Variation in the bacterial biota of the snakelocks anemone *Anemonia viridis* – natural *vs*. abnormal shifts induced by global climate change and its relevance for drug discovery

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LEGAL DETAILS

In compliance with what is stated in Decree-Law nº 216/92 of October 13th, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results here stated, as well as in their interpretation and writing of the respective manuscripts.
This thesis includes two scientific papers published in international journals ranked on ISI Web of Science and two articles in preparation, originate from the results obtained in the experimental work referenced to as:

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"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity..."

Charles Dickens

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SUMMARY

Cnidarians, namely the snakelocks anemone *Anemonia viridis* (Forskål, 1775), are good model organisms for ecological studies and important elements in marine communities. These invertebrates display a microbial biota capable of providing food and protection by the production of metabolites and other natural products. Some of these substances may be bioactive metabolites with biotechnological interest. Given the lack of studies on the natural dynamics of the microbial biota associated with sea anemones in temperate regions, one of the main goals of this thesis is to provide detailed information on how environmental drivers affect the microbial biota of *Anemonia viridis,* in south-west Europe. By acquiring this knowledge we expect to be able to understand how potential global climate change in the marine environment may disrupt the symbiotic relations between sea anemones and its bacterial populations, as well as determine if bacterial symbionts of anemones are capable of biosynthesizing bioactive compounds with biotechnological interest.

The studies presented in this thesis provide detailed information on the bacterial communities colonizing *A. viridis* and fluctuations induced by seasonal, and spatial variation, as well as physical stressors (temperature and depth) in controlled environments mimicking their natural habitat. Additionally, evidences show that environmental stressors (temperature and depth) affect not only the sea anemone – zooxanthellae symbiosis, but also associated bacterial populations. The abundance of some bacterial taxa can change significantly, with some group's even disappearing from their host anemone. Moreover, the same study reveals that bacteria in symbiosis with *A. viridis* can produce natural products and this type of biosynthesis is most active under abnormally high temperatures and full solar radiation. These observations provide important insights for future studies targeting the production of bioactive compounds by these bacteria.

RESUMO

Os Cnidários, nomeadamente a anémona-do-mar *Anemonia viridis*, são importantes elementos das comunidades marinhas sendo considerados bons organismos modelo em estudos ecológicos. Estes invertebrados exibem um biota microbiano capaz de proporcionar alimento e proteção pela produção de metabolitos e outros compostos naturais. Podendo alguns destes metabolitos ter propriedades bioativas com interesse biotecnológico. Dada a falta de estudos sobre a dinâmica natural do biota microbiano associado a anémonas-do-mar em regiões temperadas, um dos principais objectivos desta tese é fornecer informações detalhadas sobre a forma como os factores ambientais afetam o biota bacteriano da *Anemonia viridis*, no sudoeste europeu. Ao se adquirir este conhecimento, pretende ser-se capaz de entender como potenciais alterações climáticas globais no ambiente marinho, podem perturbar as relações simbióticas entre anémonas do mar e suas populações bacterianas; bem como determinar se os simbiontes bacterianos das anémonas são capazes de biosintetizar compostos bioativos com interesse biotecnológico.

Os estudos apresentados nesta tese fornecem informações detalhadas sobre as comunidades bacterianas que colonizam a *A. viridis* e flutuações induzidas pela variação sazonal e espacial; bem como por condições físicas (temperatura e profundidade) capazes de provocar stress em ambientes controlados e que simulam o *habitat* natural. As evidências demostram que estas condições físicas afectam não só a simbiose anémona-zooxanthellae, mas também as populações bacterianas associadas. A abundância de alguns taxa bacterianos podem alterar significativamente, com alguns grupos bacterianos desaparecendo mesmo da anémona hospedeira. Adicionalmente, o mesmo estudo revela que as bactérias em simbiose com *A. viridis* podem produzir produtos naturais e que este tipo de biossíntese é mais ativa sob temperaturas anormalmente elevadas e radiação solar total. Estas observações fornecem entendimentos importantes a serem usados em futuros estudos que visem a produção de compostos bioativos por estas bactérias.

CHAPTER 1

General Introduction

Parts of this chapter include excerpts from the chapter book:

Rocha J., Leal M., Calado R. (2014) Marine Bioactive Compounds from Cnidarians; Se-Kwon, K. *Springer Handbook of Marine Biotechnology.* Springer ISBN 978-3-642-53970-1

1.1. Phylum Cnidaria

Phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates that contains over 11,000 extant species (Table 1.1) (Appeltans et al., 2012). There are four significant classes of Cnidaria commonly recognized (Miller & Ball, 2008): Hydrozoa (most variable class, including hydroids, fire corals, Portuguese man-of-war, and others), Scyphozoa ("true" jellyfishes), Cubozoa (cube jellyfishes), and Anthozoa (largest class, including sea anemones, stony corals, soft corals, and others). Cnidaria get their name from the Greek word (*Cnidos*) for stinging nettle (Springer & Holley, 2012), due to cells called cnidocytes, which contain stinging organelles (nematocysts) characteristic of the phylum. Nematocysts are formed and used only by cnidarians (Daly et al., 2007). They are an ancient group with the longest fossil history of any metazoan, reaching back more than 700 million years (Conway Morris, 2000, Holstein et al., 2003).

Table 1.1. Classes and orders in the phylum Cnidaria (according to the classification proposed in the World Register of Marine Species (WoRMS) (Appeltans et al., 2012) (table from Rocha et al. (2011)).

Phylum	Class	Order	
Cnidaria (≈11,287 species)	Anthozoa (≈7500 species)	Actiniaria	Zoanthidea
		Antipatharia	Alcyonacea
		Ceriantharia	Gorgonacea
		Corallimorpharia	Helioporacea
		Scleractinia	Pennatulacea
	Cubozoa (≈36 species)	Carybdeida	Chirodropida
	Hydrozoa (≈3500 species)	Anthoathecata	Limnomedusae
		Leptothecata	Narcomedusae
		Siphonophorae	Trachymedusae
		Actinulida	
	Polypodiozoa (1 species)	Polypodiidea	
	Scyphozoa (≈200 species)	Coronatae	Semaeostomeae
		Rhizostomeae	
	Staurozoa (≈50 species)	Stauromedusae	

This group of invertebrates is found exclusively in aquatic environments, mostly in marine ecosystems. Cnidarians have simple body forms, which are usually in the form of polyp (hydroid form), adapted to a sedentary or sessile life; or medusa (jellyfish form), adapted for a floating or free-swimming existence (Figure 1.1). Most polyps have tubular bodies with a mouth at one end surrounded by tentacles. The aboral end is usually attached to a substratum by a pedal disc or other device. Polyps may live singly or in colonies (Hickman et al., 2007). For instance, a sea anemone is a single polyp, whereas corals are, in general, a colony of individual polyps, which are typically tubular and attached to a surface at their base. Both forms may occur during the life cycle in some cnidarians. Although they are mostly sessile, or at best, fairly slow moving or slow swimming, we might easily get the false impression that cnidarians were placed on earth to provide easy meals for other animals (Hickman et al., 2007).

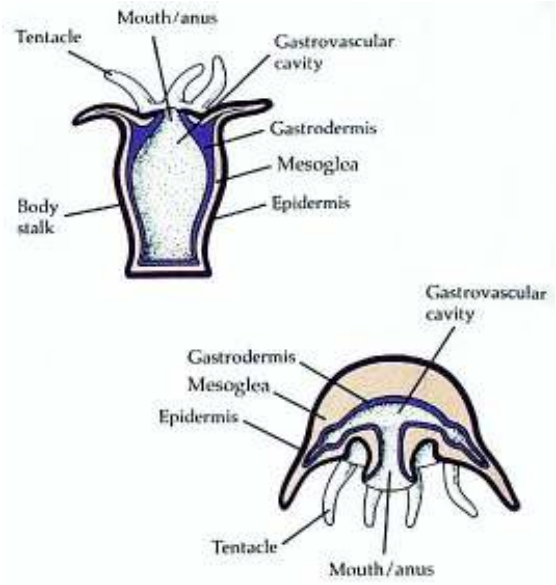

Figure 1.1. Diagram of polyp and medusa forms of cnidarians (figure from Hickman et al. (2007)).

The truth is, however, that many cnidarians are quite efficient predators of organisms that are much swifter and more complex. They manage these feats because they possess tentacles that bristle with tiny, remarkably sophisticated weapons, the nematocysts. The stinging cells are coiled structures that shoot out and inject toxins via a dart-like tip (Hickman et al., 2007).

Even though their organization has a structural and functional simplicity not found in other metazoans, they form a significant proportion of the biomass in some locations. Cnidarians are usually found in a wide geographic range: from deep waters near

hydrothermal vents to polar seabeds and tropical reefs. Some cnidarian species, mostly from Class Scyphozoa (jellyfish), are pelagic and live in the water column. In contrast, most cnidarian species are benthic, especially in warm temperature and tropical regions, as they live associated with the sediment surface.

Cnidarians sometimes live symbiotically with other animals, often as commensals on the shell or on other surface of their host. Certain hydroids and sea anemones occur on snail shells inhabited by hermit crabs, providing the crabs some protection from predators. Algae frequently live as mutuals in the tissues of cnidarians, particularly in some freshwater hydras and in reef-building corals. The presence of the algae in reef-building corals limits the occurrence of coral reefs to relatively shallow, clear water where there is sufficient light for the photosynthetic requirements of the algae (Hickman et al., 2007).

As already referred above cnidarians, like other benthic organisms, lack mechanical means to prey and have optimized their mechanisms to feed through the evolutionary history. Cnidarians display stinging cells with powerful toxins that are very helpful to disable their prey and also to drive off predators (Tardent, 1995). Besides these chemical weapons present in stinging cells, cnidarians also display other potent compounds that are useful to deter predators and keep competitors away (Faulkner, 2000, Haefner, 2003, Paul et al., 2011). For instance, tropical reefs are ecosystems with a vast biodiversity, where the substrate available for benthic cnidarian species to settle and develop is scarce (Hay, 2009). Chemical interactions between different species are thus an important mechanism for inter-specific competition, which may have dramatic consequences for the organism being outcompeted in this “chemical war”. Hence, organisms inhabiting high biodiverse tropical areas, particularly coral reefs, developed a large array of chemical compounds that have been the focus of recent bioprospecting efforts (Leal et al., 2012b).

Corals form the structure and foundation of tropical reefs, and are also important structural elements of some highly diverse deep-sea habitats. Other cnidarian species, such as sea anemones, are also very diverse and abundant in these tropical ecosystems (Sebens, 1994). These benthic cnidarians display a great variety of chemical molecules that have different biological functions. The harsh chemical and physical environmental conditions of the areas inhabited by these organisms may have been important drivers for the production of a variety of molecules with unique structural features. For instance, the incidence of predation in the majority of these organisms is low due to the toxic compounds they produce to deter predators (Lindquist, 1996). Other examples of biological functions of these compounds are defensive functions against pathogens, as well as against fouling organisms, herbivores and microorganisms (Paul & Puglisi, 2004). Such chemical compounds have been targeted by scientists searching for new chemical entities from the sea, usually known as marine natural products (MNP).

1.1.1. Class Anthozoa

Anthozoans, or “flower animals”, are polyps with a flowerlike appearance (Raven & Johnson, 2002). In these animals the free-swimming medusa stage is always absent (Bridge et al., 1995) and are exclusively marine (Fautin & Romano., 2000). They are found in both deep and shallow water and in polar seas as well as in tropical seas. These invertebrates vary greatly in size and may be solitary or colonial (Hickman et al., 2007). Class Anthozoa was traditionally considered to have two or three subclasses (Fautin & Romano., 2000). Some considered the class division made by Hyman (1940), into Alcyonaria (Octocorallia) - which include soft and horny corals, such as sea fans, sea pens, sea pansies and others - and Zoantharia (Hexacorallia) - containing sea anemones, hard corals and others (Appeltans et al., 2012, Fautin & Romano., 2000, Hyman, 1940). Others considered a third subclass, Ceriantipatharia (Dunn, 1982, Hickman et al., 2007, Wells, 1956), containing only in this case tube anemones and thorny corals. The first division was based largely on polyp symmetry and tentacle form and number, whereas the second was based on similarity of new mesenterial couples of the ceriantharian larval stage to the antipatharian polyp, on the very weak mesentery musculature in both groups, and on the insertion only in the dorsal intermesenterial space of members of both orders. Evidence from morphology (Hand, 1966) and molecular data (Berntson et al., 1999, Kayal et al., 2013) suggests Ceriantharia as an order of the subclass Zoantharia, being nowadays Ceriantipatharia a *nomen nudum* (Appeltans et al., 2012).

1.2. Sea Anemones

Named after a terrestrial flower, the anemone, sea anemones form a group of water-dwelling, filter feeding animals of the class Anthozoa, order Actinaria. Unlike most other anthozoans, sea anemones lack skeletons of any sort and are solitary (Fautin & Allen, 1992). Sea anemone polyps are larger and heavier than hydrozoan polyps and most range from 5 mm or less to 100 mm in diameter, and from 5 mm to 200 mm long, being able to grow much larger (Hickman et al., 2007). There are more than 1,000 sea anemone species found throughout the world’s oceans that run the full spectrum of colours. They can be found from poles to equator and from the deepest trenches to the shores (Fautin & Allen, 1992). They attach by means of their pedal discs to shells, rocks, timber, or whatever submerged substrata they can find, and some even burrow in mud or sand. Sea anemones are cylindrical in form with a crown of tentacles arranged in one or more circles around the mouth of the flat oral disc (Figure 1.2). The slit-shaped mouth leads into a

pharynx and at one or both ends of the mouth is a ciliated groove called a siphonoglyph, which extends into the pharynx. The siphonoglyph creates a water current directed into the pharynx, that carry in oxygen and remove wastes. The pharynx leads into a large gastrovascular cavity that is divided into six radial chambers by means of six pairs of primary (complete) septa, or mesenteries, extending vertically from the body wall to the pharynx (Figure 1.2). Openings between chambers (septal perforations) in the upper part of the pharyngeal region help in water circulation. Smaller (incomplete) septa partially subdivide the large chambers and provide a means of increasing the surface area of the gastrovascular cavity. The free edge of each incomplete septum forms a type of sinuous cord called a septal filament that is provided with nematocysts and with gland cells for digestion. In some anemones (such as *Metridium*) the lower ends of the septal filaments are prolonged into acontia threads, also provided with nematocysts and gland cells, that can be protruded through the mouth or through pores in the body wall to help overcome prey or provide defense. These pores also assist in rapid discharge of water from the body when the animal is endangered and contracts to a small size (Hickman et al., 2007, Springer & Holley, 2012).

