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# HEALTH AND DISEASE ASSESSMENT OF SHALLOW-WATER ANTHOZOANS OF THE MARINE PROTECTED AREAS OF PORTOFINO AND BERGEGGI

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# Table of Contents

# ABSTRACT

The Mediterranean Marine Animal Forests (MAFs) are among the most ecologically and structurally important marine habitats, offering critical biodiversity and ecosystem services. Nonetheless, these benthic communities are encountering unprecedented challenges due to climate change, human activities, and emerging contaminants. This project aimed to evaluate the health status of Mediterranean anthozoans in the Marine Protected Areas (MPAs) of Bergeggi and Portofino, focusing on the primary stressors, their effects, and potential solutions for the conservation and restoration of these essential ecosystems.

The research on climate change has clearly highlighted the effects of thermal stress on anthozoans, as rising sea surface temperatures have resulted in increasingly frequent marine heatwaves triggering mass mortality events within the Mediterranean benthic community, especially impacting anthozoans. Detailed histopathological analyses were conducted on three key gorgonian species (*Paramuricea clavata*, *Eunicella cavolini*, and *Leptogorgia sarmentosa*), uncovering distinct patterns of tissue damage such as exposed axial skeletons, thinning tissues, and areas of necrosis. Significantly, the study noted the presence of ciliates in the gastrodermis of *E. cavolini*, suggesting a previously underexplored interplay between thermal stress and disease processes. These findings underscore the urgent need for additional research into pathogens and environmental factors contributing to mass mortality events, to imptove our understanding of disease dynamics in Mediterranean anthozoans.

The study provided one of the first assessments of anthozoan contamination by emerging pollutants, such as phthalic acid esters (PAEs), active pharmaceutical ingredients (APIs), and UV filter compounds in the Mediterranean region. Utilizing solid-phase microextraction (SPME) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), this study documented the occurrence of these contaminants in four key anthozoan species: *Cladocora caespitosa*, *Madracis pharensis*, *Eunicella cavolini*, and *Parazoanthus axinellae*. Additionally, the presence of UV filters in *Paramuricea clavata* within and outside the Portofino Marine Protected Area (MPA) was investigated, confirming its potential for bioaccumulation. The findings revealed that all sampled specimens showed signs of contamination, with levels of bioaccumulation varying according to species, environmental conditions, and life stages. Interestingly, it was found that levels of UV filters were higher outside MPAs, highlighting their potential role in mitigating pollution. Although all

contaminants were generally detected at low concentrations, the results suggest a risk of bioaccumulation due to their environmental persistence. This underscores the importance of further research to understand the long-term effects these pollutants may have on these organisms, given their significant role in Mediterranean benthic communities.

Recognizing the urgency of protecting anthozoans and their habitats, the project also explored innovative solutions for coral restoration, as traditional restoration techniques often rely on materials that can themselves become pollutants, exacerbating environmental harm. Therefore, a novel, biodegradable coral putty from epoxidized soybean oil acrylate (ESOA) and zein has been designed and tested. This eco-friendly alternative to conventional epoxies and concretes showed quick underwater hardening and high biocompatibility, proving effective for coral transplantation. Its successful application in the Maldives served as a proof of concept, paving the way for similar efforts with Mediterranean anthozoans. This approach offers a sustainable method for supporting the recovery of these vital ecosystems while reducing ecological footprints.

Ultimately, the goal of this project was to deepen our understanding of the stressors affecting Mediterranean Marine Animal Forests (MAFs) and highlight potential solutions for their conservation. By combining research on the impacts with potential innovative solution, this study underscores the importance of a multi-disciplinary approach to protect these ecosystems. It explores the extent of the threats and efficiency of potential interventions to buy time for these ecosystems to recover and adapt to the shifting environment caused by climate change and human activities. This comprehensive strategy might be the key to preserving the unique marine biodiversity of the Mediterranean and ensuring the sustainability of its ecological functions.

# CHAPTER 1

## General introduction: Health status of anthozoans in the Mediterranean Sea in light of anthropic impact and climate change

## 1.1. MEDITERRANEAN MARINE ANIMAL FOREST: BIODIVERSITY AND VULNERABILITY

The term Marine Animal Forest (MAF) was introduced by scientists to describe multifaceted communities of living organisms with different morphologies and trophic needs that are responsible for building the framework of the environment inhabited by themselves. These communities play a crucial ecological function by creating a three-dimensional structure that provides architectural complexity to their environment to be later colonized and used as habitat or shelter by several associated species (Rossi et al., 2017; Rossi & Bramanti, 2021). These marine benthic communities comprise mainly sessile organisms, such as coral, sponges, bivalves, bryozoan, and polychaetes, that can be dominated by a single species (mussel beds or sea pen assemblages) or by several species (coral reefs and gorgonian forests). These marine habitats exhibit a remarkable diversity thanks to the high heterogeneity in environmental conditions and the presence of ecosystem engineer species that modulate resource availability for the other species, whether directly or indirectly (Gori et al., 2017; Paoli et al., 2017). Their richness and protected nature render them incredibly important also as nursery grounds for juvenile life-history stages of several species co-existing in these habitats (Cau et al., 2020).
In the marine environment, the most known examples are the tropical coral reefs, with their high biodiversity and providing habitat for countless marine species and association between them (Sebens, 1994; Buddemeier et al., 2004; Plaisance et al., 2011; Stella et al., 2011; Chen et al., 2015; Fisher et al., 2015). However, in the last decades, thanks to advances in technology, other MAFs, such as cold-water coral communities, deep Antarctic sponge grounds, or mussel beds, gained the scientific community's attention due to their ecological and commercial importance (Gutt et al., 2017; Henry & Murray, 2017). The occurrence, distribution, life history, population dynamics, trophic ecology, or physiology of the organisms that structure these communities are not always completely known or understood. Therefore, new studies are required to gain insight into their functional ecology and dynamics.

Unfortunately, anthropogenic disturbances have significantly impacted the marine environment in the last decades, considerably reducing precious biodiversity, biomass, and resilience of these ecosystems (Rossi, 2013; Hinz, 2017; Ragnarsson et al., 2017). The most impacting threat for MAFs and related marine ecosystems has been globally defined as climate change and its associated modifications. More recently, the emergence of new epizootic diseases and major outbreaks worldwide have become a significant threat almost comparable to climate change and anthropic pressure (Harvell et al., 2002; Mao-Jones et al., 2010; Hughes et al., 2017; Aeby et al., 2021; Eakin et al., 2022; Hughes et al., 2022). As a consequence, there has been significant progress on a global scale in addressing these problems in tropical coral reefs. Nonetheless, this issue can also be observed in various coastal ecosystems with fewer studies conducted, such as the Mediterranean region, where the benthic community mainly comprises Marine Animal Forest (MAFs). Indeed, Mediterranean Sea marine biodiversity is undergoing rapid alterations related to high anthropic impacts and global warming (Halpern et al., 2008; Coll et al., 2011; Bevilacqua et al., 2021), especially dramatic in such a closed and relatively small basin (Giorgi et al., 2006; Lejeusne et al., 2010).

Among the most affected organisms are Cnidarians, particularly Anthozoans, representing an ecologically dominant group in the Mediterranean benthic communities. They provide an essential contribution to marine biodiversity and serve as hosts for a multitude of associated species. Their assemblage is a perfect example of MAFs, as they are capable of colonizing extended areas, building complex three-dimensional structures that are vital for creating various biogenic microhabitats within, on, and around them throughout their lifespan or through their remains after death (Freiwald et al., 2009; Mastrototaro et al., 2010). This can occur from shallow water near the coast to the continental slope below the photic zone at depths between 100 and 1000 m, characterized by deep-water coral formations composed of different species (Boavida et al., 2019).

One of the most distinctive and emblematic habitats in this region are Gorgonian forests, formed by the assemblage of single or various gorgonian species, such as *Paramuricea clavata*, *Eunicella singularis*, *Eunicella cavolini*, *Leptogorgia sarmentosa*, and *Corallium rubrum* (Garrabou et al., 2001; Cerrano et al., 2005; Ponti et al., 2014; Betti et al., 2017; Bianchi et al., 2019; Iborra et al., 2022; Giordano et al., 2023). These species are not only recognized as iconic members of Mediterranean benthic communities but also considered among the most significant ecosystem engineers that typically create dense monospecific assemblages covering large areas of the coast (Ponti et al., 2014; Angiolillo & Canese, 2018; Gori et al., 2017). Their fan-like structure and association with various organisms, such as other cnidarians and sponges, enable them to create complex structures that are essential for providing habitat and the functionality of the coastal environment (Bo et al., 2009; Cerrano et al., 2010; Gori et al., 2011; Ponti et al., 2014; Grinyò et al., 2016; Angiolillo & Canese, 2018). Gorgonian forests are found throughout the Mediterranean region due to their ability to settle on both hard and soft bottoms, as well as their wide bathymetric and geographical distribution, which varies according to the species (Gori et al., 2011; Angiolillo & Canese, 2018). Generally, the colonies find a more stable environment in deeper areas, forming larger structures than in shallower waters (Linares et al., 2005, 2010). This distribution may be influenced by biological and environmental factors, including spawning and larval settling, sediment accumulation, and resistance to currents (Gori et al., 2011; Grinyò et al., 2016).

In addition, Scleractinians (or stony corals) are also an essential component of Mediterranean MAFs, representing the second largest group of anthozoans with at least 33 native species from eight families (Bo et al., 2017). They can be found rather commonly in the benthic community in this region, supporting a significant number of associated species thanks to the extensive carbonate skeleton constructions both in shallow and deep waters throughout continental shelf or along the margins of canyons. *Cladocora caespitosa* is the only Mediterranean zooxanthellate reef-building coral, as other hermatypic corals have been missing since Miocene (Vertino et al., 2013) and is supported by secondary bio-constructional scleractinian corals in shallow water, such as *Astroides calycularis* and *Madracis pharensis*, to increase the habitat complexity and surface topography aiming at the maximum biodiversity (Grubelić et al., 2004). *C. caespitosa* is typically found in shallow water ecosystems, forming isolated colonies or patches. In some cases, it creates aggregates of several distinct colonies called beds or large colonies of several centimeters in height called banks (Peirano et al., 1998). However, it may occasionally colonize a deeper environment (Morri et al., 1994; Kersting & Linares, 2012) thanks to biological factors, such as the ability to up-regulate heterotrophy and the presence of allelochemical defense mechanisms (Hoogenboom et al., 2010; Ferrier-Pagès et al., 2011; Kersting et al., 2013), and environmental condition such as hydrodynamic and substratum available (Chefaoui et al., 2017; Kersting et al., 2017). However, in recent decades, the larger formations have become increasingly rare due to human pressures (Kružić & Benković, 2008; Kersting & Linares, 2012) and anomalies in seawater temperature (Rodolfo-Metalpa et al., 2005; Kersting et al., 2013; Kružić et al., 2014; Jiménez et al., 2014). As a result, this species has been classified as endangered and included in the IUCN Red List (Pitacco et al., 2014; Casado-Amezúa et al., 2015).

Regrettably, these key habitat-providing species for the Mediterranean marine environment are highly vulnerable to numerous natural and anthropogenic impacts and disturbances that resulted in a steep decline of these populations (Danovaro et al., 2010; Templado, 2013; Newbold et al., 2020; Iborra et al., 2022). In 2014, the IUCN Centre for Mediterranean Cooperation, in collaboration with the International Union for the Conservation of Nature

(IUCN) Global Species Program, established a regional group of experts to complete an assessment of the conservation status of anthozoans in the Mediterranean region that resulted in the publication of the IUCN Mediterranean Red List of Anthozoans (Bo et al., 2017). This project reported a total of 150 species encompassing 26 endemics. Among them, 13% (17 species) were classified as Threatened, while 7% (10 species) Near Threatened. A significant concern arose from the fact that over 50% of the species (69) were classified as Data Deficient, highlighting a worrying lack of information about most of the species, leading to the impossibility of assessing their risk status.

## 1.2. MARINE HEATWAVES AND MASS MORTALITY EVENTS IN THE MEDITERRANEAN: PATTERNS, DRIVERS, AND IMPLICATION

The Mediterranean Sea is considered a biodiversity hotspot, with around 7% of the world's marine biodiversity and a significant presence of endemic species (Coll et al., 2011; Mannino et al., 2017). However, the increasing threats posed by anthropogenic pressures and climate change on its marine ecosystems significantly impact its biodiversity. The impact is even more substantial in this region due to its semi-enclosed nature, which has made it an “open laboratory” with the unique possibility to build and develop models for different phenomena and apply them directly in the field (Giorgi, 2006; Poloczanska et al., 2013; Halpern et al., 2015; Cramer et al., 2018; Smale et al., 2019).

This is especially true with regards to climate change and its most severe consequences, positive thermal anomalies in sea surface temperature (SST) that, if persist over time, may lead to phenomena known as marine heat waves (MHWs) that can have significant and lasting impacts (Hobday et al., 2016; Hu et al., 2024). These, in turn, have been identified as the trigger for the occurrence of mass mortality events (MMEs) affecting several marine organisms, often resulting in substantial disruptions to marine ecosystems and major implications for the benthic communities, as well as substantial economic losses in fisheries and aquaculture industry (Schaeffer & Roughan, 2017; Frölicher & Laufkötter, 2018; Oliver et al., 2018). Occasionally, the geographical scale of MHWs may increase in case of overlap with the typical warming trends of the ocean, causing an extension up to thousands of kilometers from coastal to open ocean, also facilitating a potential propagation deeper into the water column (Scannell et al., 2016; Schaeffer & Roughan, 2017). As a result, the equilibrium of the ecosystem can be reshaped entirely, leading to a rapid redistribution of marine species, mass coral bleaching, and

toxic algal blooms within a limited time frame ranging from weeks to months (Garrabou et al., 2001; Wernberg et al., 2012; Cavole et al., 2016; Hughes et al., 2017; Oliver et al., 2019).
The first globally recognized MHWs occurred in the Mediterranean Sea was in the summer of 2003, when an SST increase of 2-3°C above the seasonal mean, lasted for over a month, was registered and later associated by several studies with significant increases in air-temperature, reduction of wind stress and air-sea exchanges (Sparnocchia et al., 2006; Olita et al., 2007; Oliver et al., 2019). The same mechanism was then recognized as initiating the 2007 eastern Mediterranean heatwave, which recorded up to 5°C anomalies (Mavrakis & Tsiros, 2018). Several studies have investigated the factors behind similar events such as local oceanic and large-scale atmospheric phenomena for the Australian MHW of 2015/2016 (Benthuysen et al., 2018), the persistent multi-year “Pacific Blob” 2014–2016 (Bond et al., 2015; Di Lorenzo & Mantua, 2016), Atlantic MHW in 2012 (Chen et al., 2015), Tasman Sea MHW in 2015-2016 (Oliver et al., 2017), coastal MHWs in South Africa (Schlegel et al., 2017) and subsurface MHW intensification around Australia (Schaeffer & Roughan, 2017).

As regards the Mediterranean Sea, initially the problem gained little attention until the improvement of technologies, data availability, and observational systems showed the concrete problem of a steady increase of SST since the second half of the last century, coupled with an intensification of MMEs of benthic marine invertebrates over the same period (Rivetti et al., 2014; Marbà et al., 2015; Garrabou et al., 2019, 2022). If initially, only a few local studies linked the two phenomena in a cause-effect fashion, later an ever-increasing number of studies have been conducted to confirm and develop this theory further (Danovaro et al., 2001; Garrabou et al., 2009; Puce et al., 2009; Rivetti et al., 2014). The phenomenon has been occasionally recorded in the 1980s around the Western Mediterranean and the Aegean Sea and then more frequently from around the 2000s to date (Bensoussan et al., 2010), with a faster pace in the last 30 years, especially since 2008 (Rivetti et al., 2014; Eric et al., 2018; Garrabou et al., 2022; Iborra et al., 2022; Tignat-Perrier et al., 2022). In 1999 and 2003, the most severe and impressive MHWs and MMEs occurred, decimating several populations of more than 30 Phila of marine organisms along the Mediterranean area, especially reported in NW Mediterranean coasts of France and Italy (Cerrano et al., 2000; Rivetti et al., 2014). Since then, the problem has become highly evident, and more records have become available, revealing the increasing frequency of MHWs and MMEs both in the Mediterranean Sea and globally (Crisci et al., 2011; Kersting et al., 2013; Turicchia et al., 2018; Smale et al., 2019) at large-scale or with restricted geographic extension and/or number of species affected (Garrabou et al., 2009; Rivetti et al., 2014; Marbà et al., 2015; Rubio-Portillo et al., 2016; Darmaraki et al., 2019). It is alarming to observe that we are now experiencing heatwaves yearly, each intensifying in strength and duration. The year 2022 was particularly notable, as it recorded some of the most extreme heat levels ever documented (Bramanti et al., 2023; Martinez et al., 2023). With the data from last year and this ongoing year still pending analysis, it raises concerns about the ongoing impact of climate change and what future heatwaves may look like. The growing interest and number of studies in the Mediterranean region culminated with the creation of the mass mortality events database (MME-T-MEDNet) by the joint effort of more than 30 research institutions from 10 Mediterranean EU and non-EU countries aiming to simplify the communication, access, and sharing of information about Mediterranean MMEs and MHWs (Garrabou et al., 2019, 2022). After carefully reviewing several papers and reports, all the information has been assembled to create an unrestricted open-source dataset, ensuring transparency and clarity regarding the origin and adequate data citation, providing time series maps graphically showing the most affected area, taxa, and the thermal anomalies for each event. In addition, they have also identified the geographic and taxonomic groups that have been neglected or need to be better addressed, encouraging researchers to improve this dataset with future monitoring and research efforts by providing information for the assessment of the impact of MMEs on the biodiversity and socio-economic activities, especially with the predicted annual mean SST rise of 1.5-3°C by the end of the 21st century that will lead to acceleration and worsening of future MHW and MMEs (Somot et al., 2006; Diffenbaugh et al., 2007; Mariotti, 2015; Adloff et al., 2015; Eric et al., 2018; Oliver et al., 2018).

Focusing on the outcomes of these events, severe impacts on marine ecosystems have been documented worldwide, including biodiversity drops and topicalization of marine communities (Wernberg et al., 2012, 2016), extensive species migrations (Mills et al., 2013), stranding of marine mammals and seabirds, toxic algal blooms (Cavole et al., 2016), extensive coral bleaching (Hughes et al., 2017) and MMEs of benthic organisms (Marbà et al., 2015; Garrabou et al., 2019, 2022). In the Mediterranean Sea in particular, unprecedented mass mortality events and changes in community composition due to extreme warming were reported in the summers of 1999 (Cerrano et al., 2000; Garrabou et al., 2001; Linares et al., 2005), 2003 (Garrabou et al., 2009; Schiaparelli et al., 2007), 2006 (Kersting et al., 2013) and 2008 (Huete-Stauffer et al., 2011). Since then, MHWs have become more and more severe, longer, and spatially extended, but also closer in time, such as the event of 2012 (Kersting et al., 2013; Kruzic et al., 2014), 2015 (Marbà et al., 2015) and 2018 affecting a wide variety of species and taxa especially lethal for organisms with reduced mobility depending on both temperature and duration, with the period between 2015-2019 established as the warmest ever (Galli et al., 2017; Garrrabou et al., 2019). This forecast was beaten again in the summer of 2022 when the strongest MHW afflicted the Mediterranean Sea for over 3 months, devastatingly affecting the benthic communities (Bramanti et al., 2023; Martinez et al., 2023). The reduced interval between marine heatwaves (MHWs), which now occur each summer, worsens the situation because the organisms do not have enough time to recover their populations. Additionally, the temperature change is affecting the deeper layers of the water column, and the consequences of this will become more apparent in the coming years (Juza et al., 2022; Hamadeno & Alvera-Azcarate, 2023; Pastor & Khodoyar, 2023).

An extended literature analysis yielded over 60 publications reporting hundreds of MMEs observed from 1980 to date (Table 1). These events involved at least nine major taxonomic groups for a total of more than 90 species affected spread all over the Mediterranean Basin, especially W-NW coasts, Adriatic Sea, and Aegean Sea (Rivetti et al., 2014; Marbà et al., 2015; Garrabou et al., 2019, 2022; Hamadeno & Alvera-Azcarate, 2023; Pastor & Khodayar, 2023). Cnidaria and Porifera are by far the most affected phylum (85% of observation) and reported group (50% of the records available), followed by Bryozoa, Bivalvia, Chordata (Ascidiacea), Rhodophyta, Annelida, Chlorophyta, and Echinodermata. These results are most probably due to inter and intra-specific differences in thermal stress tolerance that make Cnidaria the most delicate taxon (Ledoux et al., 2015; Pagès-Escolà et al., 2018; Garrabou et al., 2019, 2022; Gómez-Gras et al., 2021, 2022). These phenomena have affected extremely diverse coastal areas in Mediterranean regions characterized by different substrates, from hard substrates such as rocky formations and slopes to seagrass meadows and less stable mobile substrates. However, the hard substrate was the most affected regardless of the typology; the most common depth range was between 15m and 25m (Garrabou et al., 2019, 2022; Hamadeno & Alvera-Azcarate, 2023; Pastor & Khodayar, 2023). This result may be explained by the lack of data for deeper events but also by the extreme abundance of anthozoan-dominated benthic communities in that range of depth, which contribute significantly to the biodiversity of the marine habitat in the region (Rivetti et al., 2014; Marbà et al., 2015; Wernberg, 2016). This is evident in some of the most iconic species of the Mediterranean Sea, gorgonians such as *P. clavata*, *E. cavolini*, *E. singularis*, and *C. rubrum*, as well as scleractinian *C. caespitosa*. These species comprised over one-third of the MMEs reported over the years, and as discussed earlier, they are essential in providing the complex three-dimensional habitats necessary for many other species (Bellwood et al., 2004; Rodolfo-Metalpa et al., 2005; Kersting et al., 2013; Bianchi et al., 2014; Kruzic et al., 2014; Moullec et al., 2019). Due to the unprecedented pattern observed in the last few years, with a sequence of five consecutive years of large-scale MMEs affecting multiple species, there is now a concrete possibility of future scenarios involving ecological extinctions of species with significant changes in benthic communities, loss of ecosystem functioning, and a decrease in the provision of associated services to human societies. This situation has never been reported before, both in the Mediterranean Sea and globally (Garrabou et al., 2019, 2022; Smith et al., 2021; Juza et al., 2022; Hamadeno & Alvera-Azcarate, 2023; Pastor & Khodayar, 2023), with the only relatable situation represented by a succession of recurrent large-scale coral bleaching events between 1998-2020, however not in consecutive years (Wernberg et al., 2016; Hughes et al., 2017; Muñiz-Castillo et al., 2019; Hughes et al., 2022). These events made evident the negative effects of climate change and anthropic pressure, and, therefore, it is imperative to act through the implementation of targeted conservation measures for managing changes and safeguarding the biodiversity and functionality of the Mediterranean Sea in the future.

## 1.3. NATURAL AND ANTHROPOGENIC THREATS TO MEDITERRANEAN MAF

The primary threat to the Mediterranean Sea is climate change, with its increase in temperature causing heat waves, resulting in mass mortality events and increased susceptibility to diseases, as seen in the previous sections. However, the thermal stress may also influence the cyclical development of mucilage blooms in the coastal regions. Mucilage is a thick, viscous substance that is composed of water and various dissolved organic compounds, produced by many organisms, such as plants, algae, and some types of microorganisms (Leppard, 1995; Rinaldi et al., 1995; Mistri & Ceccherelli, 1996; Giuliani et al., 2005; Sartoni et al., 2008; Giani et al., 2012; Ricci et al., 2014; Giani et al., 2016; Piazzi et al., 2018). Temperature anomalies can cause large, dense blooms of these organisms that may significantly impact the marine ecosystem by interfering with other organisms feeding, reproduction, respiration, and growth (Rinaldi et al., 1995; Cornello et al., 2005; Lorenti et al., 2005; Pugnetti et al., 2005; Danovaro et al., 2009; Montalbetti et al., 2023). Mucilage production can be influenced by various factors, such as environmental conditions, seasonal changes, and the specific biology of the organism producing it. For instance, certain phytoplankton species may produce mucilage in response to changes in light, temperature, salinity, or nutrient levels, while some seaweed species may produce it as part of their reproductive cycle (Ricci et al., 2014; Piazzi et al., 2018).

Usually, seasonal changes in environmental parameters are the primary driver of mucilage production; however, the increasingly frequent and prolonged heat waves seen in recent decades have led to several harmful mucilage blooms affecting especially gorgonians, as they provide the best support for mucilage growth with their long branches spread toward the current entangling the filamentous mucilage present in the water (Cerrano et al., 2000; Giuliani et al., 2005; Ricci et al., 2014; Caronni et al., 2016; Piazzi et al., 2018). There are three main species of algae responsible for the mucilaginous aggregates: *Nematochrysopsis marina* and *Chrysonephos lewisii* inhabiting the first 20 m of depth and respectively dominant in spring and summer; *Acinetospora crinite* occurring at greater depth and therefore affecting more *P. clavata*, *E. cavolini* and *E. singularis* than the others (Giuliani et al., 2005; Precali et al., 2005; Caronni et al., 2016, 2017; Giani et al., 2016). One of the first records available dates back to the summer of 1993, when a significant event occurred on the southern coast of Italy (Scilla, Calabria) greatly impacted a population of *P. clavata* with a high level of mortality due to colony coverage (Mistri & Ceccherelli, 1996). Thanks to the data series collected, it was possible to compare the population structure before the event (summer of 1992) and two years after the event (summer of 1995), revealing that the average density of the gorgonians found remained practically identical, suggesting a full recover in terms of numbers of colonies (Mistri & Ceccherelli, 1996). Conversely, the demographic structure of the *P. clavata* population revealed a rejuvenation of the population induced by the mucilage outbreak, as in 1995, juveniles made up 26.1% of the population, whereas, in 1992, they accounted only for 4.2% (Mistri & Ceccherelli, 1996). Ten years later, in June 2003, the European heatwave increased sea water temperature, leading to another significant outbreak of mucilage along the rocky cliffs of the Portofino Promontory in the Ligurian Sea (Precali et al., 2005; Schiaparelli et al., 2007; Lazzari et al., 2008). In this case, *C. caespitosa* was severely impacted, with 40% of the population experiencing bleaching and partial mortality due to the covering of *Acinetospora crinita* responsible for the bloom (Schiaparelli et al., 2007). Only the occurrence of a severe storm in July eventually mitigated the damage by washing the mucilage away, preventing further harm. A more recent study on the same species in Tuscany revealed that benthic mucilaginous aggregates bloom increases necrosis by about 55%, resulting in an overall loss of coverage of about 85% of living *C. caespitosa*, suggesting that benthic mucilaginous aggregates may be a serious threat also for this endemic Mediterranean hermatypic coral (De Biasi et al., 2021).

Mediterranean region is also subject to abrupt and strong natural events such as storms (wind and currents) and geological events (eruption, landslides) that may significantly impact the benthic community. However, these events are poorly investigated in the Mediterranean region, mainly due to the lack of a specific dataset and their unpredictability (Sebens, 1994; Folke et al., 2004; Harley et al., 2006; Hoeg-Guldberg & Bruno, 2010; Jimenez et al., 2012; Pages et al., 2013; Betti et al., 2017). Their impact can be direct physical damage due to increased waves and currents action or indirect due to water chemistry variation and sediment transport altering the availability of resources (Sebens, 1994; Easterling et al., 2000; Jentsch et al., 2007; Jimenez et al., 2012; Pages et al., 2013). These, in turn, may have a ripple effect throughout the benthic community, affecting the survival and reproduction of various organisms. Therefore, Mediterranean Sea storms can have a complex and far-reaching impact, with several reports available regarding the strongest that occurred in the last decades (Navarro et al., 2011; Mateo & Garcia-Rubies, 2012; Teixidó et al., 2013; Gori et al., 2017). In 2008, a significant storm-induced impact on benthic communities was reported on the Northern Catalan coast (Costa Brava), where massive removal of substrate blocks resulted in major mortality in benthic communities of gorgonians and partial burying of the meadows of *P. oceanica* (Garcia-Rubies et al., 2009; Mateo & Garcia-Rubies, 2012). Later, in 2013, downwelling induced by strong wind systems, coupled with an underwater landslide, affected the population of *C. rubrum* of the Gulf of Naples (Bavestrello et al., 2014; Toma et al., 2022). Again, in January 2015, another intense windstorm hit the Southeast coast of Cyprus, severely affecting *C. caespitosa* due to its spread occurrence in a shallow coastal environment vulnerable to disturbances, leading to various negative consequences such as macroalgae outgrowing and bacterial infection susceptibility (Crabbe et al., 2008; Pagès et al., 2013; Teixidó et al., 2013; Hidijoannou et al., 2016). The most recent and devastating manifestation was the storm on the Ligurian coast in 2018, with strong SE wind and more than 8 m high waves (Betti et al., 2020a). After comparing the *P. clavata* population in 1997, 2002, and 2016, it was found that about one-third of the colonies were prematurely dead due to detachment from the substrate from strong currents and wave action. In particular, there was a severe impact on the coral colonies living in depths up to 25 m, causing them to disappear completely. However, at depths below 30 m, the effects of the storm were less severe, resulting nonetheless in an increase in the levels of epibiosis and necrosis (Betti et al., 2020a). Additionally, the damage caused by the storm has dramatically hindered the recovery of the Portofino Promontory population, which has already been struggling due to mass mortality events in recent decades (Knowlton et al., 1981; Linares et al., 2005; Crabbe et al., 2008; Teixidó et al., 2013; Ponti et al., 2014; Betti et al., 2020a).

Unfortunately, in addition to the natural threats, several anthropogenic impacts must be considered, especially urbanization, nutrient run-off, pollution, and uncontrolled fishing, which have significantly exacerbated the decline of anthozoan populations and compromised their resilience and potential for recovery (Sebens, 1994; Bavestrello et al., 1997; Clark et al., 2016; Otero et al., 2016; Catteneo-Vietti et al., 2017; Rijnsdorp et al., 2018; Betti et al., 2020b). Uncontrolled fishing is a significant threat in the Mediterranean region, not only in terms of illegal fisheries and overfishing but also due to the destructive fishing practices, such as trawling and dredging, that cause damage to the sea floor and destroy benthic communities (Koslow et al., 2000; Tillin et al., 2006; Rooper et al., 2011; Munoz et al., 2012; Bo et al., 2014; Clark et al., 2016; Rijnsdorp et al., 2018; Betti et al., 2020b; Pitcher et al., 2022). The use of bottom trawls on hard seabed (commonly found on seamounts) typically removes the majority of the benthic fauna, especially the dominant mega-faunal components of deep-sea systems, such as corals and sponges, leading to a loss of biogenic habitats from potentially large areas, aggravated by long lifespan and slow growth rate of these organisms (Althaus et al., 2009; Munoz et al., 2012; Clark et al., 2016; Betti et al., 2020b). Most of these species are not only ecologically fundamental but also highly valuable from an economic point of view due to their underwater appeal, as exemplified by *C. rubrum,* which was extensively exploited by jewelry industries until the ban in 1994 (Tsounis et al., 2006; Cattaneo-Vietti et al., 2016, 2017).

Finally, Coma et al. in 2004 and Betti et al. in 2019 investigated the role of marine protected areas (MPAs) in the protection and health status of several benthic species. The primary outcome was the need to balance the aims of MPAs to protect and conserve natural habitats and their biological resources and the inherent appeal of tourism and associated recreational activities. In fact, in Mediterranean MPAs, additional threats to the benthic communities come from fishing, boat anchoring, and SCUBA diving. For instance, Portofino MPA (Liguria) has become one of Italy and Europe's most famous dive destinations, with a significant flow of divers, around 40000 per year (Betti et al., 2019, 2022). It has been demonstrated that mechanical damages due to high levels of recreational activity led to a threefold increase in mortality rate compared with the natural one, suggesting that the long-lived low-turnover structural components of the Mediterranean MAF ecosystems are especially vulnerable to human-related disturbance (Betti et al., 2019, 2022). Therefore, a crucial priority should be implementing strict, effective management measures and regulations, such as enlarging marine protected areas, using sustainable fishing practices, and enforcing fishing regulations to mitigate the impact of human activities on the benthic communities.

## 1.4. EMERGING CONTAMINANTS (ECs): THREATS TO MARINE ECOSYSTEMS AND KNOWLEDGE GAPS IN THE MEDITERRANEAN

The marine environment in general, even more so the Mediterranean Sea, is facing significant threats, especially climate change and anthropogenic influences such as urbanization, nutrient run-off, pollution, and uncontrolled fishing (Chen et al., 2015; Clark et al., 2016; Hughes et al., 2017; Rijnsdorp et al., 2018; Pitcher et al., 2022). Despite the rich biodiversity and crucial ecosystem services provided, human activities have increasingly jeopardized marine environment health and survival, contributing to the Anthropocene epoch characterized by widespread anthropogenic-induced environmental changes (Meybeck et al., 2003; Steffen et al., 2011; Issberner & Lèna, 2018).

In this context, one of the most impacting threats are Emerging Contaminants (ECs), which are all the substances detected in the environment that may have potential adverse effects on ecosystems and human health but for which the full extent of their impacts is not yet fully understood (Puri et al., 2023; Li et al., 2024; Wang et al., 2024). The term "emerging" highlights the ongoing research and monitoring efforts to identify and address these contaminants as their presence and potential impacts become better understood. The key sources of pollution include microplastics (MPs) and associated additives, such as phthalate esters (PAEs), along with pharmaceutical and personal care products (PPCPs), active pharmaceutical ingredients (APIs), and UV filter molecules (Blasco & Del Valls, 2008; Danovaro et al., 2008; Cózar et al., 2015; Alomar et al., 2016; Paíga et al., 2016; Brumovský et al., 2017; Pico et al., 2019; Angiolillo & Fortibuoni, 2020; Cadena-Aizaga et al., 2020; Fivenson et al., 2020; Angiolillo et al., 2021; Huang et al., 2020; Adeleye et al., 2022; Baudena et al., 2022; Hawash et al., 2023; He et al., 2023). While the occurrence and behavior of these substances in freshwater environments have been more extensively documented due to controls and studies on wastewater treatments (Hughes et al., 2012; Li, 2014; Murray et al., 2010), their dynamics in coastal and marine waters remain understudied and less understood (Arpin-Pont et al., 2014). However, this lack of information needs to be addressed because emerging contaminants can potentially cause adverse effects on several marine organisms. These effects have been documented in tropical seas with a substantial body of literature, contrary to the Mediterranean Sea region, where this issue remains relatively underexplored and under-addressed (Burns & Boxall, 2018; de Sà et al., 2018).

Generally, the response of organisms to contaminant uptake is species-specific and influenced by factors such as concentration and environmental conditions (Fossi et al., 2018; Paluselli et al., 2018; Saliu et al., 2019; Panio et al., 2020). Within the benthic community, anthozoans are particularly vulnerable, as they are typically non-selective suspension feeders that primarily consume zooplankton (Palardy et al., 2008; Houlbrèque & Ferrier-Pagès, 2009; Savinelli et al., 2020), however, there is currently limited information available with just a few studies indicating that corals can ingest microplastics (Hall et al., 2015; Allen et al., 2017; Chapron et al., 2018; Vencato et al., 2021). Other research has shown that exposure to microplastics can lead to tissue necrosis (Reichert et al., 2018), reduced feeding activity (Savinelli et al., 2020), increased bleaching (Danovaro et al., 2008), and damage to coral larvae negatively impact reproductive success (Downs et al., 2013, 2015). Furthermore, microplastics can enhance the production of reactive oxygen species (ROS), leading to increased oxidative stress in marine organisms (Montano et al., 2020; Rizzi et al., 2020, 2023; Chen et al., 2022; Montalbetti et al., 2022; Isa et al., 2022, 2023; He et al., 2023; Seveso et al., 2024).

The concentration of these compounds in marine environments is typically very low, often in the nanogram-per-liter range, yet they tend to be persistent. This raises a significant question regarding the challenge of emerging contaminants: although these substances may be present at low concentrations individually, prolonged exposure could pose risks comparable to those associated with higher concentrations over shorter periods. Therefore, improving our ability to assess pollution levels and bioaccumulation in natural environments is crucial, as ecotoxicological studies conducted in laboratories often use contaminant concentrations that are significantly higher and exposure periods that are much shorter than what is typically found in nature. While these studies are invaluable for identifying potential adverse effects, they may not fully capture the impacts of chronic, long-term exposure to low contaminant levels that organisms experience in the wild (Li et al., 2024). This issue is particularly relevant in marine ecosystems, where contaminants can persist at low concentrations but accumulate over extended periods, potentially leading to significant ecological impacts on various organisms (Tsui et al., 2014; Schneider et al., 2019; Caloni et al., 2021). In recent years, several investigations have concentrated on a range of phylogenetically diverse marine organisms, such as sponges, mussels, and fishes (Na et al., 2013; Du et al., 2015; Paluselli et al., 2018; Zhou et al., 2020; Adeleye et al., 2022; Chen et al., 2022; Rizzi et al., 2020, 2023; Hawash et al., 2023). Nevertheless, there exists a notable lack of data regarding anthozoans, especially in the Mediterranean region, where they serve as a crucial component of the marine ecosystem, limiting our comprehension of the impacts of emerging contaminants on aquatic invertebrates and their potential for bioaccumulation in tissues, transmission to offspring, and the potential carrier effects (Arnold et al., 2013; Zhou et al., 2020; Atugoda et al., 2021). Understanding the behavior, distribution, and potential impacts of these compounds is vital for developing effective management strategies to mitigate their effects on aquatic ecosystems and human health.

### 1.4.1. Plastic, microplastic, and their additives

Over the past century, the global plastic production of plastic has seen an exponential increase worldwide, reaching an unprecedented volume of approximately 350 million tons in recent years (Plastic Europe, 2021). Despite the challenge of estimating the amount of plastic in the ocean, studies indicate an accumulation of at least 8 million tons of plastic in our oceans every year, for a total of 5.25 trillion plastic particles adrift (Andrady, 2011; Jovanović, 2017). Unfortunately, the valuable properties that make plastics widely used also raise profound environmental concerns. The durability ensures persistence in the environment for extended periods, while the low density facilitates the dispersion by currents and winds, enabling plastic to travel thousands of kilometers, reaching even the most remote corners of the globe. The sum of steady production escalation, its properties, its enduring nature, and shortcomings in the disposal and discard practices (Ostle et al., 2019) made plastic materials the most pervasive and significant source of pollution, especially in marine ecosystems (Moore, 2008; Wright et al., 2013; Eriksen et al., 2014; Zhou et al., 2020). The problem also becomes more challenging considering the several pathways leading plastic into the oceans, including stormwater runoff, direct disposal, or the loss of fishing and aquaculture equipment, making it exceedingly difficult to control, monitor, and prevent (Alomar et al., 2016; Alkan et al., 2021; Kumar et al., 2021; Baudena et al., 2022; Hidalgo-Serrano et al., 2022). This issue is particularly pronounced in the Mediterranean Sea, recognized as one of the world's most significant accumulation spots of marine litter, holding around 7% of the global microplastics within just 1% of the world's marine waters (Cózar et al., 2015).

