study, is Acidobacteria, which appears to dominate the bacterial community of various HMA sponges (Hardoim and Costa, 2014; O'Connor-Sánchez et al., 2014; Slaby et al., 2017). However, information on their coding potential and physiology is still very scarce, since most of its members cannot be cultured and, in most studies, they have only been identified by their 16S rRNA gene sequences (O'Connor-Sánchez et al., 2014). Currently, the construction of metagenome-assembled genomes (MAGs) from metagenomic DNA sequences is enabling novel insights into the potential metabolism of marine sponge symbionts. In this context, Slaby et al. (2017) suggested that representatives of the phylum Acidobacteria evolved sophisticated mechanisms of defence against foreign DNA, since they present an abundance of multiple restriction-modification and toxin-antitoxin systems, which are common attributes of obligate mutualists of marine sponges (Karimi et al., 2018). Future studies of Acidobacteria MAGs shall shed more light on the patterns of nutritional exchange between these symbionts and their sponge hosts.

Pseudomonas, one of the most abundant genera in our *Chondrosia* samples, was instead not identified by Ribes et al. (2012). As well as other taxa characterising this sponge, *Pseudomonas* spp. are involved in denitrification processes, presenting a variety of denitrifying genes (Jin et al., 2015; Feng and Li, 2019). *Pseudomonas* strain MSI016, isolated from the sponge *Fasciospongia cavernosa* (Schmidt, 1862) together with other microbial taxa, also showed a high resistance to different trace elements (Cu, Pb, Hg, Co, Cd, Zn, Ni), and was suggested as an ideal bioindicator model for the monitoring of metal pollution in the marine environment (Selvin et al., 2009). A similar pattern was observed for Cu, Ni, Pb and Zn in various microbial strains isolated from *Spongia officinalis* Linnaeus, 1759 (Bauvais et al., 2015).

Considering the sedentary nature of sponges, they can live several years in the same location and accumulate metals for a long period (Selvin et al., 2009). In fact, samples of *Chondrosia* collected in Faro showed detectable concentrations of Hg and the lack of variability among samples of the same size suggest this species as an attractive bioindicator of Hg pollution. THg levels found in both cortex and choanosome were unequal, with higher concentrations in the internal region, as already observed for Hg in the same species collected in the Tuscan Archipelago (Italy) by Roveta et al. (2022). A differentiated distribution of metals was also observed in the sponge *Spheciospongia vagabunda* (Ridley, 1884), in which high Fe, Ni and Zn were distributed in patches or spots (Padovan et al., 2012). The distribution of the trace elements within sponges' body is, in fact, often heterogeneous (Batista et al., 2014), and the phenomenon is usually correlated to a different microbiota-mediated accumulation (Padovan et al., 2012). However, the THg concentration found in *Chondrosia* microbiota extracted from the two body regions are similar, in line with the lack of differences found in the microbial community composition, thus suggesting that the same microbial species bioaccumulate with the same rates. In our case, the explanation should be found in the internal organization of the sponge body. Water filtration takes place only in the choanosome (Schmitt et al., 2007), where the choanocyte chambers are present and a polarization was seen in the incorporation of foreign matter, with the choanosome being not selective and able to incorporate every kind of material and compounds (Bavestrello et al., 1998a, b), probably including metals.

From our study it is, thus, clear that the microbiota can actively bioaccumulate Hg, suggesting a possible involvement of the microbial community in the bioaccumulation process. A study conducted on the tropical sponge *Theonella swinhoei* Gray, 1868, Keren et al. (2017) also found its microbiota able to bioaccumulate different concentrations of As and Ba. However, while the *Chondrosia* microbiota displayed a lower concentration compared to the sponge fraction, Keren et al. (2017) found significantly higher metals concentrations in each extracted bacterial fraction (unicellular bacterial cells, *Entotheonella* sp. with cyanobacteria, and *Entotheonella* sp. cells) compared to the sponge fraction alone. It is hard to interpret the different patterns found between the two studies, but a few considerations can be done: (1) the analysed sponges are different species, and concentration of trace elements can vary between taxa, from phyla down to species of the same genus (Rainbow, 2002); (2) *Entotheonella* sp. is a filamentous bacterium, considered the most important microbe in *T. swinhoei*, in which it plays a fundamental role in many metabolic pathways (Wilson et al., 2014); (3) different trace elements were considered, and their bioaccumulation rates can vary depending on their nature (Simkiss and Taylor, 1995); (4) the application of different protocols in the extraction of the microbiota. Therefore, considering the observed pattern of Hg distribution between the sponge and the microbial fractions, further studies on the actual histological location of Hg inside the sponge are needed to clarify if interactions between Hg and specific functional groups exist, as demonstrated in the bath sponge *S. officinalis* (Domingues et al., 2021), and to understand in which compartment *Chondrosia* stocks the metal.