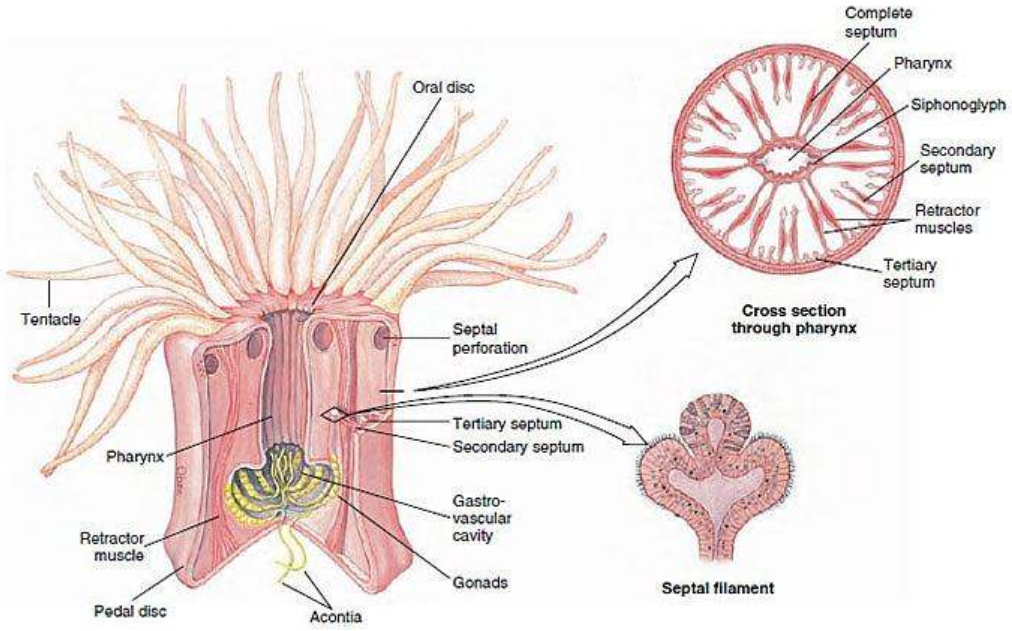

Figure 1.2. Structure of a sea anemone (figure from Hickman et al. (2007)).

Sea anemones are mostly carnivorous, and their feeding methods and digestive abilities are adapted for intake and processing of live prey (Sebens & Laakso, 1977). Food is captured in 3 different ways: 1) filter feeding, where small organic particles, both live and dead, are removed from the seawater passing over the outspread tentacles (Koehl, 1977),

2) raptorial capture, where zooplankters and motile benthic prey are caught by the tentacles (Sebens, 1981), and 3) passive capture, where sessile prey are dislodged by foraging predators or wave action and carried onto the tentacles (Sebens, 1981). Feeding behaviour in many zoantharians is under chemical control. Some respond to reduced glutathione but in certain others two compounds are involved: asparagine (the feeding activator) that causes a bending of tentacles toward the mouth, and reduced glutathione that then induces swallowing of food (Hickman et al., 2007).

Sea anemones have no central brain, but a simple network of nerves in the body wall communicates between different parts of the anemone's body. Also muscles are well developed in sea anemones and several kinds of them can be found in their tissues. They are used to contract and bend the column in various directions. These include longitudinal fibers, which occur only in the tentacles and oral disc of most species and run perpendicular to the base and serve to contract the column vertically, and circular muscles, that run around the column parallel to the base and serve to reduce the diameter of the column (Cavendish, 2010, McCloskey, 2012). The muscles are also used to expand the column and tentacles by the use of hydrostatic pressure. That is, by using the muscles to apply pressure to the water in one part of the closed coelenteron, this water is forced into other parts which subsequently appear extended and more rigid, being termed as hydrostatic skeleton (McCloskey, 2012).

Most sea anemones can glide along slowly on their pedal discs and can even crawl on their side or by using their tentacles. They can expand and stretch their tentacles in search of small vertebrates and invertebrates, which they overpower with tentacles and nematocysts and carry to the mouth. As referred, sea anemones are, for the most part, sessile creatures remaining attached to one spot. However, when disturbed or if conditions grow inhospitable, sea anemones can detach from their home and swim off in search of a more suitable location. Disturbance can be, for e.g., the touch or exposure to toxic and irritating extracts of a predatory sea star and nudibranchs (Hickman et al., 2007, Springer & Holley, 2012).

The sexes in sea anemones are separate in some species (like *Anemonia viridis;* Forskål, 1775), while most species, like the brooding anemone, are protandric hermaphrodites. Nonetheless usually only one type of gamete is reproduced at any time. The gametes develop in gastrodermal bands just behind the free edge of the mesenteries. Both sexual and asexual reproduction can occur. In sexual reproduction males release sperm to stimulate females to release eggs, and fertilization occurs. Anemones eject eggs and sperm through the mouth. Fertilization and early development may occur externally in the sea water or within the gastrovascular cavity. The fertilized egg develops into a planula larva and then into a ciliated planktonic polypoid larva in which mesentries and pharynx

appears. This polyp soon settles and becomes attached as a proper young sea anemone (Springer & Holley, 2012). Asexual reproduction is also common in sea anemones. One such method occurs by pedal laceration, in which small pieces of the pedal disc are left behind as the animal moves with the remnants forming new anemones. Many reproduce asexually by longitudinal (lengthwise) fission, and few by transverse (crosswise) fission or by budding (Hickman et al., 2007, Springer & Holley, 2012).

Anemones form some interesting mutualistic relationships with other organisms. Many species harbour symbiotic algae (zooxanthellae and zoochlorellae) within their tissues, and anemones profit from the product of algal photosynthesis (Bergschneider & Muller-Parker, 2008, Davy et al., 2012, Muller-Parker & Davy, 2001, Yellowlees et al., 2008). Hermit crabs also have developed associations with sea anemones (Brooks, 1988, Brooks, 1991, McLean, 1983, McLean & Mariscal, 1973, Ross, 1971). When hermit finds the right sea anemone, he encourages the relationship and attachment happens upon the active, tactile behaviour of one or both partners (Brooks & Mariscal, 1986, Ross, 1974). Although little information exists about how or if partners might locate each other at a distance, it has been demonstrated that chemoreception play an important role in this interaction (Brooks, 1991). It is interesting to observe that some young hermit crabs often pick up a young sea anemone to attach to their shell and they become partners for life, growing even roughly at the same rate. The crab derives some protection against predators by the anemone. The anemone gets free transportation and particles of food dropped by the hermit crab (Hickman et al., 2007). Also, certain anemonefishes (family Pomacentridae) form associations with anemones, especially in tropical Indo-Pacific waters (Cleveland et al., 2011, Elliot & Mariscal, 1997, Fautin & Allen, 1992, Holbrook & Schmitt, 2005, Mariscal, 1970, Porat & Chadwick-Furman, 2004). These fish have evolved the ability to live among the tentacles of sea anemones, even though these tentacles could quickly paralyze other fishes that touched them (Raven & Johnson, 2002). In the process of adaptation to the host, the anemonefishes chemically modify the skin mucus to prevent the anemone nematocysts from discharging (Yoshiyama et al., 1996). The anemone fishes feed on the detritus left from the meals of the host anemone, remaining uninjured under remarkable circumstances (Raven & Johnson, 2002). The anemone obviously provides shelter for the anemonefish, and the fish may help ventilate the anemone by its movements, keep the anemone free of sediment, and even lure an unwary victim to seek the same shelter (Hickman et al., 2007, Roopin et al., 2008, Szczebak et al., 2013).

1.3. Microbial communities associated with Anthozoans - a glint of these communities in sea anemones

Most eukaryotes are believed to associate with a diverse assemblage of microbial symbionts that aid in their development and health (Morrow et al., 2012). Cnidarians are simple animals with no physical barriers between the host tissue and microorganisms. Because of this they occupy an important evolutionary position for understanding direct host–microbe relations and their role in aquatic (mostly marine) ecosystems (Egan et al., 2008, Fraune & Bosch, 2007). Anthozoan cnidarians, especially sessile animals like corals, are known to host rich and diverse populations of associated bacteria. Corals have received great attention and are the most studied anthozoans in communities associations (Di Camillo et al., 2012, Egan et al., 2008). In fact it has been commonly reported the fascinating interactions between bacteria and corals in marine habitats, although most of their ecological functions remain enigmatic (Schuett et al., 2007). It is believed that coral microorganisms contribute to the overall health of the coral host (Ducklow & Mitchell, 1979, Medina, 2011, Rosenberg et al., 2007a, Rosenberg et al., 2007b). In addition, these microorganisms may be ecologically important once they appear to contribute to the ability of reef building corals to adapt to and evolve under changing environmental conditions (Reshef et al., 2006, Rosenberg et al., 2007a). It is hypothesized that corals probably coevolved with their microbial symbionts, which likely fill a critical and beneficial role in coral colony immune function. Microorganisms are found throughout the coral holobiont and appear to be regulated in part by the coral host (Morrow et al., 2012). Recently it has been suggested that diversity and types of coral-associated bacteria may also be tightly coupled with the clade of *Symbiodinium* spp. found within the coral host tissues (Littman et al., 2010, Medina, 2011). Coral microorganisms are thought to benefit the host by providing nutritional by-products, protein, and nitrogenous compounds and also by synthesizing essential vitamins (Croft et al., 2005, Lesser et al., 2007, Lesser et al., 2004, Xu & Gordon, 2003). Microbial symbionts may also protect corals from disease by preventing opportunistic infections through the occupation of otherwise available niches and by producing antibacterial agents (Koh, 1997, Ritchie & Smith, 2004, Rypien et al., 2010, Shashar et al., 1994) and other metabolites (Rocha et al., 2011). Shifts in coral microbial assemblages have been linked to bleaching (Bourne et al., 2008, Pantos et al., 2003), thermal stress (Littman et al., 2010, Thurber et al., 2008), irradiance (Muller & van Woesik, 2009), disease (Bourne et al., 2008), changes in dissolved organic nutrients (Dinsdale et al., 2008, Kline et al., 2006, Smith et al., 2006, Wang et al., 2007), and shifting pH (Medina, 2011). Shifts in both microbial diversity and metabolism have also been related to the proximity of coral to human populations (Dinsdale et al., 2008),

demonstrating that geographic location may indirectly influence coral reef health through microbial mediation. Although evidence suggests that congeneric coral species associate with similar microorganisms (Medina, 2011, Rohwer et al., 2002), the metabolic functions and specificity of coral-microbe associations are less predictable than initially hypothesized (Daniels et al., 2011, Hansson et al., 2009, Kvennefors et al., 2010, Sunagawa et al., 2010) and cannot be said that in these animals the microbial communities are species specific as deemed in Hydra (Fraune & Bosch, 2007).

While symbioses between microorganisms and some anthozoans have been widely investigated (mainly in corals), the interactions between bacteria and sea anemones are still largely unknown. In fact to date only a limited number of studies have examined the diversity of bacterial communities isolated from sea anemones (Du et al., 2010, Meron et al., 2013, Palincsar, 1989, Schuett et al., 2007, Xiao et al., 2009) and key questions concerning the phylogenetic relationship of bacterial symbionts, their ecological and physiological function, their secondary metabolites, their chemical structure and the effects of these compounds on host tissue, are still poorly known. As an example, understanding these communities and their interactions is paramount to perform an accurate estimate on the diversity of natural products that may be yielded from the sea (popularly termed as “the blue gold”). Numerous natural products primarily thought to be produced by marine invertebrates show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms (e.g., bacteria) are at least involved in their biosynthesis or are in fact the true sources of these metabolites (Proksch et al., 2002).