Research attention has been predominantly centered on micro- and nano-particles, which may arise from the direct release of microscale plastic particles by manufacturers or as a byproduct derived from the photo-oxidative degradation or weathering-induced breakdown of larger plastic items present on the ocean surface (Saliu et al., 2019; Yuan et al., 2022). Once in the water column, the density of the particles shapes the distribution pattern of microplastics; polypropylene (PP) and polyethylene (PE) due to their lower density float in the first layer of water, while polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA), and polyethylene terephthalate (PET) sink in the deeper water with their higher density (Guo & Wang, 2019). However, several observations reveal a notable deficit in floating plastic debris, implying that surface waters do not serve as the final repository for buoyant plastic debris in the ocean (Cózar et al., 2015). Instead, shore deposition, nano-fragmentation, biofouling, or ingestion are the actual potential sinks for microplastics (Cózar et al., 2015). Indeed, beaches worldwide are frequently covered with plastic debris, and most litter is usually colonized by fouling organisms, increasing the particle density and, therefore, the subsequent deposition in the benthic environment. Moreover, the size of microplastics renders them susceptible to interactions with marine biota across various trophic levels, such as potential ingestion of microplastics by amphipods, copepods, and zooplankton, with a high potential for biomagnification being prey for corals and other filter-feeding organisms within benthic ecosystems (Ferrier-Pagès et al., 2003). During this transfer process, there is also a strong potential for bioaccumulation, leading to adverse effects on the health of higher trophic level organisms and ultimately posing risks for species of human consumption (Miller et al., 2021).

However, plastic itself is not the only and most important problem. During chemical or physical degradation, it may release some additives and constituents, such as residual monomers, plastic additives, flame retardants, and other molecules, increasing the threat of toxicity (Wright et al., 2013; Zeri et al., 2018). Among them, Phthalic Acid Esters (PAEs) have recently gained most of the scientific attention, being the main additive (up to 60% of total weight) used to increase the flexibility, transparency, or longevity of plastic (Teuten et al., 2007). However, these molecules, being not covalently bound to the plastic polymers, may detach from the plastic material, becoming bioavailable in the marine environment, where they can remain for a long time due to their low solubility and lipophilicity in water (Rochman et al., 2013; Net et al., 2015; Saliu et al., 2019, 2020). This makes them highly susceptible to interactions with a variety of marine organisms, which can lead to significant negative consequences both physiologically, such as respiration, reproduction, and nutrient absorption, potentially resulting in stress or harmful changes in their health, and environmentally, such as alterations to local ecosystems, imbalances in species populations, disruption of food webs, and changes in habitat structures, ultimately affecting the overall biodiversity and stability of marine environments(Adeleye et al., 2022; Baudena et al., 2022; Hawash et al., 2023).

### 1.4.2. Pharmaceuticals and Personal Care Products (PPCPs)

Pharmaceuticals and personal care products (PPCPs) constitute a diverse group of synthetic organic compounds that have progressively become part of daily life. These compounds are components of several products and serve different functions, such as food conservation, sanitation, hygiene, beautification, and human and animal health, contributing to the efficacy of the products but also posing severe risks for different organisms and environments (Arpin-Pont et al., 2014; Ebele et al., 2017; Szopińska et al., 2022; Hawash et al., 2023).
Active pharmaceutical ingredients (APIs) are among the most prevalent and impactful groups within this broad category. They are used in human and veterinary medicine, as well as in fragrances and cosmetics (Richardson et al., 2005; Jiang et al., 2019; Xie et al., 2019a; He et al., 2019; Miller et al., 2021; Wheate, 2022). However, often they remain unregulated and are categorized as Emerging Contaminants (ECs) by the U.S. Environmental Protection Agency (EPA, 2016) or Emerging Substances by the EU NORMAN network (NORMAN, 2016). Estimating the global production of pharmaceutical and personal care products (PPCPs) is challenging due to the diverse range of products and the proprietary nature of the production data. For instance, in 2018, China alone produced approximately 18 million tons of various active pharmaceutical ingredients (APIs) and drugs (Wang et al., 2022).

Like plastic debris, PPCPs and APIs can also enter the coastal environment after disposal through various pathways, such as direct discharge, transport via rivers, domestic and industrial wastewater, aquaculture activities, agricultural runoff, and excretion by humans and animals into wastewater systems (Arnold et al., 2013; Yuan et al., 2014; Dey et al., 2019). Among the pharmaceuticals studied and detected in marine environments, the most commonly found are psychiatric drugs (such as carbamazepine, citalopram, and fluoxetine), antibiotics (including macrolides, quinolones, and sulphonamides), non-steroidal anti-inflammatories drugs (like salicylic acid, ketoprofen, and diclofenac), and β-blockers (such as carazolol, propranolol, metoprolol, and sotalol) (Mezzelani & Regoli, 2022).

As one of the most prevalent groups of emerging contaminants, the presence and adverse effects of APIs on marine organisms have received more attention from researchers globally in recent years (Zenker et al., 2014; Andreu et al., 2016; Puckowski et al., 2016; Carmona et al., 2017; Bonnefille et al., 2018; He et al., 2019; Álvarez-Ruiz & Pico, 2020; Srain et al., 2020; Zhou et al., 2020; Miller et al., 2021). This attention has been particularly heightened postpandemic when the use of pharmaceuticals has drastically increased (Van Boeckel et al., 2014; Koagouw et al., 2021). These substances are designed to target specific components of the organism and pose significant risks to various marine animals. Their chemical composition allows them to interact easily with the biological systems of these creatures, leading to a range of adverse effects. This interaction can disrupt essential physiological processes, potentially resulting in health complications, altered behaviors, or even mortality in affected species (Arpin-Pont et al., 2014; Ebele et al., 2017; Szopińska et al., 2022; Hawash et al., 2023). As a result, the impact of these substances on marine ecosystems can be profound, affecting not only individual animals but also the broader ecological balance (Mezzelani & Regoli, 2022). Despite a growing interest in APIs, studies on marine species remain limited, particularly regarding anthozoans. Consequently, further research is essential to fully understand their effects on coral cells and tissues.

### 1.4.3. UV Filters molecules

Sunscreens and UV filters represent a significant category of emerging contaminants among the wide range of pharmaceuticals and personal care products (PPCPs). While they play important roles in protecting human health, these substances also pose serious risks to reef ecosystems, especially in heavily populated tourist areas (Cadena-Aizaga et al., 2020; Mitchelmore et al., 2019, 2021). Recently, their environmental effects have attracted considerable scientific and social interest (Sanchez-Quiles & Tovar-Sanchez, 2015) due to the continuous research of new formulations with different compositions and properties by cosmetic companies each year (Tovar-Sanchez et al., 2020). Sunscreens function by establishing a physical barrier between the epidermis and solar radiation, utilizing complex formulations that consist of various molecules that either absorb, reflect, or scatter ultraviolet (UV) light, known as UV filters, that can be either organic (UV-absorbing chemicals) or inorganic substances (UV-reflecting minerals). Typically, sunscreens combine these filters to achieve broad-spectrum protection against UVA (315-400 nm) and UVB (280-315 nm) radiation, often incorporating 20 or more active compounds for sufficient efficacy (Danovaro et al., 2008; Sánchez-Quiles & Tovar-Sánchez, 2015; Watkins et al., 2021).

Unfortunately, these molecules can enter marine environments through various pathways, including wash-off from swimmers and effluents from wastewater treatment plants following usage (Tovar-Sánchez et al., 2013; Sánchez Rodríguez et al., 2015; Cadena-Azaiga et al., 2020). Consequently, it is estimated that at least 10% of global coral reefs face significant threats from UV filter exposure, with an approximate annual discharge of 6000 to 14000 tons of sunscreen into coral reef ecosystems (Danovaro et al., 2008; Downs et al., 2016). Of particular concern is the detection of a wide array of UV filter concentrations in remote areas, such as Arctic regions, which experience minimal tourist activity, suggesting that ocean currents or atmospheric pathways transport UV filters over long and short distances (Tsui et al., 2014a). The apprehensions raised by these findings have led to legislative actions in various regions. Hawaii, the Virgin Islands, Palau, Mexico, Bonaire, and Aruba have implemented prohibitions on the sale and use of sunscreens containing the most extensively studied organic UV filters (Narla et al., 2020; Levine, 2021). Currently, European legislation establishes maximum allowed concentrations for each UV filter in cosmetic products (Regulation no. 1223/2009 of the European Commission), and the European Union permits the use of 27 UV filters in concentrations ranging from 2% to 15%, of which only two are inorganic, namely titanium dioxide and zinc oxide (Cadena-Azaiga et al., 2020).

The most prevalent organic chemical UV filters include benzophenone-3 (BP-3; also known as oxybenzone), benzophenone-4 (BP-4), para-aminobenzoic acid (PABA) and PABA esters, Ethylhexyl methoxy cinnamate (EHMC), homosalate (HMS), 4-methyl benzylidene camphor (4-MBC), diethylamino hydroxy benzoyl hexyl benzoate (DHHB), and anthranilates (Chisvert et al., 2001; Danovaro & Corinaldesi, 2003; Tarazona et al., 2010; Kim et al., 2014). These chemicals typically feature an aromatic moiety with a side chain exhibiting varying degrees of unsaturation, high lipophilicity, and stability against biotic degradation (Díaz-Cruz & Barcelò, 2009; Vila et al., 2017). Conversely, the most common mineral inorganic UV filters are titanium dioxide and zinc oxide, often in nanoparticles (NPs), to ensure transparency and smoothness upon application (Lewicka et al., 2013). These mineral filters tend to be more photostable than organic ones, which can undergo photolysis, potentially transforming into hazardous by-products and harmful free-oxygen radicals that may impact the health of coral reef ecosystems and associated organisms (Danovaro et al., 2008; Downs et al., 2016; Mitchelmore et al., 2019; Tsui et al., 2014, 2017).

In literature, different studies compare the occurrence of these contaminants in various coastal regions worldwide, revealing significant levels of organic UV filter, particularly oxybenzone, in marine environments due to sunscreen use. For instance, in the US Virgin Islands, oxybenzone concentrations ranged from 1 part per million (ppm) to 90 parts per billion (ppb), with higher levels observed near coral communities and beaches frequented by more visitors (Downs et al., 2011; Bargar et al., 2015; Downs et al., 2015). Similarly, in South Carolina, concentrations of oxybenzone and octocrylene reached up to 2013 ng/L and 1409 ng/L, respectively, with elevated concentrations observed at beaches with higher usage rates (Bratkovics et al., 2015). Majorca Island exhibited high levels of oxybenzone and other sunscreen chemicals, peaking in the afternoon coinciding with peak beach attendance (Tovar-Sánchez et al., 2013), as well as Gran Canarias and Hawaii with the presence of multiple UV filters in seawater samples (Downs et al., 2015; Sánchez Rodríguez et al., 2015). Palau raised concerns about the bioaccumulation of sunscreen compounds in jellyfish and sediments, potentially impacting marine ecosystems, even in remote areas (Bell et al., 2017). These findings underscore the widespread contamination of coastal waters by UV filters and the potential ecological implications across diverse marine environments and organisms, including seagrass, shrimp, worms, sea urchins, clams, mussels, corals, fish, sea turtles, dolphins, and eggs of seabirds (Danovaro et al., 2008; McCoshum et al., 2016; Corinaldesi et al., 2017; Fivenson et al., 2020).

Some studies have indicated that sunscreen and its ingredients can negatively affect corals and other marine organisms. In particular, oxybenzone has been extensively researched, revealing documented effects such as coral bleaching and damage to coral cells across various hard coral species, particularly at elevated water temperatures (Danovaro & Corinaldesi, 2003; Danovaro et al., 2008). Additionally, it has been shown to harm and deform coral larvae (planulae), negatively impacting their DNA and reproductive success (Downs et al., 2013, 2015). Acute exposure of fish to BP-3 (benzophenone-3) is believed to cause effects similar to those observed in mammals, including endocrine disruption by altering estrogen receptor signaling pathways, reproductive problems, and declines in reproductive fitness (Downs et al., 2016). Chronic exposure may result in reduced egg production, lower embryo hatching rates, and potential gender shifts (Coronado et al., 2008). While inorganic UV filters are generally considered safer, measuring their environmental levels has proven more challenging due to the presence of naturally occurring metal oxides in aquatic systems. Nevertheless, certain types of titanium dioxide and zinc oxide nanoparticles can produce reactive oxygen species (ROS) when exposed to UV light, which poses risks of oxidative stress to marine organisms (Hanna et al., 2013; Lewicka et al., 2013). Recent research has brought attention to the potential risks associated with nanoparticles in sunscreens; however, more in-depth evaluations are essential to thoroughly understand their ecological impacts, particularly in marine environments.

## 1.5. HISTOPATHOLOGY: A VALUABLE TOOL TO ADVANCE CORAL DISEASE UNDERSTANDING AND DIAGNOSIS

In the last decades, among the multitude of threats affecting the marine environment, coral diseases have been recognized as one of the most significant factors involved in the substantial decline of coral reefs worldwide (Sutherland et al., 2004; Bruckner, 2015; Mera & Bourne, 2017; Burke et al., 2023). Starting from the first disease record in 1973 (Antonius, 1973), there has been an ever-growing number of reports in literature about disease and disease-like syndromes in reef-building corals, thanks to the better understanding and new insights into coral health linked to the concerning increase of several stressors on coral reefs. Over the years, around 40 different coral diseases have been described worldwide (Sutherland et al., 2004; Work & Meteyer, 2014; Moriarty et al., 2020; Morais et al., 2022), usually based on macroscopic features that are evident in the colonies, however just very few of them has been completely understood in the etiology and pathogen (Mohamed & Sweet, 2018). In fact, coral disease diagnosis is primarily macroscopic (extent of tissue loss, tissue color, and exposure of coral skeleton), as is evident from the names of most of them; however, it has become evident that we need to deepen our knowledge by going beyond the external macroscopic signs of coral disease that are unreliable for accurate diagnosis (Work et al., 2012, 2015; Work & Meteyer, 2014; Hawthorn et al., 2023). Therefore, coral histopathology started playing a pivotal role in elucidating the complex dynamics of coral health and disease.

The term histopathology refers to the study of disease manifestation on different tissues through the use of microscopic examination, and it is instrumental in addressing one of the major challenges in understanding coral diseases: the absence of proper descriptions of microscopic lesion morphology (Work and Aeby, 2006; Work and Mateyer, 2014; Work et al., 2012, 2015). Pathology, broadly divided into morphological and functional branches, distinguishes the manifestation signs of disease and its root causes, both essential for accurate diagnoses. In corals, for instance, a visible lesion may, upon microscopic examination, reveal cell death via autophagy, highlighting the importance of morphological pathology, while functional pathology complements this by revealing altered cellular processes, such as the activation of apoptotic pathways or fluctuations in enzyme production, which provide further clues about disease processes (Bythell et al., 2002; Downs et al., 2009).

Accurate identification of the causes of diseases, precise diagnoses, and the ability to distinguish between similar disease outcomes becomes extremely difficult without comprehensive descriptions of lesions (Work et al. 2012, 2015). Therefore, systematic and detailed descriptions of lesions at both gross and microscopic levels have become crucial to coral disease research. These descriptions help characterize the morphology of coral lesions and provide insights into their pathogenesis. Researchers can ensure clarity and accuracy in the coral disease literature by employing medical concepts and precise terminology, facilitating effective communication and knowledge dissemination within the scientific community. Due to the limitations in interpreting gross lesions and the overall understanding of coral diseases, an increasing number of studies have begun to incorporate histological techniques into their research methods, a promising step towards overcoming these limitations and advancing our understanding of coral diseases. In fact, histopathological examinations offer a direct means to assess tissue structure and identify both host-associated and invasive microorganisms, gaining profound insights into the interactions between coral tissues and the pathogens that afflict them, shedding light on the underlying mechanisms of coral disease progression (Sweet et al., 2019; Vega Thurber et al., 2020; Work et al., 2021). Moreover, histopathology is a valuable tool in investigating the physiological, pathological, and ecological aspects of coral biology, contributing significantly to our understanding of the multifaceted processes and environmental influences shaping coral health and resilience.

Given their vital importance in supporting marine biodiversity and ecosystem functioning, ecological and epidemiological research concerning coral diseases within reef ecosystems must be prioritized. By integrating histopathological techniques with comprehensive monitoring efforts, researchers can advance our understanding of coral disease dynamics and inform effective conservation strategies to preserve these vital marine habitats.

### 1.5.1. Anthozoan polyp morphology and structural adaptations

Anthozoans represent a class of exclusively marine invertebrates within the phylum Cnidaria, distinguished by a modular body structure comprised of numerous individual repeated units known as polyps. Each polyp, typically exhibiting a cylindrical shape, features an oral disc surrounded by upward-facing tentacles. The aboral end, called the basal plate, instead facilitates attachment to a solid substrate, anchoring the colony (Brusca et al., 2016). Predominantly opportunistic filter feeders, many anthozoans, particularly reef-building corals, enhance their nutritional intake through a symbiotic relationship with single-cell dinoflagellates of the genus *Symbiodinium*, more recently referred to the family Symbiodiniaceae, but commonly called zooxanthellae (Trench, 1993; LaJeunesse et al., 2018). Within this symbiosis, zooxanthellae, typically residing in cytoplasmic vacuoles within gastrodermal cells, gain protection and utilize the host's nitrogen waste and carbon dioxide, while the cnidarian host benefits from increased photosynthetic capacity, enhanced calcium carbonate production, and a vibrant coloration (Tremblay et al., 2012; Maire et al., 2021).

Polyps are situated exclusively within the outermost layer of the corallum, and the structure expands in size as new polyps arise through the budding process. Each polyp is accommodated in a calcium carbonate cup, called corallite, characterized by an outer wall known as the theca and a basal plate forming the floor (Veron, 2000; Berzins et al., 2021). As colonies develop, calcareous partitions, termed dissepiments, progressively emerge at the bases of the corallites, establishing new basal plates for the polyps (Fautin & Mariscal, 1991; Veron, 2000; Berzins et al., 2021). The spacing between polyps can vary considerably; generally, slower-growing species exhibit narrower spaces and denser partitions, whereas faster-growing, branching species display wider gaps with thinner partitions that are more susceptible to fragmentation (Galloway et al., 2007; Carricart-Ganivet et al., 2013). Radiating from the theca and the basal plate are septae, calcareous partitions that provide support to the mesenteries, which may be instrumental in species identification due to their distinctive morphology. These interstitial spaces may be colonized by fungi and protozoa; however, the impact of these organisms on coral health remains largely unknown (Marcelino et al., 2018). The portion of each polyp that extends above the theca is called the column, while the section located within the corallite cup is called the polyp base. The skeleton that separates polyps is known as the coenosteum, which interconnects the polyps through the coenenchyme, consisting of two layers divided by gastrovascular canals that remain on the surface of the skeleton in imperforate corals, while penetrates the skeleton with canals that link the corallites in perforate ones (Galloway et al., 2007). In particular, stony corals produce aragonite exoskeletons that can take on various forms, such as massive, branching, and encrusting structures. In contrast, octocorals lack a substantial exoskeleton and instead contain small calcium carbonate spicules, or sclerites, within their coenenchyme that coexist with corticocytes secreting a gorgonin axial rod, providing internal support. Desmocytes anchor tissues to this rod, with a perforated coenenchyme layer housing gastrodermal tubes (canals and solenia) connecting the polyps. The polyp wall section near the coenenchyme, reinforced with sclerites, is called the anthostele (Veron, 2000; Berzins et al., 2021).

### 1.5.2. Structural and functional anatomy of anthozoans: tissue layers, support systems, and adaptive mechanisms

At the structural and tissue level, anthozoans are soft-bodied, diploblastic metazoans characterized by two principal tissue layers: the outer epithelial layer, known as the epidermis, and the inner epithelial layer, referred to as the gastrodermis, derived from the embryonic ectoderm and endoderm, respectively. The mesoglea, a gelatinous layer primarily of ectodermal origin, separates these two layers and exhibits variability in thickness, ranging from a thin sheet to a mucoid consistency (Peters, 2016; Parisi et al., 2021). It is an amorphous matrix composed of a hydrated protein and polysaccharide polymer interspersed with collagen fibers, with variable thickness involved in sustaining the different cells and cell layers within the organism (Parisi et al., 2021). These three layers are organized around a gastrovascular cavity, which fulfills both digestive and circulatory functions, possessing a single opening that serves a dual role as both mouth and anus. This orifice, located at the oral end of each polyp, is surrounded by tentacles arranged around a flat oral disc with varying morphology, such as circular, oval, smooth, or ridged, and is occasionally encircled by a raised tissue rim referred to as the peristome (Galloway et al., 2007; Peters, 2016). In numerous scleractinian corals, the tips of the tentacles are equipped with a bulbous structure known as the acrosphere, which is densely populated with cnidocytes containing nematocysts or spirocysts that play a significant role in prey capture and defensive mechanisms. Conversely, in octocorals, the region bearing the tentacles is called the anthocodium, which typically lacks nematocysts and spirocysts. In some instances, octocorals also have an operculum composed of eight calcareous scales that cover the tentacles when retracted, thereby providing protection (Berzins et al., 2021).

In anthozoans, the oral cavity transitions into a short, muscular structure known as the actinopharynx that leads to the gastrovascular cavity, where some species exhibit one or more grooves called siphonoglyphs (no scleractinian corals). These grooves are lined with elongated epithelial cells bearing cilia, which facilitate the movement of water and nutrients into the gastrovascular cavity as well as the expulsion of waste materials (Galloway et al., 2007; Berzins et al., 2021). Within the gastrovascular cavity, structures known as mesenteries extend from the body wall towards the interior, serving to enhance the gastrodermal surface area and provide structural support, and are categorized as "complete" or "incomplete" based on their connection to the actinopharynx. The free edges of mesenteries develop into thickened coiled formations known as mesenterial or gastric filaments that play a critical role in digestion as they are equipped with ciliated epithelial cells facilitating water movement, granular gland cells responsible for secreting digestive enzymes, cnidocytes for prey capture, and adjacent phagocytic gastrodermal cells that participate in the breakdown of particulate matter (Peters, 2016; Work et al., 2024). Moreover, the mesentery regions in proximity to the body wall contain epitheliomuscular cells, which contribute to support and in the processes of polyp extension and contraction. The classification of anthozoans into two subclasses (Hexacorallia and Octocorallia) is based on the number of mesenteries present. In Hexacorallia, encompassing sea anemones and stony corals, mesenteries are typically paired and occur in multiples of six. In contrast, Octocorallia is characterized by the presence of eight complete mesenteries, which correspond to the eight tentacles featuring side branches or lateral outfoldings, as observed in soft corals and gorgonians.

An examination of the primary tissue from a histological perspective reveals that the epidermis of anthozoans consists of a singular layer of cells displaying diverse morphologies, which may include simple columnar, pseudostratified columnar, cuboidal, and, on occasion, squamous cell types. The epithelial cells forming the outermost surface in direct contact with seawater are frequently ciliated to aid in the transport of mucus and the removal of sediment (Galloway et al., 2007). This epidermal layer comprises various specialized cell types, with density and distribution varying among species, including mucocytes, nematocytes, spirocytes, ptychocytes, sensory cells, neuron-like cells, acidophilic (eosinophilic) granular gland cells, pigment cells, and epitheliomuscular cells, each fulfilling distinct functional roles (Hawthorn et al., 2023; Work et al., 2024).

In particular:

- Unicellular mucocytes found within the epidermis secrete mucus, a complex amalgamation of polysaccharides, proteins, and lipids (Crossland et al., 1980; Peters, 2016), which plays essential roles in feeding, sediment removal, and protection against desiccation and environmental stressors, such as fluctuations in salinity, temperature variations, and exposure to ultraviolet radiation (Ritchie, 2006; Bythell & Wild, 2011). Moreover, the mucus is also involved in self/non-self recognition and hosts a diverse microbial community that contributes to immune responses (Bosch & Rosenstiel, 2015).

- Cnidocytes, instead, are generally distributed singularly or in dense clusters of batteries, usually located on tentacles and body walls, and primarily involved in defense and prey capture through the production of specialized organelles known as cnidae or cnidocysts, which are characteristic of the Cnidaria phylum. These organelles are capsules containing either a solid (ptychocysts and spirocysts) or hollow tubule (nematocyst) that, when activated, is everted to capture prey or defense against predators or disturbances (nematocysts), as well as to adhere to the substrate (ptychocysts and spirocysts) (Oppengard et al., 2009; Berzins et al., 2021; Work et al., 2024). They usually possess a specialized trigger mechanism known as a cnidocil, a modified cilium that extends from the cell surface and initiates the rapid eversion of the tubule by opening an operculum in response to specific mechanical or chemical stimuli, allowing rapid uncoiling, piercing, and the release of venomous or acidic compounds to immobilize prey or deter predators (Özbek et al., 2009).

- Granular cells, despite ongoing confusion regarding their classification, are often referred to as acidophilic or eosinophilic granular cells and present challenges in identification due to their varying staining properties, granule sizes, numbers, and origins (Berzins et al., 2021; Work et al., 2024). Generally, these granular cells can be categorized into three groups: pigment cells, gland cells, and amoebocytes. In particular, in conjunction with zooxanthellae, granular pigment cells are predominantly located in the basal epidermis and contribute to color patterns, where, in some instances, they may fluoresce under specific wavelengths (Peters, 2016). On the other hand, granular gland cells, which are also known as zymogen or eosinophilic/acidophilic granular gland cells, function as secretory cells housing enzyme precursor granules that assist in food digestion within the gastrovascular cavity or externally to fight for space and growth with rival organisms. These gland cells are particularly prevalent in cnidoglandular bands within both epidermal and gastrodermal epithelia (Peters, 2016; Raz-Bahat et al., 2017).

  Lastly, granular amoebocytes, also called amoeboid chromophore cells, can be located in the epidermal and gastrodermal layers and occasionally extend into the mesoglea. The granules within these cells vary in size and may appear pink when stained with eosin or display golden to brown tones when containing melanin-like pigments, and they are believed to play a significant role in the immune responses of cnidarians (Mydlarz et al., 2008; Palmer et al., 2011). Melanin has been established as a conserved component of innate immunity with a wide prevalence among invertebrates (Nappi & Christensen, 2005; Munoz et al., 2006; Mydlarz et al., 2009; Palmer et al., 2010). In particular, melanin plays a crucial role in enhancing disease resistance in these organisms by contributing to antimicrobial defense and scavenging free radicals (Nappi & Christensen, 2005), thanks to the formation of a protective barrier, often accompanied by the aggregation of phagocytic cells (Nappi & Christensen, 2005; Palmer et al., 2008). Additionally, within anthozoans, amoebocytes are usually found to aggregate in response to various stimuli such as injury, pathogenic infections, or temperature stress (Olano & Bigger, 2000; Mydlarz et al., 2009; Palmer et al., 2010). Prior research has consistently demonstrated that an inflammatory response triggers the production of melanin pigment as a strategy to isolate and eliminate pathogens (Carella et al., 2014, 2020). Its presence suggests a vital physiological function within alcyonacean corals, offering a distinct advantage in bolstering disease resistance, particularly within the potentially vulnerable epidermal cell layer.

As regards the support system of corals, closely associated with the tissue layer described, we find the calcium carbonate skeleton in stony corals or the axial skeleton of gorgonin in octocorals. Specifically, stony corals exhibit an external basal body wall, called calicodermis, which plays a critical role in skeletal formation and comprises calicoblasts and desmocytes. Calicoblasts are responsible for producing an organic matrix and coral acid-rich proteins (CARPs) (Mass et al., 2012), which are instrumental in the crystallization of calcium and carbon dioxide into aragonite crystals, thereby promoting the deposition and formation of the skeletal structure (Mass et al., 2017). Conversely, desmocytes, typically located adjacent to the mesoglea/calicodermal boundary, represent specialized epithelial cells that adhere to the collagen fibers of the mesoglea. They facilitate attachment to the aragonite skeleton, ensuring the maintenance of skeletal integrity and supporting growth (Muscatine et al., 1997). In contrast, within gorgonians and octocorals, the mesoglea surrounds an axial skeleton essential for supporting the polyp structure and the integrity of the entire colony (Berzins et al., 2021). This axial epithelium is abundant in corticocytes and has the role of secreting the gorgonin matrix, contributing to the overall rigidity and structural support of the colony. Furthermore, desmocytes secure the tissue firmly to the axis, ensuring structural integrity and providing a connection between the polyp tissues and the internal rod that is vital for preserving the physical stability and functional capacity of gorgonian corals (Galloway et al., 2007; Peters, 2016).

Upon examining the internal layer of tissue, we find the gastrodermis, which exhibits minimal significant differences between octocorals and hexacorals. This layer lines all gastrovascular cavities, interconnecting gastrovascular canals and mesenteries, characterized by diverse cell types, including nematocytes, ciliated columnar supporting cells, collar cells, and granular gland cells (Berzins et al., 2021). As previously noted, digestion within the gastrovascular cavity is facilitated by both extracellular processes, involving enzymes, and intracellular processes, hosting absorptive cells that uptake particles through mechanisms such as phagocytosis, pinocytosis, and diffusion (Schlichter, 1982). The gastrodermis is also extremely important because of the storage of zooxanthellae, retained within vacuoles in the gastrodermis, ensuring their survival without digestion. The highest concentrations of zooxanthellae are generally located in the oral surface tissues, optimizing light absorption for photosynthesis (Downs et al., 2009). Under light microscopy, the nucleus of a zooxanthella can be identified as a deeply basophilic, oval-to-square structure, typically eccentric within the cell, characterized by permanently condensed chromatin (a dinokaryon) (Work et al., 2024). The gastrodermis, particularly in the surface and basal body walls, as well as the actinopharynx and mesenteries, can exhibit varying densities of mucocytes, with staining characteristics differing based on the application of hematoxylin, alcian blue, or alcoholic saffron, contingent upon its molecular composition and pH (Work et al., 2024). Although the specific role of mucus in polyps remains incompletely understood, it may contribute to supporting the internal bacterial microbiome (Bythell & Wild, 2011).

Finally, being among the earliest and most basic organisms in the context of evolutionary history, anthozoans are characterized by the absence of the complex systems typically observed in higher organisms (Berzins et al., 2021; Parisi et al., 2021). Nevertheless, they have developed adaptations that enable them to fulfill essential functions.

The muscular apparatus is replaced by epitheliomuscular cells, also called myoepithelial cells, distributed throughout the entirety of the epithelial layer, that rely on the mesoglea for the operational integrity of the hydrostatic skeleton, providing the necessary stiffness to maintain shape and offer resilience to support muscular actions (Parisi et al., 2021). The mesoglea, as mentioned before, can invaginate to form mesenteries that can further undergo vertical folding, resulting in structures termed mesogleal pleats, enhancing the organism's movement, bending, and retraction capacity (Seipel & Schmid, 2006). The increased surface area afforded by these pleats accommodates numerous myonemes, formations arising from the basal extensions of the plasmalemma (cell membrane) of epitheliomuscular cells. These myonemes comprise actin and myosin filaments, facilitating movement and sphincter functions surrounding the mouth. This organization along the mesogleal pleats contributes to the longitudinal retractor muscles of the polyp, with both the epidermis and gastrodermis playing significant roles in the movement and feeding mechanisms of these organisms (Berzins et al., 2021).

Furthermore, anthozoans do not possess discrete respiratory, excretory, or circulatory systems. Consequently, nutrient uptake occurs via endocytosis, absorption, or using compounds derived from symbiotic dinoflagellates, while gas exchange and excretion rely on direct membrane diffusion (Davy et al., 2012).

Regarding the nervous system, cnidarians have traditionally been perceived as having a diffuse and undifferentiated arrangement; however, they exhibit a more complex organization. This includes a precursor to the nervous system characterized by a diffuse nerve network, light-sensitive structures, statocysts, and primitive chemosensory sensors (Westfall, 2005; Kass-Simon & Hufnagel, 2015). Cnidarian nerve cells display a variety of morphologies, encompassing unipolar, bipolar, and multipolar forms, which are organized into diverse structures, including nerve nets, nerve tracts, ring nerves, giant nerves, and ganglia-like clusters (Berzins et al., 2021). The conduction systems present in cnidarians facilitate the transmission of signals from an array of sensory neurons through chemical and electrical synapses to effector systems that consist of myonemes, muscle cells, and cnidocytes (Westfall et al., 2005).

The immune system in cnidarians primarily operates via innate mechanisms, such as mucus with antimicrobial properties and amoebocytes endowed with phagocytic and biochemical functions that serve as the primary cells of the cnidarian immune response (Ritchie, 2006; Myldraz et al., 2008; Bosch & Rosenstiel, 2016). Almost all cnidarian species have amoebocytes, which perform crucial roles in various physiological processes, including antimicrobial and antioxidant functions. These cells can phagocytize and digest particulate matter and are responsible for transporting food and waste materials throughout the organism. Additionally, amoebocytes can differentiate into other cell types, contributing to the cellular diversity within cnidarian tissues (Palmer & Traylor-Knowles, 2012; Hawthorn et al., 2023; Work et al., 2021, 2024). Their substantial mobility allows them to migrate between epithelial cells and across epithelial layers, including the capacity to traverse the mesoglea. This capability underscores their significance in maintaining the health and resilience of cnidarian species, particularly in response to environmental stressors and pathogens.

In conclusion, the gonadal development and reproductive cells of cnidarians occur along the mesenteries, situated within the mesoglea between muscular layers and mesenterial filaments, resulting in the formation of oocytes and spermaries. The germ cells, which serve as precursors to gametes, originate from the gastrodermis, specifically from stem cells often referred to as totipotential, multipotential, or interstitial cells. The germ cells mature within the gastrodermis, particularly in the midsection of select mesenteries, before migrating to the mesoglea for further maturation. It is important to note that not all mesenteries participate in gamete production; some may exhibit gametogenic activities, while others do not. In species that spawn, the mesogleal and gastrodermal layers rupture, facilitating the release of gametes into the gastrovascular cavity and subsequently out of the organism through the mouth. Conversely, in brooding species, developing embryos and planula larvae are retained within the polyp until they have developed mesenteries and incorporated symbiotic dinoflagellates (Berzins et al., 2021)

## 1.6. SEARCHING FOR SOLUTION: INNOVATIVE MATERIAL FOR RESTORATION

As largely discussed before, marine animal forests are vital for the marine environment and the entire planet for their biodiversity and essential ecosystem services. However, it has also been pointed out that they are under severe threat due to climate change, pollution, and other anthropogenic pressures (Coll et al., 2010; Hughes et al., 2017). The threats and problems for the survival of these ecosystems are always clearer and more understood, but we can only do something to stop them by changing the attitude toward carbon dioxide emissions and pollution. Therefore, putting much more effort into research is necessary to buy time for the marine ecosystem to survive and leave its resilience the possibility to work out. While understanding the causes of reef degradation remains essential, the priority now lies in advancing solution-oriented strategies that optimize existing methods, reduce environmental impact, and increase efficacy on larger scales.

One of the only possibilities at our disposal is coral restoration, based on the general concept of “assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed” (Clewell et al., 2004). Several techniques have emerged as essential and practical tools for preserving coral reef ecosystems, buying time for coral populations to recover. Although restoration efforts cannot fully mitigate the pervasive impacts of climate change, an overall success rate of approximately 60–70% is estimated in a limited small scale and restricted number of coral species, and therefore, emerge as the only scalable intervention available today to support the resilience of coral reefs and maintain their essential ecological functions, underscoring the urgent need for large-scale action to contract the effects of climate change progression (Anthony et al., 2017; Boström-Einarsson et al., 2020). Additionally, restoration initiatives serve as living laboratories, allowing scientists to explore coral stress tolerance and resilience, develop new adaptive methods, and identify coral species or strains that show promise for survival under changing environmental conditions (Chamberland et al., 2017; van Oppen et al., 2017; Breed et al., 2019; Berman et al., 2023). Restoration, therefore, offers not only immediate conservation benefits but also invaluable insights for future reef management. However, scaling coral restoration to achieve meaningful conservation outcomes requires overcoming significant challenges, particularly the labor-intensive nature of many restoration techniques. Current methods, such as manual transplanting of coral fragments and installation of substrates by divers, though effective on a small scale, are costly and require considerable time and resources, such as in coral gardening with different types of nurseries, micro fragmentation or artificial reefs (Rinkevic, 2005; Levy et al., 2010; Forsman et al., 2015; Montoya-Maya et al., 2016; Bayraktarovet al., 2019; Williams et al., 2019; Boström-Einarsson et al., 2020; Hein et al., 2020; Dehnert et al., 2021; Levy et al., 2022; Berman et al., 2023). Coral restoration also benefits from integrated approaches that combine ecological science with community engagement and policy support. Local communities play a crucial role in the success of restoration projects, providing labor, monitoring, and resource management while fostering stewardship and sustainable practices (Bayraktarovet al., 2019; Hein et al., 2019). Education and outreach efforts that emphasize the ecological and economic importance of coral reefs encourage local stakeholders to participate actively in conservation initiatives, bolstering restoration success (Hein et al., 2019; Vardi et al., 2021). Policy support is equally essential for scaling restoration; governments and conservation organizations must prioritize funding for coral restoration, facilitating collaboration and ensuring that resources are directed toward impactful projects (Bayraktarovet al., 2019; Vardi et al., 2021).

Further advancements in restoration science are critical to enhance the efficacy and long-term sustainability of coral restoration. Assisted evolution techniques, which include selective breeding and genetic modification to improve coral resilience to environmental stress, offer promising avenues for coral adaptation and potentially foster coral populations that are better equipped to survive in changing environments (Santoro et al., 2021; van Oppen & Lastra, 2022). Additionally, monitoring technologies such as remote sensing and artificial intelligence (AI) support restoration efforts by providing real-time data on coral health, environmental conditions, and restoration progress. These technologies enable adaptive management strategies, allowing conservationists to track the outcomes of restoration efforts, assess ecological impacts, and optimize techniques as conditions change. Combining restoration with innovative research advances the field, providing conservationists with new tools to improve coral resilience and sustainability. One key area for improvement lies in developing eco-friendly materials that minimize pollution and ecological stress. Traditional restoration substrates, often composed of metal and plastic, are functional but can introduce pollutants into marine environments. These materials may also degrade over time, releasing micro-particles that contribute to the very stressors they aim to alleviate. To address this challenge, researchers are investigating alternative materials, including biodegradable and sustainable options, that offer a more compatible and lower-impact solution for coral restoration projects. Promising materials such as engineered biopolymers, natural limestone, and ceramic-based substrates allow corals to settle and grow without introducing harmful byproducts. Ceramics, particularly, are durable, chemically stable, and can be formed into complex structures that mimic natural reef habitats, encouraging coral larvae settlement (Levy et al., 2022; Berman et al., 2023). Biopolymers derived from renewable resources, such as algae, are another option; they decompose naturally, leaving no harmful residues, thereby supporting long-term ecosystem health (Contardi et al., 2020). By developing and integrating such eco-friendly materials, restoration efforts can reduce their ecological footprint, ensuring that the very act of restoration does not exacerbate the stressors corals face.

Coral restoration is an essential, although temporary, solution to the ongoing coral reef crisis. While it cannot address the root causes of reef degradation alone, it provides a vital buffer that allows coral populations to recover and adapt to changing conditions. By prioritizing the optimization of existing techniques, developing and implementing eco-friendly materials, and scaling restoration efforts, the scientific and conservation communities can maximize the impact of coral restoration on reef conservation. Achieving these objectives requires a solutions-oriented approach that focuses on practical, actionable interventions to support the survival and resilience of coral ecosystems in a rapidly changing world.

## 1.7. AIMS OF THE STUDY

The Mediterranean Marine Animal Forests (MAFs) are recognized as ecologically and structurally significant marine habitats that provide essential biodiversity and ecosystem services (Rossi et al., 2017; Cau et al., 2020). Nonetheless, these benthic communities are facing unprecedented challenges due to climate change, anthropogenic pressures, and emerging contaminants (Coll et al., 2011; Rossi, 2013; Hughes et al., 2017, 2022), posing a significant threat to their survival and functionality. Given the rapid and widespread occurrence of these threats, there is increasing concern that the pace of change may exceed the capacity of corals to adapt and cope with the evolving environment, leading to extensive loss of coral reef cover and the potential risk of extinction (Hoeg-Guldberg et al., 2017). The global mitigation of carbon dioxide emissions represents the only unequivocal solution to address climate change; however, achieving this objective within short timeframes appears impossible. Consequently, it is imperative to enhance our understanding of the implications for the marine ecosystem, as well as the impacts and repercussions of these changes, to identify viable solutions to alleviate the detrimental effects of climate change and human activities, and will enable these environments to survive and adapt effectively.