Conclusions

This is the first study providing a detailed description of the microbial community inhabiting the two body regions characterizing *Chondrosia reniformis*, and the first establishing the mercury accumulation relationships in a marine sponge and its associated microbiota, unveiling the total Hg content in the cortex and choanosome of this sponge species and in their corresponding microbial cell pellets.

The current investigation highlights the lack of differences in the prokaryotic community composition between the two body regions of the studied sponge. In addition, it reveals the presence of two ammonia-oxidizing archeal species, namely *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus*, in both regions of *C. reniformis'* body. These two species, together with the bacterium *Nitrosococcus* spp., another ammonia-oxidizing organism, co-dominate the whole microbial consortium, showing an enrichment in the sponge compared to the seawater. These results show that ammonium oxidation/nitrification is a key metabolic pathway taking place within *C. reniformis,* dictating nitrogen cycling within the animal, likely helping in the removal of toxic ammonium from the sponge body. In fact, having found many other denitrifying microbial species in *Chondrosia*, including several *Pseudomonas*, suggests that these may be able to pick up the NO_2 produced by the nitrifying organisms (*C. symbiosum*, *N. maritimus,* etc.) and denitrify it to N_2, closing the cycle, in line with the observations made by Ribes et al. (2012).

This work also points out the ability of the microbiota of *C. reniformis* in bioaccumulating mercury, and that the microbiota extracted from the cortex and the choanosome bioaccumulate similarly, in line with the aforementioned lack of differences in the microbial community composition, thus suggesting that the same microbial species bioaccumulate with the same rates. On the other hand, the differences in THg values recorded between the two body regions could be related to the different selectivity characterizing each region in the incorporation of foreign matter and compounds (Bavestrello et al., 1998a, b).

To conclude, considering the fundamental roles played by the microbiota in sponge secondary metabolism and the evidence that it can actively participate in the bioaccumulation process, sponges should be considered not only as bioindicators – as suggested by the international legislation (e.g., the Water Framework Directive and the Marine Strategy) – but also as useful tools in the bioremediation in metal polluted environments (Santos-Gandelman et al., 2014a, 2014b).

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CONCLUDING REMARKS

This PhD project provided new taxonomic baselines useful to assess, with future monitoring, possible changes in hydroid and sponge assemblages related to direct and indirect pressures. We will not stress enough the importance of increasing the sampling effort not only in the islands considered in this study, but in the entire Archipelago to describe the actual biodiversity of a poorly studied area. These data have also been made available to the Tuscan Archipelago National Park coordinators possibly helping stakeholders and decision makers in the implementation of new protection strategies and in the establishment of the National Park as an MPA, in line with the Italian and European legislations.

In the context of anthropogenic impacts, this work highlights the role of non-conventional benthic taxa as bioindicators of trace elements pollution, with special attention to mercury. From the systematic review and the preliminary study on different sponge species emerged the importance in identifying the more suitable species for the application as biomonitors since they better reflect the level of contamination of a specific location. Even though *Chondrosia reniformis* demonstrated to be an attractive bioindicator for mercury pollution, it will be fundamental the application of molecular and/or genetic biomarkers (e.g., metallothioneins) to understand the actual physiological effects caused by the metal.

Finally, together with a characterization of the sponge's microbial community, the analysis conducted on the THg content in its associated microbiota highlighted that the microbiome effectively bioaccumulate Hg, even though the amount in the sponge fraction is higher. Future studies are needed to understand in which histological compartment sponges accumulate TEs and the fate of metals once they enter the sponge and the microbial cells.