While both culture-dependent and independent methodologies are currently used for describing microbial diversity, the application of molecular approaches has greatly enhanced the knowledge of species diversity and population structure in natural microbial communities (Du et al., 2010). Furthermore, the development of high-throughput pyrosequencing technology has revolutionized the traditional cloning and capillary Sanger sequencing technique (Binladen et al., 2007, Margulies et al., 2005, Ronaghi et al., 1998, Rothberg & Leamon, 2008). Using simple techniques, light and electron microscope and fatty acids profiles, Palincsar et al. (Palincsar et al., 1988, Palincsar, 1989) made the first attempt published to determine bacterial symbionts in sea anemones. They described Gram-negative bacteria (suggested to belong to the genus *Vibrio*) in aggregates on the epidermis of *Aiptasia pallida*. In 2007, Schuett et al. (Schuett et al., 2007) provided information on organ-like bacterial aggregates in caverns of the tentacles epidermis of the sea anemone *Metridium senile*. In this study sequence analysis revealed three different subgroups of intra-tentacular proteobacteria. It was strongly suggested that the bacteria embedded in the aggregates were closely related to Gram-negative *Endozoicimonas*

elysicola. Additionally two bacteria were detected and assigned to be *Pseudomonas saccherophilia* and *Ralstonia pickettii*. Other three papers were published using conventional culture-dependent methods (Du et al., 2010, Williams et al., 2007, Xiao et al., 2009). The first one from Williams et al. (Williams et al., 2007) used classical methodologies to identify isolates of associated bacteria from tentacles and body tissues of *Stichodactyla haddoni*. They were able to identify eight genera: *Alcaligenes*, *Corynebacterium*, *Aeromonas*, *Sporosarcina*, *Renibacterium*, *Camobacterium*, *Oarnobacterium* and *Salinococcus*. In the other two articles the isolates were characterized by 16S rRNA gene sequence analysis. In the case of sea anemone *Anthopleura midori* (Du et al., 2010) the analysis revealed that all the strains isolated belonged to twenty two genera: *Colwellia*, *Pseudoalteromonas*, *Vibrio*, *Acinetobacter*, *Pseudomonas*, *Endozoicomonas*, *Roseovarius*, *Paracoccus*, *Loktanella*, *Leisingera*, *Sulfitobacter*, *Bacillus*, *Staphylococcus*, *Plantibacter*, *Microbacterium*, *Micrococcus*, *Joostella*, *Psychroserpens*, *Cellulophaga*, *Krokinobacter*, *Polaribacter* and *Psychrobacter* (of eight clusters: Alteromonadales, Vibrionates, Peseudomonadales, Oceanospirillales, Rhodobacterales, Bacillales, Actinomycetales, Flavobacteriales). *Pseudoalteromonas* was the dominant genus of the bacterial flora associated with the surface of this sea anemone. For the non-identified sea anemone from coast of the Naozhou island in Zhanjiang, China (Xiao et al., 2009), the sequence analysis revealed that the isolates were members of eighteen genera (*Alteromonas*, *Bacillus*, *Brachybacterium*, *Brevibacterium*, *Halobacillus*, *Halomonas*, *Nocardiopsis*, *Oceanobacillus*, *Piscibacillus*, *Planococcus*, *Pontibacillus*, *Pseudoalteromonas*, *Pseudonocardia*, *Salinicoccus*, *Salinivibrio*, *Staphylococcus*, *Vibrio*, *Virgibacillus*) belonging to three major phylogenetic groups (Actinobacteria, Firmicutes, Gammaproteobacteria). The most abundant and diverse isolates were within the phylum Firmicutes and the class Gammaproteobacteria. Finally, in 2013 a paper was published by Meron et al. (Meron et al., 2013) describing the microbial communities associated with the sea anemone *Anemonia viridis* using pyrosequencing 16S rRNA gene clone libraries. Thirteen main bacterial groups were identified: Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Candidate_division_TM, Cyanobacteria, Deltaproteobacteria, Epsilonproteobacteria, Firmicutes, Fusobacteria, Gammaproteobacteria, Planctomycetes, Verrucomicrobia. The dominant groups in the case of *A. viridis* were Gammaproteobacteria, Firmicutes and Actinobacteria, the same major phylogenetic groups determined in a non-identified sea anemone from China (Xiao et al., 2009). Although there is a long way to run to determine bacterial communities in sea anemones and their functional role in these associations, it is interesting to note that, along with the identification of associated bacterial communities, is frequent the search of antimicrobial activity. This may be due to the assumption that the production of these

metabolites may play an important role in bacteria-host interactions (Du et al., 2010); even though the cellular basis of antibacterial activity still remains largely unknown. Moreover, there is an urgent need to discover new compounds of this type to cure new and old diseases that cannot be treated with the existing drugs.

As some species of sea anemones are currently economically important, not only as marine ornamentals (Chapman et al., 1997) but also as a cuisine delicacy (Schwabe, 1979) and as producers of biotechnologically important natural products (Rocha et al., 2011), additional research efforts in these matters are necessary to gain an in depth knowledge on the true bioprospecting potential of sea anemones and their associated bacterial biota.

1.4. Global climate change and its potential impact in cnidarians

Marine ecosystems harbor a substantial fraction of the earth´s biodiversity and are maintained by the flow of energy through the food webs (Doney et al., 2012, Halpern et al., 2012). Consequently, marine communities are biological networks in which the success of species is linked, directly or indirectly through various biological interactions (e.g., predator-prey relationships, competition, facilitation, mutualism), to the performance of other species in the community. The combined effect of these interactions constitutes ecosystem function (e.g., nutrient cycling, primary and secondary productivity), through which ocean and coastal ecosystems provide the wealth of free natural benefits (Doney et al., 2012).

Anthropogenic disturbances, including climate change, are however having profound and varied consequences in marine ecosystems. The rising of atmospheric carbon dioxide (CO_2) is one of the major critical problems nowadays since its effects are globally pervasive and irreversible on ecological timescales (Natl. Res. Counc., 2011). Primary direct consequences are increasing ocean temperatures and acidity (Doney et al., 2012, Donner, 2009). Rising temperature creates an horde of additional changes, such as rising sea level, increased ocean stratification, decreased sea-ice extent and altered patterns of ocean circulation, precipitation, and freshwater input. Additionally, both warming and altered ocean circulation act to reduce subsurface oxygen (O_2) concentrations (Keeling et al., 2010). In recent decades, the rates of change have been hasty and may exceed the current and potential future tolerances of many organisms to adapt.

The direct and indirect effects of physical and chemical changes can be observed by alterations in the physiological functioning, behavior, and demographic traits (e.g., productivity) of organisms, leading to shifts in the size structure, spatial range, and

seasonal abundance of populations. These modifications, in turn, lead to altered or disrupted species interactions and trophic pathways with climate signals thereby propagating through ecosystems in both bottom-up and top-down directions.

Cnidarian species have a crucial role in marine diversity as they form both the trophic and structural foundation of coral reefs ecosystems, as well as in diverse temperate habitats (Moya et al., 2012). Coral reefs are among the most biologically rich and productive ecosystems on earth as one-quarter of all marine species associate with this specific ecosystem (Burke et al., 2012, Doney et al., 2012). The ecological impacts of changing climates and chemistry on overall marine biodiversity are potentially severe and widespread. Changes in cnidarian-dinoflagellate due to environmental stressors, such as high light and ultraviolet light radiation (Moya et al., 2012), increased temperature (Brown, 1997, Glynn, 1991), pathogen infection, pollution and changes in salinity (Ainsworth & Hoegh-Guldberg, 2008, Brown, 2000); seem to promote symbiosis dysfunction and breakdown, ultimately leading to the loss of zooxanthellae (bleaching) (Hoegh-Guldberg, 1999). Alongside, it is rare to witness the action of only one stressor, being more often observed a combination of stressors. Compounded and together they impact and reduce the abundance or impair the growth of the cnidarian and its hosts (Hughes et al., 2003).

Genetic diversity in hosts and symbionts leads to a diversity of responses to mild temperature increases, however severe temperature anomalies almost always lead to widespread bleaching and death. In the future, reefs are likely to become dominated by symbiotic associations with warm-tolerant zooxanthellae as well as bacterial populations more adapted to the new conditions, allowing some animals to survive moderate temperature increases. Nonetheless, it is necessary to perceive that potential new symbiosis may be less suitable as partners when considering other aspects of coral health, such as growth (Jones & Berkelmans, 2010). Along with corals it is well known that sea anemones are key components in some temperate communities (Muller-Parker & Davy, 2001). They are also holobionts (with symbioses with zooxanthellae and bacterial populations) known to be delicately balanced in their symbiosis and surprisingly intolerant to stresses. For this, sea anemones are recognized to be important sentinel species (Winston & Heffernan, 1999). These organisms can be used by researchers to monitor potential environmental shifts in seawaters, triggered by global climate changes. Extreme bleaching events of *Anemonia* in the Mediterranean Sea under abnormally warm water conditions (Leutenegger et al., 2007) are a good example on the suitability of these anthozoans as sentinel species. Alongside, with the disruption monitoring of the photosynthetic symbiont or bleaching effect, it is also important to monitor potential shifts in the microorganisms associated with these sea anemones to understand how environmental disturbances may shape holobionts and ultimately ecosystems.

1.5. Overview of natural product discovery from cnidarians

Research on marine natural products began in the 1950s (Bergmann & Burke, 1955), at a time when important breakthroughs on the taxonomy of marine animals took place (Blunt & Munro, 2008). This research field expanded during the 1970s and 1980s and only by the end of the 1980s and beginning of the 1990s an economically appealing activity started to take shape (Avila et al., 2008, Faulkner, 2000). Since the beginning of marine natural product research that sponges (phylum Porifera) have been recognized as the most interesting group of marine invertebrates (Osinga & Tramper, 1998). However, with the growing bioprospecting efforts and the screening of previously unexplored marine habitats and organisms, the biotechnological potential of other groups of marine invertebrates has also started to become appealing for researchers. The phylum Cnidaria is one of these groups, which is renowned by their ability to produce powerful toxins and venoms (Turk & Kem, 2009). A total of 3244 marine natural products have been described from this phylum alone since 1990 (and until 2011), which notes the importance of cnidarians for marine natural product research. Since the early 1990s that the number of new compounds from marine cnidarians has been higher than the discovery of compounds from sponges (Leal et al., 2012b), and the trend that we currently observe is still a continuous increase of natural product discovery (Figure 1.3). This shows that the bioprospecting efforts on these organisms have been continuously increasing.

The quest for new MNP from cnidarians has benefited from a renaissance since 2005, namely due to the development of new methods in analytical technology, spectroscopy and high-throughput screening (Molinski et al., 2009). It has also benefited from the failure to deliver new drug leads in significant numbers by competing technologies, such as chemical synthesis. These two different reasons may support the continuous growth of natural product discovery from cnidarians in the last decade.

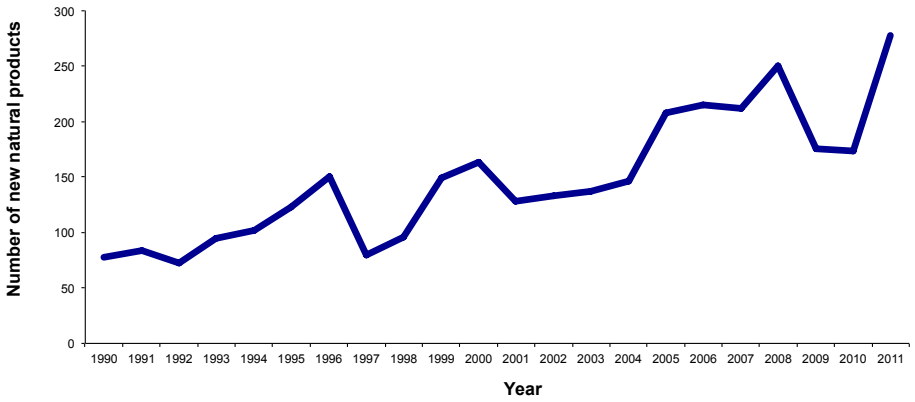

Figure 1.3. Number of new marine natural products from cnidarians discovered between 1990 and 2011 (Figure from Rocha et al. (2014)).

Bioprospecting efforts have not been evenly distributed among cnidarian taxa. From the 3244 new compounds yielded by marine cnidarian species since 1990, 99% were discovered in organisms from class Anthozoa. The remaining 1% is associated with species from class Hydrozoa. Anthozoans display a higher biodiversity, with a higher number of orders (Table 1.1). Nonetheless, 94% of the 3244 compounds were discovered in organisms from a single anthozoan order: the Alcyonacea. Only through the analysis of the taxonomic level below order, e.g., the family level is it possible to observe a more even distribution of new compounds among taxa. Figure 1.4 shows the cumulative number of natural products discovered from alcyonaceans according to family level. It is important to underline family Alcyoniidae due to the large number of new compounds discovered from species in this group, as well as the continuous increase of new compounds relatively to other Aclyonacea families.

The overall increase of new compounds associated with different Cnidaria taxa is displayed in Table 1.2. Of the most representative families from order Alcyonacea, only family Briareidae showed a small decrease on the number of new compounds discovered between the last two decades. All other families represented in Table 1.2 showed an increase between decades, which in some particular cases was relatively high (e.g., family Clavulariidae and Neptheidae). These results recorded by Leal et al. (2012b) show that the popularity of cnidarians in bioprospecting efforts continues to increase and with large numbers of new compounds being discovered every year.

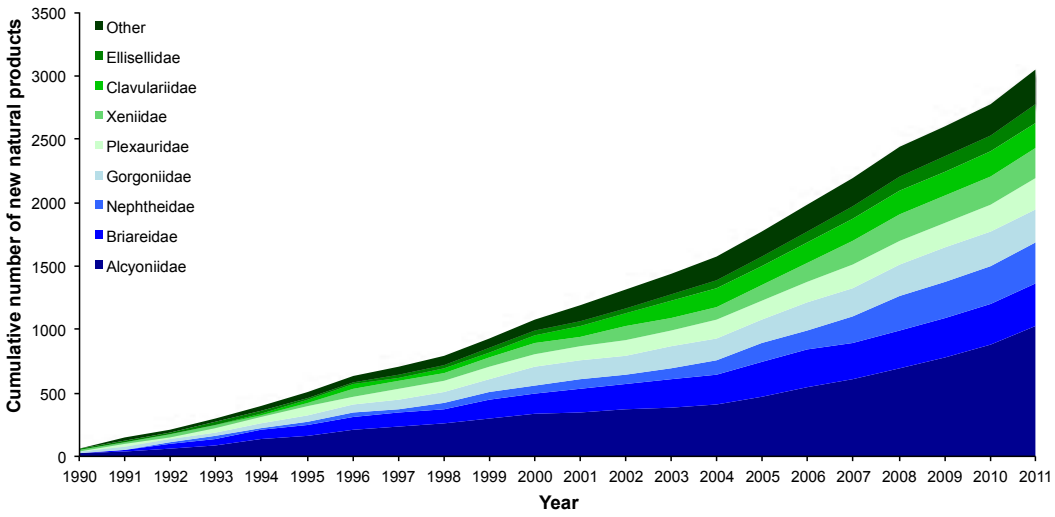

Figure 1.4. Cumulative number of new marine natural products from cnidarians according to the taxonomical level "Family". Group "Other" refers to the families Acanthogorgiidae, Anthothelidae, Coelogorgiidae, Isididae, Melithaeidae, Nidaliidae, Paragorgiidae, Paralcyoniidae, Primnoidae, Subergorgiidae and Tubiporidae (Figure from Rocha et al. (2014)).