This project aimed to conduct a comprehensive assessment of the health status of Mediterranean anthozoans within the Marine Protected Areas (MPAs) of Bergeggi and Portofino, focusing on key stressors and their impacts and exploring potential solutions for the conservation and restoration of these vital ecosystems.
To achieve this objective, the work was addressed to:

1. The evaluation and quantification of the potential bioaccumulation of different contaminants within the tissues of Mediterranean anthozoans have been conducted. This study focused on three significant contaminants: plastics and their additives, pharmaceuticals, and personal care products (especially Active Pharmaceutical Ingredients (APIs) and UV filters). Additionally, a secondary objective was evaluating the potential role of Marine Protected Areas (MPAs) in mitigating the pollution associated with these substances. The evidence gathered underscores the ecological risks connected to these contaminants, even at low concentrations, due to their persistence and potential for bioaccumulation.

2. Histopathology has been employed to investigate the effects of climate change and thermal stress on anthozoans in light of the increasing frequency of marine heatwaves that result in mass mortality events among these organisms in the Mediterranean region. Through comprehensive histopathological analyses of three key gorgonian species—*Paramuricea clavata*, *Eunicella cavolini*, and *Leptogorgia sarmentosa*—we aimed to enhance our understanding of the specific patterns of tissue damage, which include exposed axial skeletons, thinning of tissues, and necrosis in these species. Furthermore, we tried to determine whether the observed effects are solely attributable to heat stress or if a previously underexplored interaction between thermal stress and disease processes exists. This study underscores the necessity for additional research into the roles of pathogens and environmental co-factors in mass mortality events to gain a deeper insight into the disease dynamics affecting Mediterranean anthozoans.

3. Recognizing the critical need to protect anthozoans and to gain a comprehensive understanding of their impact on marine ecosystems, this project also aimed to investigate innovative solutions for coral restoration. Traditional restoration methods frequently utilize materials that can contribute to pollution, thereby worsening environmental degradation. In response to this challenge, a novel, biodegradable coral putty formulated from epoxidized soybean oil acrylate (ESOA) and zein was designed and tested in this work. This material is an eco-friendly alternative to conventional epoxies and concretes, effectively supporting coral transplantation that proved a concept that could be extended to Mediterranean anthozoans, presenting a sustainable approach to facilitating the recovery of these ecosystems while minimizing ecological footprints.

In conclusion, this project aimed to enhance our comprehension of the stressors impacting the Mediterranean marine environment and identify actionable strategies for their conservation. While addressing global stressors remains important, finding solutions to provide significant opportunities for these ecosystems to recover and adapt remains imperative. By integrating research on the effects of climate change and pollution with the application of innovative restoration materials, this study highlights the importance of a multifaceted approach to safeguarding these ecosystems.

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# CHAPTER 2

## Occurrence of phthalic acid esters (PAEs) and active pharmaceutical ingredients (APIs) in key species of anthozoans in Mediterranean Sea

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## 2.1. ABSTRACT

The Mediterranean Sea's biodiversity is declining due to climate change and human activities, with plastics and emerging contaminants (ECs) posing significant threats. This study assessed phthalic acid esters (PAEs) and active pharmaceutical ingredients (APIs) occurrence in four anthozoan species (*Cladocora caespitosa*, *Eunicella cavolini*, *Madracis pharensis*, *Parazoanthus axinellae*) using solid phase microextraction (SPME) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). All specimens were contaminated with at least one contaminant, reaching maximum values of 57.3 ng/g for the ∑PAEs and 64.2 ng/g (wet weight) for ∑APIs, with dibutyl phthalate and Ketoprofen being the most abundant. *P. axinellae* was the most contaminated species, indicating higher susceptibility to bioaccumulation, while the other three species showed two-fold lower concentrations. Moreover, the potential adverse effects of these contaminants on anthozoans have been discussed. Investigating the impact of PAEs and APIs on these species is crucial, given their critical role in the Mediterranean benthic communities.

## 2.2. INTRODUCTION

The marine environment has an incredible biodiversity and provides several important ecosystem services; however, in recent decades, human settlement and activities have significantly impacted it, making it one of the most significant threats to the marine environment. Indeed, this has led to the emergence of the term Anthropocene, which describes our geological epoch characterized by anthropogenic-induced environmental changes on a global scale (Meybeck et al., 2003; Steffen et al., 2011; Issberner & Lèna, 2018). During this period, the marine environment is facing significant risks due to global climate change and pollution, with plastic pollution being a particularly severe threat. Among the primary pollution sources there are microplastics (MPs) and their additives (PAEs), as well as personal care products (PCPs) and active pharmaceutical ingredients (APIs) (Blasco & Del Valls, 2008; Arpin-Pont et al., 2014; Cózar et al., 2015; Alomar et al., 2016; Paíga et al., 2016; Brumovský et al., 2017; Pico et al., 2019; Angiolillo & Fortibuoni, 2020; Angiolillo et al., 2021; Huang et al., 2020; Adeleye et al., 2022; Baudena et al., 2022; Hawash et al., 2023).

In terms of plastic, the past century has witnessed an exponential increase in its global production, reaching an unprecedented value of approximately 350 million tonnes worldwide in recent years (Plastic Europe, 2021). Although the exact estimation of the plastic amount in the ocean remains challenging, studies suggest that at least 5.25 trillion plastic particles are floating in the sea, and the projected amount is expected to grow even more (Andrady, 2011; Jovanović, 2017). This ever-growing increase, combined with its durability, insufficient disposal, and inadequate discarding policy (Ostle et al., 2019), has made it one of the most ubiquitous and critical sources of pollution, especially in marine ecosystems (Moore, 2008; Wright et al., 2013b; Eriksen et al., 2014; Zhou et al., 2020). This problem is further exacerbated by the fact that plastic finds multiple ways of reaching the oceans, such as stormwater runoff, direct dumping, and loss of fishing and aquaculture gear, making it extremely complex to control, monitor, and prevent (Alomar et al., 2016; Alkan et al., 2021; Kumar et al., 2021; Baudena et al., 2022; Hidalgo-Serrano et al., 2022). This is especially true in the Mediterranean Sea, considered one of the world's largest accumulation zones of marine litter, with 7% of global microplastics in just 1% of the world's marine waters (Cózar et al., 2015). The focus of concern has primarily been directed toward micro and nanoparticles, which can originate from the direct discharge of microscale plastic particles by producers or as secondary products resulting from the photo-oxidative degradation or weathering-induced breakdown of larger plastic items floating on the ocean surface (Saliu et al., 2019, 2020; Yuan et al., 2022). This aspect is critical given that toxicity of plastic materials may arise from their constituents, such as residual monomers and plastic additives, flame retardants, and other personal care products (Wright et al., 2013; Zeri et al., 2018). Among them, phthalic acid esters (PAEs) have recently gained scientific attention as essential additives (up to 60% of total weight). They are used as plasticizers during plastic production to increase flexibility, transparency, or longevity (Teuten et al., 2007). However, they are not covalently bound to the plastic polymers and, therefore, can detach from the plastic material, becoming ubiquitous and bioavailable in the surrounding environment due to their low solubility and lipophilicity in water (Rochman et al., 2013; Net et al., 2015; Saliu et al., 2019, 2020).

Instead, pharmaceuticals and personal care products (PPCPs) are synthetic organic compounds that have become increasingly important over the years in various aspects of daily life, ranging from food production and conservation to sanitation, and also in relation to our health (Arpin-Pont et al., 2014; Ebele et al., 2017; Szopińska et al., 2022; Hawash et al., 2023). They comprise several organic contaminants, among which active pharmaceutical ingredients (APIs), used in human and veterinary medicine, or also in fragrances, UV protectors in sunscreen agents, and cosmetics ingredients, are one of the most present and impacting groups (Richardson et al., 2005; Jiang et al., 2019; Xie et al., 2019a; He et al., 2019; Miller et al., 2021; Wheate, 2022). Often, they are not yet regulated and are referred to as Emerging Contaminants (ECs) by the EPA (EPA, 2016) or Emerging Substances by the EU NORMAN network (NORMAN, 2016). Unfortunately, similar to plastic debris, after disposal, APIs can enter the coastal environment through various pathways such as direct discharge, riverine transport, domestic or industrial wastewater, aquaculture, agricultural runoff, and excretion by humans and animals into wastewaters (Arnold et al., 2013; Yuan et al., 2014; Dey et al., 2019). To date, the pharmaceuticals most extensively studied and detected include psychiatric drugs (carbamazepine, citalopram, and fluoxetine), antibiotics (macrolides, quinolones, and sulphonamides), non-steroidal drugs, and anti-inflammatories (salicylic acid ketoprofen and diclofenac), and β-blockers (carazolol, propranolol, metoprolol, and sotalol) (Mezzelani & Regoli, 2022). As one of the most common groups of emerging contaminants, their presence and adverse effects on the marine environment has recently gained attention of researchers and have been investigated globally in different studies (Zenker et al., 2014; Andreu et al., 2016; Puckowski et al., 2016; Carmona et al., 2017; Bonnefille et al., 2018; He et al., 2019; Álvarez-Ruiz & Pico, 2020; Srain et al., 2020; Zhou et al., 2020; Miller et al., 2021), especially after the pandemic period when the use of pharmaceutical has increased drastically (Van Boeckel et al., 2014; Koagouw et al., 2021).

Although the occurrence and fate in freshwater are well documented for many of those substances due to controls and studies on wastewater treatments (Hughes et al., 2012; Li, 2014; Murray et al., 2010), their behavior in coastal and marine waters is much less studied and understood (Arpin-Pont et al., 2014; Gaw et al., 2014). This aspect requires further investigation since plastic, together with its additives and pharmaceuticals, may cause adverse effects and consequences on marine organisms, as documented in tropical seas with a consistent body of literature, contrary to the Mediterranean Sea region, where the issue remains less studied and addressed (Burns & Boxall, 2018; de Sà et al., 2018). In general, the response of organisms to contaminants uptake is species-specific and is influenced by factors such as concentration and environmental conditions (Fossi et al., 2018; Paluselli et al., 2018; Saliu et al., 2019; Panio et al., 2020). Within the benthic community, anthozoans are particularly affected as they are typically regarded as non-selective suspension feeders that primarily feed on zooplankton (Palardy et al., 2008; Houlbrèque & Ferrier-Pagès, 2009; Savinelli et al., 2020). However, their interactions with microplastics and APIs, to an even lesser extent, have not been thoroughly studied. While a few studies have shown the ingestion of microplastics by corals

(Hall et al., 2015; Allen et al., 2017; Chapron et al., 2018; Vencato et al., 2021), tissue necrosis induced by microplastic exposure (Reichert et al., 2018), and reduced feeding activity (Savinelli et al., 2020), there is currently still very limited information about emerging contaminants. In the last year, different studies focused on phylogenetically different marine organisms, such as sponges, mussels, and fishes respectively (Na et al., 2013; Du et al., 2015; Paluselli et al., 2018; Zhou et al., 2020; Adeleye et al., 2022; Chen et al., 2022; Rizzi et al., 2020, 2023; Hawash et al., 2023), but there is a substantial scarcity of data on anthozoans. Therefore, the lack of comprehensive studies regarding the presence of PAEs and APIs impacting the benthic community responsible for most of the Mediterranean marine animal forest is limiting our understanding of the effects of emerging contaminants on aquatic invertebrates, including the potential to bioaccumulate them within their tissues, transfer them to offspring, and the potential carrier effects of both PAEs and APIs (Arnold et al., 2013; Zhou et al., 2020; Atugoda et al., 2021).

Therefore, this study aims to investigate, for the first time, the occurrence of phthalic acid esters (PAEs) and active pharmaceutical ingredients (APIs) in four key species of Mediterranean benthic anthozoans: *Cladocora caespitosa*, *Eunicella cavolini*, *Madracis pharensis*, and *Parazoanthus axinellae*. Specifically, the study tested the presence and concentration of five PAEs, namely dibutyl phthalate (DBP), benzyl butyl phthalate (BBzP), diethyl phthalate (DEP), Bis (2-ethylhexyl) phthalate (DEHP), and dimethyl phthalate (DMP) (Supplementary materials, Table 1); and 7 APIs commonly used, namely Amisulpride, Metoprolol, Propylphenazone, Carbamazepine, Clarithromycin, Ketoprofen and Diclofenac (Supplementary materials, Table 2) and investigated any possible difference in their species-specific bioaccumulation. Since these species play a crucial role in forming the habitat of benthic communities in the Mediterranean coastal environment, the investigation of the potential impact posed by PAEs and APIs may provide an important insight into the health status of the benthic communities and improve their management strategies.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Study area and sampling

Underwater surveys were conducted in May 2023 to investigate the presence and concentration of PAEs and APIs in the Northwestern Mediterranean Sea, precisely in the coastal area of

Paraggi (Figure 1), near Santa Margherita Ligure (Liguria, Italy, 44°18'41.0"N; 9°12'47.4"E), an area which is part of the Portofino Marine Protected Area (MPA) established in 1999.
The rationale behind the choice of the sampling site was beyond accessibility and permit, but it encompasses the close proximity to areas significantly influenced by human activities and potential pollution, considering various factors, including the extent of anthropogenic activities along the coastline, the presence of tourists and residents in the area, and the intensity of boat traffic (Dobler, 2002; Bevilacqua et al., 2021). Moreover, the choice of species in this study was guided by their widespread distribution and abundance across the Mediterranean region (Bianchi & Morri, 2000; Cerrano et al., 2006; Sini et al., 2015; Betti et al., 2017; Bianchi et al., 2019; Kersting et al., 2023), resulting in highly accessible sampling without exerting undue pressure on the local benthic community.

A total of 20 samples (Table 3), 5 for each species (*Cladocora caespitosa*, *Madracis pharensis*, *Eunicella cavolini*, and *Parazoanthus axinellae*) (Figure 2), were collected by SCUBA diving along the slope of the bay at a depth ranging from 5-25 m and with water temperature between 19-21°C. Each sample was collected, immediately wrapped in aluminum foil, and preserved in glass vials to avoid cross-contamination of PAEs from other materials.

### 2.3.2. SPME-LC-MS/MS analysis

For the determination of PAEs and APIs in the coral tissue, we used solid phase microextraction (SPME) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) following a method we previously described (Saliu et al., 2020a, 2020b). Briefly, for each sample, two distinct C18 SPME fibers (Sigma-Aldrich 57234-U) were used, one for PAEs and one for APIs extraction. These fibers were inserted into the anthozoan tissue for 30 min in both cases. Then, the desorption of the extracted analytes was carried out using methanol:water mixture, respectively, at 90:10 for PAEs and 80:20 for APIs. LC-MS analysis was performed with a TSQ Quantum Access Max LC/MS instrument (ThermoScientific) equipped with an ESI interface and a triple quadrupole mass analyzer. Analytes were detected and quantified by operating the mass spectrometer in the selected reaction. For QA/QC, a total of 6 procedural blanks were run to establish the limit of quantitation (LOQs) of the method, and an in-house quality control sample was prepared and analyzed to determine recoveries and precision. During sample manipulation, particular care was taken to limit phthalates background contamination. Precisely, we followed measures previously described in the literature (Fierens et al., 2012; Panio et al., 2020).

In particular, the following PAEs and APIs were tested: dibutyl phthalate (DBP), benzylbutyl phthalate (BBzP), diethyl phthalate (DEP), Bis (2-ethylhexyl) phthalate (DEHP), and dimethyl phthalate (DMP) (Supplementary materials, Table 1); Amisulpride, Metoprolol, Propylphenazone, Carbamazepine, Clarithromycin, Ketoprofen and Diclofenac (Supplementary materials, Table 2).

### 2.3.3. Details of the LC-MS/MS method applied

Analyses were carried out employing an LC-MS/MS TSQ Quantum Access Max instrument (ThermoFisher) equipped with a UHPLC/HPLC chromatograph, an electron spray ionization interface ESI, and a triple quadrupole mass analyzer. The chromatographic separation of the analytes was performed on a Thermo Scientific Accucore C-18 aQ column (100 mm × 2.1 mm I.D., 2.6 μm).

For PAEs, the elution was carried out with a binary mixture applying isocratic condition: 6% of mobile phase in pump A (water with 0.1% of formic acid) and 94% of mobile phase in pump B (methanol with 1% of water). The flow rate was set up at 0.7 mL/min.

For APIs, the elution was carried out following an elution gradient program and employing a binary mixture: water with 0.1% formic acid in pump A and methanol with 1% water in pump B. The Flow rate was set at 0.5 mL/min. For the first minute of elution, pump B's percentage was kept at 20%, then raised from 30 to 70% for the subsequent 4 minutes, followed by a 5-minute linear gradient to 96%, and then maintained at 96% for 5 minutes.

In both cases, sample introduction was performed using an autosampler in partial loop mode (40 μL). The injection volume was set up at 10 μL. The ESI-MS interface was operated in the positive ionization. The spray voltage was set up at 3500 V, the vaporizer temperature at 350°C, and the capillary temperature at 270°C. Sheath gas pressure was set up at 50 arbitrary units, auxiliary gas pressure at 15 arbitrary units, and ion sweep gas pressure at two arbitrary units. Mass spectrometry analyses were performed in selected reaction monitoring mode (SRM). For each target analyte, one qualifier and one quantifier were researched. The selected transitions for APIs and PAEs are reported in Tables S1 and S2. Collision gas pressure was set at 1.0 m Torr and the cycle time at 0.6 s. Data acquisition and processing were performed using the software Xcalibur (Thermo Scientific).

### 2.3.4. QA/QC and blank controls

Due to the limited availability of marine invertebrates for QA/QC, tests were carried out using agarose gel samples spiked at 1% (w/v) with the native standard solution. Previous studies have already proven the validity of agarose gel in mimicking in vitro the free diffusion of analytes in cnidaria tissues (Saliu et al., 2020). Specifically, the QC sample was prepared by weighing 12 g of agarose gel, which was then dissolved in hot phosphate buffer (pH 7.4) and allowed to solidify at room temperature for three hours. Just before solidification, the material was divided into two aliquots, and each aliquot was spiked with 600 ng of the PAEs and APIs reference mixture (respectively) and vortexed for 10 min to homogenize the analyte distribution in the gel. The final QC sample, resulting in a nominal concentration level of 100 ng/g, was then divided into six aliquots and used to determine recoveries and precision by back-calculation (considering the nominal values).

In addition, six procedural blanks obtained from empty vials were analyzed to determine background contamination levels. The limit of quantitation (LOQs) of the method was established from the replicated blanks analysis considering the mean plus six times the standard deviation, as previously reported in Panio et al. 2020. Especially during the preparation of the sample for PAEs analysis, special care was taken to limit contamination of the samples since these compounds are known to be ubiquitous and to occur in chemical laboratories. Before use, glassware and vials were rinsed with dichloromethane, heated at 450°C for at least four hours, and covered with aluminum foil. Syringes and spatula were rinsed carefully with dichloromethane. No laboratory gloves were used during the sample preparation, and analyses were carried out in a dedicated air cabinet with laminar flow. During the operation, personnel used protective cotton lab coats, and the contact with plastic material was extremely limited.

### 2.3.5. Statistical analysis

All the data obtained were tested for normality with Kolmogorov–Smirnov tests. In case the normal distribution and homogeneity of variance were violated, Kruskal–Wallis and Mann–Whitney *U* tests were performed to analyze the mean differences in the concentration of contaminants among the species. Data are presented as the arithmetic mean ± standard deviation and in ng/g wet weight unless stated otherwise. All the statistical analyses performed for this study were conducted using IBM SPSS 28 Software (IBM SPSS 28, New York, NY, USA).

## 2.4. RESULT AND DISCUSSION

### 2.4.1. Occurrence of PAEs and APIs in anthozoans

Analysis of blank controls showed an average of 0.4 ± 0.5 ng/g for $\sum PAEs$ and 0.2 ± 0.1 ng/g for $\sum APIs$. The resulting Limit of Quantification (LOQs) were 0.9 ng/g and 2.1ng/g for the different PAEs and 0.2 ng/g and 0.5 ng/g for the different APIs. Analysis of the quality control sample showed good recoveries (86 – 103%) and precision (4 – 15%).

Analysis of the 20 specimens showed the occurrence of at least one type of PAEs and APIs in each of the samples analyzed (Table 4 and 5). The only exception was two samples (PA0523CLA4 and PA0523EUN4) found to have PAEs levels below the detection limit for all the PAEs searched. For APIs, Amilsulpride, Propyphenazone, and Clarithromycin resulted below the detection limit for all the samples and were therefore excluded from the statistical analyses. Overall, the total amount of APIs (517.8 ng/g wet weight) found was higher compared to PAEs (344.5 ng/g wet weight) in the 20 samples analyzed (Figure 3a). Table 1 summarizes the results with the range and average concentration for each species analyzed. The average concentration of PAEs in all the samples was 17.2 ± 11.4 ng/g with a maximum value of 57.3 ng/g, while the average concentration of APIs in all the samples was 25.9 ± 16.9 ng/g with a maximum value of 64.2 ng/g (Figure 3b). Among them, the most abundant contaminants were Ketoprofen and DBP, with 16.9 ± 17.0 ng/g and 10.3 ± 5.5 ng/g, respectively, while all other contaminants were detected at concentrations less than half of the most abundant ones within each of the two classes (Figure 3c).

### 2.4.2. Statistical analysis and comparison of PAEs and APIs in anthozoan

Comparing the results obtained for all the species, *P. axinellae* showed the highest concentration for both contaminants, with bioaccumulation capacity more than two-fold higher than the other species (Figure 4; Table 6). Indeed, the molecules detected in *P. axinellae* accounted for almost 50% of the total contaminants found in both classes of contaminants. Looking at PAEs, *M. pharensis* and *C. caespitosa* followed in terms of mean concentration of contaminants, while *E. cavolini* showed lower values (Figure 4). In general, *P. axinellae* exhibited the highest concentration of every contaminant analyzed, with the only exception of BBzP, the only PAEs that had a higher concentration in *C. caespitosa* and Carbamazepine, the only molecule not found in *P. axinellae* (Table 4, Table 5 and 6). In addition, *E. cavolini* was the only species showing no sign of contamination by two out of four PAEs (DEP and DEHP; Table 5 and 6). Moreover, *P. axinellae* again showed the highest overall concentration of APIs, while the other three species showed similar concentration values for all the compounds. Interestingly, Diclofenac and Carbamazepine were the only contaminants displaying a similar concentration among all the species analyzed. However, all the mean differences between contaminants in the different species were not statistically significant (Kruskal–Wallis test on PAEs, p = 0.170, $p > 0.05$; Kruskal–Wallis test on APIs, p = 0.738, $p > 0.05$).

This absence of species-specific differences in contaminants distribution among the analyzed species may have two possible explanations. On one hand, despite belonging to different orders and having distinct characteristics, these anthozoans may exhibit a common bioaccumulation pattern. In fact, detoxification, excretion, and uptake mechanisms are thought to be similar and evolutionarily conserved within this class of organisms (Miller et al., 2007; Parisi et al., 2020). On the other hand, the elevated intraspecific variability observed may reflect differences among individuals of the same species in terms of various life stages. In fact, colonies collected at different life stages may potentially present a variation in metabolic activity and detoxification capability (Dullo, 2005; Sawall & Al-Sofyani, 2015; Murphy & Richmond, 2016; Bythell et al., 2017; Linsmayer et al., 2020). In particular, it is plausible that younger individuals invest a significant amount of their energy toward growth, leading to higher metabolic rates and enhanced detoxification abilities compared to older colonies.

Moreover, while acknowledging the speculative nature of this assertion and the further investigation needed to validate it, we cannot exclude the possibility that the micro-environment might exert an influence on the ability of individuals to bioaccumulate differently. Hence, the variation should be taken into consideration, as the concentration of contaminants may be influenced not only by their sources but also by the different characteristics of the sites along the coast and their associated environmental conditions (Hartmann et al., 2017). To comprehensively understand contaminant dynamics in a specific environment, it is crucial to account for all the combined effects that may influence the process of intake of contaminants, such as exposure levels, proximity to the contamination source, water currents, temperature, and depth, that significantly impact the spatial distribution and quantity of contaminants present in the water column (Carson et al., 2011; Lee et al., 2014; Hartmann et al., 2017). In addition, the analyzed species exhibit different growth morphologies: *M. pharensis* is an encrusting coral, *C. caespitosa* is a massive coral, *P. axinellae* presented small, extruded polyps, and *E. cavolini* characterized by branching growth that exposed them differently to the water and contaminants. Moreover, *M. pharensis* and *P. axinellae* colonize more sheltered areas, contrary to *C. caespitosa* and *E. cavolini,* which colonize open spaces on the shallow bottom and on the slope directly exposed to current, respectively. In this context, the observed higher bioaccumulation of *Parazoanthus axinellae* may be explained by its location in the coastal environment, usually inhabiting sheltered areas like caves or overhangs shielded from direct sunlight. This characteristic may account for the significantly higher susceptibility of this species to all analyzed contaminants, possibly due to reduced hydrodynamics, which promotes the accumulation of pollutants, leading to prolonged exposure at higher concentrations available to corals, which in turn may promote their absorption. Conversely, the other species analyzed are usually found directly exposed on the slopes and rock formations, which are more subject to currents, waves, and overall hydrodynamics and, consequently, reduce the interaction time with the contaminants. As a result, they exhibited approximately half the concentration of most compounds, with *Eunicella cavolini* being the only species that even lacked the presence of two of the investigated PAEs. However, it is fundamental to understand that coral physiological processes are understudied, and therefore, the variability in bioaccumulation may not be given solely by the interaction with water but also by intrinsic biological and physiological characteristics unique to each species.

### 2.4.3. Contamination of Mediterranean Sea anthozoan and adverse effects

To the best of our knowledge, this study is the first to report the occurrence of PAEs and APIs in anthozoans in the Mediterranean Sea. Due to the semi-enclosed nature of its basin, this region is recognized as one of the most severely impacted by human activities and plastic pollution (Giorgi et al., 2006; Lejeusne et al., 2010; Coll et al., 2011; Bevilacqua et al., 2021). Human settlements and activities, including fish farming for antibiotics (Zou et al., 2011) and antiparasitic drugs (Rico & Van den Brink, 2014), as well as recreational activities for sunscreen UV filters and pharmaceuticals being washed into the water (Bachelot et al., 2012; He et al., 2019; Madizikela et al., 2020; Branchet et al., 2021; Miller et al., 2021; Wheate, 2022), all contribute to the contamination of these coastal environments. However, little data on APIs in seawater has been published (Arnold et al., 2013; Klosterhaus et al., 2013; Ojemaye & Petrik, 2018; Madizikela et al., 2020; Srain et al., 2020; Branchet et al., 2021), and studying their impact on the marine environment is challenging due to the limitations posed by the dilution and diffusion in a vast medium like seawater, also compounded by its complex hydrodynamics (Arpin-Pont, 2014; Hawash et al., 2023).

Unfortunately, all the coral samples analyzed were contaminated with PAEs, APIs, or, in most cases, both. In particular, they showed a higher average concentration of APIs (25.9 ± 16.9 ng/g) than PAEs (17.2 ± 11.3 ng/g), suggesting a higher capability of APIs accumulation in their tissue. However, if a different susceptibility to those contaminants exists or if it simply involves variations in the concentration of these molecules in the seawater, it should be clarified with further studies. Previous studies conducted in both freshwater and marine environments have demonstrated that the concentration of contaminants in the water may change based on the level of human activity in the specific area (Arnold et al., 2013; Stefanakis et al., 2020; Gogoi et al., 2018; Sadutto et al., 2021). In this particular case, both PAEs and APIs were found in all specimens, as expected, due to the significant human impact in the sampling area situated near the heavily frequented Santa Margherita Ligure, in the proximity of Genova harbor, one of the busiest ports in the Mediterranean region (Dobler, 2002; Bevilacqua et al., 2021). Moreover, it has also been demonstrated that the concentration of contaminants varies seasonally, not only in response to tourist activities but also due to environmental factors, such as precipitation, temperature, and sunlight that affect the degradation, adsorption, and concentration in the marine environment (Rodríguez-Navas et al., 2013; Pavlidou et al., 2014; Moreno-González et al., 2015; Alygizakis et al., 2016; Zhao et al., 2017; Mezzelani et al., 2018; Čelić et al., 2019; Adeleye et al., 2022; Faranda et al., 2023).
In addition, while our study provides valuable insights, it is essential to acknowledge the limitations associated with the singular sampling point and the constrained number of samples per species. Hence, to comprehensively address the influence of different sources and their relative relevance, future studies should explore differences among individual samples and consider factors such as seasonal variations, distance to contaminants, and spatial and depth distributions, particularly concerning anthozoans.

After comparing the findings of this study with the available literature, it became evident that the levels of PAEs in Mediterranean waters generally tend to be lower than those observed in the organisms being investigated, with a range reported for Mediterranean Sea at 130-1330 ng/L for PAEs and 0.1 – 2.7 ng/L for APIs (Madzikela et al., 2020; Adeleye et al.,2022; Raguso et al., 2022). This suggests that anthozoans may absorb and accumulate some of these compounds in their tissue. The obtained concentrations of PAEs were comparable to those found in association with microplastic worldwide in various organisms, such as sponges, clams, mussels, shrimps, sea urchins, fishes, and turtles (Boerger et al., 2010; Wright et al., 2013; Devriese et al., 2015; Van Cauwenberghe et al., 2015; Bordbar et al., 2018; Anastasopoulou & Fortibuoni, 2019; Sala et al., 2021; Esposito et al., 2022; Raguso et al., 2022; Rios-Fuster et al., 2022; Saliu et al., 2022; Rizzi et al., 2020, 2023; Squillante et al., 2023), as well as in anthozoans in other regions (Saliu et al., 2018, 2019; Montano et al., 2020; Isa et al., 2023; Raguso et al., 2022), confirming the accumulation of PAEs in different organisms and tissues. As our study is the first to investigate APIs concentration in anthozoans, there is no direct comparison with prior literature available. However, similarly to the findings regarding PAEs, the values detected for APIs were comparable with the ones mentioned in several works reporting variable concentrations in marine environments in general (Hawash et al., 2023) and in the Mediterranean Sea (Brumovský et al., 2017). Moreover, the presence of various APIs has been reported in studies on different organisms, such as clams, mussels, crustaceans, fishes, mammals (Vernouillet et al., 2010; Lahti et al., 2012; Na et al., 2013; Devriese et al., 2015; Du et al., 2015; Moreno Gonzales et al., 2016; Van Cauwenberghe et al., 2015; Paluselli et al., 2018; Zhou et al., 2020; Adeleye et al., 2022; Chen et al., 2022; Hawash et al., 2023), confirming their presence and accumulation in marine organisms. This research has been conducted on different organisms not only to determine pharmaceuticals with potential bioaccumulation but also to identify the most suitable species for use as biomarkers in monitoring these compounds. As a result, most of the studies are focused on model organisms that are sessile, easy to retrieve, with broad geographical distribution and filter feeders, and thus continuously exposed to contaminants. However, these compounds are subject to bioaccumulation, leading to studies on more complex organisms positioned higher in the food web, such as sea snails, crustaceans, and fishes, arriving in some cases to iconic species such as mammals.

In the field of emerging contaminants, it is important to understand their ecological and physiological impact on marine fauna. They represent one of the most dangerous threats due to their widespread occurrence and their ability to affect a wide range of non-target species, as they are specifically designed to be biologically reactive at very low concentrations, making them able to interfere with biochemical and physiological processes with long-term effects on marine ecosystems still unknown and understudied (Mezzelani & Regoli, 2022). Microplastics have been studied extensively in marine organisms, including few studies on reef-building corals (Saliu et al., 2019; Montano et al., 2020; Isa et al., 2022; Raguso et al., 2022), but research on specific types of contaminants like PAEs and APIs is still lacking in the Mediterranean Sea. This scarcity of research may derive from the difficulty in investigating the effects of these contaminants in long-term, environmentally relevant settings (Adeleye et al., 2022). The available data suggest that exposure to low concentrations of APIs over a long period can cause sub-lethal effects on marine organisms, from simple sponges to the more complex vertebrates, in terms of behavior, reproduction, feminization, and reduced feeding and body weight (Corcoran et al., 2010; Brodin et al., 2014; Chopra & Kumar, 2018; Adeleye et al., 2022; Rizzi et al., 2023). While aquatic populations may not experience mortality or complete loss of functionality at low exposure concentrations, sub-lethal effects could still be detrimental.

In general, APIs are released directly into the water, making them bioavailable, and therefore, corals, as suspension feeders, may come into direct contact with both contaminants analyzed in the study, either through feeding or direct interactions. Feeding occurs through their tentacles, used to capture plankton and small organic matter, making the ingestion of microplastics and other organic molecules possible (Chapron et al., 2018). Additionally, they may consume prey that is contaminated as well (Procter et al., 2019). Therefore, the presence of both types of contaminants in the analyzed species' tissues may be due to active ingestion or internalization transfer into the tissue (Saliu et al., 2019, 2020). Nevertheless, there are no studies on these processes, and further investigation is needed to understand whether the levels of PAEs and APIs found in the coral tissues are related to direct ingestion or other mechanisms. Plastics have already been recognized for their detrimental effects on numerous species, but the ingestion of plastic additives such as PAEs emerges as a significant concern for coral health (Hall et al., 2015; Allen et al., 2017; Chapron et al., 2018; Reichert et al., 2018; Vencato et al., 2021). In fact, they may adversely affect coral energetics, growth, and health, causing changes in photosynthetic performance, symbiosis with zooxanthellae, tissue bleaching, and necrosis (Reichert et al., 2018; Tang et al., 2018; Syakti et al., 2019; Huang et al., 2020; Lanctôt et al., 2020; Savinelli et al., 2020; Mendrik et al., 2021) endangering their survival and resilience (Hall et al., 2015; Allen et al., 2017; Chapron et al., 2018; Okubo et al., 2018; Rades et al., 2022). They may also affect mucus production and alter gene expression, as seen in the main biomarkers of coral homeostasis, such as heat shock protein (Hsp), Catalase (CAT), and superoxide dismutase (SOD), and therefore on the level of protein, lipid, and reactive oxygen species (ROS) metabolism at the cellular level (Mendrik et al., 2021; Chen et al., 2022; Doering et al., 2023; Montalbetti et al., 2022, 2023).

Regarding APIs, there is a limited number of studies about their effects on marine species, and no studies are related directly to anthozoans; therefore, more research is needed to understand their impact on coral cells and tissue properly. In the present study, two out of four (Diclofenac and Ketoprofen) contaminants analyzed were Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), which are analgesics commonly used to alleviate pain and inflammation (Meek et al., 2010; Parolini et al., 2011), commonly found in marine organisms, from benthic invertebrates to top predators with increasing concentration due to their biomagnification effect (Mezzelani et al., 2018). Their mechanism of action causes the inhibition of the enzymes involved in the cascade signaling process to produce prostaglandins for the activation of immune responses and, therefore, can result in modulation of the immune response, leading to excessive inflammatory cascades, accumulation of activated macrophages, and ulceration (Thomas & Rosenstiel, 2015; Mezzelani & Regoli, 2022). In addition, they may also influence the antioxidant system while also hampering the function of enzymes responsible for detoxification (Bosch & Philip Rosenstiel, 2015; Tarrant, 2015; Woodley et al., 2016). Specifically, the active ingredients inhibit the reaction involved in the formation of different molecules involved in the response processes to pain, inflammation, regulation of blood flow, coagulation, and synthesis of protective gastric mucosa (Fent et al., 2006; Meek et al., 2010; Parolini et al., 2011). Moreover, they may also influence inflammatory responses, leading to functional impairment or disruption of the simple cell organization of the main tissue of corals due to vacuole formation and accumulation, as already demonstrated in mesentery tissue (Chen et al., 2022). However, research has demonstrated that water dilution alone does not effectively prevent bioaccumulation in aquatic organisms, and therefore, even if concentrations found are considerably lower than those harmful to humans, potential adverse consequences in non-target species cannot be excluded, particularly considering the long-term exposures and chronic effects (Xie et al., 2017). The two additional APIs contaminants detected were Carbamazepine and Metoprolol. The former is a psychotropic drug highly present in marine biota due to its refractory properties, resulting in an extremely long average half-life in aquatic ecosystems (Bu et al., 2016; Zhu et al., 2019; Mezzelani & Regoli, 2022), known to cause oxidative stress that can lead to reproductive impairment (Mezzelani et al., 2018). The latter is a beta-blocker typically used to treat heart and pressure-related diseases and may result in reduced lysosomal membrane stability and affect various physiological functions, including gonad development (Franzellitti et al., 2011, 2013). Although in this case, the concentrations were lower than other APIs, they may still have adverse effects on reproductive functionality and contribute to the accumulation of oxidative stress on corals over an extended period. However, to date, the available data on the impact of pharmaceuticals on non-target species remain fragmented and scarce, requiring further research to understand the specific mechanisms involved and thus validate these presumptions.

## 2.5. CONCLUSIONS

In conclusion, considering the crucial role of anthozoans in forming the benthic habitat of Mediterranean coastal communities, detecting their contamination calls for more comprehensive investigations into the potential impact of PAEs and APIs on species within this group. Given the ever-growing use and widespread presence of plastic and drugs, the scarcity of research on their environmental presence and biological implications on anthozoans highlights a significant knowledge gap. Understanding their biological effects and consequences in marine ecosystems is a complicated and multifaceted challenge, especially because organisms are typically exposed to complex chemical mixtures where individual compounds can interact through various mechanisms, leading to either synergistic or antagonistic feedback affecting the cellular and physiological responses and, as a consequence, also the population dynamics and the overall functioning of the ecosystem.

It is crucial to note that this study represents a preliminary exploration aimed at examining concentrations and potential species-specific differences in bioaccumulation. This research lays the groundwork for future studies, where the dataset should be expanded both in terms of sample quantity and analyzed sites. Additionally, the next steps should incorporate variables such as specific sampling points within the coral tissue (polyp or coenenchyma) or consider mucous secretion in the analysis, as well as all the processes involved in addition to sorption, such as diet, respiration, detoxification mechanisms. Moreover, considering environmental factors such as seasonal variations, distance to contaminants, and spatial and depth distributions will be essential for a more comprehensive understanding of contaminant dynamics in coral ecosystems.

Anthozoan suspension feeders are likely facing these impacts that may act synergistically with climate-driven events responsible for mass mortalities. Thus, it is essential to understand better the long-term impacts of sublethal concentrations of PAEs and APIs under diverse environmental conditions, as they are fundamental organisms in the benthic community of the Mediterranean Sea. This is particularly important in light of the future growing pollution scenario and the need for better disposal management and pollution control in coastal areas.