In conclusion, the implementation of monitoring programmes on marine coastal communities, together with the use of benthic organisms as biomonitors of changing in species composition or of pollutants are essential to determine the health status of these ecosystems for the achievement of the GES, as suggested by the Marine Strategy Framework Directive. This study represents an advancement on the taxonomic and ecological knowledge of benthic communities, providing additional data to address the main inputs of the major European Directives (e.g., MSFD, WFD, Biodiversity Strategy for 2030) to protect coasts, seas, and oceans.

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ADDITIONAL CONTRIBUTIONS

In this last section are provided other publications, not included in the PhD thesis, and the research products that have been shown as contributions to national and international conferences, presented during the PhD period.

Peer-reviewed publications

Roveta C., Annibaldi A., Domenichelli F., Gregorin C., Gridelli S., Pantano V., Vagnoni F., Puce S. 2022. Single and combined effects of two trace elements (Cd and Cu) on the asexual reproduction of *Aurelia* sp. polyps. *Aquatic Ecology,* 1-7. https://doi.org/10.1007/s10452-021-09940-8

Roveta C., Annibaldi A., Vagnoni F., Pulido Mantas T., Domenichelli F., Gridelli S., Puce S. 2020. Short-term effects of environmental factors on the asexual reproduction of *Aurelia* sp. polyps. *Chemistry and Ecology, 36*(5), 486-492. https://doi.org/10.1080/02757540.2020.1735375

Roveta C., Pica D., Puce S. 2019. The cnidome of *Olindias muelleri* (Cnidaria: Hydrozoa: Limnomedusae) from South Adriatic Sea. *Zoomorphology, 138*(4), 437-442. https://doi.org/10.1007/s00435-019-00460-6

Roveta C., Bavestrello G., Montefalcone M., Pica D., Puce S. 2019. Asymmetrical distribution of *Distichopora violacea* (Cnidaria: Hydrozoa) in four Maldivian atolls. *The European Zoological Journal, 86*(1), 9-19. https://doi.org/10.1080/24750263.2018.1551943

Pulido Mantas T., Varotti C., **Roveta** C., Palma M., Innocenti C., Giusti M., Benabdi M., Trainito E., Mačić V., Gambi M.C., Cerrano C.. Mediterranean Sea shelters for the Gold Coral *Savalia savaglia* (Bertoloni, 1819): an assessment of potential distribution of a rare parasitic species. *Marine Environmental Research,* minor revisions.

Afghan A., Cerrano C., Luzi G., Calcinai B. Puce S., Pulido Mantas T., **Roveta** C., Di Camillo C.G. 2020. Main Anthropogenic Impacts on Benthic Macrofauna of Sandy Beaches: A Review. *Journal of Marine Science and Engineering, 8*(6): 405. https://doi.org/10.1080/02757540.2020.1735375

Contributions at national and international conferences

Roveta C., Annibaldi A., Calcinai B., Girolametti F., Illuminati S., Pulido Mantas T., Truzzi C., Puce S. 2021. Annual variations of Hg levels in two Mediterranean sponge species. Conference Proceedings of the XVII Italian-Hungarian Symposium on Spectrochemistry: Current approaches in Health and Environmental Protection. Turin (Italy), 14-18 June. ISBN: 9788897655077

Roveta C., Girolametti F., Calcinai B., Pica D., Truzzi C., Illuminati S., Annibaldi A., Puce S. 2019. Hg contamination of the sponges of the Integral Nature Reserve of Montecristo. 37th Congress TUMA -UNIVPM & Società Chimica Italiana. Ancona (Italy), 19-20 September.

Vattese J., Puce S., Dominichelli F., Gridelli S., **Roveta** C., Fernndez T.V., Musco L. Together or alone? Protocooperative predation may be an evolutionary driver promoting gregarism in cnidarian polyps. 8a Congress SIBE 2019 -Italian Society for Evolutionary Biology. Padova (Italy), 01-04 September.

Roveta C., Annibaldi A., Vignoli F., Pulido Mantas T., Domenichelli F., Gridelli S., Puce S. Short-term effects of mercury on the asexual reproduction of *Aurelia* sp. polyps. 1st MS SeaDay - ARPAT & Società Chimica Italiana. Livorno (Italy), 06-07 June.