The high chemical diversity associated with cnidarians may be related to the high biodiversity displayed by this group. While about 11,000 cnidarian species are currently known (Appeltans et al., 2012), new compounds have only been recorded from 337 species (distributed over 117 genera). This means that only ~3.1% of cnidarian biodiversity has yielded new chemical compounds. This does not necessarily mean that the remaining ~97% of cnidarian species do not display any different compounds.

Table 1.2. Number of new compounds discovered in the most representative taxa of phylum Cnidaria in the 1990s and 2000s decades (adapted from Leal et al. (2012b)).

Taxon	New compounds in the 1990s	New compounds in the 2000s	Decade variation of new compounds (%)
Phylum Cnidaria	1031	1773	+72%
Class Anthozoa	1017	1758	+73%
Sub-class Octocorallia	963	1715	+78%
Order Alcyonacea	934	1694	+84%
Family Alcyoniidae	293	489	+67%
Family Briareidae	158	156	- 1%
Family Clavulariidae	41	150	+266%
Family Gorgoniidae	109	165	+51%
Family Nephtheidae	58	227	+291%
Family Plexauridae	97	99	+2%
Family Xeniidae	72	147	+107%

Most likely, this is a result of the preference of scientists to search for new chemical entities that have been focused on a relatively low number of species. For instance, the most popular species among the Alcyoniidae are *Clavularia viridis*, *Briareum excavatum* and *Antillogorgia elisabethae* (Figure 1.5), which have been important cnidarians in the history of marine natural products research (Leal et al., 2012b). Diversification of bioprospected species has been relatively low, as it is possible to observe in Figure 1.5. This figure plot the number of new compounds discovered in cnidarian species since 1990 and sort that information according to the number of new compounds discovered in each species. The uneven result among bioprospected species is clearly observed. As most cnidarian species displaying a high number of new compounds inhabit tropical areas, this may suggests that bioprospecting efforts have been biased toward these particular species, probably driven by previous studies showing the high chemical diversity displayed by such taxa (Leal et al., 2012b). Although the assumption that all cnidarian species display similar chemical diversity is incorrect, Figure 1.5 shows that a large number of new molecules associated to other cnidarians are yet to be unravelled. This is particularly evident if considering that compounds currently known were discovered from only~3% of total cnidarian biodiversity.

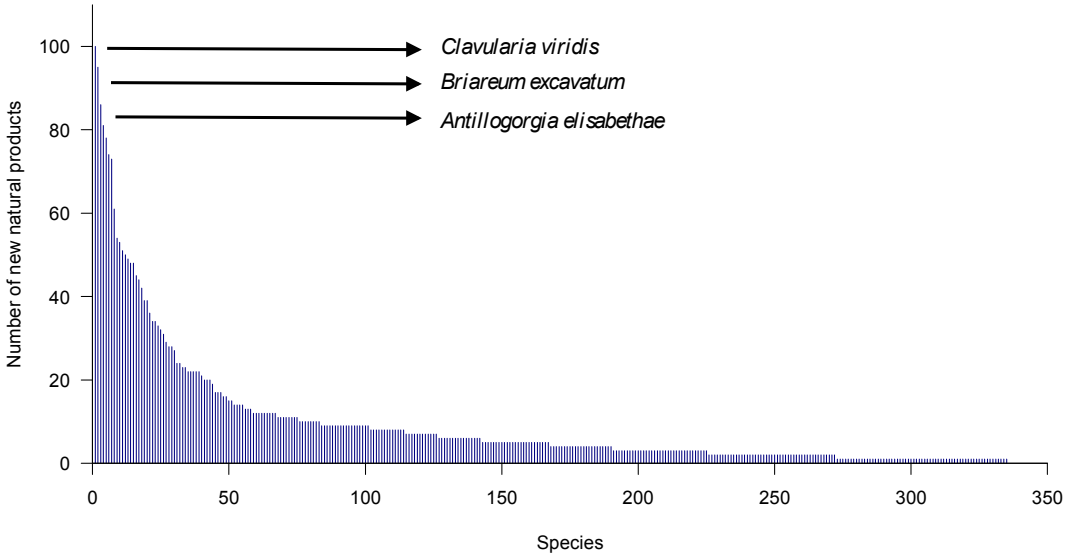

Figure 1.5. Number of new natural products discovered per cnidarian species. Each bar corresponds to a single species (total of 337 species). The three species yielding the highest number of natural products are indicated (Figure from Rocha et al. (2014)).

Another issue that should be noted is biodiscovery hotspots of new cnidarian compounds. Although biogeography is by itself a well-studied topic, its investigation in marine natural products research is still scarce. This is probably justified by the lack of precise geographical information on collection sites. However, recent studies already started to address this topic, and clearly reveal bioprospecting efforts to be biased towards tropical areas (Leal et al., 2012a, Leal et al., 2012b). New molecules discovered from cnidarian species over the past decades have mostly resulted from bioprospecting on Asian territories close to tropical areas, particularly in Taiwan, Japan and China. Remarkably, 50% of such new molecules from cnidarians discovered since 1990 resulted from organisms collected in the marine environment surrounding these two territories.

The compounds discovered in cnidarian species belong to various chemical groups, although the majority are terpenoids. Leal et al. (2012a) showed that in the last decade, 66% of the compounds discovered in cnidarians were terpenoids, which contrasts with the relatively lower percentage of discovered alkaloids (10%), steroids (9%), aliphatic compounds (8%) and carbohydrates (6%). This data shows that particular chemical groups, such as terpenoids in the case of cnidarians, have been unquestionably more popular among researchers searching for new compounds. Terpenoids are secondary metabolites that are not directly involved in critical physiological processes. These compounds often play a role in interspecific and other ecological interactions, displaying a

wide array of known bioactivities and biological functions (McClintock & Baker, 2001, Paul, 1992, Paul et al., 2007). Directing new investigations according to the results of this and previous studies, by preferentially targeting molecules of this chemical group (Munro et al., 1999), increased the researchers chances for successful drug discovery and consequent patenting and commercialization. Several examples are already described for the application of terpenoids in the pharmaceutical and food industry due to their potential and effectiveness as medicines and flavor enhancers (Munro et al., 1999, Zwenger & Basu, 2008).

Besides directed bioprospecting efforts towards particular groups of organisms and chemical structures, researchers have also been narrowing their searches on particular molecules, with emphasis to the type and relevance of bioactivity displayed, in order to identify the most promising targets for their drug discovery pipelines (Rocha et al., 2011). These marine molecules exhibit various types of biological activities, such as anti-inflammatory, antitumor, antimalarial, etc. It is not surprising that over the past 40 years major advances in the discovery of marine drugs have been recorded in clinical trials for cancer (Hill & Fenical, 2010). Although there are several marine bioactive compounds in preclinical and clinical trials, only a relatively small number of molecules have reached this stage of the drug discovery pipeline. This process is very complex and encompasses several steps: target identification and validation, assay development, lead identification and optimization, pre-development, preclinical development, clinical research phase I to phase III, regulatory approval and phase IV (post-approval studies) (Mayer et al., 2010, Molinski et al., 2009, Paul et al., 2010). In effect, the drug discovery pipelines normally takes 10-15 years from the first to the last step involving high investments.

New drugs derived from marine natural products isolated from cnidarians may still be several years away, but it is unquestionable that more chemical entities, besides toxins and venoms, will be recorded from cnidarians (Rocha et al., 2011). In conclusion, this diverse group of marine invertebrates is destined to play a major role in the pursuit of new drugs from the sea.

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CHAPTER 2

Optimization of preservation and processing of sea anemones for microbial community analysis using molecular tools

Optimization of preservation and processing of sea anemones for microbial community analysis using molecular tools

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2.1. Abstract

For several years, knowledge on the microbiome associated with marine invertebrates was impaired by the challenges associated with the characterization of bacterial communities. With the advent of culture independent molecular tools it is possible to gain new insights on the diversity and richness of microorganisms associated with marine invertebrates. In the present study, we evaluated if different preservation and processing methodologies (prior to DNA extraction) can affect the bacterial diversity retrieved from snakelocks anemone *Anemonia viridis*. Denaturing gradient gel electrophoresis (DGGE) community fingerprints were used as proxy to determine the bacterial diversity retrieved (H'). Statistical analyses indicated that preservation significantly affects H'. The best approach to preserve and process *A. viridis* biomass for bacterial community fingerprint analysis was flash freezing in liquid nitrogen (preservation) followed by the use of a mechanical homogenizer (process), as it consistently yielded higher H'. Alternatively, biomass samples can be processed fresh followed by cell lyses using a mechanical homogenizer or mortar and pestle. The suitability of employing these two alternative procedures was further reinforced by the quantification of the 16S rRNA gene; no significant differences were recorded when comparing these two approaches and the use of liquid nitrogen followed by processing with a mechanical homogenizer.

2.2. Introduction

Phylum Cnidaria is a large, diverse and ecologically important group of relatively simple organisms that is widely distributed in marine environments (Daly et al., 2007). Research on marine cnidarians experienced a significant advance over the last decades with the growing awareness on the vulnerability of certain key ecosystems (e.g. coral reefs) driven by direct or indirect anthropogenic actions (Carpenter et al., 2008, Hoegh-Guldberg et al., 2007, Hughes et al., 2003). Additionally, with the intensification of bioprospecting of marine invertebrates for drug discovery, as well as other biotechnological applications, researchers have started to target cnidarians in their quest for marine bioactive compounds (Leal et al., 2012, Rocha et al., 2011). Alongside with this new trend, there are growing evidences that microbes associated with marine invertebrates may be the true producers of such bioactive compounds or, at least, be partially involved in the process of biosynthesis of some of these molecules (Shnit-Orland & Kushmaro, 2009). Several of these compounds are secondary metabolites produced by symbiotic microorganisms in chemical mediation and/or defense of interaction among marine

microorganisms (Paul & Puglisi, 2004). The microbiome of certain marine invertebrates may represent a remarkable proportion of the holobiont biomass, with anthozoan cnidarians being no exception and hosting abundant and diverse communities of bacteria (Di Camillo et al., 2012). Certain species able to secrete mucus may reach microbial concentrations up to 1000-fold higher than those observed in seawater (Rosenberg et al., 2007). While microbial communities associated with tropical reef building corals are already starting to be unraveled, those colonizing other groups of anthozoans are still largely unknown (La Riviere et al., 2013). For several years, this gap of knowledge has been mainly due to the challenges associated with the characterization of bacterial communities using culture dependent approaches (Fuhrman & Campbell, 1998). Only a small fraction of microbial symbionts can be cultured outside its cnidarian host using conventional culture media. The advent of culture independent molecular technologies (e.g. Denaturing Gradient Gel Electrophoresis (DGGE) and high–throughput DNA sequencing), made possible to overcome these bottlenecks and reveal the diversity and richness of microorganisms associated with marine invertebrates in general (Cleary et al., 2013, Di Camillo et al., 2012, White et al., 2012). Anthozoan cnidarians are no exception to this breakthrough (Bourne et al., 2013, La Riviere et al., 2013, Lee et al., 2012). Regardless of the potential associated with the use of high–throughput DNA sequencing to profile the microbiome of marine invertebrates, the final results achieved are still largely dependent on the quality and quantity of DNA extracted from collected samples (Simister et al., 2011). DNA quality and quantity is known to vary with the procedures employed for preserving and processing samples, as well as on the reliability of the DNA extraction method (Nagy, 2010, Simister et al., 2011).

Sea anemones, namely those hosting endosymbiotic photosynthetic dinoflagellates, are recognized to be important sentinel species (Winston & Heffernan, 1999). These organisms may help researchers to monitor potential environmental shifts in temperate coastal waters triggered by global climate changes. Extreme bleaching events of *Anemonia* in the Mediterranean under abnormally warm water conditions (Leutenegger et al., 2007) are a good example on the suitability of these anthozoans as sentinel species. In light of the hologenome theory (Rosenberg et al., 2007), these anemones should be considered as holobionts (Margulis & Fester, 1991), a complex symbiosis between the cnidarian animal, its photosynthetic microalgae (e.g., zooxanthellae) and its complex community of associated microorganisms that play a key role on the overall health of the cnidarian host. Therefore, it is important to monitor potential shifts in the microorganisms associated with these sea anemones to understand how environmental disturbances may shape anemone individuals and populations.

Despite the existence of reports on the suitability of processing techniques to preserve samples and extract microbial DNA from marine invertebrates (e.g. Ferrara et al., 2006, Simister et al., 2011), only a few studies are currently available on sea anemones (Meron et al., 2013, Pinto et al., 2000). Given the current state of the art on this topic and the complexity/specificity of this biological matrix, we consider that it is relevant to standardize a protocol that can allow researchers to extract good-quality DNA in order to perform a reliable analysis of the bacterial communities associated with sea anemones. In line with this goal, here we used the snakelocks anemone *Anemonia viridis* (Forskål, 1775) as a model species to evaluate how different preservation and processing approaches could affect the quality of extracted DNA and the molecular profiles of bacterial communities retrieved from these organisms.