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## 2.7. TABLES

**Table 1.** Overview of the APIs molecules analyzed.

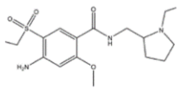
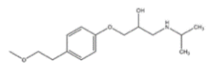
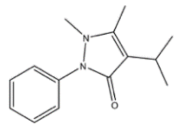
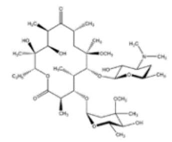
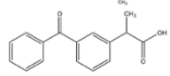
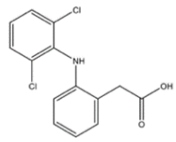
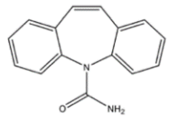
| Name | Molecular formula | Molecular mass | Precursor ion (polarity) | Product ion (CE) | Structure |
|---|---|---|---|---|---|
| Amilsulpride | $C_{17}H_{27}N_3O_4S_4$ | 369.48 | 370.08 (+) | 242.00 (27) -196.00 (42) |  |
| Metoprolol | $C_{15}H_{25}NO_3$ | 267.37 | 268.07 (+) | 116.09 (17) – 74.16 (21) |  |
| Propylphenazone | $C_{14}H_{18}N_2O$ | 238.14 | 231.03 (+) | 189.90 (21) – 56.20 (32) |  |
| Clarithromycin | $C_{38}H_{69}NO_{13}$ | 747.96 | 748.25 (+) | 590.24 (18) – 157.92 (26) |  |
| Ketoprofen | $C_{16}H_{14}O_3$ | 254.28 | 254.96 (+) | 208.90 (13) – 77.12 (39) |  |
| Diclofenac | $C_{14}H_{11}Cl_2NO_2$ | 296.15 | 295.87 (+) | 249.80 (15) – 213.90 (36) |  |
| Carbamazepine | $C_{15}H_{12}N_2O$ | 236.27 | 236.80 (+) | 193.90 (19) – 192.01 (24) |  |

**Table 2.** Overview of the PAEs molecules analyzed.

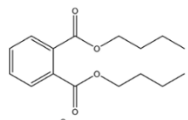
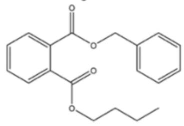
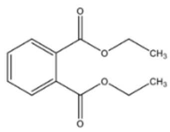
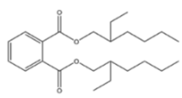
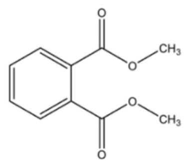
| Name | Molecular formula | Molecular mass | Precursor ion (polarity) | Product ion (CE) | Structure |
|---|---|---|---|---|---|
| Dibutyl Phthalate (DBP) | $C_{16}H_{22}O_4$ | 278.35 | 279.14 (+) | 149.09 (19) – 121.12 (30) |  |
| Benzylbutyl Phthalate (BBzP) | $C_{19}H_{20}O_4$ | 312.36 | 313.11 (+) | 149.11 (21) – 91.32 (29) |  |
| Diethyl Phthalate (DEP) | $C_{12}H_{14}O_4$ | 222.24 | 223.08 (+) | 149.08 (18) – 121.13 (28) |  |
| Bis (2-ethylhexyl) Phthalate (DEHP) | $C_{24}H_{38}O_4$ | 390.56 | 391.18 (+) | 149.11(25) – 121.09 (27) |  |
| Dimethyl Phthalate (DMP) | $C_{10}H_{10}O_4$ | 194.19 | 194.84 (+) | 163.09 (11) – 77.26 (33) |  |

**Table 3.** List of collected specimens and collection parameters.

| Collected specimen resume | | | | | | |
|---|---|---|---|---|---|---|
| **Sample ID** | **Species** | **Sampling date** | **Site** | **Depth (m)** | **Habitat** | **Health status** |
| PA0523CLA1 | *Cladocora caespitosa* | 30/05/22 | Paraggi | 5 | Rocky bottom | Healthy |
| PA0523CLA2 | *Cladocora caespitosa* | 30/05/22 | Paraggi | 7 | Rocky bottom | Healthy |
| PA0523CLA3 | *Cladocora caespitosa* | 30/05/22 | Paraggi | 6 | Rocky bottom | Healthy |
| PA0523CLA4 | *Cladocora caespitosa* | 30/05/22 | Paraggi | 6 | Rocky bottom | Healthy |
| PA0523CLA5 | *Cladocora caespitosa* | 30/05/22 | Paraggi | 8 | Rocky bottom | Healthy |
| PA0523EUN1 | *Eunicella cavolini* | 30/05/22 | Paraggi | 21 | Rocky slope | Healthy |
| PA0523EUN2 | *Eunicella cavolini* | 30/05/22 | Paraggi | 23 | Rocky slope | Healthy |
| PA0523EUN3 | *Eunicella cavolini* | 30/05/22 | Paraggi | 23 | Rocky slope | Healthy |
| PA0523EUN4 | *Eunicella cavolini* | 30/05/22 | Paraggi | 25 | Rocky slope | Healthy |
| PA0523EUN5 | *Eunicella cavolini* | 30/05/22 | Paraggi | 25 | Rocky slope | Healthy |
| PA0523MAD1 | *Madracis pharensis* | 30/05/22 | Paraggi | 20 | Rocky slope | Healthy |
| PA0523MAD2 | *Madracis pharensis* | 30/05/22 | Paraggi | 20 | Rocky slope | Healthy |
| PA0523MAD3 | *Madracis pharensis* | 30/05/22 | Paraggi | 20 | Rocky slope | Healthy |
| PA0523MAD4 | *Madracis pharensis* | 30/05/22 | Paraggi | 20 | Rocky slope | Healthy |
| PA0523MAD5 | *Madracis pharensis* | 30/05/22 | Paraggi | 20 | Rocky slope | Healthy |
| PA0523PAR1 | *Parazoanthus axinellae* | 30/05/22 | Paraggi | 17 | Overhang | Healthy |
| PA0523PAR2 | *Parazoanthus axinellae* | 30/05/22 | Paraggi | 17 | Overhang | Healthy |
| PA0523PAR3 | *Parazoanthus axinellae* | 30/05/22 | Paraggi | 17 | Overhang | Healthy |
| PA0523PAR4 | *Parazoanthus axinellae* | 30/05/22 | Paraggi | 17 | Overhang | Healthy |
| PA0523PAR5 | *Parazoanthus axinellae* | 30/05/22 | Paraggi | 17 | Overhang | Healthy |

**Table 4.** Active pharmaceutical ingredients (APIs) concentrations (ng/g wet weight) detected in the anthozoans.

| APIs | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample ID** | **Amilsulpride** | **Metoprolol** | **Propylphenazone** | **Carbamazepine** | **Clarithromycin** | **Ketoprofen** | **Diclofenac** |
| PA0523CLA1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 6.3 |
| PA0523CLA2 | n.d. | 4.5 | n.d. | 3.0 | n.d. | 19.5 | n.d. |
| PA0523CLA3 | n.d. | n.d. | n.d. | 3.0 | n.d. | n.d. | 6.2 |
| PA0523CLA4 | n.d. | 4.5 | n.d. | 3.0 | n.d. | n.d. | 6.3 |
| PA0523CLA5 | n.d. | n.d. | n.d. | 3.0 | n.d. | 16.5 | n.d. |
| PA0523EUN1 | n.d. | 4.5 | n.d. | n.d. | n.d. | n.d. | 6.3 |
| PA0523EUN2 | n.d. | 4.5 | n.d. | 3.1 | n.d. | n.d. | 6.3 |
| PA0523EUN3 | n.d. | n.d. | n.d. | 3.0 | n.d. | 16.8 | 6.3 |
| PA0523EUN4 | n.d. | 4.5 | n.d. | 3.1 | n.d. | n.d. | n.d. |
| PA0523EUN5 | n.d. | n.d. | n.d. | 3.1 | n.d. | 19.1 | 6.3 |
| PA0523MAD1 | n.d. | n.d. | n.d. | 3.1 | n.d. | 14.5 | 6.3 |
| PA0523MAD2 | n.d. | n.d. | n.d. | 3.1 | n.d. | 20.2 | n.d. |
| PA0523MAD3 | n.d. | 4.5 | n.d. | 3.0 | n.d. | n.d. | 6.3 |
| PA0523MAD4 | n.d. | n.d. | n.d. | 3.1 | n.d. | n.d. | 6.6 |
| PA0523MAD5 | n.d. | n.d. | n.d. | n.d. | n.d. | 20.4 | 6.3 |
| PA0523PAR1 | n.d. | n.d. | n.d. | n.d. | n.d. | 57.4 | 6.6 |
| PA0523PAR2 | n.d. | 4.5 | n.d. | n.d. | n.d. | 40.4 | 6.4 |
| PA0523PAR3 | n.d. | 4.5 | n.d. | n.d. | n.d. | 42.4 | 6.4 |
| PA0523PAR4 | n.d. | 4.5 | n.d. | n.d. | n.d. | 32.1 | 6.4 |
| PA0523PAR5 | n.d. | 4.5 | n.d. | n.d. | n.d. | 39.5 | n.d. |

**Table 5.** Phthalic acid esters (PAEs) concentrations (ng/g wet weight) detected in the anthozoans.

| | PAEs | | | |
|---|---|---|---|---|
| **Sample ID** | **DEP** | **BBzP** | **DBP** | **DEHP** |
| PA0523CLA1 | n.d. | 1.4 | 12.0 | n.d. |
| PA0523CLA2 | n.d. | 1.3 | n.d. | n.d. |
| PA0523CLA3 | 6.2 | 15.1 | 8.6 | 7.3 |
| PA0523CLA4 | n.d. | n.d. | n.d. | n.d. |
| PA0523CLA5 | n.d. | 2.7 | 12.6 | n.d. |
| PA0523EUN1 | n.d. | n.d. | 16.7 | n.d. |
| PA0523EUN2 | n.d. | n.d. | 7.4 | n.d. |
| PA0523EUN3 | n.d. | n.d. | 9.4 | n.d. |
| PA0523EUN4 | n.d. | n.d. | n.d. | n.d. |
| PA0523EUN5 | n.d. | 0.8 | n.d. | n.d. |
| PA0523MAD1 | n.d. | n.d. | 7.7 | n.d. |
| PA0523MAD2 | 12.4 | n.d. | 25.9 | n.d. |
| PA0523MAD3 | n.d. | 1.9 | 7.2 | n.d. |
| PA0523MAD4 | n.d. | 2.1 | n.d. | n.d. |
| PA0523MAD5 | n.d. | n.d. | 6.9 | 11.0 |
| PA0523PAR1 | 9.8 | 3.2 | n.d. | 44.1 |
| PA0523PAR2 | n.d. | 0.8 | 24.8 | 11.1 |
| PA0523PAR3 | n.d. | n.d. | 22.2 | n.d. |
| PA0523PAR4 | 5.7 | n.d. | 21.5 | n.d. |
| PA0523PAR5 | n.d. | n.d. | 23.7 | n.d. |

**Table 6.** Range and mean concentration of PAEs and APIs in the four coral species analyzed expressed in ng/g (n.d. = non-detected).

| | PAEs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DEP | | BBzP | | DBP | | DEHP | |
| **SPECIES** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** |
| *Cladocora caespitosa* | 1.3 ± 2.8 | 0 – 6.2 | 4.1 ± 6.1 | 0 – 15.1 | 6.6 ± 6.2 | 0 – 12.6 | 1.5 ± 3.3 | 0 – 7.4 |
| *Eunicella cavolini* | n.d. | n.d. | 0.2 ± 0.3 | 0 – 0.8 | 6.7 ± 7.0 | 0 – 16.7 | 0.0 ± 0.0 | n.d. |
| *Madracis pharensis* | 2.5 ± 5.6 | 0 – 12.5 | 0.8 ± 1.1 | 0 – 2.1 | 9.6 ± 9.7 | 0 – 25.9 | 2.2 ± 4.9 | 0 – 11.0 |
| *Parazoanthus axinellae* | 3.1 ± 4.5 | 0 – 9.9 | 0.8 ± 1.4 | 0 – 3.3 | 18.5 ± 10.4 | 0 – 24.9 | 11.1 ± 19.1 | 0 – 44.2 |

| | APIs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Metoprolol | | Carbamazepine | | Ketoprofen | | Diclofenac | |
| **SPECIES** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** |
| *Cladocora caespitosa* | 1.8 ± 2.5 | 0 – 4.6 | 2.4 ± 1.4 | 0 – 3.0 | 7.2 ± 9.9 | 0 – 19.5 | 3.8 ± 3.5 | 0 – 6.4 |
| *Eunicella cavolini* | 2.7 ± 2.5 | 0 – 4.5 | 2.5 ± 1.4 | 0 – 3.2 | 7.2 ± 9.9 | 0 – 19.2 | 5.1 ± 2.8 | 0 – 6.4 |
| *Madracis pharensis* | 0.9 ± 2.0 | 0 – 4.6 | 2.5 ± 1.4 | 0 – 3.2 | 11.1 ± 10.4 | 0 – 20.5 | 5.1 ± 2.8 | 0 – 6.6 |
| *Parazoanthus axinellae* | 3.6 ± 2.0 | 0 – 4.6 | n.d. | n.d. | 42.4 ± 9.3 | 0 – 57.5 | 5.2 ± 2.9 | 0 – 6.7 |

## 2.8. FIGURES

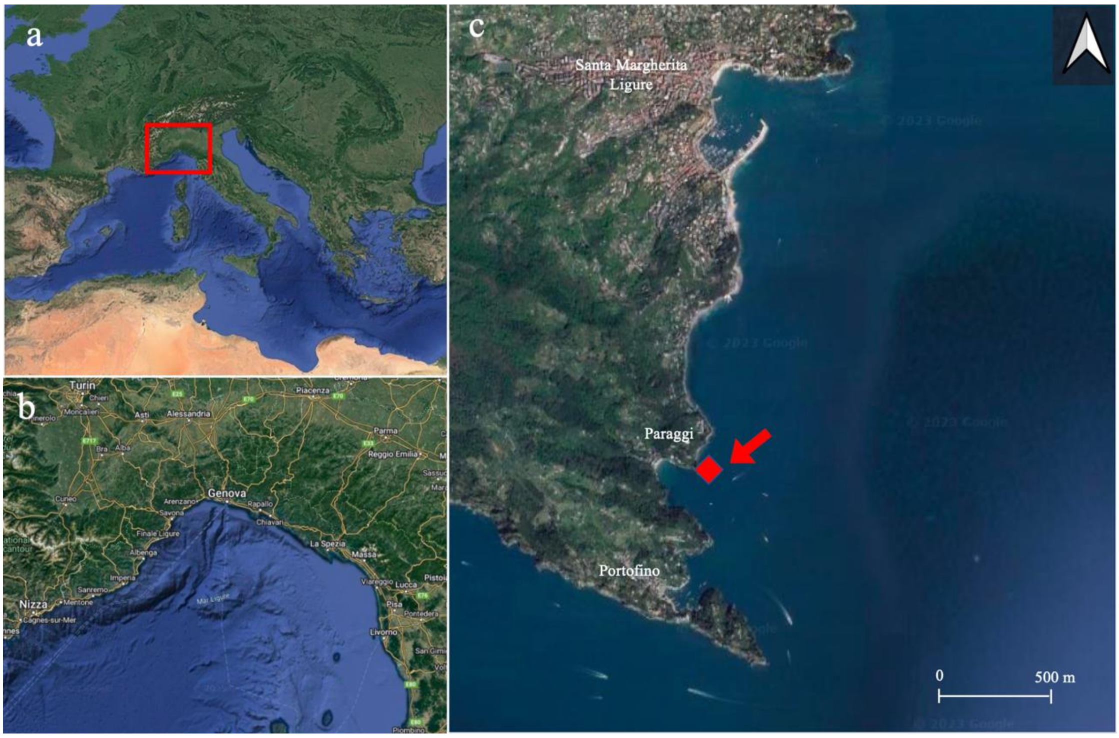



**Figure 1.** Geographical contextualization of the study area, Paraggi in Portofino Marine Protected Area, Liguria, Italy (44°18'41.0"N; 9°12'47.4"E), highlighting the sampling site: **a)** Mediterranean Sea; **b)** close up on Northwestern Mediterranean Sea; **c)** close up on Portofino Marine Protected Area with the sampling site highlighted.

Maps made from MapTiler, GoogleHybridMap, and GoogleSatelliteMap loaded into QGIS.

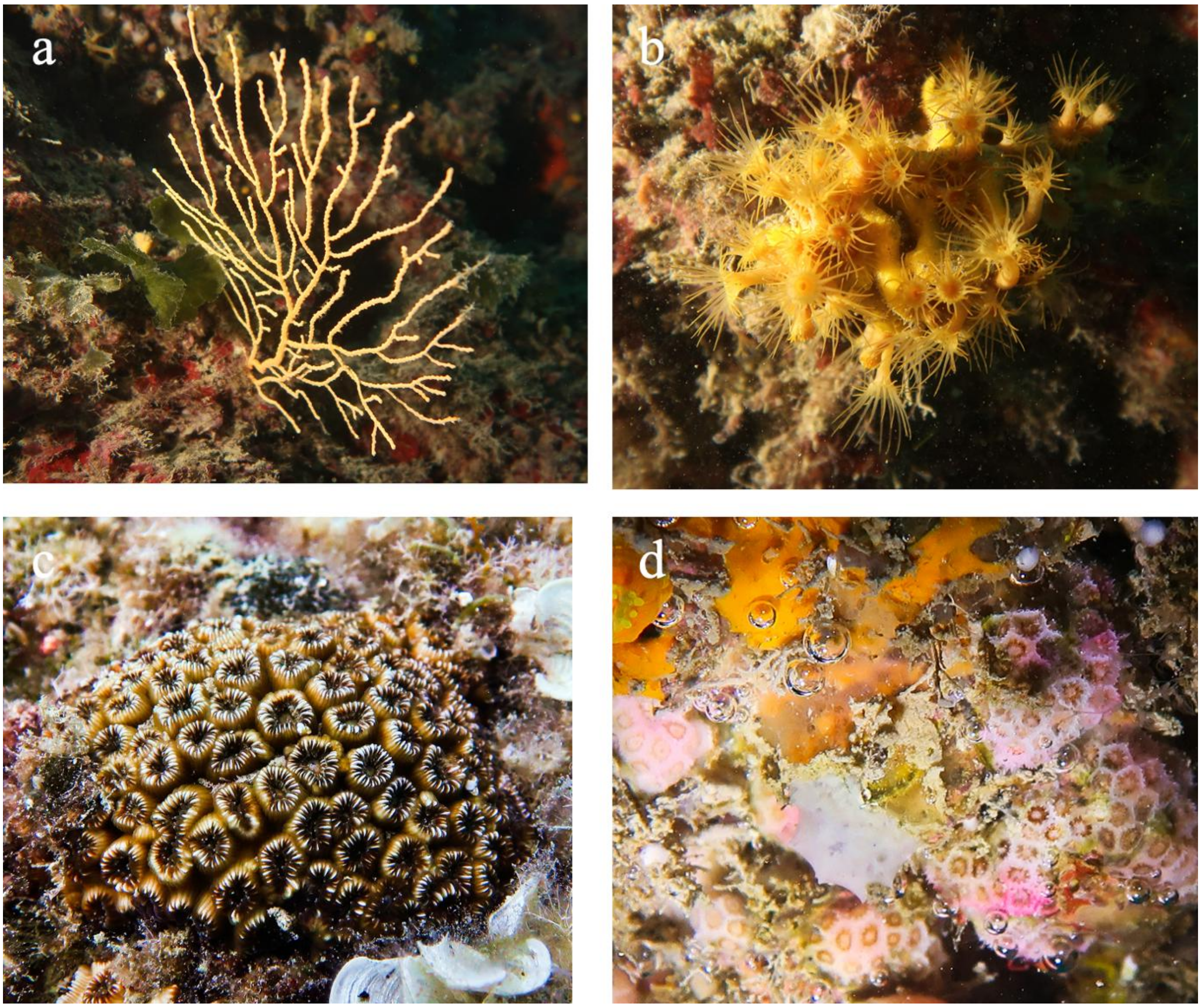


**Figure 2.** Overview of the four anthozoans species considered for the study: **a)** *Eunicella cavolini*; **b)** *Parazoanthus axinellae*; **c)** *Cladocora caespitosa*; and **d)** *Madracis pharensis*.

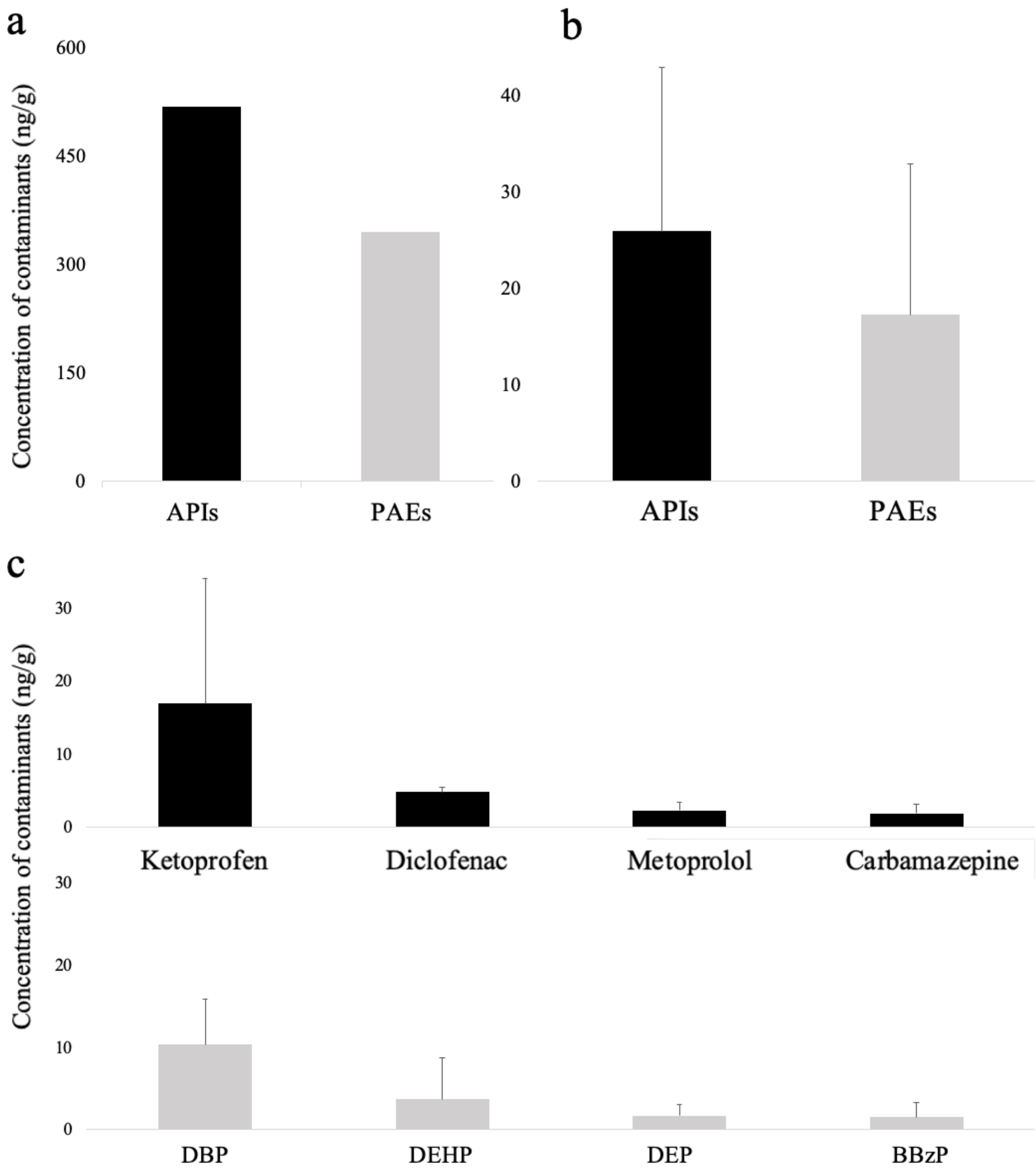



**Figure 3.** Overview of the comparison between APIs and PAEs: **a)** Overall total amount of contaminants; **b)** Overall average of contaminants; and c) Average concentration of each contaminant.

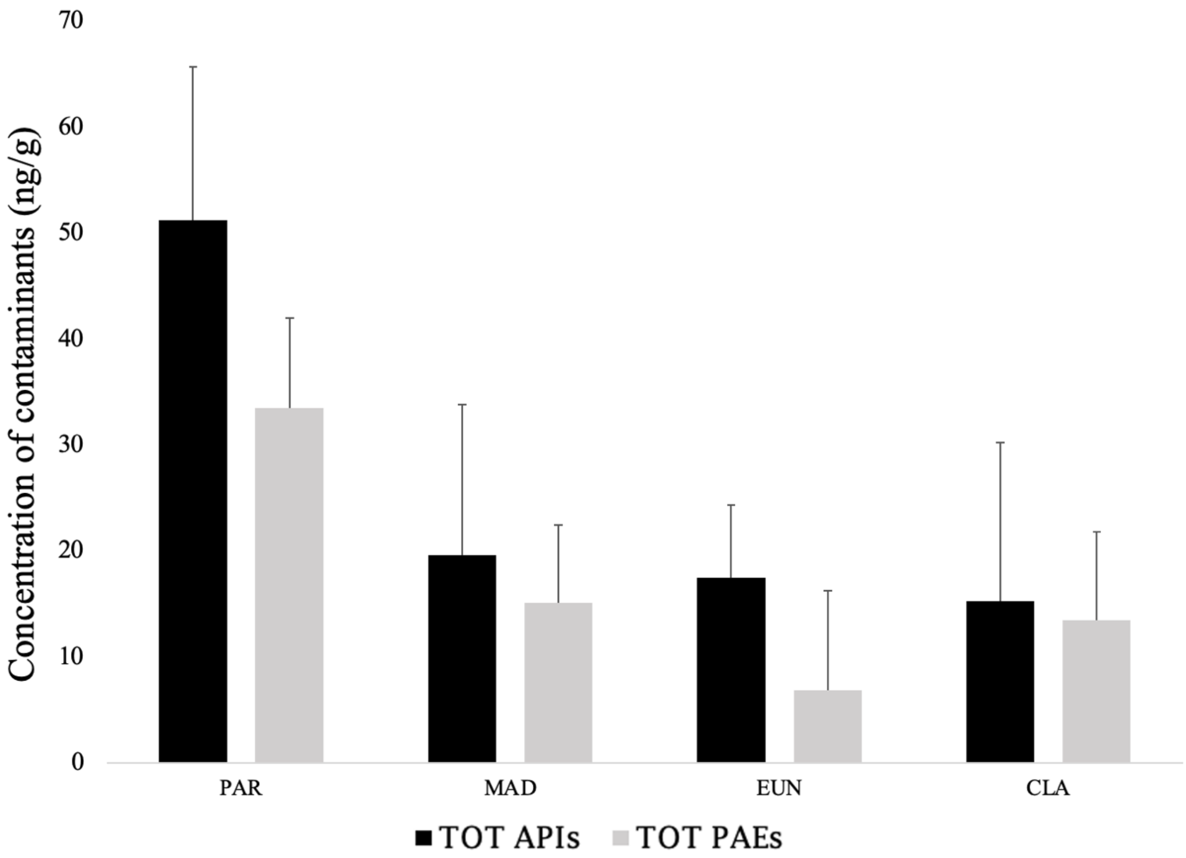



**Figure 4.** The average total concentration of both classes of contaminants per species.

# CHAPTER 3

## Silent sunscreen contaminants: occurrence of UV-filter molecules in *Paramuricea clavata* and the potential mitigating role of Marine Protected Area in the Mediterranean Sea

This work is submitted as Brief Report:

Gobbato, J., Becchi, A., Parmegiani, A., Collina, E., Lasagni, M., Saliu, F., Galli, P., and Montano, S. Silent sunscreen contaminants: occurrence of UV-filter molecules in *Paramuricea clavata* and the potential mitigating role of Marine Protected Area in the Mediterranean Sea. *Coral Reef, European Coral Reef Symposium Special Issue*

## 3.1. ABSTRACT

The marine environment and its vital biodiversity are increasingly threatened by anthropogenic pollution, particularly UV-filter compounds in sunscreen formulations. These contaminants enter coastal waters directly through recreational activities or indirectly via wastewater, posing ecological risks, especially in densely populated or touristic regions. Despite several studies documenting the adverse effects of UV filters on marine life, including anthozoans with enhanced coral bleaching, impaired reproduction, and increased oxidative stress, research in the Mediterranean region remains limited.

This study investigates the occurrence of UV filters in *Paramuricea clavata* within and outside the Portofino Marine Protected Area (MPA) in the Northwestern Mediterranean Sea. Findings confirm the potential for bioaccumulation of oxybenzone in *P. clavata*, possibly influenced by environmental conditions, with higher contaminant levels outside the MPA, highlighting the potential protective role of MPAs in mitigating bioaccumulation. Although overall low concentrations were detected, results indicate potential bioaccumulation and environmental persistence of UV filters, suggesting the need for further research to understand their long-term residency and impact on marine ecosystems.

## 3.2. INTRODUCTION

The marine environment holds a universally recognized significance for our planet, serving as a vital habitat for millions of species (Fisher et al., 2015) and delivering several ecosystem services to coastal communities (Costanza et al., 2014; Chen et al., 2015). However, climate change and human activities increasingly threaten these invaluable ecosystems, primarily through pollution and habitat degradation (Hoegh-Guldberg et al., 2017; Hughes et al., 2017, 2018). One of the most impacting anthropic threats is the contamination of coastal waters from emerging contaminants such as plastic, pharmaceuticals, and personal care products that are posing significant risks to the marine environment, particularly in densely populated and tourist-heavy regions (Cadena-Aizaga et al., 2020; Mitchelmore et al., 2019, 2021). In this context, the environmental impact of sunscreens has received considerable scientific attention in recent years (Sanchez-Quiles & Tovar-Sanchez, 2015), prompting research into new formulations and rheological properties to minimize their ecological impact (Tovar-Sanchez et al., 2020).

Sunscreens protect the skin from harmful solar radiation by providing a physical barrier through molecules known as UV filters, which absorb, reflect, or scatter UV light, typically combined to provide broad-spectrum protection against both UV-A (315-400 nm) and UV-B (280-315 nm) radiation, with formulations often containing over 20 compounds (Danovaro et al., 2008; Witkins & Sallach, 2021). Unfortunately, these molecules can enter coastal waters either directly from swimmers wash-off or indirectly via wastewater treatment effluents following production or excretion (Tovar-Sánchez et al., 2013; Sánchez Rodríguez et al., 2015; Cadena-Azaiga et al., 2020). As a result, coral reefs worldwide face threats from UV filter exposure, with an estimated release of 6000 to 14000 tons of sunscreen lotion yearly (Danovaro et al., 2008; Tsui et al., 2017). These raised concerns that led to regulations and bans on certain sunscreens in different regions (Fivenson et al., 2020; Narla et al., 2020) and several studies worldwide on the most prevalent organic chemical UV filters including oxybenzone, benzophenone, para-aminobenzoic acid (PABA), cinnamates, homosalate, camphor, and anthranilates (Chisvert et al., 2001; Danovaro & Corinaldesi, 2003; Kim & Choi, 2014; Fivenson et al., 2020); as well as the most common mineral inorganic UV-filters titanium dioxide and zinc oxide (Lewicka et al., 2013). These works underscore the widespread contamination of coastal waters by UV filters and the potential ecological implications across diverse marine environments and organisms (McCoshum et al., 2016; Corinaldesi et al., 2017; Fivenson et al., 2020).

Among them, anthozoans, as sessile non-selective suspension feeders, are especially vulnerable to interaction with several contaminants. Yet, insufficient studies have been conducted worldwide on their impact and adverse effects, especially in the Mediterranean region. The most studied is oxybenzone, which has been demonstrated to cause adverse effects such as enhanced bleaching (Danovaro & Corinaldesi, 2003; Danovaro et al., 2008) and damage to coral larvae (planulae) with negative impacts on reproductive success (Downs et al., 2014, 2015). Inorganic UV filters are generally considered safer; however, certain types of titanium dioxide and zinc oxide nanoparticles increase the production of reactive oxygen species (ROS) under UV illumination, enhancing the oxidative stress to marine organisms (Hanna et al., 2013; Lewicka et al., 2013; Downie et al., 2023; He et al., 2023).

Few studies have examined the occurrence of UV-filter contamination in the Mediterranean Sea; therefore, a significant gap remains concerning the Italian coast and its benthic communities.

To address this gap, our study investigated the presence of UV filters in *Paramuricea clavata*, one of the most iconic and ecologically important species in Mediterranean ecosystems. Additionally, we evaluated whether the regulation within the Marine Protected Area (MPA) had a potential mitigating effect on preserving these organisms from this type of contamination. Our research aimed to provide new valuable insights to improve the protection of marine biodiversity in the Mediterranean region.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Study area and sampling

In the summer of 2023, two underwater surveys were conducted to investigate the presence and concentration of UV filter molecules in *Paramuricea clavata* in the Northwestern Mediterranean Sea at sites within and outside the Portofino Marine Protected Area (MPA) in the coastal area of Punta Chiappa, near Camogli, Liguria (Italy) (Figure 1a). The Portofino MPA encompasses diverse coastal environments across multiple municipalities, each subject to considerable tourism pressures throughout the year. These pressures influence protected and non-protected areas, with significant implications for marine biodiversity, water quality, and the overall resilience of local ecosystems (Dobler, 2002; Bevilacqua et al., 2021).

Our sampling was conducted at two distinct sites within the Portofino Marine Protected Area (MPA), which were selected to highlight the variation in human impact both within and outside the protected zone. Specifically, Site A was located outside the MPA, in close proximity to a cliff that is frequently visited by people engaged in recreational activities such as swimming, sunbathing, and snorkeling (Figure 2a). Furthermore, this site is relatively sheltered from strong currents due to the adjacent promontory, resulting in a more stable and less variable aquatic environment characterized by a patchy presence of *P. clavata* (Figure 2b). The significant daily presence of tourists at Site A may lead to localized disturbances and potential pollutant inputs, as observed in previous studies (Mitchlemore et al., 2019; Cadeina-Aizaga et al., 2022). Conversely, Site B is situated within the protected zone of the Portofino MPA, positioned slightly further offshore, where only regulated scuba diving activities are permitted to limit the direct human impact on the area (Figure 3a). Additionally, Site B is more exposed to hydrodynamic forces, which promote enhanced water exchange, potentially mitigating some effects of human disturbance, characterized by a rich *P. clavata* forest (Figure 3b). This site, thus, serves as a comparative baseline for evaluating contamination variance between the two sites. For the analyses, *P. clavata* was selected due to its wide distribution and abundance (Figure 1b), reducing the pressure exerted on the local benthic community, and for its essential ecosystem engineer role across the Mediterranean, making it a valuable proxy for anthozoans in the region (Rossi et al., 2017; Iborra et al., 2022). A total of 20 samples were collected by SCUBA diving between 15-30 m depth, wrapped in aluminum foil, transferred in glass vials to prevent contamination, and stored at -20°C.

### 3.3.2. SPME-GC-MS/MS analysis

The UV-filter determination in coral tissue was performed using solid-phase microextraction (SPME) followed by gas chromatography coupled with mass spectrometry (GC-MS), according to the method described by Yılmazcan et al. 2015. Briefly, for each sample, a C18 SPME fiber (Sigma-Aldrich 57234-U) was used for the extraction. GC analyses were conducted on an Agilent 8860 instrument equipped with a 5977B single quadrupole detector, with analytes detection and quantification operated in selected ion monitoring. In details, SPME sampling was performed in direct immersion mode (DI) following an adaptation of the method described by Ocaña-Rios (Ocaña-Rios et al., 2019). Extraction time was set up at 30 min at 25 °C. After sampling, the fiber was withdrawn, rinsed with deionized water, and then desorbed with 250 µL of acetonitrile (500 rpm, 1 h) twice for complete recovery of the analytes. The extracts were combined and evaporated to dryness with a gentle flow of nitrogen and derivatized with 40 µL of MSTFA (60 °C for 30 min); after cooling, 10 ng of internal standard was added and analyzed by GC-MS.

Under the optimized conditions, the SPME-GC/MS method showed a linear response in the concentration range of 0.5–100 ng/g with correlation coefficients of 0.990–0.999. The LOQ calculated from the matrix-matched calibration curves resulted in 0.012 µg/g d.w. for oxybenzone and 0.005 µg/g for octinoxate. Surface water samples were filtered through a 1.2-µm glass-fiber filter and acidified with 0.1% (v/v) 3 M HCl, and the analysis was conducted using the protocol reported by Yılmazcan Ö et al. 2015. In particular, the UV filters tested included Oxybenzone (2-hydroxy-4-methoxybenzophenone), Octinoxate (ethylhexyl methoxycinnamate), Oxybenzone-(phenyl-d5), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA).

### 3.3.3. Details of the GC-MS method applied

GC-MS analyses were carried out using an Agilent 8860 instrument equipped with a 5977B single quadrupole detector. The GC injector was operated in splitless mode at 260 °C. The injection volume was 1 µL. Analytes were separated with a non-polar HP-5MS UI (Agilent Technologies, USA) capillary column with 5% phenyl/95% dimethyl polysiloxane and dimensions of 30 m × 0.250 mm, film thickness 0.25 µm. Helium (purity > 99.999%, Sapio, Italy) was used as carrier gas at a constant low rate of 1 mL/min. The oven temperature was programmed for a total analysis time of 20 min per sample as follows: 2 min at 50°C, then increased to 110 °C at 20°C/min, then further increased to 300°C at 50 C/min, which was held for 5 min. Mass spectrometric detection was performed by applying a Scan Speed of 0.1 amu sec-1 and a Scan rate of 40-450 amu. The detector was set at a transfer line temperature of 230 °C and the ion source to 200°C, using a positive ion detection mode. Mass spectra were acquired in the electron impact mode at 70 eV, and the following measurements were performed in SCAN/SIM acquisition. Retention times and identifying and quantifying ions selected for the target compounds are shown in Table 1. The samples were analyzed in triplicate, and blank runs were performed before and after each analysis.

### 3.3.4. Chemicals and materials

C18 SPME fiber probes (45 µm, 1.5 cm coating length) were purchased from Sigma Aldrich (Milano, Italy. The fibers were conditioned according to instructions provided by the supplier. UV filter chemicals (oxybenzone (2-hydroxy-4-methoxybenzophenone) and octinoxate (ethylhexyl methoxycinnamate), Oxybenzone-(phenyl-d5), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), hydrochloric acid (37%) sodium chloride, HPLC-grade methanol and acetonitrile were purchased from Merck Chemicals S.P.A (Milano, Italy, www.sigmaaldrich.it). UV filter stock solutions were prepared in methanol at 10 mg/mL and stored at 4ºC. Working standard solutions were prepared by dilution of stock solutions in Ultra-pure water. Agarose gel spiked at 0.5, 1, 2, 10, 25, 50, and 100 ng/g was used as a reference for the matrix-matched calibration.

### 3.3.5. Statistical analysis

All the data obtained were tested for normality with Kolmogorov–Smirnov tests. As the normal distribution and homogeneity of variance were violated, Mann-Whitney tests were performed to compute the mean differences in the concentration of contaminants between the sampling sites. Data are presented as the arithmetic mean ± standard error and in ng/g wet weight unless stated otherwise. All the statistical analyses were performed using IBM SPSS 28 Software (IBM SPSS 28, New York, NY, USA).

## 3.4. RESULTS

Oxybenzone and/or octinoxate were detected in 6 of the 20 corals surveyed (30%), while in the remaining specimens, the instrumental response was below the method detection limit (Tab. 1). Notably, only one sample collected within the MPA showed a detectable level of contamination, while the frequency of detection was higher outside the MPA (50%; Table 2). Moreover, the analyses of seawater samples also revealed that the concentration of UV filters was below detection limits, confirming bioaccumulation within coral tissues.

Overall, the total amount of UV filters detected was 180 ng/g (wet weight), with oxybenzone showing a higher concentration than octinoxate (Figure 4a) but with maximum values of 31 ng/g and 39 ng/g, respectively. The average concentrations were also comparable, with Oxybenzone at 5.4 ± 2.3 ng/g and octinoxate at 3.7 ± 2.1 ng/g (Figure 4b).

Samples were collected both inside and outside its boundaries to test the influence of MPA. The total amount detected was 15 ng/g inside and 165 ng/g outside the MPA (Figure 5a). On average, higher concentrations of both contaminants were found outside the MPA, with oxybenzone at 9.2 ± 3.9 ng/g compared to the 7.3 ± 3.9 ng/g of octinoxate (Figure 5b). Interestingly, octinoxate was not detected inside the MPA, while oxybenzone was present in a lower concentration, precisely 1.5 ± 1.5 ng/g (Figure 5b).