2.3. Methods

Sample collection, preservation and processing

Five snakelocks anemones *A. viridis* were collected at low tide, in the intertidal region of Praia da Aguda (41°02'51.06"N; 8°39'14.20"W), Arcozelo, Portugal, in November 2011 and individually stocked in sterile plastic bags for immediate transportation to the laboratory.

Each of the five sea anemones collected was fragmented into 9 similar sized pieces using sterile scalpels blades along their radial axis; each piece included similar amounts of anemones body and tentacles, as well as a similar wet weight. A factorial experimental design employing three levels of preservation (samples used fresh, frozen at -80 ºC and flash frozen with liquid nitrogen) and three levels of processing (non-homogenized samples, samples homogenized with mortar and pestle and samples homogenized with a mechanical tissue homogenizer) was tested prior to DNA extraction. Briefly, this factorial design allowed us to evaluate 9 different experimental treatments, each with five independent replicates: Fr_NH (fresh samples non homogenized, where fresh samples were used directly for extraction of nucleic acids without any further processing or preservation); Fr_H (fresh samples were processed with the Omni Tissue Homogenizer (Omni International, Kennesaw, Georgia, USA) and used for DNA extraction without any further treatment); Fr_MP (fresh samples were processed with the mortar and pestle and used for DNA extraction without any further treatment); LN_H and LN_MP (samples were first preserved by flash freezing in liquid nitrogen and kept at -80 °C and then homogenized with the Omni Tissue Homogenizer or mortar and pestle, respectively, prior to DNA extraction); F-80_H and F-80_MP (samples were first frozen and kept at -80 °C

and then homogenized with the Omni Tissue Homogenizer or mortar and pestle, respectively, prior to DNA extraction); LN_NH (samples were flash frozen in liquid nitrogen and kept at -80 °C and used for DNA extraction without any further processing); and F-80_NH (samples were frozen and kept at -80 °C and used for DNA extraction without any further processing) (see Figure 2.1 for a schematic representation of the experimental design).

Extraction of nucleic acid

Nucleic acids were extracted from 0.5 g of sea anemone samples from each experimental treatment described above. All samples were homogenized using FastPrep® (Qbiogene Inc., USA) bead-beating system in combination with a mixture of beads (0.10 g Zirconia beads (0.1 mm) + 0.20 g glass beads (0.25 – 0.5 mm) + 0.20 g glass beads (0.75 – 1.0 mm) + 2 glass beads (2.85 – 3.45 mm)) (ROTH, DE) and Buffer SLX Mlus from E.Z.N.A.™ Soil DNA Kit (Omega Bio-Tek Inc., USA). Extraction was performed according to the instructions provided by the manufacturer. DNA was determined using Qubit™ dsDNA HS Assay Kits for Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) (see Supplementary Table ST1).

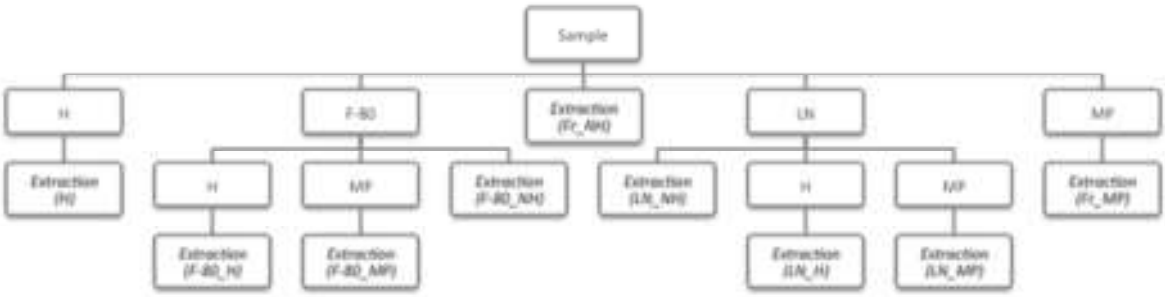

Figure 2.1. Schematic representation of the experimental design employed to evaluate the effect of different processing and preservation approaches on bacterial diversity retrieved after performing bacterial DNA extraction and amplification of snakelocks anemone *Anemonia viridis*. Fr – Fresh; NH – non-homogenized; H – maceration with homogenizer; MP - maceration with mortar & pestle; F-80 – freezing and preservation at -80 °C; LN - freezing with liquid nitrogen followed by preservation at -80 °C.

Bacterial community diversity

Bacterial community composition was evaluated by performing a DGGE based on DNA (16S rRNA gene). The bacterial fingerprints yielded by the DGGE were used as a proxy to evaluate the diversity of the bacterial community retrieved from sea anemones handled

according to each of the preservation and processing combinations described above. NESTED PCR was used for a more efficient amplification of 16S rRNA gene fragments of bacterial genomic DNA extracted from sea anemone. In the first PCR, the universal bacterial primers F-27 (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and R-1492 (5′-TACGG(C/T)TACCTTGTTACGACTT-3′) were used to amplify c. 1500 bp of the 16S rRNA gene (Heuer et al., 1997, Weisburg et al., 1991). The PCR reaction mixtures (25 µL) consisted of DNA template (1 µL), DreamTaq PCR Master Mix (2X) (12.5 µL) (Fermentas, Thermo Fisher Scientific Inc., USA), bovine serum albumin (BSA, 2.0 mg/mL) and forward (0.1 µM) and reverse primers (0.1 µM). After 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94 °C, 45 s at 56 °C and 1.3 min at 72 °C, the PCR was finished by an extension step at 72 °C for 10 min. The amplicons obtained from the first PCR were then used as templates for a second PCR with bacterial DGGE primers F984-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACC TTAC-3') and R1378 (5'- CGGTGTGTACAAGGCCCGGGAACG -3') (c. 473 bp) (Heuer et al., 1997). For these PCR reactions it was used the same quantity of DNA template, DreamTaq PCR Master Mix (2X), forward and reverse primers and 4% acetamide. Cycling conditions were of 4 min at 94 °C for denaturation, 25 thermal cycles of 1 min at 95 °C, 1 min at 53 °C and 2 min at 72 °C, with an extension step at 72 °C for 7 min to finish the PCR reactions. All PCR amplification products were examined by electrophoresis on 1.0% agarose gels containing GelRed (Biotium Inc., USA). DGGE analysis was carried out on a DCode universal mutation detection system (Bio-Rad). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel (40-58% of denaturants) containing 6-10% acrylamide. The run was performed in 1x Tris-acetate–EDTA buffer at 58 °C at a constant voltage of 160 V for 16 h. DGGE gels were silver-stained according to (Riesner et al., 1989). The solutions used were 10% (vol/vol) ethanol plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared developing solution containing 0.01% (wt/vol) sodium borohydride, 0.15% formaldehyde, 1.5% (wt/vol) NaOH, and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were documented with a Molecular Imager chemiDoc XRS+ digitalize system (Bio-Rad). A total of five DGGEs were performed and analyzed: four DGGEs to cover all the samples of the nine experimental procedures, and one DGGE including the procedures yielding the highest values of Shannon's index of diversity (H') (see below on Statistical analysis).

DNA quantification using Real-time PCR

As Real-time PCR is an expensive laboratory procedure, it was solely used to determine the copy numbers of the 16S rRNA gene from the three preservation and processing combinations yielding the highest H' values. The assays were performed with a StepOne

real time PCR system (Applied Biosystems). The samples were quantified by determining the threshold cycle value and by comparing it to a standard curve to determine the copy number. Bacterial primers 968F (5´-AACGCGAAGAACCTTAC-3´) and 1401R (5'-CGGTGTGTACAAGACCC-3´) were used to amplify 16S rRNA gene fragments (c. 433 bp) from template DNA (Nübel U, 1996). The real-time PCR master mix (20 µL) contained template DNA (1 µL), 2x SYBR Green Master Mix (Apllied Biosystems) and forward (0.1 µM) and reverse (0.1 µM) primers. The amplifications were carried out as following: initial denaturation (10 min at 95 °C) was followed by 40 cycles of 15 s at 94 °C, 30 s at 53 °C, and 30 s at 72°C and completed by fluorescence data acquisition at 80 °C to dissociate the primers dimers. Product specificity was confirmed by melting point analysis (55 °C to 95 °C with a plate read every 0.5 °C).

A fragment of the 16S rRNA gene amplified with primers U27 and 1492R (ca. 1450 bp) (Weisburg et al., 1991), was used as a standard for the calibration curve. After amplification the standard was purified using the Geneclean II kit (MP Biomedical, France) and quantified with the Quant-iT dsDNA high sensitivity assay kit (Invitrogen, USA) and the Qubit fluorometer (Invitrogen, USA). The gene copy number in the initial standard curve was calculated considering the DNA content, the length of the fragment and the average weight of a base pair (650 Da). A standard curve was constructed by producing a ten times dilution series from 10^8 to 10^1 target gene copies per µL.

Sample copy numbers were log transformed and normalize to DNA input.

Statistical analysis

Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). Shannon's index of diversity (H'), was determined as $H' = - \sum pi \ln pi$, where pi is often the proportion of individuals belonging to the each species in the dataset of interest. The existence of significant differences in Shannon's index of diversity (H') values calculated from each experimental treatment was investigated by using a two-way ANOVA (with processing and preserving procedures being used as the categorical factors and the test also accounting for interactions between both factors). The assumptions of normality and homogeneity of variance were verified by using the Shapiro-Wilks and Levene's test, respectively. Post hoc Tukey HSD test was used whenever significant differences at $p < 0.05$ were recorded. The two-way ANOVA was performed using the software STATISTICA® 7 (StatSoft Inc., USA).

A Principal Coordinates Analysis (PCO) was performed representing differences among experimental treatments along the first two axes. The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on

compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A similarity/difference matrix was latter constructed using the Euclidean distance. This multivariate statistical test was performed using Primer 6.1 with PERMANOVA add-on (Primer-E Ltd, UK).

Significant differences among treatments selected for DNA quantification using Real-time PCR were tested using a one-way ANOVA using also the software STATISTICA® 7 (StatSoft Inc., USA). The assumptions of normality and homogeneity of variance were verified by using the Shapiro-Wilks and Bartlett's test, respectively.

2.4. Results

The two-way ANOVA revealed that there was no significant (P = 0.496) interaction between the processing and preserving procedures that were tested and that the categorical factor processing did not significantly (P = 0.347) affected the values of Shannon's index of diversity (H') calculated from the bacterial fingerprints generated from DGGE. However, the categorical factor preserving significantly (P = 0.022) affected H' values.

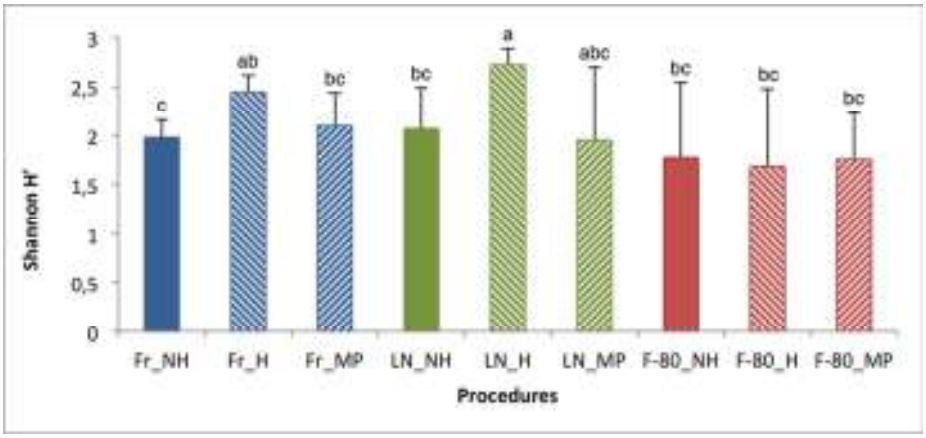

Figure 2.2. Shannon's index of diversity (H') calculated from DGGE community profiles of Bacteria detected on snakelocks anemone *Anemonia viridis* from each experimental treatment. Values presented are means (+s.d.) of five independent replicates. Fr – Fresh (blue); NH – non-homogenized (full colored); H – maceration with homogenizer (pinstripe right); MP - maceration with mortar & pestle (pinstripe left); LN - freezing with liquid nitrogen followed by preservation at -80 °C (green); F-80 – freezing and preservation at -80 °C (red). Different letters represent significant differences (Tukey's test, P < 0.05).

Experimental treatments employing freezing at -80 ºC differed significantly from those processing fresh samples or samples flash frozen with liquid nitrogen (P = 0.017 and P = 0.025, respectively). The highest average H' value (± s.d.) was displayed by LN_H (H' = 2.72 ± 0.17), while the lowest was that of F-80_H (H' = 1.68 ± 0.79) (Figure 2.2).

The bacterial fingerprints recorded in the DGGE of the three experimental treatments promoting the highest H' (in descending order, LN_H, Fr_H and Fr_MP) are illustrated in Figure 2.3.

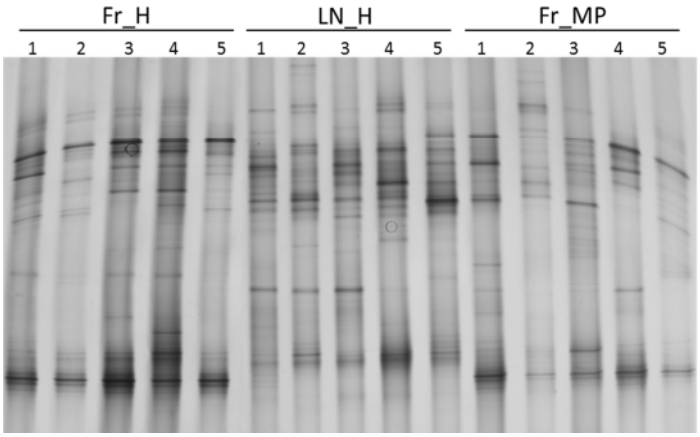

Figure 2.3. Denaturing gradient gel electrophoresis (DGGE) based analysis of bacterial community composition in the snakelocks anemone *Anemonia viridis* (cropped image, full-length gel is presented in Supplementary Fig. S1). The DGGE gel presented compares community fingerprints of 16S rRNA gene fragments amplified from DNA for the three experimental procedures displaying the highest H' values calculated from DGGE community profiles of Bacteria detected in samples of snakelocks anemone *Anemonia viridis*: fresh samples processed with homogenizer (Fr_H); samples frozen with liquid nitrogen followed by processing with homogenizer (LN_H); and fresh samples processed with mortar & pestle (Fr_MP). Equal numbers represent samples originating from the same anemone.