## 3.5 DISCUSSIONS

Given the semi-enclosed nature of the Mediterranean basin, which is one of the most heavily impacted regions by anthropogenic activities and pollution (Giorgi et al., 2006; Coll et al., 2011; Bevilacqua et al., 2021), we expected to find similar contaminant concentrations to those detected in other anthozoan species and marine organisms worldwide (Gago-Ferrero et al., 2012; Labille et al., 2020; Mitchelmore et al., 2021; Cadena-Aizaga et al., 2022; Witkins & Sallach, 2021; Gobbato et al., 2024). However, despite the significant presence of human activities, only a minority of the sample exhibited detectable contamination (Tab. 1), with considerable variability in contaminant levels among them. These findings suggest that while UV contamination may be minimal, it remains a concern, as low-level contamination was still observed. Seawater analysis also revealed UV filter concentrations below detection limits, underscoring the potential for bioaccumulation of these contaminants in *P. clavata*. Indeed, to our knowledge, this study represents the first documentation of these types of pollutants in this species and region. The significant role of this species within the Mediterranean benthic community underscores the alarming nature of the current lack of evidence and results, claiming a more comprehensive and diffuse approach to address these issues effectively.

Accurately assessing the occurrence and impact of emerging contaminants in marine environments is extremely challenging due to the vastness of seawaters and the consequent dispersion and dilution of pollutants in the water columns (Brumovsky et al., 2017; Hartmann et al., 2017). Therefore, further research with a larger dataset is essential to have a proper picture of the situation because the low detection rates may be influenced by limitations in the sensitivity of the method (Ramos et al., 2015) or specific factors that may impact the efficacy of detection in *P. clavata*. Moreover, the primary mechanism of internalization of these pollutants in anthozoan is thought to be ingestion but remains unknown, further complicating our understanding of their impacts. Indeed, we may hypothesize that detoxification and excretion mechanisms of *P. clavata* also influence the extent of internalized contaminants. However, without a comprehensive understanding of the processes involved, bioavailability and exposure duration likely play critical roles. These factors are highly variable in a complex and dynamic environment like the marine one (Hartmann et al., 2017; Gogoi et al., 2018; Stefanakis & Becker, 2020), as reflected by our findings, which showed significant intraspecific variability. Similar variability has been observed for other categories of contaminants and anthozoan species in previous studies, where metabolic activity and detoxification capacity were proposed to be influenced by the different life stages of the individuals (Murphy & Richmond, 2016; Bythell et al., 2017; Linsmayer et al., 2020; Gobbato et al., 2024).

The differences in contaminant levels inside and outside the MPA revealed important insight. As previously discussed, bioavailability and exposure seem to be the most critical factors influencing bioaccumulation, suggesting that MPA regulations and management could potentially mitigate the internalization process of these compounds. Specifically, our sampling site inside the MPA is within Zone B protected area, where most activities are prohibited and only licensed operators are permitted limited access, contrasting significantly with the more accessible and heavily frequented site outside the MPA. This increased human presence and activity in the latter likely contributed to higher contaminant input, explaining the trend in higher amounts found in *P. clavata* outside the MPA. A comprehensive understanding of contaminant dynamics requires taking into consideration multiple interacting factors, including exposure levels, proximity to contamination sources, and hydrodynamics. These factors significantly influence the spatial distribution and concentration of pollutants in the water column (Carson et al., 2011; Lee et al., 2014; Labille et al., 2020; Miller et al., 2021; Wheate, 2022). Our findings support this theory, demonstrating that Site B, more sheltered from hydrodynamic influences due to its proximity to the promontory, exhibited a more stable and less variable aquatic environment, resulting in higher contamination levels. Moreover, the choice of *P. clavata* extended our sampling to greater depths (15 – 30 m) compared to previous studies reporting UV filter contamination that generally stopped up to 10 meters (Corinaldesi et al., 2018; Mitchlemore et al., 2019; Cadena-Aizaga et al., 2022) or even only evaluated for ecotoxicological analyses in laboratory tanks (Downs et al., 2015; He et al., 2019). Therefore, further studies need to consider a broader range of sites, depths, and seasonal variation, as it is expected to have a higher impact in the summer due to enhanced anthropic impact.

These findings highlight the importance and need for further research to explore how specific regulatory measures and environmental conditions within MPAs influence contaminant dynamics. Thus, this study highlighted the potential of marine protected area regulations to mitigate the bioaccumulation of pollutants in marine ecosystems and the protective role such areas may play against anthropogenic contamination. Further and continued investigation across diverse MPAs and regions will be essential for developing a broader understanding of bioaccumulation processes and informing strategies to safeguard vulnerable species like *P. clavata*, ultimately contributing to improved management and conservation of marine biodiversity. Additionally, improving the tools to assess pollution levels and bioaccumulation in the natural environment is crucial, as ecotoxicological studies conducted under laboratory conditions often use contaminant concentrations significantly higher and in much shorter exposure periods than what is typically found in nature. While these studies are invaluable for identifying potential adverse effects, they may not fully capture the impacts of chronic long-term exposure to low contaminant levels that organisms experience in the wild (Li et al., 2024). This is particularly relevant in marine ecosystems, where contaminants can persist at low concentrations but accumulate over extended times, potentially leading to substantial ecological impacts on different organisms (Tsui et al., 2014; Schneider et al., 2019; Caloni et al., 2021). The few studies focusing on anthozoans and close related organisms suggest similar concerns, with evidence of occurrence (Saliu et al., 2018, 2019, 2022; Montano et al., 2020; Rizzi et al., 2020, 2023; Raguso et al., 2022; Isa et al., 2022; Gobbato et al., 2024) and detrimental effects, such as enhanced bleaching (Danovaro & Corinaldesi, 2003; Danovaro et al., 2008), reduced reproductivity (Downs et al., 2014, 2015), and increased oxidative stress (Hanna et al., 2013; Lewicka et al., 2013; Downie et al., 2023; He et al., 2023; Isa et al., 2024). In conclusion, further research is necessary to determine whether bioavailability, physiological processes, or other environmental factors are the predominant drivers of contaminant internalization and accumulation in *P. clavata* and similar anthozoans. This study provides the first evidence of UV filter contamination in this species and region, highlighting a previously underestimated environmental concern. These findings are essential for refining management strategies and enhancing conservation efforts, which may play a key role in reducing pollutant exposure and promoting healthier ecosystems in the Mediterranean region. Expanding our understanding of these dynamics will allow for the development of more effective protection measures to improve the potential benefits of MPAs.

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## 3.7. TABLES

**Table 1**. Retention times and selected ions for the analysis of the target compounds by DI-SPME-GC-MS

| **Compound** | **Retention time** | **Molecular Weight (g/mol)** | **Monitored m/z** |
|---|---|---|---|
| Octinoxate | 11.3 | 290.4 | 204, 249, 362 |
| Oxybenzone | 12.9 | 228.2 | 242, 285, 300 |

**Table 2**. Overview of the UV filter concentration in the sample analyzed. (Bdl = below detection limit).

| Sample | MPA site | Oxybenzone (ng/g) | Octinoxate (ng/g) |
|---|---|---|---|
| OUT_S1 | A | 27 | 15 |
| OUT_S2 | A | 18 | Bdl |
| OUT_S3 | A | Bdl | 7 |
| OUT_S4 | A | Bdl | Bdl |
| OUT_S5 | A | 16 | 12 |
| OUT_S6 | A | Bdl | Bdl |
| OUT_S7 | A | Bdl | Bdl |
| OUT_S8 | A | 31 | 39 |
| OUT_S9 | A | Bdl | Bdl |
| OUT_S10 | A | Bdl | Bdl |
| IN_S1 | B | Bdl | Bdl |
| IN_S2 | B | Bdl | Bdl |
| IN_S3 | B | Bdl | Bdl |
| IN_S4 | B | 15 | Bdl |
| IN_S5 | B | Bdl | Bdl |
| IN_S6 | B | Bdl | Bdl |
| IN_S7 | B | Bdl | Bdl |
| IN_S8 | B | Bdl | Bdl |
| IN_S9 | B | Bdl | Bdl |
| IN_S10 | B | Bdl | Bdl |

## 3.8. FIGURES

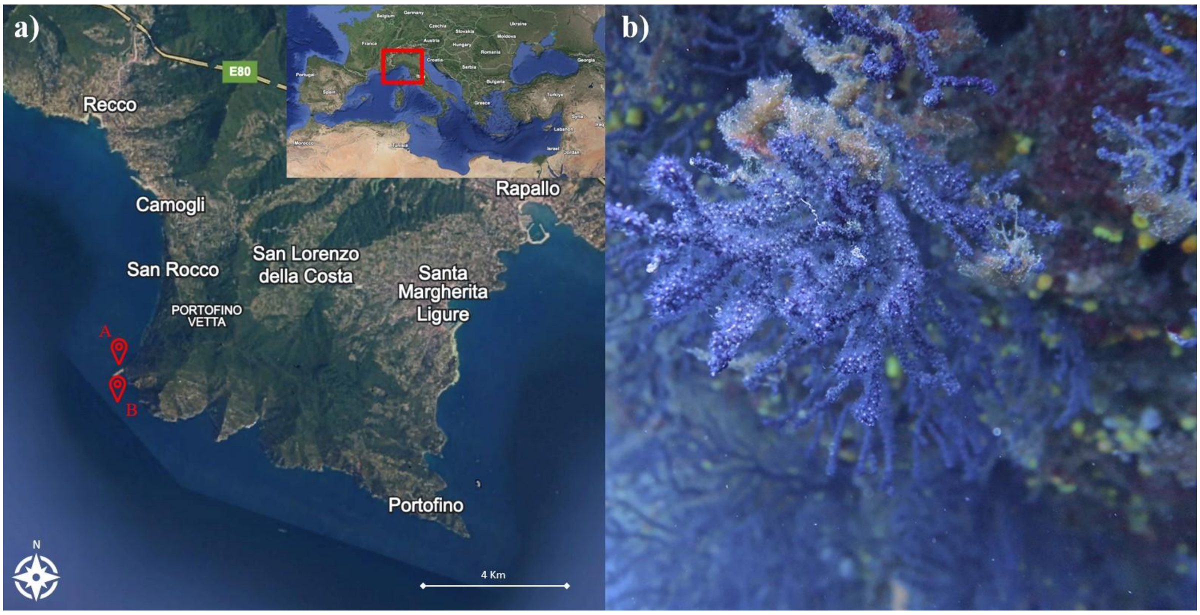


**Figure 1.** Overview of the sampling sites and species sampled: a) Geographical contextualization of Portofino Marine Protected Area (MPA) with the sampling sites highlighted, made from GoogleHybridMap and GoogleSatelliteMap loaded into QGIS; b) example of a colony of the *Paramuricea clavata* found in the sampling sites.

**Figure 2.** Overview of Site B, located outside the Portofino MPA in close proximity to a cliff that is frequently visited by people engaged in recreational activities such as swimming, sunbathing, and snorkeling: **a)** detail of people sunbathing on the cliff and swimming above the sampling site; **b)** detail of the patch of *P. clavata* from which the samples were collected.

**Figure 3.** Overview of Site A, located inside the Portofino MPA further offshore where only regulated scuba diving activities are permitted to limit the direct human impact on the area: **a)** detail of the diving boat in navigation toward the site; **b)** detail of the rich *P. clavata* forest present, from which the samples were collected.

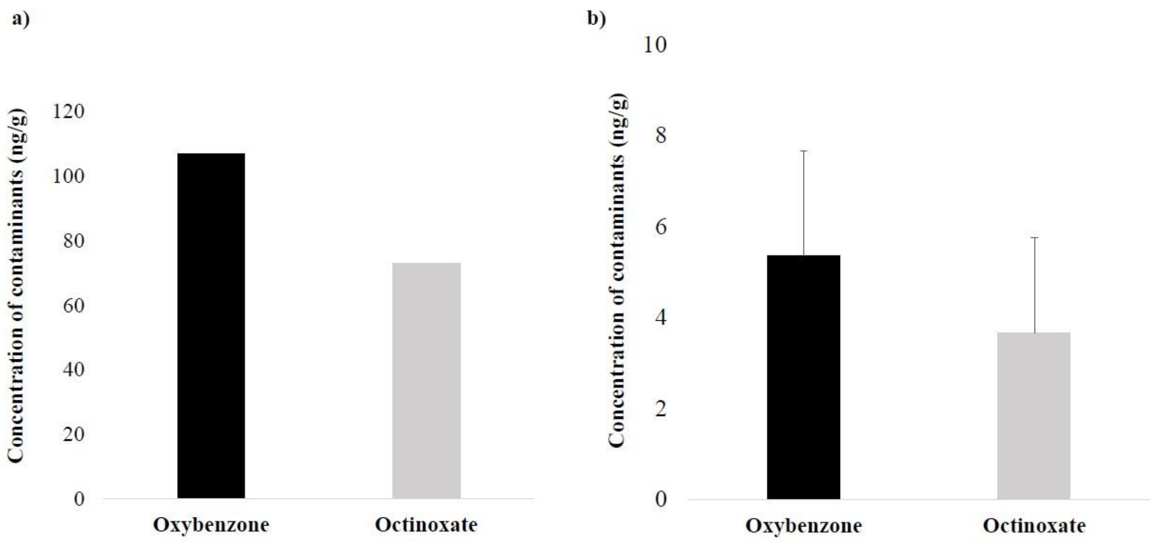


**Figure 4.** Concentrations of UV filter contaminants in *Paramuricea clavata* tissues: overall **a)** total and **b)** average concentration of contaminants express in ng/g (wet weight).

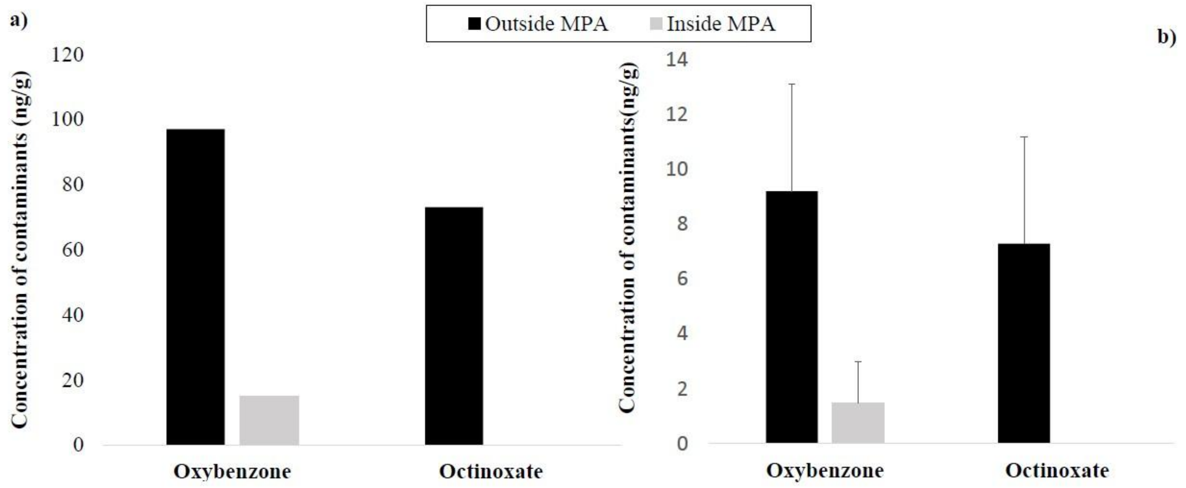


**Figure 5.** Concentrations of UV filter contaminants in *Paramuricea clavata* tissues inside and outside the Portofino MPA: overall **a)** total and **b)** average concentration found outside (black) and inside (grey) the Portofino Marine Protected Area, express in ng/g (wet weight).

# CHAPTER 4

## Pathology of tissue loss in three key gorgonian species in the Mediterranean Sea

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## 4.1. ABSTRACT

The Mediterranean is known for its marine biodiversity, especially gorgonian forests. Unfortunately, these are experiencing rapid declines due to climate change, manifested by repeated marine heat waves resulting in mass mortality events since the early 1990s. To better understand why gorgonians are declining, more systematic approaches to investigate the exact causes are needed, and pathology may aid in this goal.

We described gross and microscopic pathology of tissue loss in three key gorgonian species in the Mediterranean region, *Paramuricea clavata*, *Eunicella cavolini*, and *Leptogorgia sarmentosa*, that were all experiencing various degrees of acute to subacute tissue loss characterized by exposed axial skeleton sometimes partly colonized by epibionts and thinning of adjacent tissues. The most significant variety of lesions was seen in *P. clavata,* followed by *L. sarmentosa* and *E. cavolini.* For all species, dissociation of gastrodermal cells was the dominant microscopic lesion, followed by necrosis of the gastrodermis. Ciliates invading gastrodermis and associated with necrosis of polyps were seen only in *E. cavolini.* Epidermal tissue loss was seen only in *L. sarmentosa,* while *P. clavata* was distinguished by a prominent inflammatory response and unidentified dark round structures within the tentacle epidermis and gastrodermis with no host response. Further work to understand the cause of death in gorgonians is needed, particularly to elucidate the role of ciliates and environmental co-factors or infectious agents not visible on light microscopy, as well as applications of additional tools such as cytology.

## 4.2. INTRODUCTION

The Mediterranean Sea is known for its incredible biodiversity, notable by gorgonian forests, a complex underwater ecosystem that provides habitat for a significant number of associated organisms (Gori et al., 2017, 2019; Rossi et al., 2017). Unfortunately, marine ecosystems in the Mediterranean have been facing a significant decline in biodiversity and health status over the past decades due to a multitude of threats, such as human impact and pollution, global climate change, and ocean acidification (Jackson et al., 2001; Carpenter et al., 2008; Pandolfi et al., 2011; Rossi et al., 2013; Hughes et al., 2017; Bevilacqua et al., 2021). Among these threats, climate change stands out as a major contributor to the deterioration of the Mediterranean marine environment (Kim et al., 2019; Pisano et al., 2020; Garrabou et al., 2022). In the region, anthozoans dominate the benthos exemplified by gorgonian forests that are iconic members and ecosystem engineers of Mediterranean benthic communities creating dense monospecific assemblages covering large areas of the coast (Paoli et al., 2017; Gori et al., 2019). This ecosystem is threatened by marine heat waves that appear to be increasingly frequent over the past two decades, beginning with the significant heatwave of 1999 (Cerrano et al., 2000; Rivetti et al., 2014; Garrabou et al., 2022). These heat waves expose gorgonians to prolonged thermal stress that, in turn, can trigger mass mortality with disrupting effects on entire marine ecosystems and significant implications for benthic communities (Garrabou et al., 2001; Crisci et al., 2011; Oliver et al., 2018; Darmaraki et al., 2019a, 2019b; Garrabou et al., 2019, 2022).

Despite extensive ecological research on the long-term patterns and mortality trends associated with climate change impacts on gorgonians, there remains a knowledge gap in understanding the biological processes responsible for the deterioration of their health and resultant mortality. This scarcity of studies may derive from the challenges in defining the disease etiology, progression, and transmission. Although many studies claim the identification of infectious causes of cnidarian disease (Rosenberg & Ben Haim, 2002; Sutherland et al., 2004; Sweet & Séré, 2016; Montano et al., 2020), few of those are supported by morphologic evidence at the microscopy level (Work & Aeby, 2006; Work & Meteyer, 2014). Without such descriptions, it becomes difficult to accurately identify the causes of diseases or develop an understanding of disease pathogenesis (Work & Meteyer, 2014; Hawthorn et al., 2023). Therefore, comprehensive and systematic descriptions of lesions at both gross and microscopic levels are gaining importance (Work & Rameyer, 2005; Becker et al., 2023).

We are aware of two studies looking at the histology of gorgonians in the Mediterranean. Carella et al. (2014) examined two sea fans (*Eunicella singularis* and *E. cavolini*) from the Gulf of Naples and saw histologic evidence of thickening of the axial skeleton with molecular signatures of bacteria. In a subsequent paper, Carella et al. (2020) documented nodular lesions interpreted as melanin and amyloid deposits in these same corals. Here, we add to the knowledge of pathology in gorgonians by describing gross and microscopic similar pathology in two additional species (*Leptogorgia sarmentosa* and *Paramuricea clavata*) from northwest and southwest Italy.

## 4.3. MATERIALS AND METHODS

Samples from three different gorgonian species manifesting tissue loss were collected in three locations off the coast of Italy between August and October 2022 (Figure 1): three *Paramuricea clavata* from the Marine Protected Area of Bergeggi (Liguria, Italy, 44°14'04.3"N; 8°26'46.6"E); five *Eunicella cavolini* from the coast of Paraggi in the Marine Protected Area of Portofino (Liguria, Italy, 44°18'41.0"N; 9°12'47.4"E); five *Leptogorgia sarmentosa* and seven *Paramuricea clavata* from Scilla (Calabria, Italy, 38°15'22.5"N; 15°42'44.9"E).
Before sampling, photographs were taken of the individuals displaying varying degrees of tissue loss, exposed axial skeleton, and, in some cases, overgrowth with turf algae (Figure 2). Based on what is known about gross lesions in corals (Work & Aeby 2006), gross lesions showing overgrowth of skeleton by turf algae or other biota were judged more chronic than those showing bare skeleton alone. The specimens were collected during SCUBA diving activities by removing small fragments of the branches showing normal to abnormal tissue, then placed in zip-lock bags; paired apparently normal tissues were not collected. Upon reaching the surface, each sample was immediately fixed in plastic tubes filled with Z-fix solution (Sigma-Aldrich™, St. Louis, Missouri, USA). During collection, a roving diving technique was used to examine the sampled colonies and the surrounding area for potential predators that could cause similar injuries or tissue loss.

Before processing, each sample was photographed and trimmed into ~3 cm long fragments to include both the tissue loss interface and a healthy segment. The samples were then decalcified with Cal-Ex-II Fixative Decalcifier solution (Fisher Scientific™, Hampton, New Hampshire, USA) for 24 to 48 hours. Then, the samples were placed in tissue cassettes and embedded in paraffin. The resulting wax blocks were then cut into 4 to 5 μm thick sections with a rotary microtome, mounted on glass microscope slides, and stained with hematoxylin and eosin (H&E). To visualize suspected fungi or melanin in the gorgonin skeleton, the proteinaceous matrix that makes up gorgonin (Ehrlich, 2019), Grocott's Methenamine Silver (GMS) and Fontana-Masson (FM) staining were performed respectively (Prophet et al., 1992). The control for melanin was a melanoma from a dog (Figure 3, left), while the control for fungi was a bird lung infected with *Aspergillus* (Figure 3, right). The sections were examined with an Olympus BX43 microscope, and photomicrographs were taken using a Lumenera Infinity 3 camera.

## 4.4. RESULTS

All 20 specimens analyzed had gross evidence of tissue loss. However, based on our analyses during the collection, no evidence of predators directly on the colonies or in the surrounding area could explain this tissue loss.

Grossly, lesions for *P. clavata* and *L. sarmentosa* were similar in that they manifested as abrupt cessation of tissues at branch tips, revealing mostly unfouled axial skeleton, suggesting that tissue loss was a recent event (Figures 2A-D). We concluded this because bare substrates in marine environments tend to become colonized by turf algae and other epibionts quickly; examples include subacute tissue loss in corals (Work & Aeby, 2006). In contrast, tissue loss in *E. cavolini* was a more diffuse process encompassing branch tips and bases, leading to exposure of the axial skeleton with deposition of amorphous unidentified dark material on the bare skeleton, suggesting a more chronic process. Moreover, intact tissue adjacent to areas of tissue loss manifested varying degrees of indistinct pink discoloration (Figures 2E and 2F). All species had an abrupt transition from healthy to tissue loss.

On histology, several tissue changes were observed. The apparently normal polyps consisted of tentacles over a mesoglea penetrated by solenia lined by gastrodermis and bare cavities (sclerites) atop an axial skeleton composed of gorgonin (Elrich, 2019) with variably sized cavities (Figure 4A). The most common lesion across all species was dissociation (Figure 4B) or necrosis (Figures 4B-C) of gastrodermis. In less severe changes, the gastrodermis showed vacuolation (Figure 4D). Occasionally, necrosis of the epidermis with tissue loss (Figure 4E) was seen in *L. sarmentosa*. Additionally, unidentified dark amorphous structures were seen in the epidermis and gastrodermis of tentacles of *P. clavata*, with no associated host response (Figure 4F).

Notably, *P. clavata* was also distinguished by an inflammatory response consisting of eosinophilic granular cells infiltrating mesoglea, sometimes associated with algae growth in the axial skeleton (Figure 5A) or vacuolated gastrodermis (Figure 5B). In *E. cavolini* ciliates were found colonizing the epidermis (Figure 5C) or gastrodermis (Figure 5D), occasionally associated with necrosis of the gastrodermis (Figure 5E) or the entire polyp (Figure 5F).

Microscopic changes in the axial skeleton were generally limited to the presence of inflammatory cells adjacent to the deposition of gorgonin (Figure 6A) that stained negative with Fontana-Masson (Figure 6B), as compared with Figure 3 (left). In cases where live tissue overlaid the axial skeleton, algae infiltration was accompanied by deposition of gorgonin (Figure 6C), whereas this was not evident in areas of bare axial skeleton colonized by epibionts (Figure 6D) or microbial mats (Figure 6E). Within the cavities of the axial skeleton, finely fibrillar material was found in both bare and live tissue-overlaid areas (Figure 6E), staining positive with silver stain (Figure 6F), but not matching the size or morphology expected for fungi (Figure 3, right).

Among the species analyzed, the highest variety of lesions was seen in *P. clavata,* followed by *L. sarmentosa* and *E. cavolini*. Finally, the inflammatory response was seen only in *P. clavata*, epidermal necrosis was seen exclusively in *L. sarmentosa*, and ciliates were present only in *E. cavolini* (Table 1).

## 4.5 DISCUSSIONS

Similar tissue loss on the tips of gorgonians branches has been reported in the last decades during prolonged thermal stress periods and mass mortalities events, where, in some cases, some recovery has been noted both in terms of population health and individuals affected by tissue loss (Cerrano et al., 2000; Carella et al., 2014; Turicchia et al., 2018; Canessa et al., 2023). Indeed, this type of lesion on the tissue of gorgonians has been observed and potentially associated with thermal stress in previous research (Carella et al., 2014; Carella et al., 2020; Garrabou et al., 2022). Here, we wanted to understand better whether the observed patterns of tissue loss across different species could be attributed solely to thermal stress or if other factors, such as pathogens or predation, might also contribute to this phenomenon.

The most significant lesion seen in all sea fans was necrosis and vacuolation of the gastrodermis, which likely led to tissue sloughing and gross lesions observed in the field in this study. Necrosis of gastrodermis is a common host response in other cnidaria with tissue loss of unexplained origin, and it has been seen in corals with tissue loss from the Caribbean (Landsberg et al., 2020) and the Pacific (Work and Aeby, 2011). In Caribbean Sea fans (*Gorgonia ventalina*) with purpling lesions, Becker et al. (2023) saw gastrodermal necrosis and amoebocytic infiltrates along with deposition of gorgonin around algae invading skeleton similar to changes seen here. Inflammation in gorgonians is also well documented (Mydlarz et al., 2008). Carella et al. (2014) examined *E. cavolini* and *E. singularis* in the Gulf of Naples using histology, which showed inflammation in tissues and cyanobacteria presence detected by molecular assays and histology. In subsequent investigations, Carella et al. (2020) concluded that nodules found in sea fans were the result of infection with cyanobacteria, leading to the deposition of melanin in the axial skeletons; however, the organisms they showed in their figures were more compatible with plant cells with cell walls (algae), something we observed here invading the axial skeleton of gorgonians. We were unable to convincingly show staining of melanin in sea fan axial skeletons here, at least, compared to positive controls. Becker et al. (2023) stated they saw melanin in Caribbean Sea fans but without a confirmatory stain, while Carella et al. (2020) showed staining of the axial skeleton with Fontana-Masson that was not all that different from our specimens and did not reflect the staining expected in our positive control. Although melanin deposition can play an important role in the defense mechanisms of invertebrates (Nappi & Christensen, 2005), whether melanin plays such a role in sea fans remains to be confirmed.

The only foreign organisms associated with host changes at the cellular level were infiltrates of algae in the axial skeleton associated with varying degrees of gorgonin deposition. Similar changes were seen in Caribbean Sea fans (Becker et al., 2023), and based on the presence of what appears to be macroalgae in the figures of Carella et al. (2020), nodules in *E. cavolini* and *E. singularis* from the Gulf of Naples may be responses to algal overgrowth of axial skeleton. Indeed, algal overgrowth of the gorgonian axial skeleton leading to nodule formation has been known since the early 1980s (Goldberg et al., 1984; Morse et al., 1981). Unlike Becker et al. (2023), we did not observe convincing evidence of fungal infection in gorgonians. The presence of cavities in the axial skeleton with a framework of finely fibrillar material that, here, stained positive with silver, seems to be a normal part of the skeletal structure of Mediterranean gorgonians; similar cavities have been seen in other species from the region (Carella et al., 2014; Carella et al., 2020). The unidentified dark bodies in the tentacles of *P. clavata* were similar to those seen by Carella et al. (2020), who judged them to be cells; however, we were unable to visualize nuclei in the structures seen here, thus their identity remains uncertain.

The presence of ciliates exclusively in *E. cavolini* was intriguing, particularly in cases where ciliates were seen colonizing intact epidermis and invading intact gastrodermis and associated with necrosis of polyp tissue and gastrodermis (Figure 5). Invasion of intact tissue layers suggests that ciliates are not mere detritivores and could play a potential role in the pathogenesis of tissue loss in this species. However, apparently normal tissues were not collected in this study, so we cannot be certain that these are pathogenic. Becker et al. (2023) saw ciliates in apparently normal and lesioned tissues of Caribbean Sea fans and saw ciliates settling on the epidermis; however, they did not have any conclusions regarding their role in causing lesions. This should be addressed in future work because ciliates invading tissues and associated with tissue loss have been seen in other corals like *Montipora capitata* (Work et al., 2012) and *Acropora sp*. (Bourne et al., 2008).

Additional histopathology investigations, including examination of apparently normal tissues to aid in the interpretation of lesions, would benefit future histopathology studies in Italian gorgonians. Other methods, such as cytology, may also help understand the morphology and pathogenesis of lesions in this important group of animals (Work et al., 2024). As gorgonian forests and other marine ecosystems face increasing threats, gaining a deeper understanding of the processes and causes leading to tissue damage becomes imperative for effectively conserving this fragile ecosystem, which is fundamental for the Mediterranean benthic community.

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## 4.7. TABLES

**Table 1**. Enumeration of presence/absence of each type of lesion (n.d. = non-detected).

| LESION TYPE | ***Eunicella cavolini*** **(n=5)** | ***Leptogorgia sarmentosa*** **(n=5)** | ***Paramuricea clavata*** **(n=10)** | **TOTAL** |
|---|---|---|---|---|
| Gastrodermal dissociation | 5 | 5 | 10 | 20 |
| Gastrodermal necrosis | 3 | 3 | 6 | 12 |
| Ciliates | 4 | 0 | 0 | 4 |
| Round bodies tentacles | 0 | 0 | 4 | 4 |
| Epidermal tissue loss | 0 | 3 | 0 | 3 |
| Gastrodermal vacuolation | 0 | 0 | 3 | 3 |
| Granular cell infiltrates mesoglea | 0 | 0 | 3 | 3 |
| Algae in skeleton | 0 | 2 | 1 | 3 |
| Total | 12 | 14 | 51 | 38 |

## 4.8. FIGURES


**Figure 1. Geographical contextualization of the study area highlighting the sampling site.** **A)** Mediterranean Sea with Liguria and Calabria coasts highlighted; **B)** Ligurian Sea; **C)** Scilla area with the sampling site highlighted (38°15'22.5"N 15°42'44.9"E); **D)** Bergeggi Marine Protected Area with the sampling site highlighted (44°14'04.3"N; 8°26'46.6"E); **E)** Portofino Marine Protected Area with the sampling site highlighted (44°18'41.0"N; 9°12'47.4"E). Maps made from MapTiler, GoogleHybridMap, and GoogleSatellite loaded into QGIS.

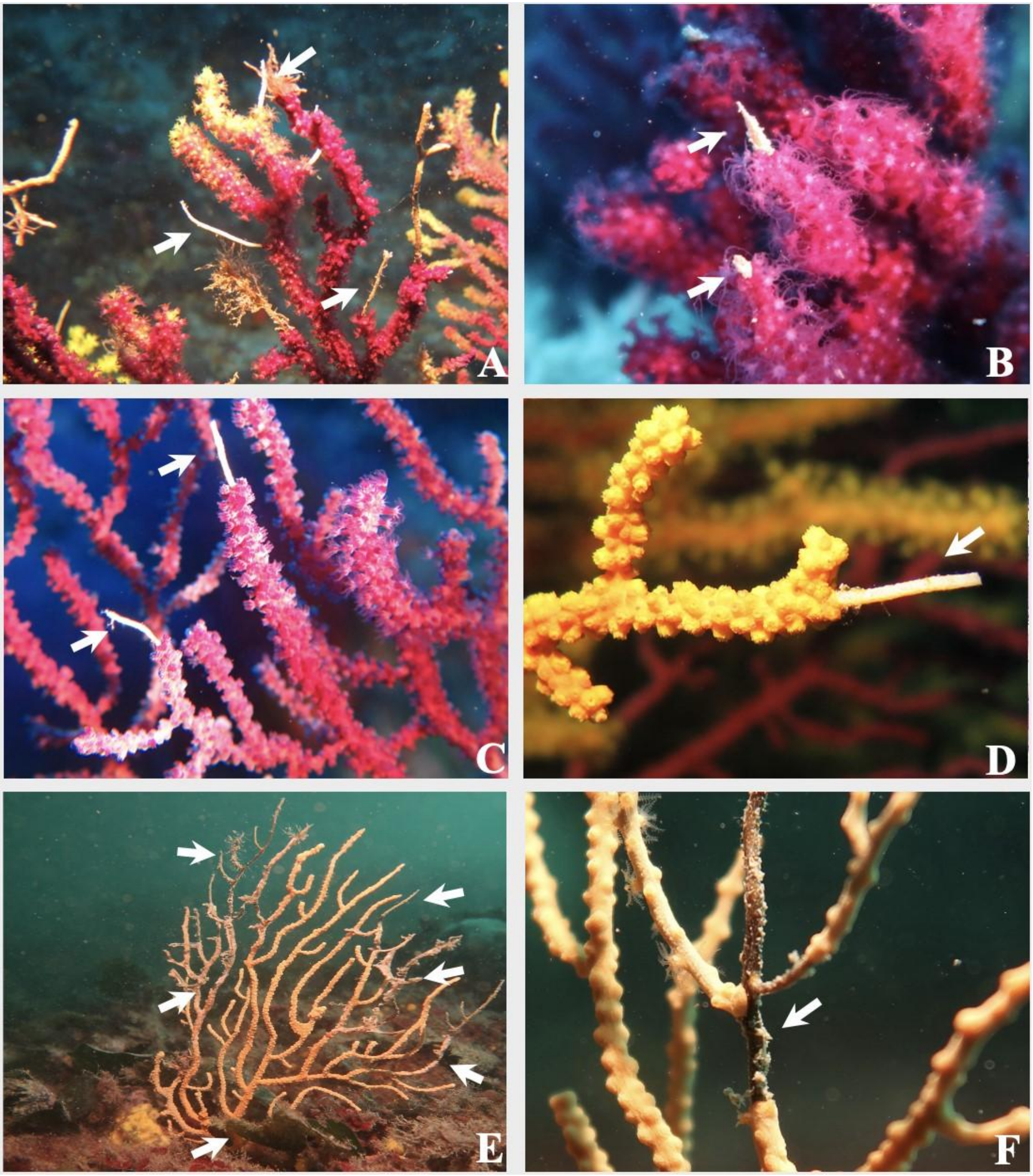


**Figure 2. Tissue loss overview in wild Mediterranean anthozoan colonies. A)** colony-wide view and **B)** close-up of tissue loss at apical branches *P. clavata*; note exposed axial skeleton (arrow); **C)** colony-wide and **D)** close-up of *L. sarmentosa* showing tissue loss at apical branches; note bare axial skeleton (arrow); **E)** colony-wide view of extensive tissue loss on *E. cavolini* colony both basally and apically, showing axial skeleton with overgrowth by amorphous material and suggesting a more chronic process (arrows); **F)** close-up detail depicting tissue loss in *E. cavolini*; note segmental area of tissue loss exposing axial skeleton with overgrowth by black material (arrow).

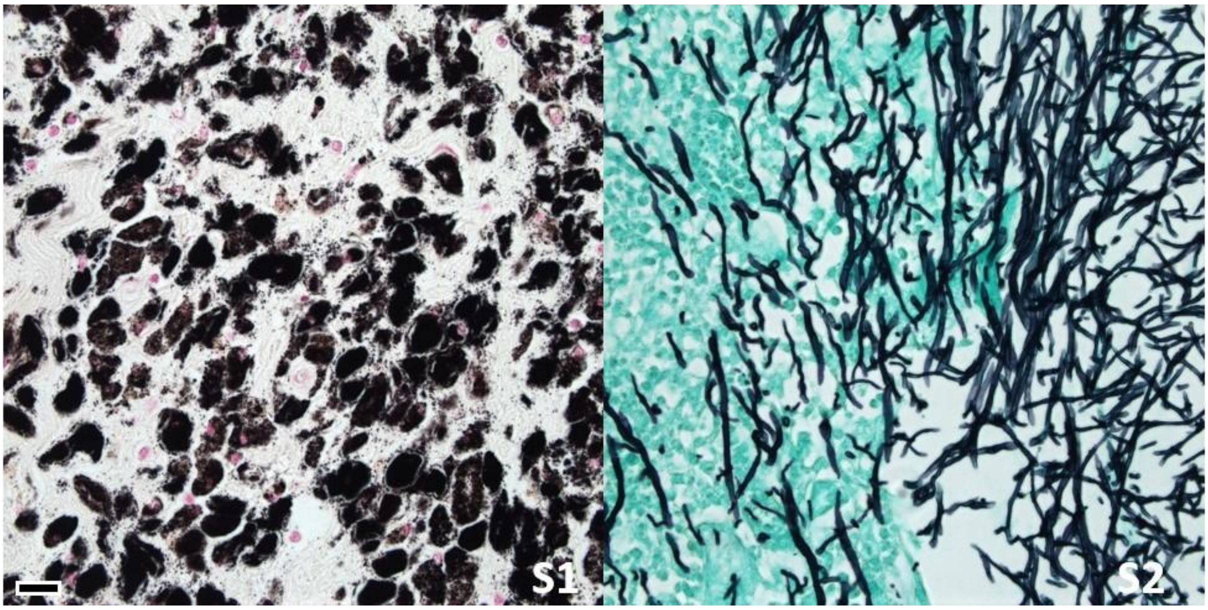


**Figure 3. Staining controls. left)** Dog melanoma stained with Fontana-Masson; black staining of melanocytes; **right)** Bird lung stained with Grocott's Methenamine Silver; black filamentous branching fungal hyphae; bar = 20 µm.

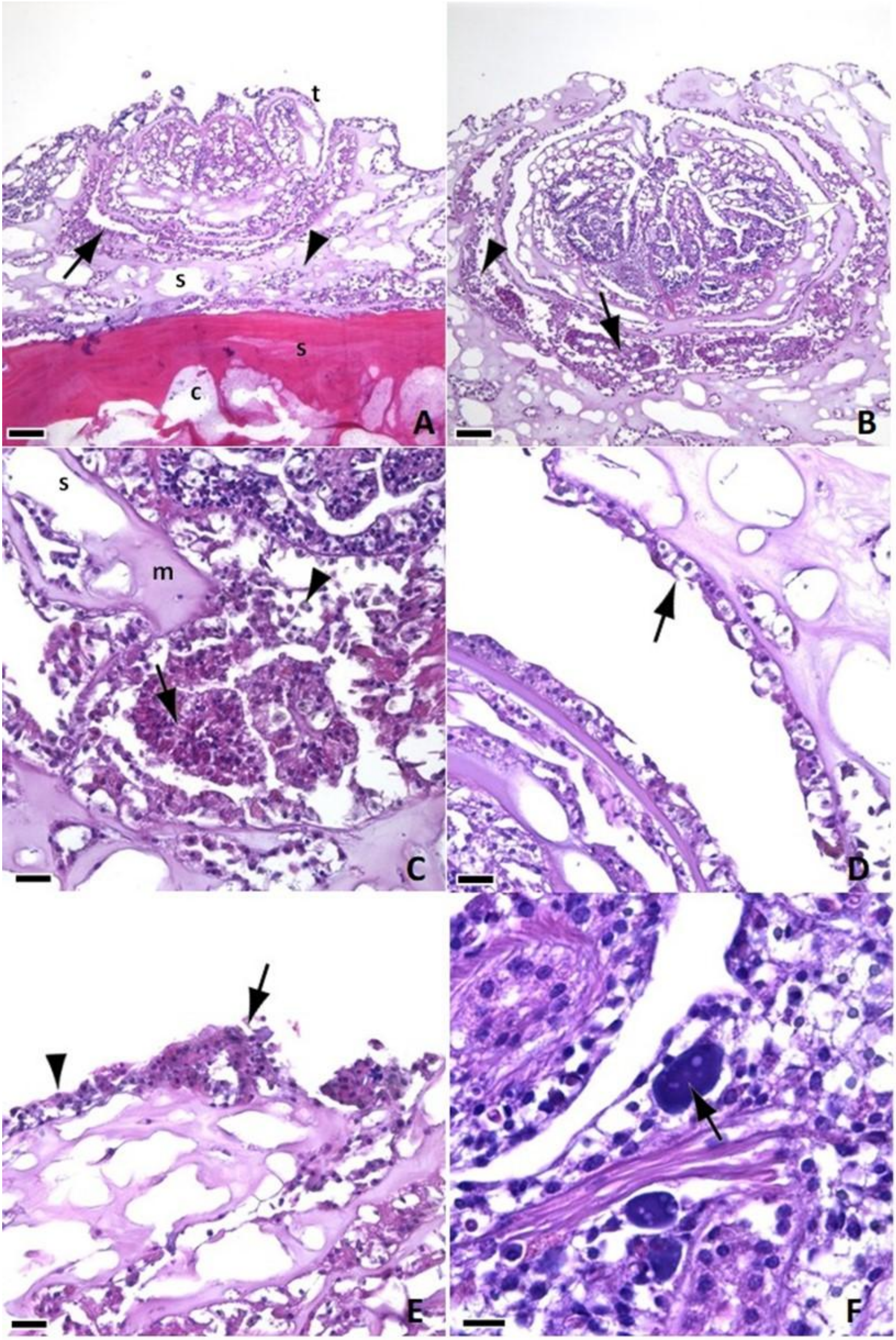
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**Figure 4. Histology of sea fans collected from Italy showing acute to subacute tissue loss, hematoxylin and eosin.** **A)** *L. sarmentosa* apparently normal polyp atop axial skeleton (s) containing cavities (c); note tentacle (t), sclerites (s) within mesoglea (arrowhead) and gastrodermis (arrow) lining solenia; bar = 50 μm; **B)** *L. sarmentosa* polyp; note necrotic gastrodermis (black arrow) characterized by clumps of hypereosinophilic material within solenia, dissociating (arrowhead) and necrotic (arrow) gastrodermal cells; bar = 50 μm; **C)** Details of necrotic gastrodermis sloughing into solenia; note clumps of hypereosinophilic debris with karyorrhectic and pyknotic nuclei (arrow) along with dissociating gastrodermal cells (arrowhead); (m) mesoglea, (s) sclerite; bar = 10 μm; **D)** *P. clavata*; note vacuolation of gastrodermis (arrow); bar = 10 μm; **E)** *L. sarmentosa*; note epidermal necrosis (arrow) with exposure of underlying mesoglea and contrast with more intact epidermis (arrowhead); bar = 20 μm; **F)** *P. clavata*; note unidentified amorphous dark bodies with variably sized cavities within the gastrodermis and epidermis of the tentacle (arrow); bar = 10 μm.

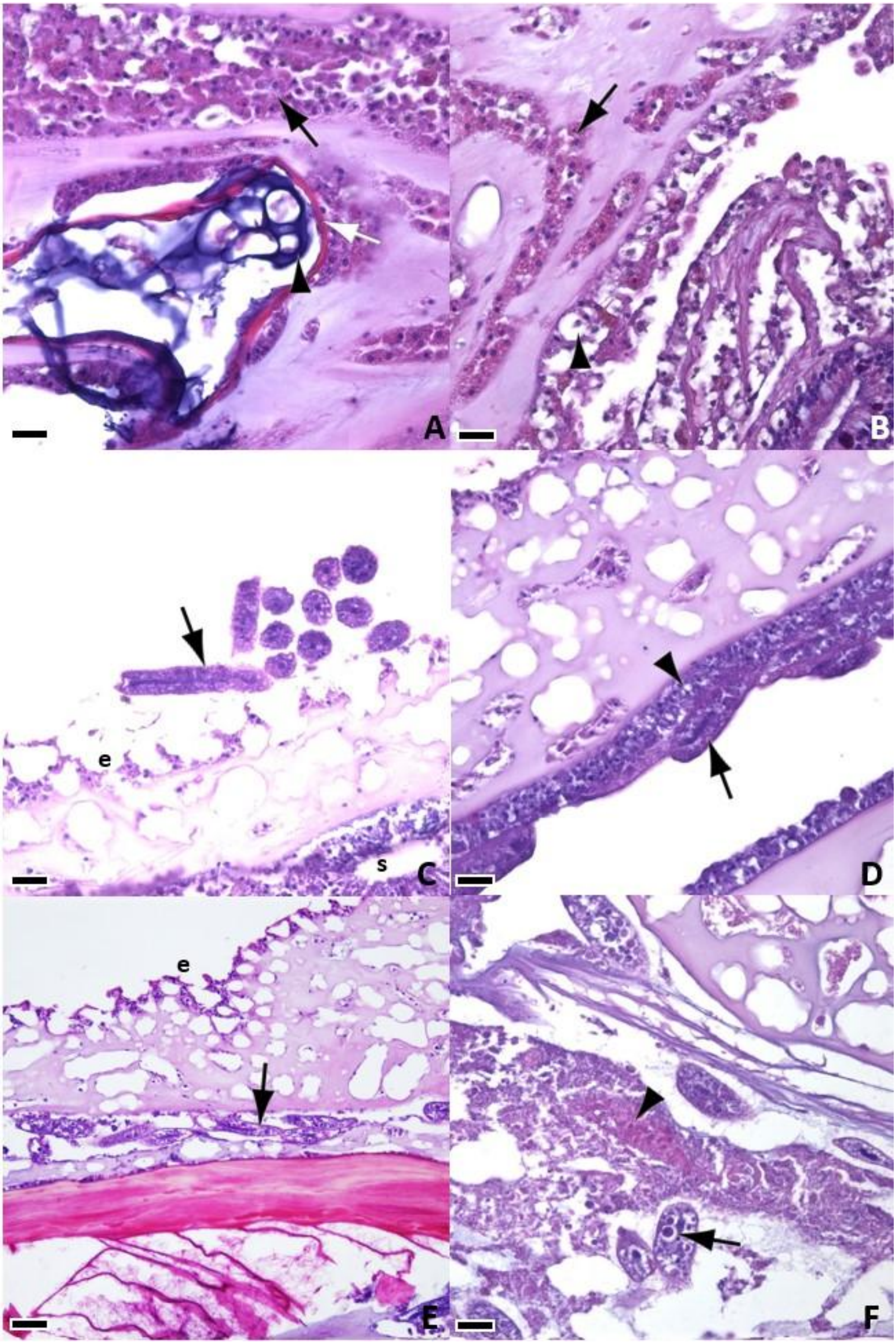
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**Figure 5. Histology of inflammation and ciliates in sea fans from Italy with tissue loss, hematoxylin and eosin; bar = 10 µm all plates. A)** *P. clavata*; note infiltrates of eosinophilic granular cells in mesoglea (arrow) adjacent to infiltrates of algae with cell walls (arrowhead) with deposition of gorgonin (white arrow); **B)** *P. clavata*; note infiltrates of eosinophilic granular cells in mesoglea (arrow) and vacuolation and dissociation of gastrodermis (arrowhead); **C)** *E. cavolini*; note ciliates (arrow) on top of the intact epidermis (e) and solenia (s) surrounded by gastrodermis; **D)** Close-up of ciliates (arrow) invading intact gastrodermis (arrowhead); **E)** *E. cavolini*; note ciliates (arrow) among gastrodermal cell debris within solenia and intact epidermis (e); **F)** Necrotic polyp with ciliates; note clumps of eosinophilic debris (arrowhead) associated with ciliates, one of which has ingested material (arrow).

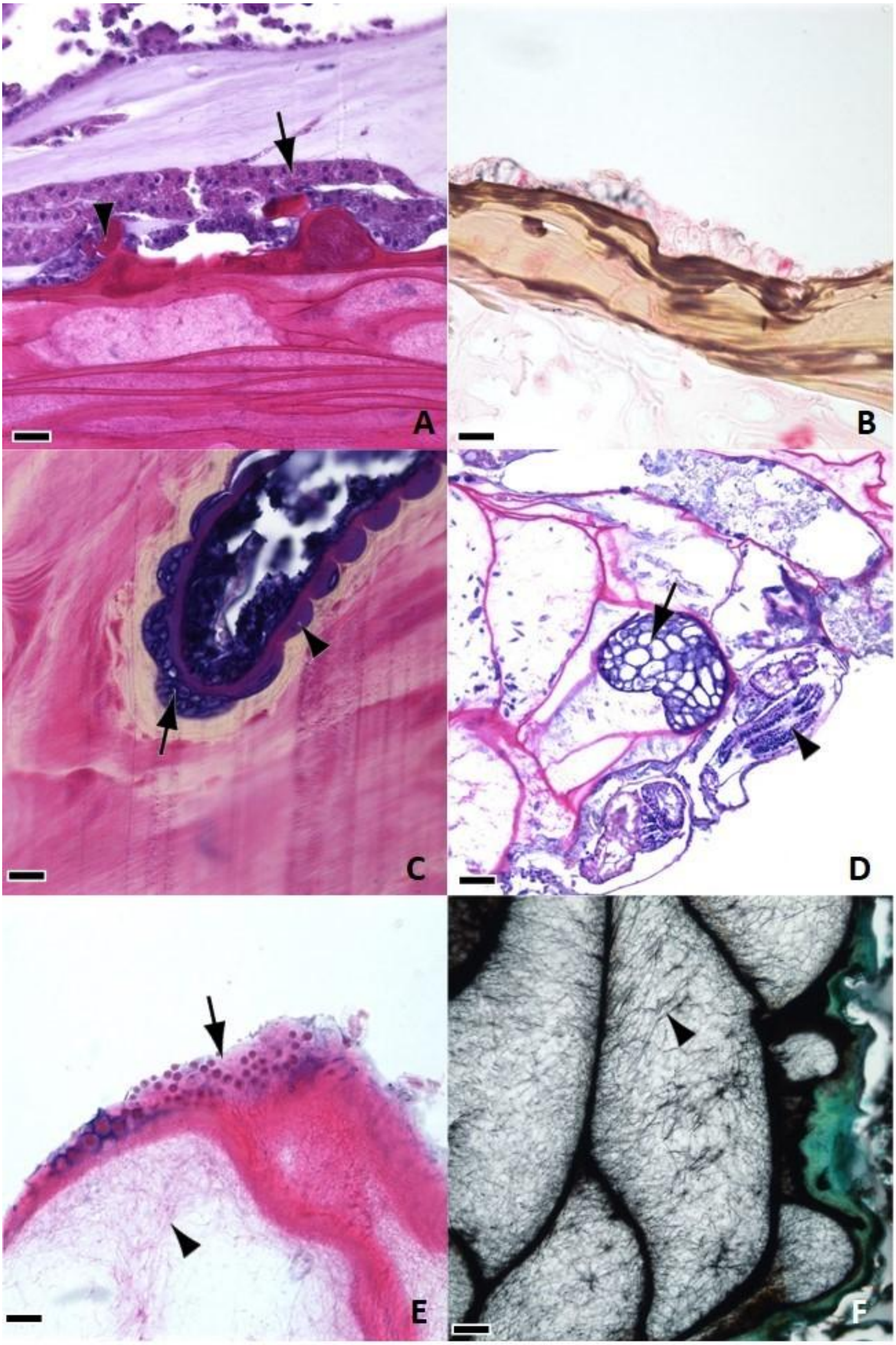
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**Figure 6. Histology of axial skeleton of sea fans with tissue loss collected from Italy showing acute to subacute tissue loss, hematoxylin and eosin on all panels, except B (Fontana-Masson) and F (Grocott's Methenamine Silver).** **A)** *P. clavata*; note infiltrates of granular mesogleal cells (arrow) and deposition of gorgonin (arrowhead); bar = 10 μm; **B)** Fontana-Masson stain of deposition of gorgonin; note lack of staining for melanin (compare with Fig. S1); bar = 10 μm; **C)** *L. sarmentosa*; algal infiltrates in axial skeleton overlaid by live tissue; note structures with cell walls (arrow) and deposition of gorgonin (arrowhead); bar = 10 μm; **D)** *L. sarmentosa*; deposition of epibionts on the bare axial skeleton; note algae in the skeletal matrix (arrow) and unidentified metazoan (arrowhead) on the surface; bar = 50 μm; **E)** *L. sarmentosa*; note microbial mat of unicellular structures on the surface of the bare axial skeleton (arrow) along with finely fibrillar material within space in the axial skeleton (arrowhead); bar = 10 μm; **F)** *L. sarmentosa*; note silver stain of filaments in E that do not fit the morphology of fungi characteristic of parallel walled branching structures with septa (Fig. S2); bar = 10 μm.

# CHAPTER 5

## Underwater Quick-hardening Vegetable oil-based Biodegradable Putty for Coral Reef Restoration and Rehabilitation

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## 5.1. ABSTRACT

Coral reefs are threatened by climate change and the effects of human activity on the marine environment. Researchers are attempting to rescue this fragile ecosystem through coral restoration actions (coral gardening, micro-fragmentation, etc.), and a common step in these procedures is transplanting the new coral colonies into coral reefs. To do that, commercial concrete or epoxy resins, also called putty, are utilized, highlighting different concerns about their mechanical and hardening performances and their impact and fate once released into the environment. Hence, this study presents a new biodegradable epoxidized soybean oil acrylate (ESOA)/zein-based coral putty capable of quick hardening underwater as an eco-friendly alternative for transplanting new coral colonies in the reef. The coral putty comprises two components: a radical initiator and a radical accelerator. Once the two components are mixed, the coral putty becomes hard underwater in 20–25 minutes, showing a hardening timescale much faster than other commercial products. The coral putty is biocompatible when applied to the coral *Stylophora pistillata* in aquaria, and *Acropora tenuis* corals are out-planted on the reef in the Maldives, demonstrating how this new class of vegetable-oil-based materials can be a suitable alternative to epoxy resins and concretes commonly used in coral restoration procedures.

## 5.2. INTRODUCTION

Nowadays, climate changes and anthropogenic impacts threaten coral reefs, and the risk of losing a significant part of this irreplaceable biodiverse ecosystem is no longer a futuristic prediction. Indeed, recent estimations foresee that if global warming exceeds 1.5°C, 70–90% of reef corals are at risk of being lost, while if it exceeds 2.0°C above pre-industrial temperatures, this percentage may reach 99% (Hoegh-Guldberg et al., 2018; Knowlton et al., 2021; Voolstra et al., 2023). Researchers have been designing new practices to heal or rescue corals from massive mortality events caused by global and local threats (Contardi et al., 2020, 2021, 2023; Santoro et al., 2021; Wangpraseurt et al., 2022; Roger et al., 2023). At the top of the list is coral reef restoration, which is based on the general concept of "assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (Clewell et al., 2004). Moreover, various advanced techniques (e.g., asexual or sexual propagation and substrate enhancement methods) that allow the planning of coral restoration actions at the local, regional, or global scale are flourishing worldwide (Omori, 2019; Boström-Einarsson et al., 2020). It has been estimated that this approach has an overall success rate of around 60-70% but is limited to a small scale and a restricted number of coral species, thus calling for large-scale action to contract the effects of climate change progression (Bayraktarov et al., 2019; Boström-Einarsson et al., 2020). Currently, about half of all restoration projects use the cost-effective “coral gardening approach” (Bayraktarov et al., 2019; Boström-Einarsson et al., 2020). Usually, in this two-step process, coral fragments derived from donor colonies or collected on the seabed are first reared under favorable growing conditions (i.e., an optimal combination of exposure to light, sedimentation, water flow, temperature, etc.) in artificial *in-situ* or *ex-situ* structures before being transplanted to the target (degraded) restoration site (Rinkevich, 1995, 2000; Epstein et al., 2001). Structures such as tables, frames, trees, and other mid-water floating in-situ nurseries have demonstrated remarkable efficiency in accomplishing the task of rearing thousands of asexually produced fragments at low costs (Shaish et al., 2008; Levy et al., 2010; Dehnert et al., 2021). Apart from that, an alternative approach is the production of artificial reefs, which are benthic structures that serve as substrates for the attachment and growth of corals and other marine organisms, providing habitats and helping in the restoration of degraded ocean ecosystems (Berman et al., 2023a). This strategy is quickly becoming popular thanks to 3D printing, which allows the design and fabrication of accurate, fine, and tunable structures for coral growth (Levy et al., 2022; Berman et al., 2023b). Lastly, micro-fragmentation is an emerging technique in which fragments coming from the same colony are placed close to each other on a flat substrate. This asexual propagation technique can accelerate up to ten times the growth rate of these fragments and is more cost-effective than other restoration techniques (Page et al., 2018; Mostrales et al., 2022).

The common step to all of these restoration strategies is the out-plantation of the “new” coral colonies onto the reef. Currently, several tools can be involved in the coral attachment procedure, such as nails, cable ties, coral clips, monofilament fishing lines made of synthetic materials that alter the environment, as well as marine cement and epoxy glue, which do not ensure biodegradation and suitable stability over time (Gomez et al., 2010; Hollarsmith et al., 2012). Among them, paste-like, malleable, two-component epoxy materials that harden at room temperature and are activated when the two components are mixed, named putties, have been broadly used (Gomez et al., 2010). They have been applied for out-plant coral colonies (Hollarsmith et al., 2012) or fixing the corals to specific supports in the aquaria to enhance their growth and health (Musco et al., 2017). However, those materials are not always explicitly designed for the attachment of corals but for various applications that vary from the protection

of boats and oil platforms from corrosion, repair/rebuild of plastic/metallic pipes or parts made of wood, fiberglass, metal, concrete, and glass, to sculpting, craft and home décor projects. For this reason, such epoxy putties, synthesized from non-renewable petroleum sources, may present mechanical properties and durability underwater that do not match the requirements for eco-friendly coral transplantation. Indeed, after their functional lifetime, not being biodegradable, they persist in the environment for centuries and result in plastic waste accumulation (Jiang et al., 2024). Moreover, most of the commercial epoxy resins are not biodegradable and are crosslinked using diglycidyl ether of bisphenol A (DGEBA) as a curing agent, usually indicated in the list of the ingredients as "bisphenol-A-(epichlorhydrin) epoxy" (Kumar et al., 2018; Jiang et al., 2024). These compounds raise serious concerns regarding their toxicity for human beings and the environment, especially when used extensively (Krijgsheld & Van der Gen, 1986; Radon et al., 2006; Ramakrishnan & Wayne, 2008; Liu et al., 2019; Gonzalez et al., 2021).
Another option is marine concrete, but it cannot be mixed underwater, has a slow hardening rate, and is usually applied using pastry bags or adapted guns to squeeze it out on the reef surface (Frias-Torres et al., 2019; Unsworth et al., 2021). Cyanoacrylate gel glues have also been tested, showing quick curing, but the difficulty of handling them underwater, their poor mechanical properties, and the dislodgment risk allow their application only for small coral pieces and in protected environments (no waves, no currents) (Dizon et al., 2008). The fact that epoxy putties and concrete can also take hours to harden is another point to consider. During the hardening procedure, the users should keep the transplanted coral stable and protect it from waves or general sea motions that can alter its position; therefore, an additional subsequent check of the out-planting process should be carried out the day after to verify its success (Dizon et al., 2008; Boström-Einarsson et al., 2020).

Hence, the materials developed to support the rescue and restoration of coral reefs call for new advanced strategies. Furthermore, following the guidelines given by the Sustainable Development Goals (SDG) set by the United Nations, the design of novel, sustainable, and eco-friendly technologies is mandatory for preserving life below water (SDG 14). A proper sustainable and scalable coral restoration plan should involve using biodegradable and non-toxic materials derived from natural renewable sources or waste biomass. These concepts, already used in other fields and underwater applications (Aracri et al., 2021a and 2021b; Zaidi et al., 2021), can also be utilized to design novel coral putties.

Herein, we present an epoxidized soybean oil acrylate (ESOA)/zein-based putty prepared using a commercial planetary centrifugal mixer as a new eco-friendly tool for coral reef restoration. ESOA derives directly from the corresponding plant oil, which is considered a bio-based feedstock. Its abundance, renewability, and environmental benignity make it ideal for the design of thermosetting materials (Zhang et al., 2017; Zych et al., 2020). Zein, a protein derived from corn with tunable hydrophobicity and biodegradability, has been widely used in different fields such as food packaging and biomedicine, and more recently, even as a tunable matrix for the underwater delivery of antioxidants into corals (Kasaai, 2018; Contardi et al., 2023; Fiorentini et al., 2023).

The morphology, hardening kinetics, and mechanical and thermal properties of the developed coral putty were characterized, and its performance was compared to the commercial DGEBA-based epoxy resins IamSub and Aquamend, commonly used for coral restoration and for fixing corals in aquaria, as explained before. Our coral putty, meaning the crosslinked matrix with the filler zein, showed a much quicker hardening rate compared to the two other commercial putties, and its two components were stable both during the mixing process and after 24 hours of immersion in the seawater. The coral putty and the pristine crosslinked ESOA matrix (without the zein) proved biodegradable in the seawater-based Biochemical Oxygen Demand (BOD) test. At the same time, IamSub and Aquamend putties did not show any evidence of biodegradation after 30 days. Finally, our coral putty was first applied on *Stylophora pistillata* in a controlled system context and then also in the natural environment in the Maldives for out-planting *Acropora tenuis* colonies back onto the reef.

## 5.3. MATERIALS AND METHODS

### 5.3.1. Materials

Epoxidized soybean oil acrylate (ESOA), dibenzoyl peroxide Luperox® A75, zein, and sodium hydroxide were purchased from Sigma-Aldrich and used as received. Para-toluidine ethoxylate tertiary amine commercialized with the name of Bisomer® PTE was kindly provided by GEO® Speciality Chemicals. IamSub was shipped from Veneziani Zetagi (Colorificio Zetagì srl - Via Pasubio, 41, 36051 Olmo di Creazzo (Vi), Italy, https://www.zetagi.it/iam-sub/). At the same time, Aquamend was purchased from Amazon and produced by Polymeric Systems, Inc. (47 Park Avenue, Elverson, Pennsylvania, United States, www.polymericsystems.com).

### 5.3.2. Coral Putty Preparation

The coral putty is designed as a two-component system (A + B). Component A contains dibenzoyl peroxide (radical initiator), and component B contains para-toluidine ethoxylate tertiary amine (accelerator). Both components were prepared in the same way using THINKY Mixer (https://www.thinkymixer.com/en-us/) running a mixing program for 10 s at 1200 rpm, 10 s at 1400 rpm, 10 s at 1600 rpm, 10 seconds at 1800 rpm, and 50 s at 2000 rpm in a plastic cup provided by the same company. Firstly, either 0.4% w/w of the radical initiator was homogenized with 49.6% w/w of ESOA (for component A) or 0.3% w/w of the accelerator with 49.7% w/w of ESOA (for component B) using two mixing cycles. Zein powder was sifted through a 50µm mesh before use. Subsequently, 50% w/w of zein powder was added as a filler to adjust viscosity and homogenized using three mixing cycles to obtain a moldable yellow putty (Figure 1A). The final coral putty was obtained by mixing components A and B by hand in a 1:1 weight ratio for around 2 min and a subsequent 30-minute-long wait for its hardening. Coral putty without zein, containing only the ESOA crosslinked with the initiator and the accelerator, was also used as a reference sample for the characterization.

### 5.3.3. Scanning Electron Microscopy (SEM)

The morphology of the samples was analyzed by Scanning Electron Microscopy (SEM) using a variable pressure microscope JOEL JSM-649LA (JEOL, Tokyo, Japan) equipped with a tungsten thermionic electron source and working in high vacuum mode, with an acceleration voltage of 5 kV. The specimens were coated with a 10 nm thick film of gold utilizing a Cressington Sputter Coater – 208 HR (Cressington, Watford, UK).

### 5.3.4. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Infrared spectra were acquired using an ATR accessory (MIRacle ATR, PIKE Technologies) with a diamond crystal coupled to an FTIR spectrometer (Vertex 70v FT-IR, Bruker). All spectra were recorded between 4000 and 600 cm$^{-1}$, with a 4 cm$^{-1}$ resolution, accumulating 128 scans.

### 5.3.5. Thermogravimetric Analysis (TGA)

The thermal degradation behavior of the coral putty and coral putty with no zein, ESOA, and zein was determined by thermogravimetric analysis (TGA) using a TGA Q500 instrument (TA Instruments, USA). Measurements were carried out using 3–5 mg of sample in a platinum pan under inert $N_2$ flow (50 mL min$^{-1}$) in a temperature range from 30 to 800 °C and with a heating rate of 10 °C min$^{-1}$. The weight loss and its first derivative were acquired simultaneously as a function of time/temperature.

### 5.3.6. Dynamic Thermal Mechanical Analysis (DTMA)

Dynamic thermal mechanical analysis (DMTA) was performed using a DMTA TA Q800 equipped with a compression clamp in time sweep mode. The coral putty, IamSub, and Aquamend samples were tested at 28 °C to simulate the water temperature in the coral reef environment. Experiments were performed in a single-frequency oscillation mode with a frequency of 10 Hz and a displacement amplitude of 0.1% in auto tension offset control. Data were recorded every two minutes, and the curing dynamics were evaluated from the evolution of the storage modulus, loss modulus, and tan delta as a function of time.

### 5.3.7. Compression Test

The mechanical properties of cured putties were characterized by uniaxial compression using a 3365 Instron dynamometer equipped with a 2kN load cell. Putties were cured in a cylindrical shape (approximately 10 mm in height and 10 mm in diameter) and tested at the displacement rate of 5 mm/min (n = 7). From the stress-strain curves, Young's modulus was evaluated as the slope of the initial linear region; depending on the fracture mechanism, the material strength was also assessed, in the samples that showed strong shattering, as the peak stress before the drop at failure.

### 5.3.8. Stability underwater

Both components of the coral putty (A and B), IamSub and Aquamend, were placed separately in vials filled with seawater to evaluate their stability in the marine environment. Their condition was monitored for 2 hours, and pictures were acquired. Then, the seawater from each sample was analyzed using a Varian Cary 6000i Scan UV-visible spectrophotometer (Walnut Creek, California, USA), and the transparency of the solutions was obtained by reading the absorbance at 600 nm and calculated as follows: $T(\%) = 10^{-Abs} * 100$

Similarly, the two components of each putty were mixed in 700 mL of seawater in a crystallizer. Photographs of the water before and after the blending were acquired. Finally, after mixing, the seawater from each sample was analyzed utilizing the UV-visible spectrophotometer.

### 5.3.9. Water Contact Angle (WCA)

The coral putty, IamSub, and Aquamend's water contact angles (WCA) were measured at room temperature using a contact angle goniometer OCA-20 (DataPhysics, Instruments GmbH, Filderstadt, Germany). Deionized water droplets of 5 µL were laid on the surface, and the contact angle was calculated from the side view with the help of the software. To ensure repeatability, seven different measurements were taken for each sample.

### 5.3.10. Biochemical Oxygen Demand (BOD) Test

Biochemical oxygen demand (BOD) was used to investigate the biodegradability of the samples in the marine environment, which can be easily determined by monitoring the oxygen consumption in a closed respirometer. In detail, samples were cut into small pieces (around 5 mm side squares), and about 100 mg of material was added to 432 mL of seawater as the single carbon source. Seawater was chosen as a liquid medium to mimic realistic environmental conditions. It already contained microbial consortia and the saline nutrients needed for their growth. The experiment was conducted in triplicate at room temperature inside amber glass bottles with a volume of 510 mL, hermetically closed with the OxiTop® measuring head. Sodium hydroxide was added as a $CO_2$ scavenger to sequestrate carbon dioxide produced during biodegradation. Biotic consumption of the oxygen present in the free volume of the system was measured as a function of the decrease in pressure. Raw oxygen consumption data (mg $O_2$/L) were corrected by subtracting the mean values of the blanks obtained by measuring the seawater's oxygen consumption without any test material. After this subtraction, values were normalized on the mass of the individual samples and referred to 100 mg of material (mg $O_2$/100 mg material). Finally, the means of the triplicates were calculated and plotted versus time.

### 5.3.11. Application on corals and biodegradation in aquarium

The reef-building coral *Stylophora pistillata* was used as the test species (Meziere et al., 2021; Contardi et al., 2023). Experiments were performed with 30 fragments (~6 cm in length) obtained from six large mother colonies raised inside tanks of the Aquarium of Genova (Genova, Italy). Before testing, fragments were kept under controlled conditions in an acclimatization 3100 L tank (photoperiod was 12 h:12 h light:dark) to recover for one month. To improve recovery, colonies were fed daily with a food mixture solution containing the unicellular algae *Tetraselmis suecica* and zooplankton *Brachionus plicatilis* belonging to the phylum Rotifera. During the day (from 8:00 to 15:00), the tank was set up as a semi-open system, constantly supplied (about 300 L/hour) with UV-filtered seawater pumped from the 50-m-deep outside the Foranea dam of Genova. The temperature was constantly kept at 25 °C. Corals were illuminated with an HQI lamp (400 W 10.000 K Nepturion BLV) at an average irradiance of 250 μmol photons $m^{-2}$ $s^{-1}$. After 15:00, the tank was set up as a closed system, where the water circulation pump (Argonaut av150-2dn-sb 220v) was set between 10 and 13 $m^3$/h to ensure a complete change of water every 25 minutes. Once taken by the pump, the water from the tank was passed through a filtration system consisting of a sand filter (0.4 mm, Astralpool ARTIC) and a UV sterilization system (Panaque 750 s, with four 40 W lamps embedded), and it was subsequently reinserted into the tank. Moreover, 50 L of calcium hydroxide solution at a concentration of 18 g/L was added dropwise every night to facilitate the calcification of corals, enhancing their growth. Chemical-physical parameters (pH, nitrates, nitrites, ammonium, phosphates, salinity) of the water tank were monitored twice a week.

Nubbins were divided into two experimental groups (n = 15). Each group was fixed on dowels Fischer using coral putty (n = 5), IamSub (n = 5), and Aquamend (n = 5). This latter is a common procedure used in aquaria for growing nubbins (Jantzen et al., 2013; Leal et al., 2016; Pontes et al., 2023). The second group of nubbins was fixed shaping the putties (Coral putty (n = 5), IamSub (n = 5), and Aquamend (n = 5)) around them and placed at the bottom of the tanks. The health condition of the nubbins was monitored for 3 months. Specifically, coral nubbins were regularly photographed (three times per week) to monitor their health status on a visual-morphological level. The following stress parameters were considered as present or absent: polyp retraction, production of mucus by the coral, presence of possible necrosis in the tissue, and presence of eventual tissue bleaching. Furthermore, to confirm the optimal state of health and growth, the live coral tissue grown on the material applied and the three-dimensional growth was evaluated through image analyses.

### 5.3.12. Application on corals in the Maldives

In May 2022, the coral putty underwent on-site testing and was monitored until April 2023 on Magoodhoo Island, Faafu Atoll, Republic of Maldives (Figure 2). The lagoon was chosen as the out-planting location due to its favorable environmental conditions, as it was protected from seasonal storms and had better exposure to currents in relation to the reef orientation. A sheltered rocky substrate at a depth of six meters was chosen, which was protected from direct exposure to currents but had sufficient light to facilitate coral growth. *Acropora tenuis*, a fast-growing and robust species commonly used in restoration projects, was chosen for experimentation, and five colonies were harvested. All five colonies were out-planted in a linear configuration to have consistent conditions.

Before the dive, equal amounts of coral putty components A and B were prepared in bead form and placed into separate bags to facilitate the underwater mixing process. Upon arrival at the site, the flat surface of the reef rock was carefully brushed to remove algae and sediment to improve the adhesion of the coral putty and ensure the successful attachment of the coral colonies. The shape was studied for each colony to find the optimal amount of product and the number of contact points to use in the out-planting process. Two equal parts of coral putty components A and B were mixed by hand for approximately one minute and then carefully applied to the rock, ensuring a solid adherence to the substrate. The base of the coral was then inserted and secured by shaping the coral putty around the base.

Over the following month, monitoring the out-planted corals involved regular visits to assess their stability on the substrate and capture images. These photographs were used to evaluate the progress of each out-planted colony, assessing its stability against currents, the growth capacity over the product, overall growth, the extent of live tissue coverage, and general health status.

## 5.4. RESULTS AND DISCUSSION

### 5.4.1. Coral putty design and fabrication

The coral putty was designed as a typical two-component system (A + B), and its crosslinking and hardening are based on the polymerization of acrylates present in the ESOA vegetable oil. Both components were prepared using a planetary centrifugal bubble-free mixer (Figure 1A). Component A contains dibenzoyl peroxide (radical initiator) and component B contains para-toluidine ethoxylate tertiary amine (accelerator). Both components are stable at room temperature when kept separately and can be stored for several months. After mixing them in a weight ratio of 1:1, the tertiary amine accelerator triggers the decomposition of dibenzoyl peroxide into benzoyl radicals that start the polymerization of acrylates, causing the putty to harden (Figure 1B and Figure 3). Zein was selected as a natural, hydrophobic filler to adjust the viscosity of the putty, which led to an easy-to-handle moldable paste.

### 5.4.2. Morphological, chemical and thermal characterization

The morphologies of the components A and B, the crosslinked oil without zein (named crosslinked ESOA), and the coral putty were investigated by SEM (Figures 4A-D). Components A and B showed the zein powder well-dispersed in the ESOA (Figures 4A and 2B). No clusters of the peroxide were detected in component A. Crosslinked ESOA presented a smooth and compact surface (Figure 4C). The crosslinked coral putty presented again the dispersion of the zein powder into the ESOA and no phase separation or precipitation of any of the ingredients from components A and B was evident after the mix, suggesting a good blend of the two components in the coral putty (Figure 4D).

To confirm the successful crosslinking through acrylate polymerization, spectra of ESOA, crosslinked ESOA, coral putty, component A, component B, and zein were obtained and analyzed using ATR-FTIR (Figure 4E). Specifically, the ESOA spectrum showed the following vibrations modes: a weak O-H stretching mode at 3508 $cm^{-1}$ of the small amount of hydroxyl group in the later side of the chains; asymmetric and symmetric $CH_2$ and $CH_3$ stretching modes at 2955, 2926, 2870, and 2855 $cm^{-1}$ connected with the aliphatic chain; C=O stretching mode of the esters between the fatty acid and the glycerol at 1740 $cm^{-1}$; C=O stretching mode of the acrylic ester in the chain at 1722 $cm^{-1}$; acrylic C=C stretching modes of the acrylic group (trans and cis isomers) at 1636 and 1618 $cm^{-1}$; $CH_2$ and $CH_3$ bending modes at 1465 and 1373 $cm^{-1}$, respectively; C-O-C stretching modes for the acrylic ester and the fatty ester at 1188 and 1157 $cm^{-1}$, respectively; wagging terminal $CH_2$ of the acrylic group mode at 809 $cm^{-1}$; rocking $CH_3$ of the fatty chains mode at 723 $cm^{-1}$ (Liu et al., 2019).

Changes connected with the crosslinking process can be observed in the spectrum of the crosslinked matrix without the zein filler. To highlight these changes, the two spectra were normalized in the function of rocking $CH_3$ of the fatty chain mode at 723 $cm^{-1}$, which is not involved in the reaction being the terminal part of the fatty chains (Figure S2). An intense reduction of the signals of the acrylic group at 1636, 1618, and 809 $cm^{-1}$ compared to the ESOA sample was observed. The C=O directly connected to the acrylic group was much less intense. Therefore, after crosslinking, it cannot be distinguished from the C=O of the fatty acid, suggesting a potential shift at higher wavenumbers due to the lack of the reacted acrylic group. Indeed, the peak is centered at 1728 $cm^{-1}$. Likewise, the C-O-C signal at 1188 $cm^{-1}$ drastically reduced its intensity compared to the ESOA spectrum, less intense than the C-O-C stretching mode of the fatty acid chain. These changes confirmed the successful crosslinking reaction, which directly involves the acrylic group, as schematized in Figure 1B.

The crosslinked matrix with the filler zein coral putty presented an overlapping of the peaks of ESOA and zein. Zein peaks cover the acrylic doublet, but the other signals of the occurred crosslinking can be observed. Indeed, the C=O stretching modes were overlapped and centered at 1728 $cm^{-1}$, the C-O-C of the fatty acid chain was more intense than the ones of the acrylic ester, and the wagging terminal $CH_2$ of the acrylic group was less intense than the rocking $CH_3$ of the fatty chain mode. This was also confirmed in the spectra of components A and B, where these peaks were similar to the pristine ESOA. Also, the typical peaks of zein can be recognized here with the following vibration modes: N-H stretching occurs at 3288 $cm^{-1}$; O-H stretching at 3201 $cm^{-1}$; the typical C-H aromatic and aliphatic stretching vibration from 3090 to 2850 $cm^{-1}$, which confirmed the presence of aromatic and aliphatic groups of the zein amino acids; the Amide I centered at 1646 $cm^{-1}$, Amide II at 1516 $cm^{-1}$, and Amide III at 1446 $cm^{-1}$ characteristic of the peptide bond; and C-N stretching at 1235 $cm^{-1}$. Finally, the difference in the amount of acrylic acid between the two components and the coral putty was calculated to quantify the yield of the crosslinking reaction. To obtain this, the area under the curve (AUC) of the acrylic group at 1636 and 1618 $cm^{-1}$ for the components A and B and the coral putty was calculated. Then, an average of the AUC of components A and B was obtained and divided by the AUC of coral putty to obtain the consumed quantity of the acrylic group after the crosslinking reaction, which resulted in 64%.

The thermal stability and how it could be potentially affected by the crosslinking process was investigated by the TGA analysis (Figure 4F). The thermogravimetric curve of zein showed the initial weight loss related to the moisture evaporation at around 50 °C, and its main degradation temperatures were centered at 280 and 310 °C which can be associated with either the breakdown of the amino acids or the peptide bonds (Dhandayuthapani et al., 2012). The pristine ESOA began losing weight around 200 °C and showed two main weight loss peaks at around 384 and 415 °C. In the crosslinked condition, the first main peak of weight loss was anticipated to be 379 °C, but the decomposition of ESOA was not deeply affected by the crosslinking. A similar absence of changes was also observed by Meng et al., who studied acrylate crosslinking mechanisms (Meng et al., 2020). On the other hand, in the developed coral putty, the initial zein degradation was increased until 323 °C, while the first main peak of the oil weight loss was anticipated at 366 °C, and the second one was slightly shifted to 420 °C. Apparently, the crosslinking seems to have a protective effect on zein, which is probably protected by the crosslinked structure of the cured oil.