The first two axis of the PCO explained 65.1% of the variability recorded in the bacterial fingerprints of the three experimental treatments yielding the highest H' (Figure 2.4). Samples from treatment LN_H are clearly clustered apart from those where samples were processed fresh (Fr).

Real-time PCR quantification of 16S rRNA gene did not reveal the existence of any significant differences between the three procedures yielding the highest H' (P = 0.008).

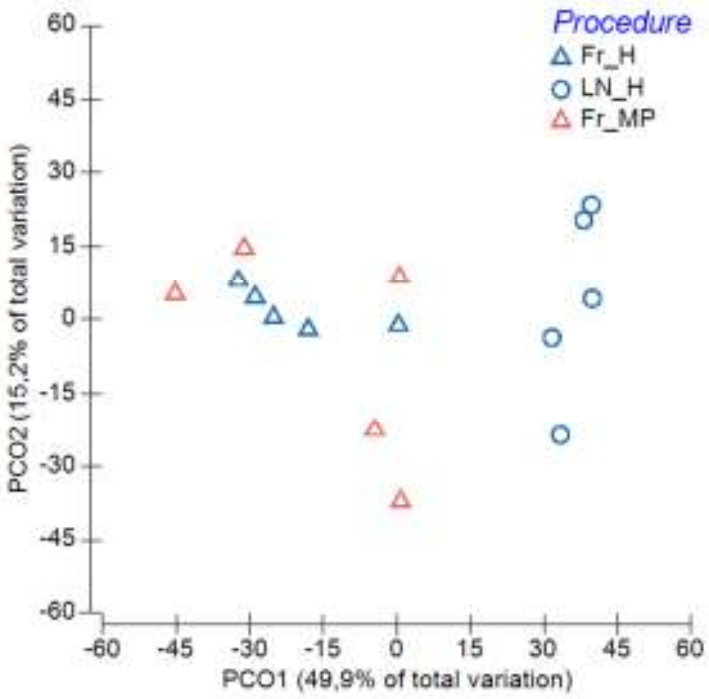

Figure 2.4. PCO of the three experimental procedures displaying the highest H' values calculated from DGGE community profiles of Bacteria detected in samples of snakelocks anemone *Anemonia viridis*: fresh samples processed with homogenizer (Fr_H); samples frozen with liquid nitrogen followed by processing with homogenizer (LN_H); and fresh samples processed with mortar & pestle (Fr_MP).

2.5. Discussion

The present study reveals that the use of community fingerprinting approaches such as PCR-DGGE is a robust technique to assess and/or optimize processing and preservation methodologies of biological samples destined for microbial communities analysis using molecular tools (Cleary et al., 2012, Lai et al., 2006). While it is true that PCR-DGGE only detects the more abundant taxa present in the sample being analyzed it also provides an excellent high-throughput tool for comparative community structure analysis, it allows researchers to determine and compare the relative abundance of different bacterial populations, and therefore compare procedures, without the need to use more expensive and labor intensive techniques (Cleary et al., 2012, Liu, 2010, Neilson et al., 2013). Indeed, by using this approach it was possible to verify that there are no significant interactions between preservation and processing procedures employed for samples of *A. viridis* meant to be used in bacterial diversity analysis using molecular techniques.

Preservation was recorded to significantly affect H' of bacterial communities retrieved from sea anemones, as already recorded for sponges (Simister et al., 2011). It is now recognized by researchers that the preservation technique employed for marine invertebrate samples is a key point for molecular analysis of microbial communities (Dawson et al., 1998, Gray et al., 2013). In the present study it was possible to show that flash freezing and homogenizing (LN_H) collected samples consistently yielded the highest bacterial diversity from snakelocks anemones (Figure 2.2). It was also possible to verify that sea anemones tissue can also be processed fresh (e.g. Fr_H and Fr_MP) with satisfactory results if researchers have the constraint of not being able to flash freeze samples with liquid nitrogen.

According to the 16S rRNA gene quantification results from Real-time PCR, any of the three procedures was considered a suitable option to obtain bacterial DNA for molecular studies of bacterial communities from sea anemones.

The best results achieved in our study through the flash freezing of collected samples are in line with the fact that at such extremely low temperatures no DNA degradation occurs through enzymatic activity (Abad et al., 2008, Nagy, 2010, Post et al., 1993, Reiss et al., 1995); in this way the bacterial diversity retained is close to that present at sampling time.

Liquid nitrogen can be difficult to obtain and transport in remote locations and keeping samples frozen while in transit can at times be a challenging task. However, our results support the fact that flash freezing is indeed the most efficient approach when aiming to preserve biological samples from invertebrates for molecular analysis of their microbial communities (Dawson et al., 1998, Gray et al., 2013, Reiss et al., 1995). Successfully retrieving microbial communities associated with these marine animals can be of paramount importance for biotechnological (Leal et al., 2013) and/or ecological (Meron et al., 2013) purposes. The extraction method can affect the diversity of microorganisms retrieved from sea anemones (Simister et al., 2011). Nonetheless, as the extraction method employed in the present study displays a good compromise between the quantity and quality of extracted DNA, processing costs and processing time per sample, we recommend researchers to use our methodology. In the future, the use of standardized procedures for processing and preserving collected samples of sea anemones will allow researchers to perform reliable comparisons by ensuring homogeneity between studies. Moreover, it also makes possible the use of less expensive approaches (e.g. DGGE) to compare shifts in the relative abundance of the microbiome associated with these marine invertebrates.

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Author Contributions

Conceived and designed the experiments: JR, NCMG, RC. Performed the experiments: JR. Analyzed the data: JR, NCMG, RC. Contributed reagents/materials/analysis tools: RC. Wrote the paper: JR, FJRCC, LP, NCMG, RC.

Additional Information

The authors declare no competing financial interests.

CHAPTER 3

Bacterial communities associated with snakelocks anemone *Anemonia viridis* - natural variability and its relevance for experimental studies

3.1. Introduction

Marine ecosystems harbor a substantial fraction of the Earth's biodiversity and comprise invertebrate animals known to host a considerable abundance and diversity microorganisms (e.g., Olson & Kellogg, 2010, Shnit-Orland & Kushmaro, 2009, Sweet et al., 2011). In fact, the microbiome of certain marine invertebrates may represent a remarkable proportion of the holobiont biomass (Di Camillo et al., 2012).

Marine organisms are known to have specific associations with a range of microorganisms (Thakur & Muller, 2005); however little is known about the microbial affiliation and diversity associated with such marine organisms (Menezes et al., 2010). Their mode of association (mutualism, commensalism or parasitism) and specific physiological functions are often unidentified (Schuett & Doepke, 2010). Nonetheless, at present, our knowledge on this topic goes little beyond the recognition that these complex microbial communities are extremely important components for functionally healthy ecosystems (Ainsworth et al., 2010, Menezes et al., 2010).

Sea anemones (phylum Cnidaria) can be found globally in a multitude of environmental conditions and all exhibit mutual relationships with numerous microorganisms. These invertebrate animals possess endobiotic bacteria harbored within the column, tentacles and the mucus surface layer (Schuett & Doepke, 2010). Microbial diversity surveys in sea anemones indicate a wide variety of heterotrophic bacteria (Du et al., 2010), with a high percentage probably being host-specific species or groups (Schuett & Doepke, 2010).

Sea anemones, specifically those hosting endosymbiotic photosynthetic dinoflagellates, are recognized to be important sentinel species (Winston & Heffernan, 1999). These organisms may help researchers to monitor potential environmental shifts in temperate coastal waters triggered by global climate changes.

3.2. Characterization of *Anemonia viridis* (Forskål, 1775)

The use of snakelocks anemone *Anemonia viridis* (Forskål, 1775) as a sentinel species is documented (Leutenegger et al., 2007). With the growing awareness of its importance there is still a gap in knowledge concerning this species as a holobiont, namely its bacterial communities. This chapter in particular aims to answer questions that may be raised when designing an experimental procedure with this species.

Snakelocks anemones, *A. viridis*, comprises two morphotypes: one with green coloration due to the photosynthetic zooxanthellae algae growing in the tentacle tissues, sometimes with the tentacle tips purple; and other with creamy-brown coloration. These animals have

some of the longest tentacles of all sea anemones. With over 200 sticky tentacles, the snakelocks anemone grows to 20 cm across and eight cm tall. The tentacles are lined with venomous stinging cells called cnidocyte.

A. viridis, is found in temperate shallow water throughout the Mediterranean Sea and north along Portugal, Spain, and France to the southern and western coasts of Great Britain. It may also occur along the African coast south of the Straits of Gibraltar. They commonly occur attached to a hard bottom in shallow waters and/or intertidal pools. Dispite, these anemones have already been found at 20 m depth, they rarely occur below 10-12 meters (Eaker, 2003). By commonly occurring above 12 m depth, this species avoids interspecific conflicts with other invertebrates and position them closer to sunlight that provides energy for their endosymbiotic microalgae (e.g., the zooxanthellae).

3.3. Similarity of bacterial populations associated with the whole body of snakelocks anemone *Anemonia viridis* and solely its tentacles

Most of the studies conducted to determine bacteria in sea anemones use only one part of the animals, the tentacles (Meron et al., 2013, Schuett et al., 2007). However, this approach may not be the most suitable to determine the composition of the bacterial community of snakelocks anemones. To ascertain if this theory is accurate we compared the bacterial communities present in the entire animal (body and tentacles) *vs* its tentacles; we formulated the following null hypothesis: there are no differences between composition of the bacterial community from whole *A. viridis* and its tentacles.

3.3.1. Methods

Sample collection, processing and extraction of nucleic acid

Five snakelocks anemones *A. viridis* and 1 L of seawater were collected at low tide, in the intertidal region of Praia da Aguda (41°02'51.06''N; 8°39'14.20''W), Arcozelo, Portugal, in May 2011 and individually stocked in sterile plastic bags for immediate transportation to the laboratory.

Half of the tentacles, for each sea anemone, were excised using sterile scalpels blades and processed separately of the remaining tentacles and body. Samples were homogenized using a mechanical tissue homogenizer (Omni Tissue Homogenizer (Omni International, Kennesaw, Georgia, USA)) followed by FastPrep® (Qbiogene Inc., USA) bead-beating system in combination with a mixture of beads (0.10 g Zirconia beads (0.1

mm) + 0.20 g glass beads (0.25 – 0.5 mm) + 0.20 g glass beads (0.75 – 1.0 mm) + 2 glass beads (2.85 – 3.45 mm)) (ROTH, DE) and Buffer SLX Mlus from E.Z.N.A.™ Soil DNA Kit (Omega Bio-Tek Inc., USA). Extraction was performed according to the instructions provided by the manufacturer.

The seawater sample was processed using E.Z.N.A.® Water DNA Kit (Omega Bio-Tek Inc., USA) and extraction was performed according to the instructions provided by the manufacturer.

Bacterial community diversity

NESTED PCR was used for a more efficient amplification of 16S rRNA gene fragments of bacterial genomic DNA extracted from sea anemone. In the first PCR, the universal bacterial primers F-27 and R-1492 were used to amplify c. 1500 bp of the 16S rRNA gene (Heuer et al., 1997, Weisburg et al., 1991). The PCR reaction mixtures (25 µL) consisted of DNA template (1 µL), DreamTaq PCR Master Mix (2X) (12.5 µL) (Fermentas, Thermo Fisher Scientific Inc., USA), bovine serum albumin (BSA, 2.0 mg/mL) and forward (0.1 µM) and reverse primers (0.1 µM). After 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94 °C, 45 s at 56 °C and 1.3 min at 72 °C, the PCR was finished by an extension step at 72 °C for 10 min. The amplicons obtained from the first PCR were then used as templates for a second PCR with bacterial DGGE primers F984-GC and R1378 (c. 473 bp) (Heuer et al., 1997). For these PCR reactions it was used the same quantity of DNA template, DreamTaq PCR Master Mix (2X), forward and reverse primers and 4% acetamide. Cycling conditions were of 4 min at 94 °C for denaturation, 25 thermal cycles of 1 min at 95 °C, 1 min at 53 °C and 2 min at 72 °C, with an extension step at 72 °C for 7 min to finish the PCR reactions. All PCR amplification products were examined by electrophoresis on 1.0% agarose gels containing GelRed (Biotium Inc., USA). Bacterial fingerprints were yielded by performing a DGGE based on DNA (16S rRNA gene) and used as a proxy to evaluate the bacterial community retrieved from sea anemones. DGGE analysis was carried out on a DCode universal mutation detection system (Bio-Rad). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel (40-58% of denaturants) containing 6–10% acrylamide. The run was performed in 1x Tris-acetate–EDTA buffer at 58 °C at a constant voltage of 160 V for 16 h. DGGE gels were silver-stained according to (Riesner et al., 1989). The solutions used were 10% (vol/vol) ethanol plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared developing solution containing 0.01% (wt/vol) sodium borohydride, 0.15% formaldehyde, 1.5% (wt/vol) NaOH, and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were documented with a Molecular Imager chemiDoc XRS+ digitalize system (Bio-Rad).

Statistical Analysis

Bacterial fingerprints were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent relative similarities by comparing the variation in species abundance and composition among treatments. Shannon's index of diversity (H'), was determined as $H' = - \sum pi \ln pi$, where pi is often the proportion of individuals belonging to the each species in the dataset of interest.

3.3.2. Results

Five replicates were processed for PCR-DGGE analysis fitting as entire animal and tentacles (Figure 3.1). Seawater from the same location was also processed. ANOSIM from the PCR-DGGE profiles showed there are significant differences between the compositions of bacterial communities retrieved from the entire animal and its tentacles ($R = 0.024$, $P = 0.031$). Shannon-Weaver diversity index shows that diversity is higher using the entire animal ($H'_{entire\ animal} = 1.5 \pm 0.51$; $H'_{tentacles} = 1.17 \pm 0.52$).

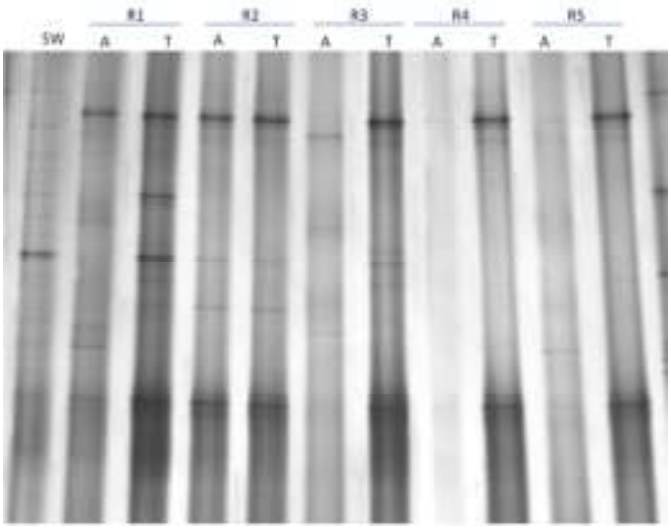

Figure 3.1. PCR-DGGE fingerprints of the five replicates. Numbers represent the replicate (R), A represent the entire animal (body and tentacles), T represents tentacles and SW represents the seawater sample.

3.3.3. Discussion

The use of only tentacles in studies may prevent the havoc of certain habitats (e.g. by preventing massive harvesting and habitat destruction) since sea anemones have the ability to regenerate damaged or lost parts of their bodies (Holstein et al., 2003). However, and according to our results, there are significant differences between the entire animal and its tentacles when performing studies addressing the diversity of bacterial communities composition. We therefore recommend the used of the whole animal as some bacteria or bacterial groups may be excluded when using only tentacles.

3.4. Similarity of bacterial populations associated with the two morphotypes of the snakelocks anemone *Anemonia viridis*

There are two well-known morphotypes of *A. viridis* that have been previously named as *A. sulcata* var. *smaragdina* and *A. rustica* being nowadays accepted as synonyms of *A. viridis* (Caparkaya et al., 2010). The color differences among these morphotypes are mainly due to the presence or absence of green fluorescent proteins (GFP) and pink pigments. The morphotype displaying these compounds exhibits green tentacles with pink tips, while the other morphotype shows a pale creamy-brown color (Caparkaya et al., 2010). Both morphotypes host an endosymbiotic microalgae commonly known as zooxanthellae. Apart from its photosynthetic symbiont, both morphotypes exhibit a mutual relationship with numerous microorganisms. Being the same species, it is important to determine if the bacterial communities composition associating with the two morphotypes are similar and thus is indifferent to use either one of these morphotypes to study the microbial biota of *A. viridis*. The null hypothesis formulated is as follows: there are no differences in the bacterial communities associated with the green and brown morphotypes of the snakelocks anemones *A. viridis.*

3.4.1. Methods

Sample collection, processing and extraction of nucleic acid

Five snakelocks anemones from each of the two morphotypes (green and brown) of *A. viridis* were collected at low tide, in the intertidal region of Praia da Aguda (41°02'51.06"N; 8°39'14.20"W), Arcozelo, Portugal, in November 2011. After stocked individually in sterile plastic bags, the animals were transported immediately to the laboratory for processing

and extraction of nucleic acid. The procedure was directed as described in section "Sample collection, processing and extraction of nucleic acid" from 3.3.1. Methods.

Bacterial community diversity

Bacterial community composition was evaluated according to described in section "Bacterial community diversity" from 3.3.1. Methods.

Statistical Analysis

Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). Shannon's index of diversity (H'), was determined as $H' = - \sum pi \ln pi$, where pi is often the proportion of individuals belonging to the each species in the dataset of interest. The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent relative similarities by comparing the variation in species abundance and composition among treatments.

3.4.2. Results

Five replicates of each morphotype were processed for PCR-DGGE analysis. Shannon-Weaver diversity index shows that diversity is similar between the two morphotypes of the snakelocks anemone *A. viridis* ($H'_{brown\ morph.}$ = 2.09 ± 0.15; $H'_{tgreen\ morph.}$ = 1.95 ± 0.32), and two main bands are characteristic of the two morphotypes (Figure 3.2). ANOSIM from the PCR-DGGE profiles showed there are significant differences between the bacterial communities composition retrieved from the two morphotypes (R = 0.252, P = 0.008).

3.4.3. Discussion

In this study the null hypothesis was rejected, as differences were recorded between the compositions of the bacterial community displayed by the two morphotypes of the snakelocks anemones *A. viridis*; nonetheless this result appears to be due to natural variability. Also, the bacterial diversity recorded was similar.

To choose between the green and the brown morphotype, when analysing the composition of the bacterial communities retrieved from *A. viridis,* other questions (such

as morphotype abundance in the sampling site) must be considered (e.g., to avoid unnecessary impacts associated with the collection of specimens for research studies).

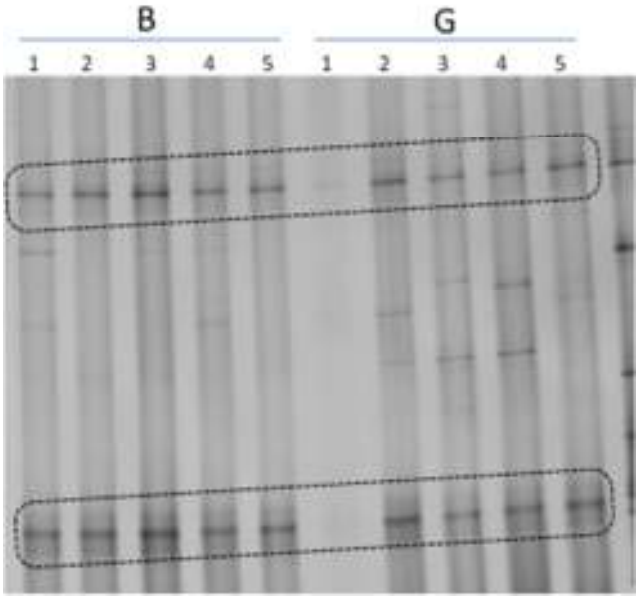

Figure 3.2. PCR-DGGE fingerprints for the two morphotypes of *A. viridis*. Numbers represent the replicate (R), B represent the brown morphotype of *A. viridis* and G represents the green morphotype.

3.5. Micro-spatial variation analysis of bacterial populations associated with the snakelocks anemone *Anemonia viridis*

Sea anemones can be found globally and in a multitude of depths and environmental conditions. With the increasing search for new sources of natural products (Leal et al., 2012, Rocha et al., 2011), it is vital to fully understand how these microorganisms vary within the organisms, not only at a macro scale but also at a micro scale and between geographical locations. In this study the bacterial community composition of *A. viridis* was analyzed between tide pools from the same geographic site and the following null hypothesis was formulated: there are no differences between the compositions of bacterial communities retrieved between tide pools.

3.5.1. Methods

Sample collection, processing and extraction of nucleic acid

Snakelocks anemones of *A. viridis* from four different tide pools (n = 5 *per* tide pool) locate in the same geographical site were collected at low tide, in the intertidal region of Praia da Aguda (41°02'51.06"N; 8°39'14.20"W), Arcozelo, Portugal, in November 2011 and individually stocked in sterile plastic bags for immediate transportation to the laboratory.

Processing and extraction of nucleic acid of the twenty animals, was conducted as described in section “Sample collection, processing and extraction of nucleic acid” from 3.3.1. Methods.

Bacterial community diversity

Bacterial community composition was evaluated according to described in section “Bacterial community diversity” from 3.3.1. Methods.

Statistical Analysis

Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). Total species (S), Pielou’s evenness index (J’) and Shannon’s index of diversity (H’) were determined. The raw data matrix was log (x +1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent relative similarities between tide pools.

3.5.2. Results

Five replicates from each tide pool were processed for PCR-DGGE analysis. Table 3.1 summarizes the results obtained for total species (S), Pielou’s evenness (J’) and Shannon-Weaver diversity indexes. Shannon-Weaver diversity index shows that diversity is similar between the four tide pools and Pielou’s evenness index indicates that community samples are quite even in each tide pool. Global R from ANOSIM (R = 0.194, P = 0.023) showed there are significant differences between the compositions of bacterial communities retrieved between tide pools.

3.5.3. Discussion

Although results do not support the null hypothesis postulated and indicate there are differences in bacterial community composition of *A. viridis* between tide pools from the same geographic site, they are due to natural variability. Snakelocks anemones from each tide pool may have been originated by asexual reproduction, characteristic of this species, and thus transfer its communities to the next generation of sea anemones allowing

continuity and possible expansion of the populations. This fact can be of extreme importance when considering bacteria as the true producers of bioactive compounds (Shnit-Orland & Kushmaro, 2009).

Table 3.1. Bacterial community analysis from PCR-DGGE fingerprints of *A. viridis* from four tide pools.

Tide Pool	S	J'	H'	ANOSIM	
				R-value	P-value
A	7,8	0,86 ± 0,06	1,94 ±0,21		
B	7,6	0,84 ± 0,09	1,65 ± 0,28		
C	8,2	0,85 ± 0,06	1,72 ± 0,44		
D	8,0	0,88 ± 0,02	1,78 ± 0,29		
A,B				0,188	0,0103
A,C				0,124	0,0143
A,D				0,36	0,0024
B,C				-0,076	0,0722
B,D				0,284	0,0056
C,D				0,18	0,0143

3.6. Seasonal variation of bacterial populations associated with the snakelocks anemone *Anemonia viridis*

Determining and understanding temporal stability (namely between seasons) is as important as to fully comprehend how microorganisms vary within the organisms and between geographical locations. In this study the composition of the bacterial community of *A. viridis* was analyzed in two different seasons: spring *vs* fall. The null hypothesis formulated is as fallows: there are no differences between bacterial communities composition retrieved from the two seasons.

3.6.1. Methods

Sample collection, processing and extraction of nucleic acid

Four snakelocks anemones of *A. viridis* were collected at low tide, in the intertidal region of Praia da Aguda (41°02'51.06"N; 8°39'14.20"W), Arcozelo, Portugal, in the spring and

fall of 2011 (n = 4 *per* season). Samples were individually stocked in sterile plastic bags for immediate transportation to the laboratory.

Processing and extraction of nucleic acid of the eight animals, was conducted as described in section “Sample collection, processing and extraction of nucleic acid” from 3.3.1. Methods.

Bacterial community diversity

Bacterial community composition was evaluated according to described in section “Bacterial community diversity” from 3.3.1. Methods.

Statistical Analysis

Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). Shannon’s index of diversity (H’), was determined as $H' = - \sum pi \ln pi$, where pi is often the proportion of individuals belonging to the each species in the dataset of interest. The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent relative similarities by comparing the variation in species abundance and composition among treatments. A dendogram of PCR-DGGE fingerprints (Bray-Curtis Similarity) was constructed.

3.6.2. Results

Four replicates from season (spring and fall) were processed for PCR-DGGE analysis. Shannon-Weaver diversity index shows that diversity of the bacterial communities in the snakelocks anemone *A. viridis* is higher in the spring (H'_{spring} = 2.09 ± 0.10; H'_{fall} = 1.74 ± 0.18). ANOSIM from the PCR-DGGE profiles showed that there are significant differences between bacterial communities composition retrieved from the two seasons (R = 0.896, P = 0.029). The dendogram of PCR-DGGE fingerprints two clusters, one for each season (Figure 3.3)

3.6.3. Discussion

Studies in the past decade concluded that bacterial communities composition associated with marine organisms were spatially and seasonally stable (Anderson et al., 2010, Hentschel et al., 2002, Rohwer et al., 2002). However, our results reveal that changes in the composition of bacterial communities occur during seasons, even in those where differences in environmental parameters are less extreme, (e.g. spring and fall, in opposition to summer and winter). The results obtained in this study are in turn, in accordance with several studies that demonstrate shifts in bacterial communities when extreme events or changes in physical factors (e.g., temperature and radiation) occur (Higuchi et al., 2013, Ravindran et al., 2013, Simmons et al., 2008).

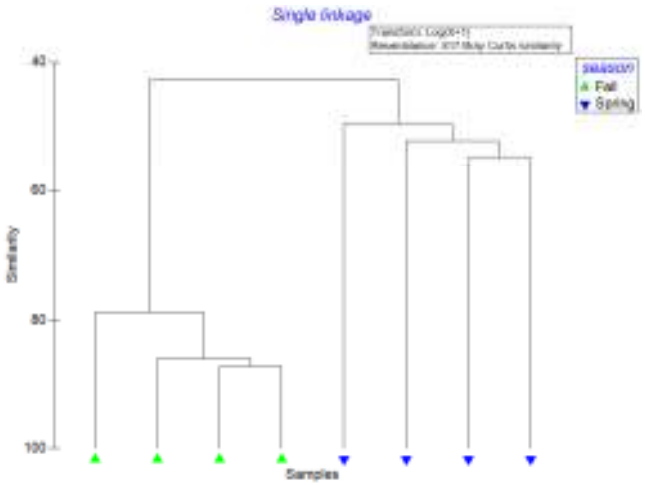

Figure 3.3. Dendogram of PCR-DGGE fingerprints (Bray-Curtis Similarity) showing samples from the two seasons tested.