### 5.4.3. Mechanical properties

The hardening time after mixing the two putty components can be crucial for successful coral out-planting. Indeed, it should be slow enough to give the operator time to shape the paste at the out-planting site and then place the coral inside it. At the same time, it should be fast enough to ensure reliable support for the coral before it can be eradicated due to its weight, waves, or interaction with other organisms.

For this reason, the hardening of IamSub, Aquamend, and coral putty was evaluated using DMA by monitoring the variation of Storage and Loss Modulus (E' and E'', respectively) and Tan δ over time at the control temperature of 28 °C, as this is a typical ocean temperature in the tropical zone where most corals live. During the thermoset curing, the crossover point (where E' and E'' cross each other or where the Tan $\delta = 1$) can be considered as a liquid-solid transition (Roller, 1986; Liang & Chandrashekhara, 2006; Zych et al., 2020). The DMA results for IamSub, Aquamend, and coral putty are reported in Figure 10. Both storage and loss modulus of IamSub and Aquamend slightly increased during the curing, but no crossover point was observed even after 80 min, suggesting that none of the materials became solid or hardened during that time (Figures 5A and 5B). On the other hand, the storage modulus of the coral putty increased faster than the loss modulus (Figure 5C), and the crossover point was observed after around 20–25 minutes, suggesting a transition from liquid to solid and hardening of the material. Similarly, the value of Tan δ remained above one along the measurements for IamSub and Aquamend, while it quickly decreased below one for the coral putty.

Additionally, compression tests were carried out to investigate the compression strength and modulus of the IamSub, Aquamend, and coral putty (Figures 6A and 6B). IamSub showed a Young's Modulus (YM) of 84.0 ± 8.0 MPa and compression strength (CS) of 18.0 ± 0.8 MPa. Similarly, Aquamend had a YM of 264.5 ± 4.5 MPa and a CS of 14.0 ± 2.5 MPa, while coral putty performed with a YM of 28.5 ± 10.0 MPa and a CS of 2.3 ± 0.5 MPa. Although coral putty had lower YM and CS values, they are comparable to commercial bricks or ice (Parashar & Parashar, 2012; Bonath et al., 2013), making the coral putty strong enough to support a coral despite its vegetable origin.

### 5.4.4. Interaction with water and biodegradability

To reach the reef area for the out-planting, the operator has to bring the paste underwater, keeping the two components separated, and then mix them *in-situ*. To achieve that, the paste needs to be stable in the seawater, avoiding the entrance of water into the paste, and should not release components into the environment. To evaluate such parameters, the two components labeled A and B of coral putty, IamSub, and Aquamend were immersed in seawater for 2 hours. As can be noticed in Figure 7A, the water turbidity implies that component A of IamSub and Aquamend released some macroscopic ingredients, suggesting instability and potential risk of environmental pollution. On the other hand, the two components of coral putty were macroscopically stable during their stay in seawater. For further confirmation of such observations, the remaining seawater solutions were spectroscopically analyzed, and the transparency degree was 100 and 99.9 % for the components A and B of coral putty, respectively, 85 and 98 % for the components A and B of IamSub, respectively, and 91 and 99 % for the components A and B of Aquamend, respectively.

Another essential feature is the stability of the putties during their underwater mixing. For this reason, the components (A + B) of the putties were blended inside a crystallizer full of seawater. The seawater resulted in having a transparency of 99.7, 60.9, and 93.5 % in the case of the coral putty, IamSub, and Aquamend, respectively, with IamSub being the most unstable, turning seawater into an opaque light blue suspension. Instead, Aquamend did not release a high amount of ingredients, while coral putty had excellent stability performance during the mixing (Figure 8).

WCA analyses were carried out to investigate the nature of the surface when exposed to water for the IamSub, Aquamend, and coral putty, and the results are reported in Figure 7B. IamSub presented a WCA of 104°, while Aquamend had a higher value of 110°. Coral putty showed the highest WCA value of 113°. Such results revealed that the hydrophobicity of the tested materials is comparable to that of commercial products.

The biodegradation of the ESOA crosslinked (ESOA oil + radical initiator + radical accelerator, without fillers), coral putty (zein filler), IamSub, Aquamend, and cellulose was evaluated by BOD test in seawater (Figure 7C). Cellulose was used as the control sample and as expected, showed high biodegradability that was detected already after one day, with a final BOD value of around 62 mg $O_2$/100 mg. Coral putty started degrading after 10 days and reached a BOD value of 23.5 mg $O_2$/100 mg after 30 days. The two components A and B started the biodegradation after 3-4 days and reached BOD values of 63.9 and 43.1 mg $O_2$/100 mg after 30 days, respectively, similar to the ones of cellulose (Figure 9), as these materials, mainly composed of zein and ESOA, are not crosslinked, and therefore microorganisms can have easier access to biodegrade them. Crosslinked ESOA was also biodegradable, reaching, though, a lower BOD value (6.5 mg $O_2$/100 mg) after 30 days since it does not contain the highly biodegradable zein. Nevertheless, the fact that the material partially biodegrades within 30 days shows that the crosslinked ESOA undergoes biodegradation. Finally, IamSub and Aquamend showed no sign of biodegradation during the test period, as expected from synthetic commercial epoxy resins.

### 5.4.5. Application onto corals in the aquarium

The ability of our Coral Putty to act as a coral support was first validated in the aquarium. Nubbins of *Stylophora pistillata* were fixed on dowels Fishers using coral putty and monitored for three months. Photographs of the nubbins stabilized with the coral putty were taken after the application at 0, 30, and 90 days respectively (Figures 10A-D). As a reference and comparison, nubbins fixed with IamSub and Aquamend were also monitored (Figures 11 and 12).

The quick hardening property of the coral putty ensured immediate suitable stability of the nubbins to the support. This performance was comparable to IamSub and Aquamend (Figures 11 and 12). Nubbins treated with the three putties did not show any alteration of colors, polyps' condition, or tissue necrosis, and normal growth occurred during the observation period

(Figures 10, 11, and 12). To further evaluate the performance of the developed coral putty, a simulation of the shape required for a proper attachment to the reef was made with only the putty as a support. Also in this case, the same procedure was followed for IamSub and Aquamend for comparison. Results showed that the corals attached with coral putty again did not display any color change or signal of stress throughout the 3 months of analyses (Figures 10E-H), showing features comparable to the ones of commercial products (Figures 11 and 12). The overall growth was normally occurring, highlighted by the numerous apical corallites starting to grow from the main branches of the corals (Figure 10H).

### 5.4.6. Application onto corals in the field

Finally, coral putty was used for an on-field out-planting action in the Maldivian coral reefs in May 2022. The procedure is shown in Figure 13A and consists of choosing the coralline substrate, cleaning the substrate with a brusher, taking component A and component B, properly mixing, placing the well-mixed coral putty on the coralline substrate, and the proper attachment of a new colony. The overall process lasts around 3 minutes. Afterward, a test to simulate strong waves and to avoid the dislodgment of corals was performed. Specifically, the diver moved his/her arm and hand close to the transplanted fragment to generate mechanical stress. After 20 minutes from the transplantation made with the coral putty, the fragments were stably attached to the reef. *Acropora tenuis* colonies were transplanted on the reef and left for 12 months (Figure 13B). The colonies remained fixed on the out-planting point, and the corals grew during the monitoring period in accordance with the natural growth rate of hard coral in nature (Anderson et al., 2017; Mahmoud et al., 2019; Weil et al., 2020; Prabowo et al., 2022). These results demonstrate the versatility of the coral putty for its utilization not only as support in aquaria but also in direct out-planting of corals *in-situ*.

The ease of use and the hardening time make the coral putty extremely manageable in water for the time necessary to shape it effectively to fit the contact points provided by the different fragments in relation to the available positions on the reef to be restored. Indeed, coral putty offers significant advantages over traditional support materials and techniques, such as cement, which provides limited flexibility at contact points, extended preparation time, longer hardening periods, experienced operators, and requires additional monitoring of the success. Similarly, bicomponent epoxy generally used for coral restoration is stickier and more difficult to mix and shape, as well as slower in hardening, requiring more time to ensure the attachment of corals, reducing the success rate of out-planting sessions. Therefore, coral putty presents an opportunity to enhance the process in terms of both the number of colonies that can be out-planted per dive and the success rate of the out-planting itself, as corals are securely attached and better equipped to withstand currents and wave energy.

Furthermore, the monitoring period revealed that the putty has no visible impact on coral health. Initially, the colony, being an opportunistic fragment partially covered by sand at the bottom, displayed some pale areas due to sedimentation (Figure 13B, May 2022). However, following the out-planting in a favorable reef location, it exhibited optimal growth, recovering from its previous pale state. Moreover, the coral putty appears to integrate well with the environment, where algae have quickly colonized it within a few days. This integration does not impede coral growth or pose any disturbance to the surrounding organisms, as suggested by the healthy coral colony visible at the bottom of each image (Figure 13), which remains unaffected.

## 5.5. CONCLUSION

The out-planting phase is the last step of most coral gardening techniques. During this phase, corals are moved back into the reef ecosystem after growing in *ex-situ* or *in-situ* nurseries. Specifically, corals are moved to rescue areas where the reef was altered due to local increases in temperature, new human infrastructures, the spread of diseases, or pollutants in the water (Goergen et al., 2020). The success of this procedure is around 65 % on average (Boström-Einarsson et al., 2020) and the current methodologies used to contrast this phenomenon present several drawbacks. Indeed, commercial epoxy resin or concrete, adapted from other applications to fix corals on the reef, has a slow hardening rate, is not biodegradable, and is made of artificial substances that can potentially release toxic components into the environment. Therefore, the current challenge is to design and produce biocompatible and biodegradable quick-hardening materials for transplanting corals with low environmental impact. Moreover, they should be manageable and provide enough time for the operator to shape them around corals and fix them to the natural substrate.

Herein, we presented the first biodegradable coral putty based on ESOA and filled with zein, a hydrophobic protein derived from corn. The coral putty was designed to contain a radical initiator and a radical accelerator, respectively. Once the two components are mixed in the convenient weight ratio 1:1, the curing reaction is triggered, and the coral putty becomes hard in around 20-25 minutes, in contrast with commercial epoxy resins or concretes used for the same purpose, which can even take hours or days to harden. Such commercial materials have a higher compression strength (around 13 – 50 MPa; Abdalla et al., 2019) compared to the 2 MPa of the coral putty; however, as proved in this work, such high mechanical strength is unnecessary for this type of application. Of note, the high mechanical performance of commercial bisphenol A-based epoxies derives from their aromatic structure and comes along with toxicity to aquatic organisms, and impedes biodegradability. Furthermore, both components of the coral putty presented excellent stability in seawater before mixing and curing, unlike some ingredients of the two commercial products, especially the IamSub, which were diffusing in the seawater not only when they were just left in it but also upon mechanical stress during the mixing. At the same time, the cured coral putty had a WCA of around 130° and resulted in being biodegradable as evaluated by the BOD test, while both the IamSub and the Aquamend did not show any signs of biodegradation. The technical features of the coral putty and the commercial epoxy resins evaluated in this work are reported in Table 1.

Finally, yet importantly, the ability of our coral putty to fix nubbins of *Stylophora pistillata* was confirmed in aquarium for a period of 6 months without showing any adverse external effects on the corals. Similarly, it was successfully used to out-plant *Acropora tenuis* colonies on coral reefs in the Maldives, and after one year, the corals grew with no macroscopic stress signals. Moreover, the overall process of transplantation lasted around 3 minutes, and after 20 minutes, the fragments were stably anchored to the reef. This performance can significantly impact the success of coral restoration when compared to other materials like commercial epoxy resins or concrete, which can take several hours to a full day to set. This extended hardening time means divers have to return the following day to check on the status of the fragments. (Frias-Torres et al., 2019).

In conclusion, this work seeks to showcase the potential of sustainable, bio-based, and biodegradable materials for coral out-plantation and management both in aquaria and in natural settings. These materials provide performance that is comparable to that of commercial products currently used for these purposes, which are often designed for different applications. In addition, materials based on renewable and natural ingredients, such as vegetable oils and proteins, can replace epoxy resins in coral restoration actions, thus reducing the overall total amount of non-biodegradable materials released in the seawater. Hence, material science and marine ecology can be united in the design and fabrication of more sustainable, eco-friendly, and biodegradable tools that can ameliorate coral restoration but, at the same time, rescue the environment in the long term.

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## 5.7. TABLES

**Table 1.** Summarizing the overall properties of IamSub, Aquamend, and Coral Putty.

| Properties | IamSub | Aquamend | Coral Putty |
|---|---|---|---|
| **Compression Strength** | 18.00 MPa | 14.00 MPa | 2.30 MPa |
| **Rate of hardening** | 3-4 hours | 1-2 hours | 20-25 min |
| **Macroscopic release of Components in the environment during the mixing** | Yes | Yes | No |
| **Water Contact Angle** | 104° | 110° | 113° |
| **Biodegradability** | No | No | Yes |

## 5.8. FIGURES

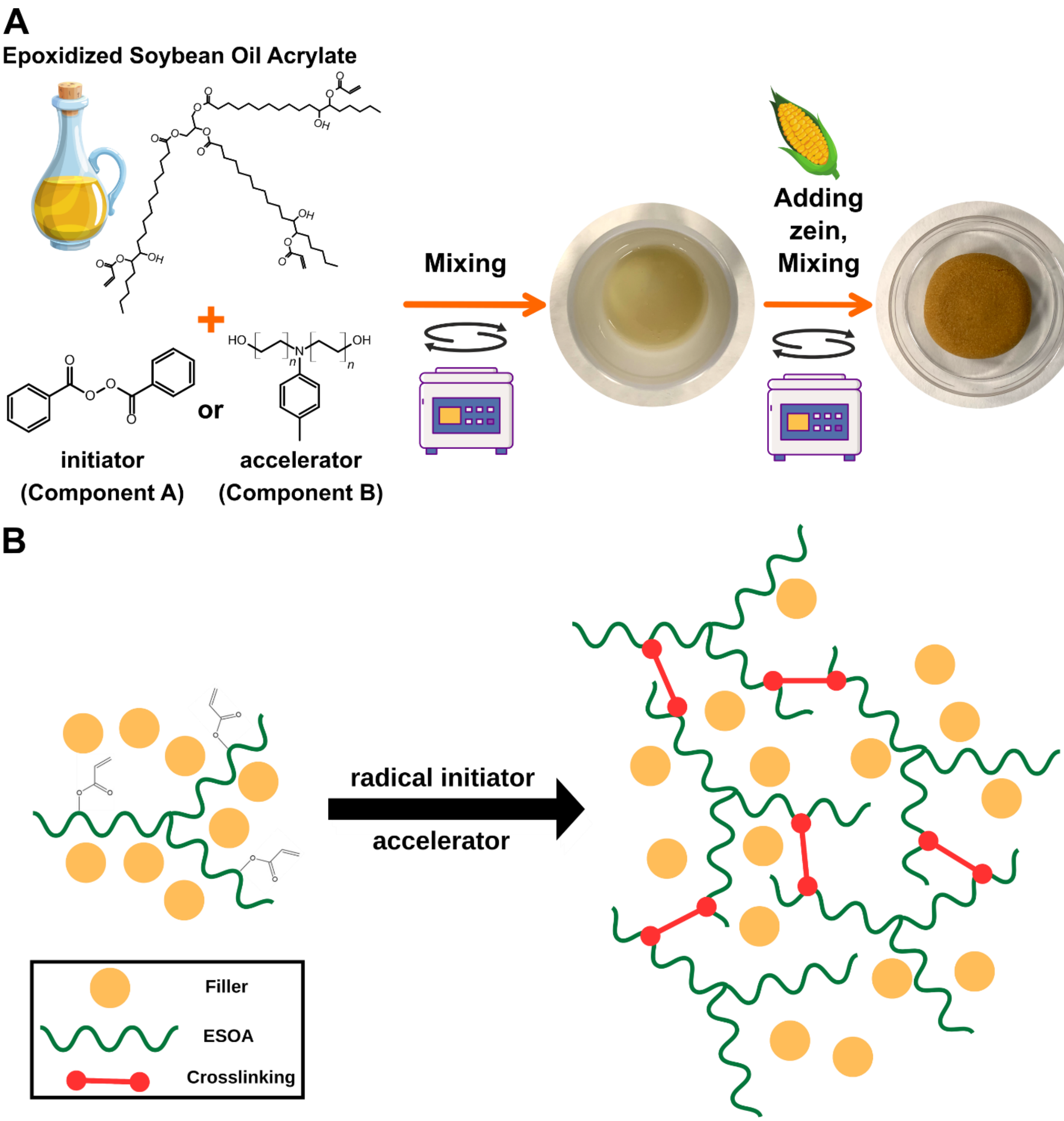


**Figure 1. Preparation and cross-linking. A)** Scheme of the preparation of coral putty; **B)** Scheme of the crosslinking occurring inside the coral putty after mixing components A and B.

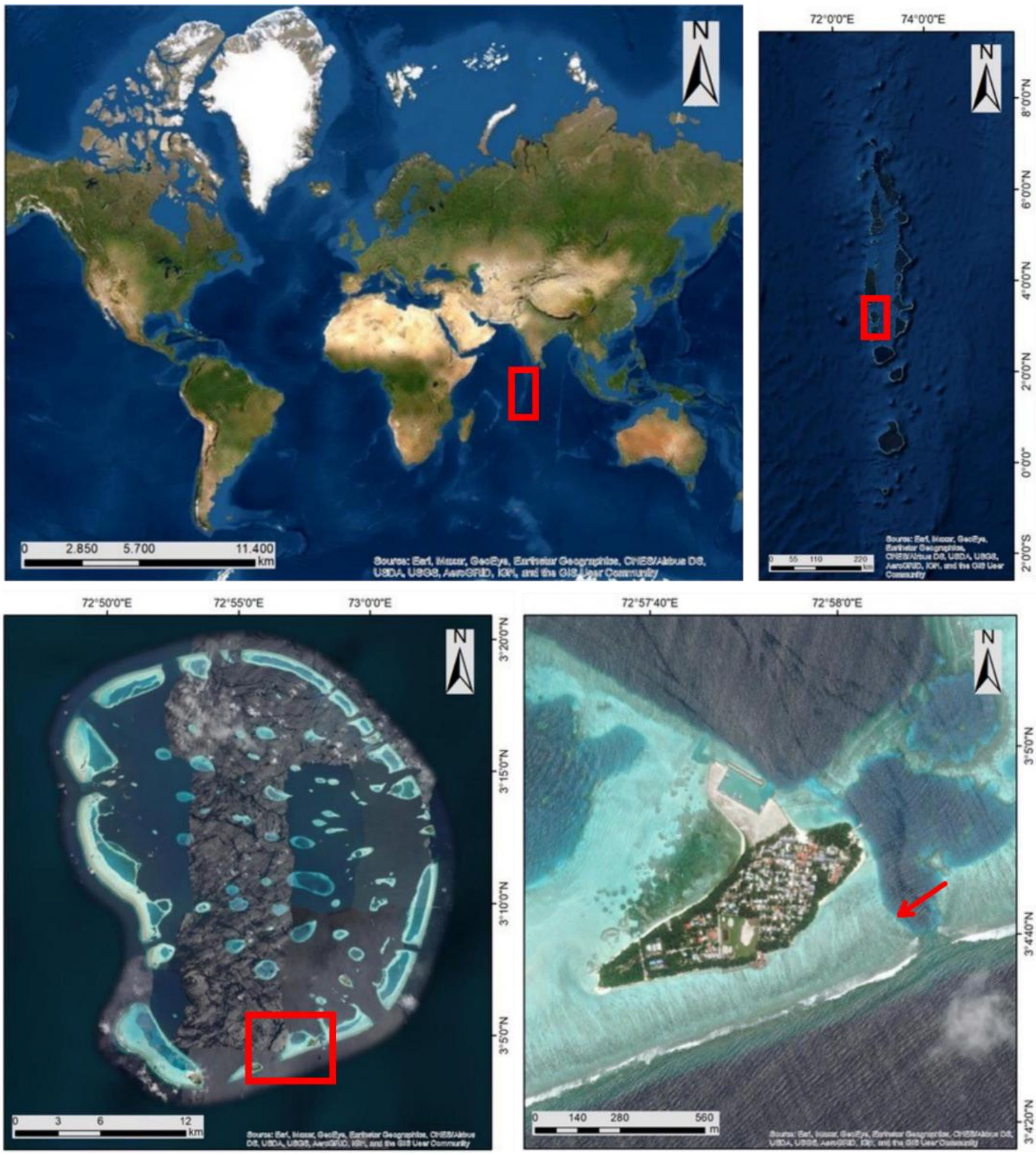


**Figure 2**. **Study area**. **A)** Republic of Maldives; **B)** Faafu Atoll; **C)** Magoodhoo Island, out-planting site (arrow; 3°04′ N, 72°57′ W). The map was made using Google satellite and loaded into QGIS.

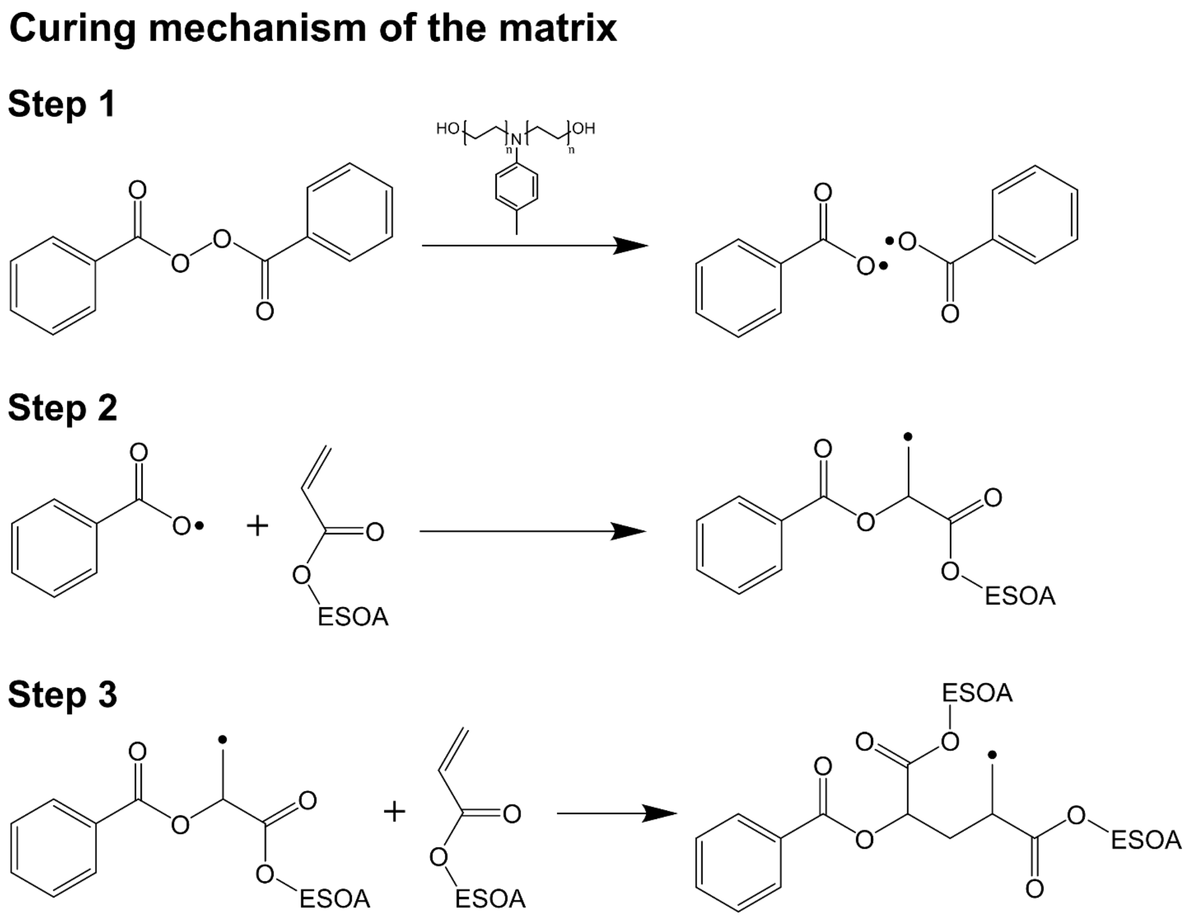


**Figure 3. Crosslinking mechanism.** Scheme of the curing mechanisms inside the vegetable oil matrix.

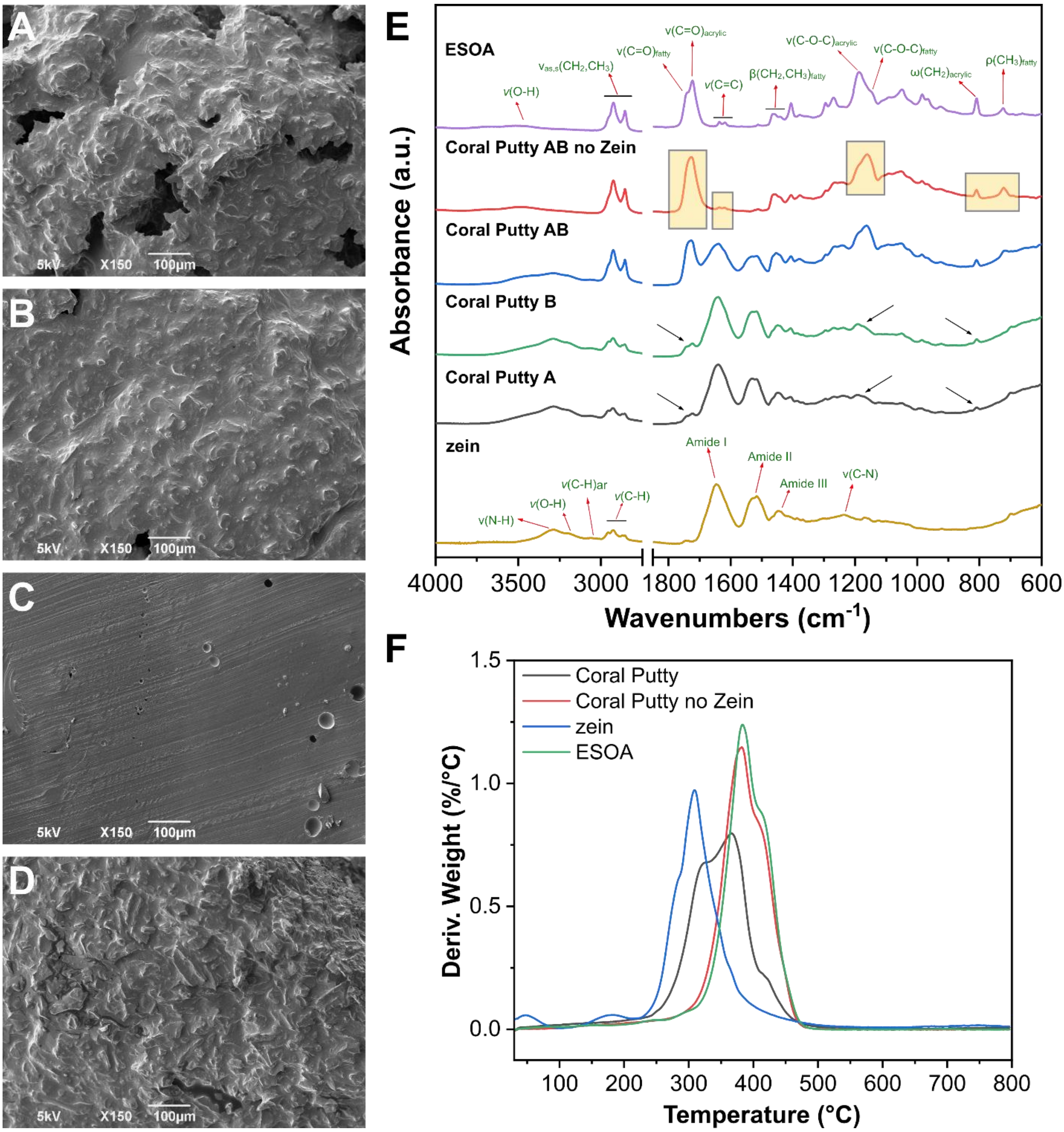


**Figure 4. Morphological, chemical, and thermal analysis. A)** SEM images of component A; **B)** SEM images of the component; **C)** SEM images of coral putty without zein; and **D)** SEM images of coral putty; **E)** ATR-FTIR spectra for ESOA, coral putty without zein, coral putty, component B, component A, and zein; **F)** Derivative thermogravimetric curves for coral putty, coral putty without zein, zein, and ESOA.

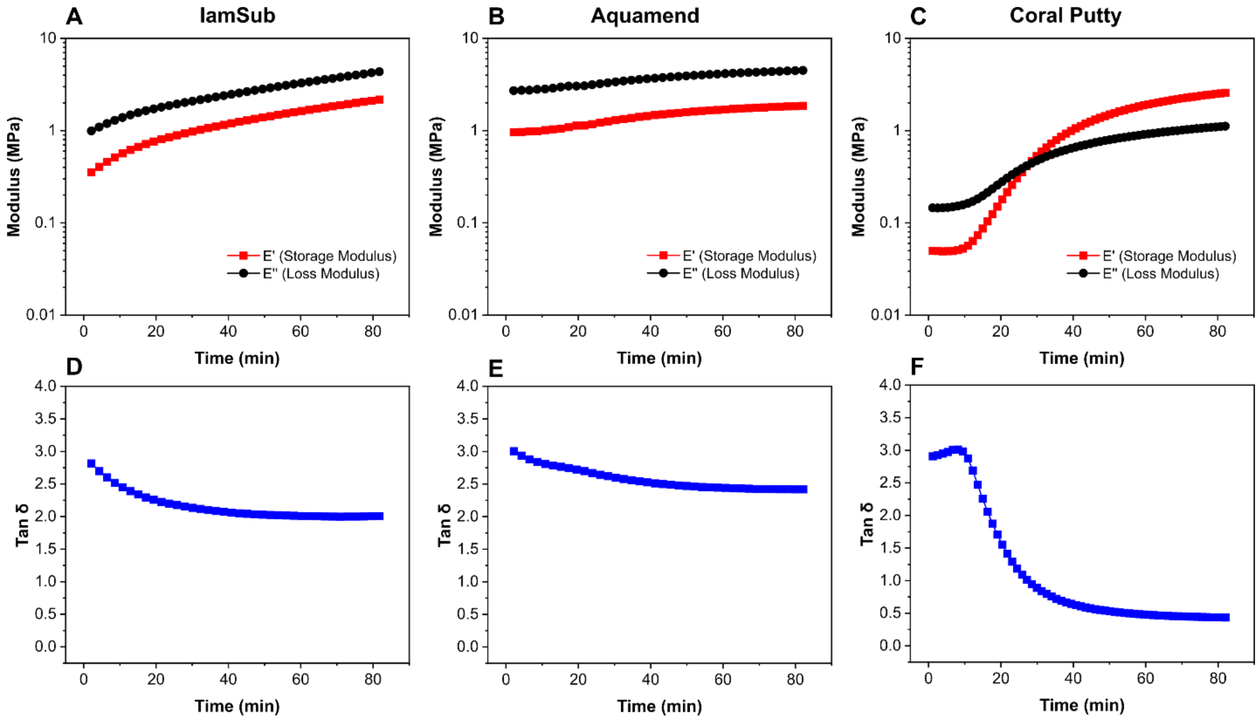


**Figure 5. DMA measurements. A-C)** Storage and loss modulus values for IamSub, Aquamend, and coral putty at 28 °C, respectively; **D-F)** Tan δ values for IamSub, Aquamend, and coral putty at 28 °C, respectively.

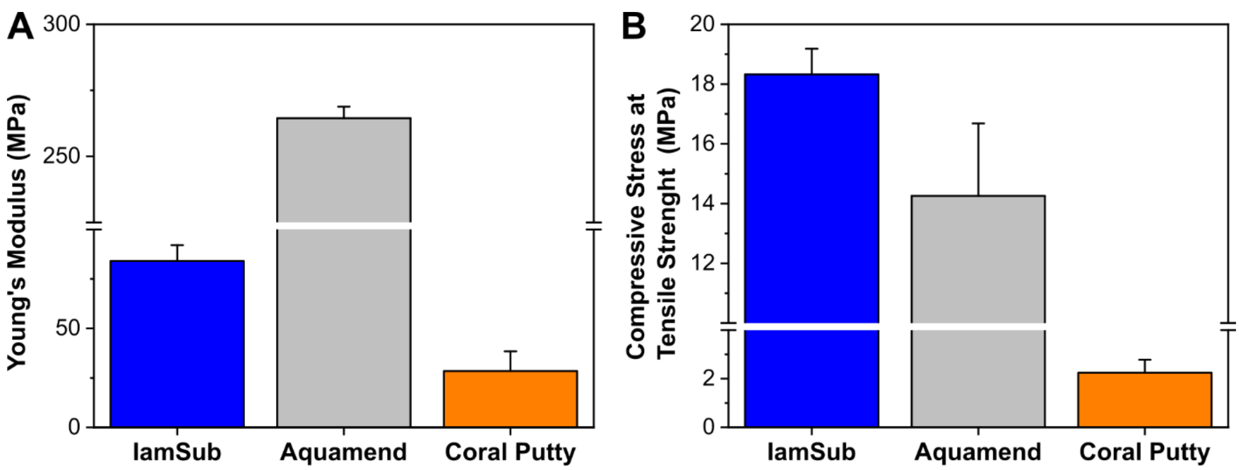


**Figure 6. Compression Test. A) and B)** Young's Modulus and compressive stress at tensile strength, respectively, for the IamSub, Aquamend, and coral putty samples

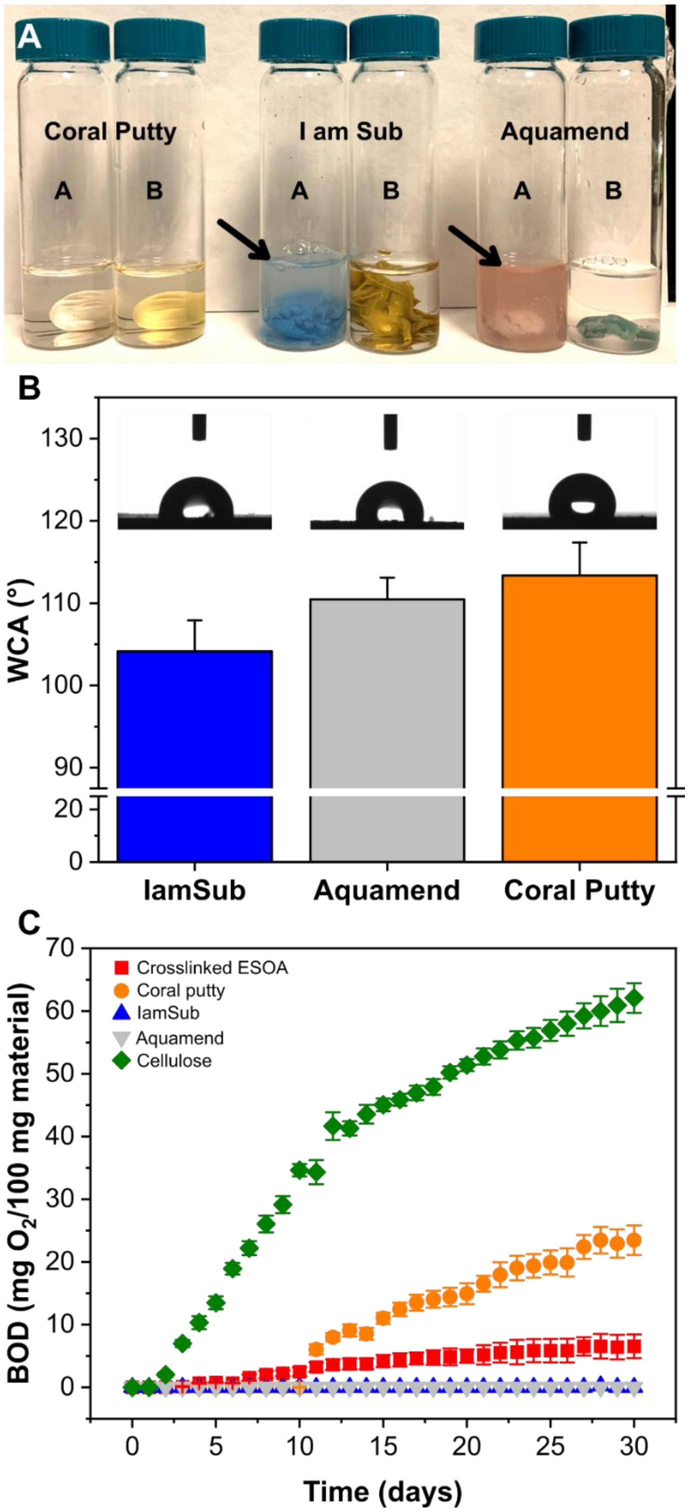


**Figure 7. Interaction with water. A)** Dissolution test of the components A and B of coral putty, IamSub, and Aquamend (from left to right); **B)** WCA values for IamSub, Aquamend, and coral putty; **C)** Biochemical oxygen consumption (mg $O_2$/100 mg material) as a function of the time (days) for coral putty without zein, coral putty (with zein as filler), IamSub, Aquamend, and cellulose.

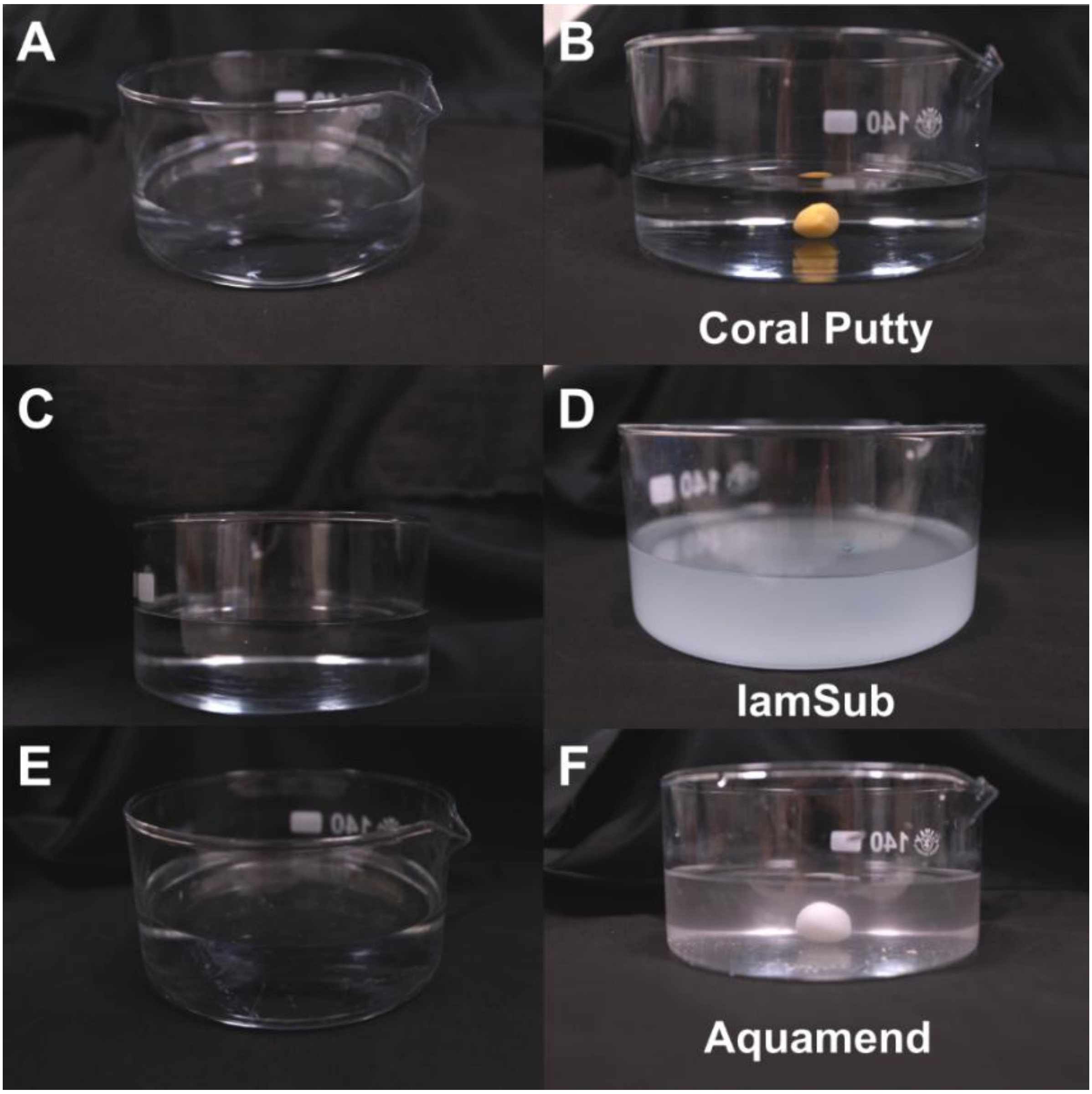


**Figure 8. Test mixing underwater. A) and B)** Photographs of seawater before and after the mixing of coral putty; **C) and D)** Photographs of seawater before and after the mixing of IamSub; **E) and F)** Photographs of seawater before and after the mixing of Aquamend.

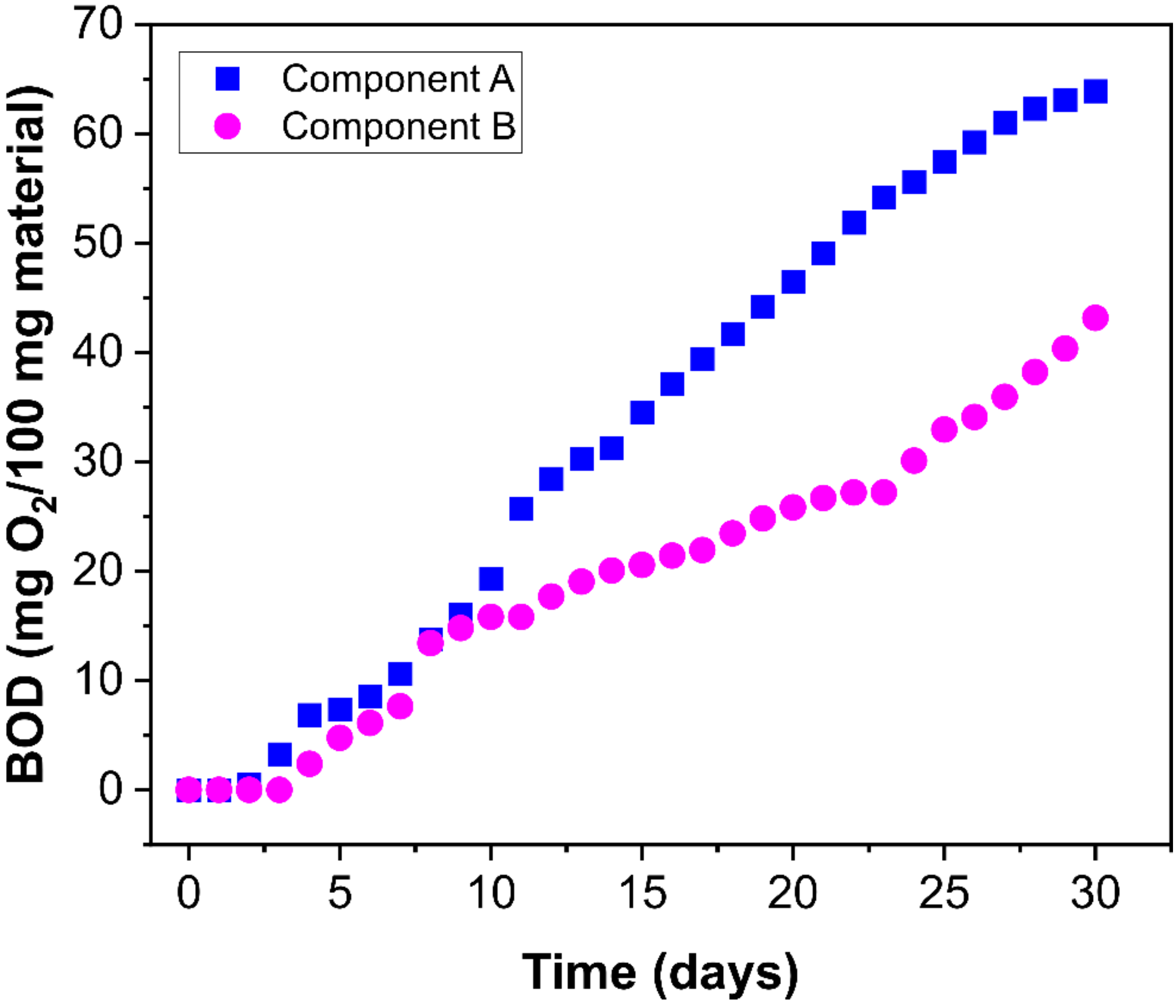


**Figure 9. BOD test for the single components.** Graph of the BOD test for the components A and B of coral putty.

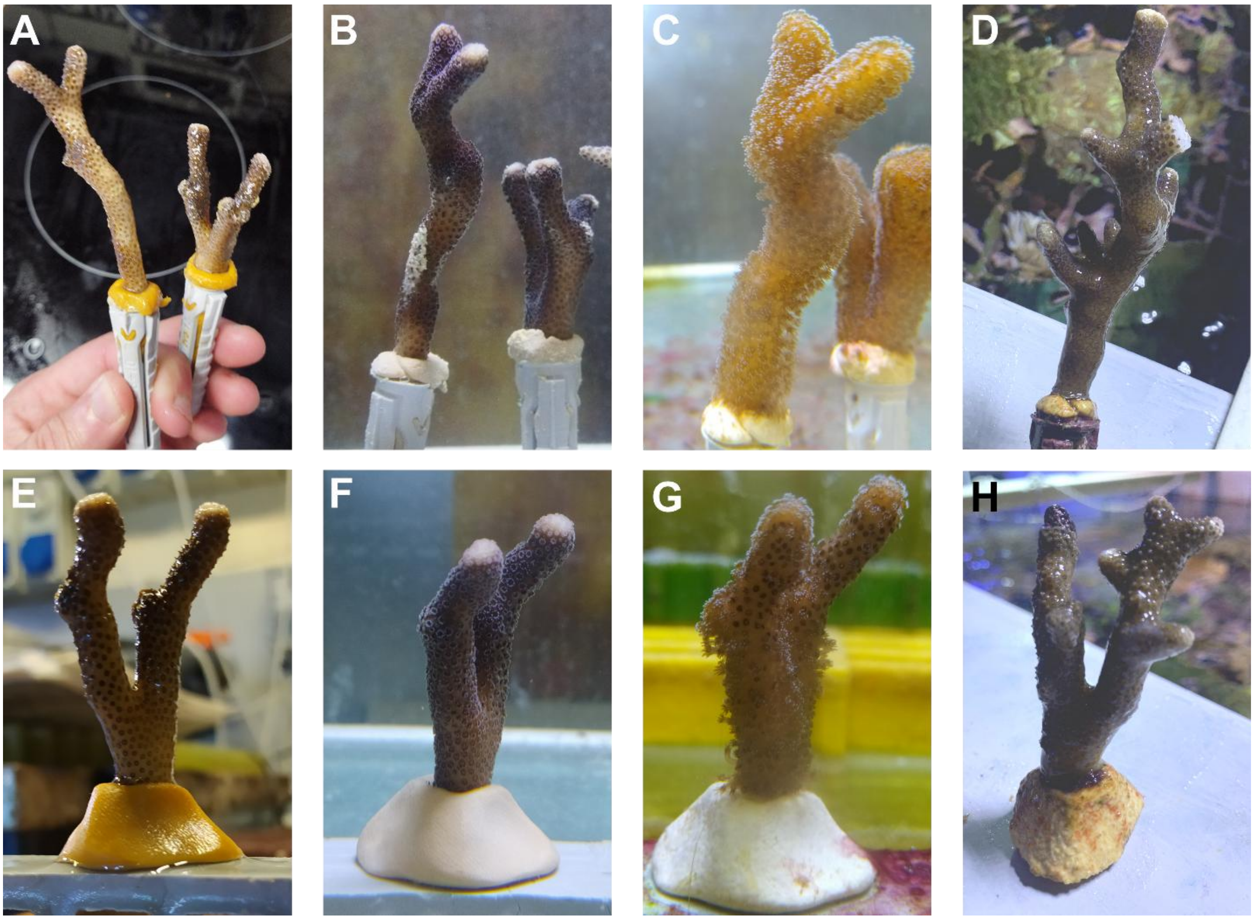


**Figure 10. Coral putty in aquarium. A-D)** Photographs of a *Stylophora pistillata* nubbin fixed with the coral putty inside a dowel Fischer as support immediately after the application and at 0, 30, and 90 days, respectively; **E-H)** Photographs of a *Stylophora pistillata* nubbin fixed directly with the coral putty without support immediately after the application, and at 0, 30, and 90 days, respectively. In both cases, 4 grams of coral putty were used, and the nubbins were inserted around 2 cm into the putty.

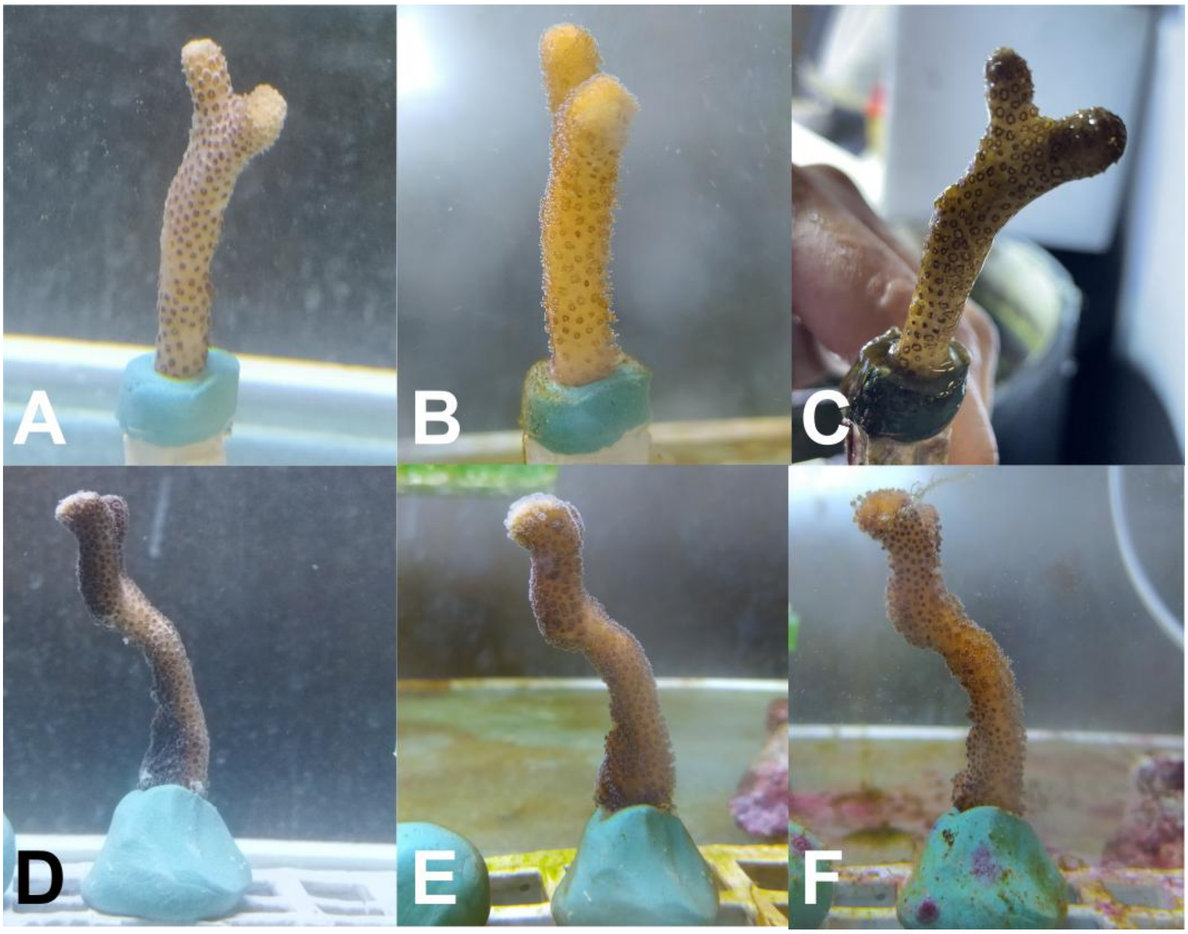


**Figure 11. IamSub in aquarium. A-C)** Photographs of a *Stylophora pistillata* nubbin fixed with the IamSub inside a dowel Fischer as support after 0, 30, and 90 days, respectively; **D-F)** Photographs of a *Stylophora pistillata* nubbin fixed with IamSub as support after 0, 30, and 90 days, respectively.

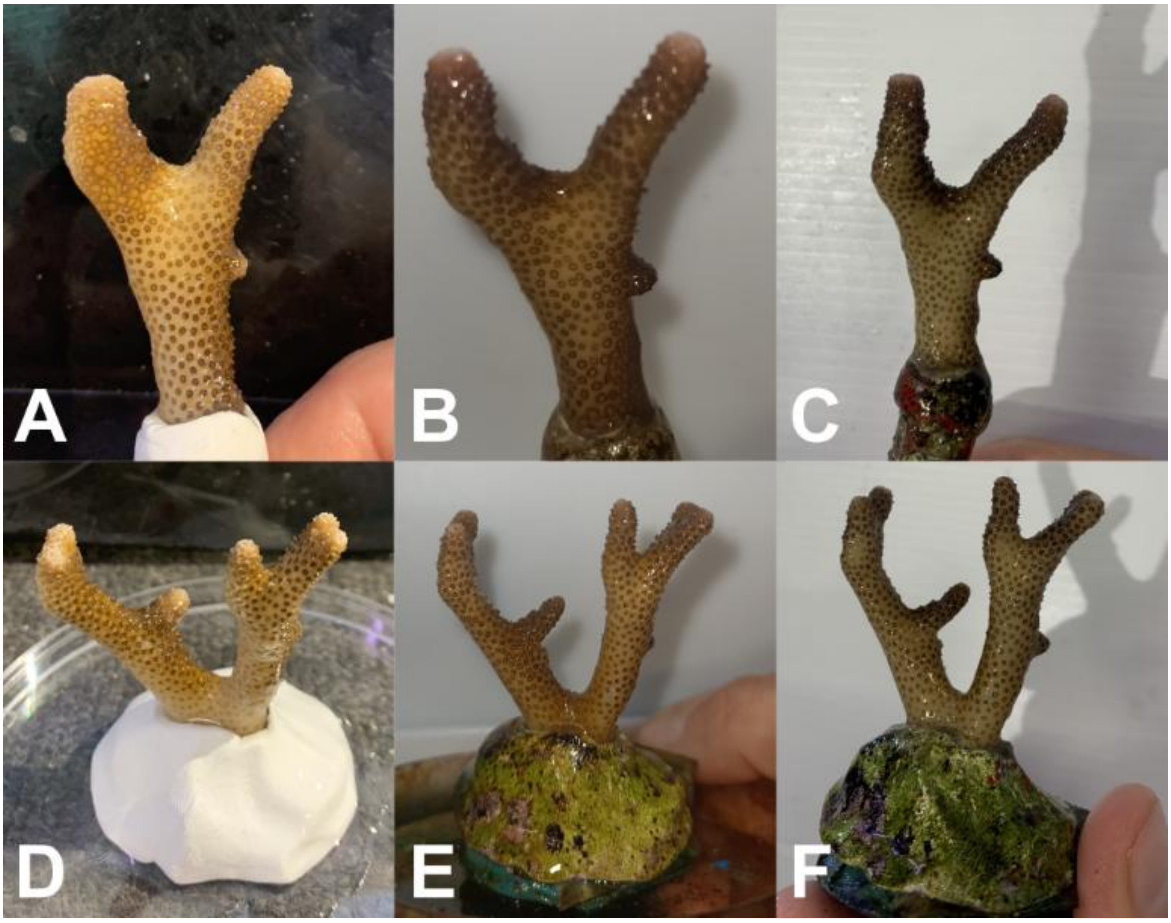


**Figure 12. Aquamend in aquarium. A-C)** Photographs of a *Stylophora pistillata* nubbin fixed with the Aquamend inside a dowel Fischer as support after 0, 30, and 90 days, respectively; **D-F)** Photographs of a *Stylophora pistillata* nubbin fixed with Aquamend as support after 0, 30, and 90 days, respectively.


**Figure 13. On-field application. A)** Frames extracted from an underwater video of the on-field coral transplantation action using coral putty, where four grams were used for small fragments, and the coral fragments were inserted into the putty for 2-3 cm; **B)** Out-planted coral nubbin in the Maldives immediately, after 6 and 12 months. The red arrows and the white lines indicate approximately the position of the coral putty. Eight grams of coral putty were used for bigger fragments, such as the one reported in Figure B, and the coral fragments were inserted into the putty for 3 cm.

# CHAPTER 6

## Conclusions.

## 6.1. GENERAL CONCLUSIONS

It has been clearly stated that the biodiversity of the Mediterranean Sea is under threat due to the combined effects of climate change and anthropic impacts and disturbances. It has been discussed how these threats disrupt the delicate balance of these essential ecosystems, resulting in the loss of critical habitats, such as marine animal forests, with potentially dire consequences for the ecosystems, the organisms, and the millions of people who depend on them for their livelihoods. Climate change, human pressure, and diseases threaten anthozoan communities worldwide, with no sign of lessening in the prediction for the next years, resulting in degraded marine ecosystems, unprecedented decline in health and biodiversity, and loss of ecosystem services they provide humankind. Therefore, generating novel data on current marine diversity and associated threats in the Anthropocene is essential to finding a new way to mitigate this loss and establish effective conservation strategies. It is now imperative, without doubt, as we have reached the point where large-scale bleaching events and MHWs have become so common to be considered a new normality.

The present work aimed to comprehensively evaluate the health status of anthozoans in the Mediterranean Sea, focusing on the major threats posed by climate change, anthropogenic pressures, and contaminants. It is well known that marine animal forests are facing a variety of stressors that jeopardize their survival worldwide, mainly due to diseases, climate-induced stress, and human activities. While similar threats are anticipated in the Mediterranean region, this study revealed that disease occurrence in Mediterranean anthozoans is less impacting and relatively understudied, likely due to limited research efforts in the area. However, other factors, such as thermal stress and contamination by emerging pollutants, have been demonstrated to significantly contribute to the decline in anthozoan health in the Mediterranean Sea and to have a more important impact than previously thought.

First, the work confirmed that thermal stress is a primary driver of tissue loss in anthozoans, likely resulting from the increasingly frequent marine heat waves associated with climate change. Our research into three key Mediterranean gorgonian species, *Paramuricea clavata*, *Eunicella cavolini*, and *Leptogorgia sarmentosa*, showed distinct patterns of tissue damage and pathology, leading to acute to subacute tissue loss, characterized by exposed axial skeletons and tissue thinning. Interestingly, our histopathological analyses revealed the presence of ciliates invading the gastrodermis in *E. cavolini*, suggesting a potential involvement of disease processes in conjunction with thermal stress neglected before. These observations improve the limited knowledge of disease dynamics in Mediterranean anthozoans and highlight the need for further research to unravel the roles of various pathogens and environmental stressors in these mass mortality events, which are always more frequent in the region.

In addition to climate change, emerging contaminants have emerged as a substantial threat to the biodiversity of the Mediterranean Sea. The study provided the first evidence of phthalic acid esters (PAEs), active pharmaceutical ingredients (APIs), UV filter molecules within Mediterranean anthozoan tissues, and their potential bioaccumulation. Using advanced analytical techniques such as solid-phase microextraction (SPME) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), the presence of these contaminants in four anthozoan species (*Cladocora caespitosa*, *E. cavolini*, *Madracis pharensis*, and *Parazoanthus axinellae*) have been demonstrated. Every specimen showed contamination by at least one of these emerging contaminants, with the highest concentrations reaching 57.3 ng/g for the total PAEs and 64.2 ng/g (wet weight) for the total APIs, with *P. axinellae* exhibiting the highest levels of contamination. Moreover, this study posed the basis for hypotheses on the processes controlling the internalization of these contaminants, which seemed to vary depending on the individuals' species, environmental conditions, and life stages. Additionally, the study looked into the presence of UV filters in *Paramuricea clavata* within and outside the Portofino Marine Protected Area (MPA), confirming its potential for accumulating substances such as oxybenzone. The higher contaminant levels outside the MPA highlighted the potential protective role of MPAs in mitigating bioaccumulation, while the potential bioaccumulation and environmental persistence of UV filters suggested the need for further research to understand their long-term residency and impact on marine ecosystems. These findings emphasize the pressing need to understand the long-term ecological impacts of PAEs, APIs and UV filters on anthozoans and their associated benthic communities because even if at minimal concentration, the contaminants are continuously entering the marine environment. These findings corroborate the notion that pollution from emerging contaminants is a pervasive issue in the Mediterranean region, often neglected, potentially leading to negative effects on marine biodiversity. Given the crucial ecological role of anthozoans in structuring benthic communities and supporting marine biodiversity, addressing the issue of contamination must become imperative.

In an era when marine ecosystems face pressing risks of decline due to climate change, habitat degradation, and pollution, extensive research has been conducted to enhance our knowledge of these threats, comprehend the physiological responses of marine organisms, and elucidate the intricate interactions between environmental stressors and aquatic life. Despite recognizing the importance of studying environmental threats worldwide, comparatively limited research and effort has been dedicated to the Mediterranean region, as often highlighted in this work.

It is increasingly evident that improving our understanding of these threats and challenges is essential; however, such efforts primarily assist in documenting the decline of these vital ecosystems and not saving them. There is an urgent need to shift our focus toward finding and implementing practical solutions. It is known that no viable long-term solution can be realized unless we drastically change immediately human behaviors, particularly in reducing greenhouse gas emissions and controlling pollution. Nevertheless, we do have tools at our disposal to potentially buy time for some anthozoan species to recover and adapt, thereby offering a chance for the survival of these ecosystems.

One of them is restoration activities, which have been demonstrated to be a locally effective approach for aiding the recovery of threatened anthozoan populations or regions. However, for restoration to be successful and sustainable nowadays, it must be coupled with innovation in restoration techniques and materials. Conventional restoration methods often employ materials that can become themselves harmful to the environment. Recognizing this, in this work, a novel, biodegradable coral putty based on epoxidized soybean oil acrylate (ESOA) and zein has been developed, which can harden underwater in 20–25 minutes, faster than other environmentally hazardous commercial products. This new material offers an eco-friendly alternative for coral transplantation, minimizing additional environmental impacts during restoration efforts while providing an easy-to-use method that can enhance the productivity and efficacy of the action. This new coral putty resulted biocompatible when tested with the coral *Stylophora pistillata* in aquaria and successfully supported the out-planting of *Acropora tenuis* colonies in the Maldives reef ecosystem. The putty's performance highlights the potential of using biodegradable and renewable materials in coral restoration, which could be extended to other regions, such as anthozoans in the Mediterranean. This approach mitigates the ecological footprint of restoration activities and aligns with broader conservation goals of promoting sustainability and reducing reliance on petroleum-derived and polluting materials.

In conclusion, this thesis provides new insights into the threats faced by Mediterranean anthozoans, particularly concerning thermal stress and pollution by emerging contaminants. While our work adds to the growing body of knowledge on the ecological impacts of climate change and human activities, it also underscores the critical need for action. Beyond simply understanding the mechanisms of decline, it is time to actively pursue solutions that offer respite to these vulnerable ecosystems. Developing eco-friendly restoration materials such as the ESOA/zein-based coral putty presents a promising example.

However, it must be part of a more significant, coordinated effort that includes reducing pollution, controlling emissions, and promoting sustainable practices. Massive collaborations within the scientific community are necessary to coordinate efforts and fill the gaps that still exist in understanding the vulnerability of biodiversity to multiple stressors and how these impacts affect the functionality and connections within the marine environment. International collaborations are also necessary to propose a network of solutions and develop more effective management in the ongoing climate change and human development scenario. Implementing prevention measures, monitoring, and restoring action became a priority to slow down the process and, with time, maybe reverse the decline of anthozoans in the Mediterranean region. All of these measures should be coupled with the possibility of building and/or enlarging ecologically representative MPA networks that may enhance the adaptation capacity of different organisms, mitigate threats, and help build resilience for these ecosystems. Only through a comprehensive approach can we hope to safeguard the future of the Mediterranean's marine biodiversity and the ecological functions it supports.

## APPENDIX

### Abstract of the peer-reviewed papers published during the PhD program

# I. PAPERS RELATED TO THE PHD PROJECT

## I.1. Occurrence of phthalic acid esters (PAEs) and active pharmaceutical ingredients (APIs) in key species of anthozoans in Mediterranean Sea

Gobbato, J., Becchi, A., Bises, C., Siena, F., Lasagni, M., Saliu, F., Galli, P., and Montano, S.

*Marine Pollution Bulletin* – (2024) – 200, 116078 – DOI: 10.1016/j.marpolbul.2024.116078

The Mediterranean Sea's biodiversity is declining due to climate change and human activities, with plastics and emerging contaminants (ECs) posing significant threats. This study assessed phthalic acid esters (PAEs) and active pharmaceutical ingredients (APIs) occurrence in four anthozoan species (*Cladocora caespitosa*, *Eunicella cavolini*, *Madracis pharensis*, *Parazoanthus axinellae*) using solid phase microextraction (SPME) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). All specimens were contaminated with at least one contaminant, reaching maximum values of 57.3 ng/g for the ∑PAEs and 64.2 ng/g (wet weight) for ∑APIs, with dibutyl phthalate and Ketoprofen being the most abundant. P. axinellae was the most contaminated species, indicating higher susceptibility to bioaccumulation, while the other three species showed two-fold lower concentrations. Moreover, the potential adverse effects of these contaminants on anthozoans have been discussed. Investigating the impact of PAEs and APIs on these species is crucial, given their key role in the Mediterranean benthic communities.

## I.2. Pathology of tissue loss in three key gorgonian species in the Mediterranean Sea

Gobbato, J., Work, T.M., Facchinelli, M.P., Siena, M.F., Montalbetti, E., Seveso, D., Louis, Y.D., Galli, P., and Montano, S.

*Journal of Invertebrate Pathology* – (2024) – 207, 108197 – DOI: 10.1016/j.jip.2024.108197

The Mediterranean is known for its marine biodiversity, especially gorgonian forests. Unfortunately, these are experiencing rapid declines due to climate change manifested by repeated marine heat waves resulting in mass mortality events since the early 1990s. To better understand why gorgonians are declining, there is a need for more systematic approaches to investigate exactly why they might be dying, and pathology may aid in this goal.

We described gross and microscopic pathology of tissue loss in three key gorgonian species in the Mediterranean region, *Paramuricea clavata*, *Eunicella cavolini*, and *Leptogorgia sarmentosa*, that were all experiencing various degrees of tissue loss characterized by exposed axial skeleton and thinning of adjacent tissues. On light microscopy, *L. sarmentosa* showed partial ablation and dissociation of gastrodermis, while *P. clavata* showed cellular vacuolation and a skeletal axis devoid of tissue coverage. *E. cavolini* showed diffuse coagulation necrosis of the gastrodermis, resulting in the loss of cellular organization. Deposition of gorgonin that stained weakly for melanin was also a notable host response illustrating a potential component of innate immunity in this group of invertebrates and its potential significance in bolstering disease resistance. Ciliates were found only within necrotic tissues in *E. cavolini* and were likely secondary scavengers. We saw no evidence of infectious agents visible on light microscopy as a primary cause of death. Further work to understand the cause of death in gorgonians might focus on the role of environmental co-factors or infectious agents not visible on light microscopy or applications of additional tools such as cytology.

## I.3. Underwater Quick-hardening Vegetable oil-based Biodegradable Putty for Coral Reef Restoration and Rehabilitation

Zych, A., Contardi, M., Rinaldi, C., Scribano, V., Isa, V., Kossyvaki, D., Gobbato, J., Ceseracciu, L., Lavorano, S., Galli, P., Athanassiou, A., and Montano, S.

*Advance Sustainable Systems* – (2024) – 2400110 – DOI: 10.1002/adsu.202400110

Coral reefs are threatened by climate change and the effects of human activity on the marine environment. Researchers are attempting to rescue this fragile ecosystem through coral restoration actions (coral gardening, micro-fragmentation, etc.), and a common step in these procedures is transplanting the new coral colonies into coral reefs. To do that, commercial concrete or epoxy resins, also called putty, are utilized, highlighting different concerns about their mechanical and hardening performances and their impact and fate once released into the environment. Hence, this study presents a new biodegradable epoxidized soybean oil acrylate (ESOA)/zein-based coral putty capable of quick hardening underwater as an eco-friendly alternative for transplanting new coral colonies in the reef. The coral putty is composed of two components: a radical initiator and a radical accelerator. Once the two components are mixed, the coral putty becomes hard underwater in 20–25 minutes, showing a hardening timescale much faster than other commercial products. The coral putty is biocompatible when applied to the coral *Stylophora pistillata* in aquaria, and *Acropora tenuis* corals are out-planted on the reef in the Maldives, demonstrating how this new class of vegetable-oil-based materials can be a suitable alternative to epoxy resins and concretes commonly used in coral restoration procedures.

## II. OTHER PAPERS

### II.1. Temporal patterns in coral disease prevalences at Thudufushi Island, Maldives, 2010–2022

Bises, C., Gobbato, J., Lainati, N., Dehnert, I., Siena, F.M., Seveso, D., Montalbetti, E., Louis, Y.D., and Montano, S.

*Diseases of Aquatic Organisms* – (2024) – 159 – DOI: 10.3354/dao03807

Coral reefs are lately suffering a fast decline in biodiversity due to the coupled effect of climate change and disease outbreaks, which in recent decades have been reported with higher frequency and shorter intervals. Limited studies have been conducted on coral diseases in the Maldives resulting in the impossibility of assessing the temporal trend in their dynamics. In this context, we evaluated the change in the distribution, prevalence, and host range of 4 diseases, namely black band disease (BBD), brown band disease (BrB), skeletal eroding band (SEB), and white syndrome (WS), in the reef system around Thudufushi Island after an interval of 12 years since the last assessment. In this period, the overall disease prevalence increased, except for BrB, with SEB showing the most severe increase in 2022 in comparison to 2010. The overall average prevalence of coral diseases is approximately 2%, indicating an increase of about 0.7% since 2010. Diseased coral colonies were found in all the investigated sites, with the east site being the most affected and SEB emerging as the most prevalent disease across all the investigated sites. The affected colonies belong to 13 genera, with *Psammocora* genus showing the highest overall mean disease prevalence. This study depicted a basic temporal trend in disease prevalence that confirms an increase in coral diseases in the region and calls for a dedicated national monitoring protocol to better understand and predict future coral disease dynamics at regional scales.

### II.2. New evidence of grey reef sharks (*Carcharhinus amblyrhynchos*) displaying chafing behaviors on whale shark (*Rhincodon typus)* individuals in the Maldives

Gobbato*, J., Parmegiani*, A., Seveso, D., Galli, P., and Montano, S.

*Marine Biodiversity* – (2024) – 54, 34 – DOI: 10.1007/s12526-024-01430-y

* Co-first authorship

Sharks rubbing against ocean floor or rocks are common events in marine environments, while instances of interspecific shark chafing behavior have been observed much less frequently. This behavior has garnered scientific interest in recent years and usually involves a smaller shark rubbing against a larger species, utilizing dermal denticles to rid itself of parasites or dead skin cells. Despite the costs and benefits of this behavior not yet fully understood, we report new evidence of grey reef sharks (*Carcharhinus amblyrhynchos;* Bleeker, 1856) engaging in chafing behavior against whale sharks (*Rhincodon typus;* Smith, 1828) in the Maldives. This behavior suggests that grey reef sharks may engage in chasing behavior for cleaning purposes or take advantage of larger sharks as an anti-predation tactic. These records contribute to shed light on previously unexplored aspects of elasmobranch interspecies interactions and highlight the need for further research into this topic. The prevalence of such behavior across different regions suggests its potential significance in the ecological dynamics of the shark population.

**II.3. Heavy metal and trace element concentrations in the blood of scalloped hammerhead sharks (*Sphyrna lewini*) from La Paz Bay, México**

Whitehead, A.D., Gayford, J.H., Pancaldi, F., Gobbato, J., Boldrin, G., Tringali, M., Ketchum, J.T., Gàlvan-Magaña, F., Seveso, D., and Montano, S.

*Marine Pollution Bulletin* – (2024) – 201, 116155 – DOI: 10.1016/j.marpolbul.2024.116155

Sharks are particularly susceptible to bioaccumulation due to their life history characteristics and trophic position within marine ecosystems. Despite this, studies of bioaccumulation over only a small proportion of extant species. In this study, we report concentrations of trace elements and heavy metals in blood samples of *Sphyrna lewini* for the first time. We report high concentrations of several trace elements and heavy metals, with concentrations of some elements exceeding the limit determined safe for human consumption. High elemental concentrations may reflect biochemical differences between blood plasma and other tissues; however, they may also be symptomatic of high levels of exposure triggered by anthropogenic activities. We also provide evidence of elemental accumulation through ontogeny, the nature of which differs from that previously reported. Ultimately, this baseline study increases our understanding of interspecific and intraspecific variation in bioaccumulation and ecotoxicology in elasmobranchs, which may prove important in ensuring adequate management.

### II.4. Physical and cellular impact of environmentally relevant microplastic exposure on thermally challenged *Pocillopora damicornis* (Cnidaria, Scleractinia)

Isa, V., Seveso, D., Diamante, L., Montalbetti, E., Montano, S., Gobbato, J., Lavorano, S., Galli, P., and Louis, Y.D.

*Science of the Total Environment* – (2024) – 918, 170651 – DOI: 10.1016/j.scitotenv.2024.170651

Microplastic pollution is an increasing threat to coral reefs, which are already strongly challenged by climate change-related heat stress. Although it is known that scleractinian corals can ingest microplastic, little is known about their egestion and how microplastic exposure may impair corals at physiological and cellular levels. In addition, the effects of microplastic pollution at current environmental concentration have been little investigated to date, particularly in corals already impacted by heat stress. This study investigated the combined effects of these environmental threats on *Pocillopora damicornis* from a physical and cellular perspective. Colonies were exposed to three concentrations of polyethylene microplastic beads (no microplastic beads: [No MP], 1 mg/L: [Low MP]; 10 mg/L: [High MP]), and two different temperatures (25 ◦C and 30 ◦C) for 72 h. No visual signs of stress in corals, such as abnormal mucus production and polyp extroflection, were recorded. At [Low MP], beads adhered to colonies were ingested and egested. Moreover, thermally stressed colonies showed a lower adhesion and higher egestion of microplastic beads. Coral bleaching was observed with increased temperature and microplastic bead concentration, as indicated by a general decrease in chlorophyll concentration and Symbiodiniaceae density. An increase in lipid peroxidation was measured in colonies exposed to [Low MP] and [High MP], and an up-regulation of stress response gene hsp70 was observed due to the synergistic interaction of both stressors. Overall, our findings showed that heat stress still represents the main threat to *P. damicornis*. At the same time, the effect of microplastics on coral health and physiology may be minor, especially at control temperature. However, microplastics could exacerbate the impact of thermal stress on cellular homeostasis, even at [Low MP]. While reducing ocean warming is critical for preserving coral reefs, effective management of emerging threats like microplastic pollution is equally essential.

## II.5. Widespread Occurrence of Growth Anomalies in the Republic of Maldives

Bises, C., Dehnert, I., Aeby, G.S., Dennis, M.M., Gobbato, J., Hodge, J., Staiger, M., Siena, F.M., Galli, P., and Montano, S.

*Diversity* – (2024) – 16, 15 – DOI: 10.3390/d16010015

In the last decades, there has been a concerning increase in the frequency and severity of coral disease outbreaks on a global scale, resulting in significant damage to the coral reef ecosystem and biodiversity. Growth anomalies (GAs) have been increasingly observed, with significantly higher occurrences in larger and older coral colonies compared to their smaller counterparts. However, there is a notable lack of knowledge and reports regarding growth anomalies in the Maldivian region. Here, we provide the first evidence of four distinct growth anomalies on three coral species, respectively on *Acropora* sp., *Montipora* sp., and *Pachyseris speciosa*, observed across four different locations across three atolls within the Maldivian Archipelago.

## II.6. First record of the bull shark *Carcharhinus leucas* (Valenciennes, 1839) from the Maldivian archipelago, central Indian Ocean

Parmegiani*, A., Gobbato*, J., Seveso, D., Galli, P., and Montano, S.

*Journal of Fish Biology* – (2023) – 1, 6 – DOI: 10.1111/jfb.15518

* Co-first authorship

Verified records of the bull shark *Carcharhinus leucas* are lacking in the Maldives. This study provides the first confirmed evidence of 23 sightings observed from 2013 to 2023 in the central and southern atolls of this archipelago. Most of the sightings occurred close to inhabited areas, where food waste is often discarded into the water, or in several dive sites, suggesting the presence of this species in different locations around central and southern atolls. Although further research is required to thoroughly investigate the *C. leucas* population in the Maldives, this report documents and confirms its presence in this region.

## II.7. Spatial Ecology of the Association between Demosponges and *Nemalecium lighti* at Bonaire, Dutch Caribbean

Gobbato, J., Magrini, A., Garcìa-Hernàndez, J.E., Virdis, F., Galli, P., Seveso, D., and Montano, S.

*Diversity* – (2022) – 14, 607 – DOI: 10.3390/d14080607

Coral reefs are among the most biodiverse marine ecosystems and one of the richest in terms of associations and species interactions, especially those involving invertebrates such as corals and sponges. Despite that, our knowledge about cryptic fauna and their ecological role remains remarkably scarce. This study aimed to address this gap by defining for the first time the spatial ecology of the association between the epibiont hydrozoan *Nemalecium lighti* and the Porifera community of shallow coral reef systems at Bonaire. In particular, the host range, prevalence, and distribution of the association were examined in relation to different sites, depths, and dimensions of the sponge hosts. We report *Nemalecium lighti* to be in association with 9 out of 16 genera of sponges encountered and 15 out of 16 of the dive sites examined. The prevalence of the hydroid–sponge association in Bonaire reef was 6.55%, with a maximum value of over 30%. This hydrozoan is a generalist symbiont, displaying a strong preference for sponges of the genus *Aplysina*, with no significant preference in relation to depth. On the contrary, the size of the host appeared to influence the prevalence of association, with large tubular sponges found to be the preferred host. Although further studies are needed to better understand the biological and ecological reason for these results, this study improved our knowledge of Bonaire's coral reef cryptofauna diversity and its interspecific associations.

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