3.7. Conclusions

The four studies presented in this chapter were performed considering there are almost no reports regarding bacterial communities in snakelocks anemones *A. viridis* (Meron et al., 2013). Although bacterial symbionts communities of *A. viridis* were not determined, these new data brings new insights on how future studies and experiences should be conducted to better understand the bacterial communities composition and their role with the host symbiont and the environment.

Thereby in future studies samples should include tentacles and body of the sea anemone. Bacterial communities are species specific (morphotype independent) and stable enough

to be transferred to the next generations of sea anemones. Moreover, environmental parameters affect the bacterial communities populations and so the interactions between host-symbionts and the production of bioactive compounds.

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CHAPTER 4

Use of an experimental life support system to predict the effects of temperature and depth in the bacterial communities associated with *Anemonia viridis*

4.1. Introduction

Extreme environmental events, such those associated to global climate change, are often associated to the disruption of symbiotic associations of cnidarians with a range of micro and macroorganisms. Cnidarian–dinoflagellate endosymbiosis are common in the marine environment and have a key role in marine biodiversity: they form both the trophic and structural foundation of coral reefs ecosystems and are also a key component in some temperate communities (Muller-Parker & Davy, 2001). Nonetheless, these symbioses appear to be delicately balanced on the edge of dysfunction and surprisingly intolerant to stresses as high light and ultraviolet light radiation (Moya et al., 2012), increased temperature (Brown, 1997, Glynn, 1991), pathogen infection, pollution and changes in salinity (Ainsworth & Hoegh-Guldberg, 2008, Brown, 2000). Alone or combined, these factors seem to promote symbiosis dysfunction and breakdown and, ultimately, lead to the loss of zooxanthellae or so-called bleaching (Hoegh-Guldberg, 1999). In corals, ultraviolet radiation (UVR) has been shown to be an agent leading to bleaching, either directly (Gleason & Wellington, 1993) or in synergy with temperature (Lesser, 2010, Lesser & Shick, 1989), and thus contributing to the worldwide degradation of coral reef ecosystems. In sea anemones, ultraviolet radiation exposure alone holds little impact and induces limited transcriptomic response. On the other hand thermal stress combined or not with UVR, induces symbiosis disruption and bleaching (Moya et al., 2012). In this case the transcriptomic response to thermal stress is immediate, transient and potentiated by ultraviolet radiation in the sea anemone.

Considering the above, sea anemones (namely those hosting endosymbiotic photosynthetic dinoflagellates) are recognized to be important sentinel species (Winston & Heffernan, 1999). These organisms can be used by researchers to monitor potential environmental shifts in temperate coastal waters triggered by global climate changes. Extreme bleaching events of *Anemonia* in the Mediterranean under abnormally warm water conditions (Leutenegger et al., 2007) are a good example on the suitability of these anthozoans as sentinel species. Additionally, these anemones should also be considered as holobionts (Margulis & Fester, 1991), due to its complex symbiosis between the cnidarian animal, its photosynthetic microalgae (e.g., zooxanthellae) and its intricate community of associated microorganisms that play a key role on the overall health, defense and nutrition of the cnidarian host (Paul & Puglisi, 2004). Alongside, with the disruption monitoring of the photosynthetic simbiont or bleaching effect, it is important to monitor potential shifts in the microorganisms associated with these sea anemones to understand how environmental disturbances may shape anemone individuals and/or populations.

Radiation and temperature are by far the stress factors most studied. However there is a lack of knowledge on how the symbionts (photosynthetic microalgae and microbial communities) of the cnidarian host behave with the expected increase of seawater temperature. Considering the inverse exponential relationship between depth and UV irradiance, we designed an experiment where two temperatures (T) and two UVR-PAR radiations (R) were combined (thus being used as proxies for depth) to enabled four treatments to be tested: R at 100% (as proxy of a shallow depth) and T at 15 ºC (R100T15, standard conditions in the wild), R at 50% (as proxy for deeper waters where *A. viridis* can still occur) and T at 15 ºC (R50T15, radiation as the stress factor), R at 100% and T at 28 ºC (R100T28, temperature as the stress factor), and R at 50% and T at 28 ºC (R50T28, radiation and temperature as the stress factors). The temperature stress factor (T at 28 ºC) was elected as this abnormal temperature can occur in tide pools in days where the solar radiation is high, and/or great breadth tides are observed. This stress is less probable to occur in depth, hence the use of 50% of radiation, but there are still records that report this abnormal conditions (e.g. bleaching events in the Mediterranean Sea (Leutenegger et al., 2007)).

4.2. Methods

Sample collection

Thirty-two snakelocks anemones of the brown morphotype of *Anemonia viridis* (Forskål, 1775) were collected at low tide, in the intertidal region of Praia da Aguda (41º02'51.06''N; 8º39'14.20''W), Arcozelo, Portugal, in January 2012. Samples were stocked in sterile plastic bags and transported immediately to the laboratory.

Experimental life support system and experimental design

To evaluate the dynamics of the bacterial communities associated with snakelocks anemones in normal conditions against extreme events, we used an experimental life support system (ELSS, Figure 4.1) (Coelho et al., 2013), capable of simulate different radiation (R, UVR (50 and 100% of the total fluorescent tube intensity) + PAR, according to Coelho et al. (2013)) and temperatures (T, 15 ± 1.5 and 28 ± 1 ºC). The ELSS is divided into two frames of 16 microcosms (32 in total) (glass tanks 25 cm high, 28 cm length and 12.4 cm width, each with a maximum functional water volume of approximately 3 L), which enabled the use of four treatments simultaneously: R at 100% and T at 15 ºC (R100T15, normal condition), R at 50% and T at 15 ºC (R50T15), R at 100% and T at 28 ºC (R100T28) and R at 50% and T at 28 ºC (R50T28). Each treatment comprised eight

independent replicates containing four snakelocks anemones. All replicates were arranged in a randomized split-plot design. To mimic photoperiod and light conditions at Portuguese latitudes a 14 h diurnal light cycle was simulated and one tidal cycle per day was programed to renew half of the synthetic saltwater present in the microcosms. Salinity was adjusted to simulate the conditions recorded at the sampling location (34 ppm ± 1.5) and pH was kept among 7.6 - 8.2.

Snakelocks anemones were acclimated to the experimental control setting for 5 days, before being subjected to the stress conditions for 10 days. During all the time the animals were not feed.

Figure 4.1. Lateral front view of the experimental life support system (image by Joana Rocha).

Processing and extraction of nucleic acid

Nucleic acids were extracted from 0.5 g of sea anemone samples from each experimental treatment described above. All samples were homogenized using a mechanical tissue homogenizer (Omni Tissue Homogenizer (Omni International, Kennesaw, Georgia, USA)) followed by FastPrep® (Qbiogene Inc., USA) bead-beating system in combination with a mixture of beads (0.10 g Zirconia beads (0.1 mm) + 0.20 g glass beads (0.25 – 0.5 mm) + 0.20 g glass beads (0.75 – 1.0 mm) + 2 glass beads (2.85 – 3.45 mm)) (ROTH, DE) and Buffer SLX Mlus from E.Z.N.A.™ Soil DNA Kit (Omega Bio-Tek Inc., USA). Extraction was performed according to the instructions provided by the manufacturer.

Bacterial community diversity

Bacterial community composition was evaluated by performing a DGGE based on DNA (16S rRNA gene). DGGE fingerprinting was used prior to pyrosequencing, to compare bacterial community compositions among samples, and later, the data was complemented

with a more-in-depth barcoded pyrosequencing analysis of composite samples. NESTED PCR was used for a more efficient amplification of 16S rRNA gene fragments of bacterial genomic DNA extracted from sea anemone. In the first PCR, the universal bacterial primers F-27 (Weisburg et al., 1991) and R-1492 (Heuer et al., 1997) were used to amplify c. 1500 bp of the 16S rRNA gene. The PCR reaction mixtures (25 µL) consisted of 1 µL of DNA template, 12.5 µL DreamTaq PCR Master Mix (2X) (Fermentas, Thermo Fisher Scientific Inc., USA), 2.0 mg/mL bovine serum albumin (BSA) and 0.1 µM of forward and reverse primers. After 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94 °C, 45 s at 56 °C and 1.3 min at 72 °C, the PCR was finished by an extension step at 72 °C for 10 min. The amplicons obtained from the first PCR were then used as templates for a second PCR with bacterial DGGE primers F984-GC (c. 473 bp) (Heuer et al., 1997). For these PCR reactions it was used the same quantity of DNA template, DreamTaq PCR Master Mix (2X), forward and reverse primers and 4% acetamide. Cycling conditions were of 4 min at 94 °C for denaturation, 25 thermal cycles of 1 min at 95 °C, 1 min at 53 °C and 2 min at 72 °C, with an extension step at 72 °C for 7 min to finish the PCR reactions. All PCR amplification products were examined by electrophoresis on 1.0% agarose gels containing GelRed (Biotium Inc., USA). DGGE analysis was carried out on a DCode universal mutation detection system (Bio-Rad). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel (40-58% of denaturants) containing 6–10% acrylamide. The run was performed in 1x Tris-acetate–EDTA buffer at 58 °C at a constant voltage of 160 V for 16 h. DGGE gels were silver-stained according to (Riesner et al., 1989). The solutions used were 10% (vol/vol) ethanol plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared developing solution containing 0.01% (wt/vol) sodium borohydride, 0.15% formaldehyde, 1.5% (wt/vol) NaOH, and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were documented with a Molecular Imager chemiDoc XRS+ digitalize system (Bio-Rad).

Statistical Analysis

Bacterial fingerprints of each denaturing gradient gel were normalized using the GelCompar 4.0 software (Applied Maths, Belgium), as described by Smalla et al. (2001). The raw data matrix was log (x + 1) transformed prior to the statistical analysis in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson et al., 2008). A similarity/difference matrix was latter constructed using the Euclidean distance. A one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent relative similarities by comparing the variation in species abundance and composition among treatments. A Principal Coordinates Analysis (PCO) was performed representing differences among experimental

treatments along the first two axes. This multivariate statistical test was performed using Primer 6.1 with PERMANOVA add-on (Primer-E Ltd, UK).

Pyrosequencing and Sequence analysis

A barcoded pyrosequencing approach was used for the compositional analyses of bacterial communities (one composite sample per treatment). Fragments of the 16S ribosomal RNA (rRNA) gene were sequenced for each sample with primers V3 Forward (5′-ACTCCTACGGGAGGCAG-3′) and V4 Reverse (5′-TACNVRRGTHTCTAATYC-3′) using the 454 Genome Sequencer FLX Titanium (Life Sciences Roche Diagnostics Ltd, UK).

The barcoded pyrosequencing libraries were analysed using the Quantitative Insights Into Microbial Ecology (QIIME, Caporaso et al., 2010) software package (http://www.qiime.org/, last accessed 2012-11-19) on a computer running the BioLinux operating system (http://nebc.nerc.ac.uk/tools/bio-linux/bio-linux-6.0, last accessed 2012-11-19). In QIIME, fasta and qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set below 300 bp after removal of forward primers and barcodes, backward primers were removed using the 'truncate only' argument, and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. In addition to user-defined cut-offs, the split_libraries.py script performs several quality-filtering steps (http:// qiime.org/scripts/split_libraries.html). OTUs were selected using the recently developed UPARSE clustering method and chimera check (Edgar, 2013) and the most recent Greengenes database (http://greengenes.secondgenome.com/downloads/database/13_5) as reference database. In the present study, we used *de novo* checking and reference-based chimera checking using a reference fasta file ('99_otus.fasta') from the Greengenes 13_5 release. Representative sequences were selected using the pick_rep_set.py script in QIIME using the 'most_abundant' method. Reference sequences of OTUs were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the rdp method (Wang et al., 2007). In the assign_taxonomy.py function, we used a fasta file containing reference sequences from the Greengenes 13_5 release as training sequences for the rdp classifier. We used a modified version of the taxonomy file supplied with the Greengenes 13_5 release to map sequences onto the assigned taxonomy. Finally, we used the make_otu_ table.py script in QIIME to generate a square matrix of OTUs by samples.

Identification of closely related organisms and phylogeny of abundant OTUs

Closely related organisms of numerically abundant OTUs (≥100 sequences) were identified using the NCBI BLAST command line "blastn" tool with the − db argument set to nt (Zhang et al., 2000). BLAST was also used to obtain sequences of bacteria, which were included in a bootstrap consensus phylogenetic tree based on 1,000 replicate trees along with representative sequences belonging to most abundant OTUs. The tree was built with the Mega5 Program (http://www.megasoftware. net/; last checked 2014-07-08) (Tamura et al., 2013) using the neighbor-joining algorithm with Jukes-Cantor corrections (Pires et al., 2012).

Metagenome Analysis

In the present study, we use PICRUSt (Langille et al., 2013) to predict the metagenome of each sample. PICRUSt is a bioinformatics tool that uses marker genes, in this case 16S rRNA, to predict metagenome gene functional content. These predictions are precalculated for genes in databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) and Clusters of Orthologous Groups of proteins (COG). In the present study, we used the KEGG database. Output of PICRUSt consists of a table of functional counts, i.e., KEGG Pathway counts by sample. Note that because of functional overlap, some orthologs can be represented in multiple pathways. Since KOs can belong to several pathways, we used the categorize_by_function.py script in PICRUSt to collapse the PICRUSt predictions at the level of the individual pathways.

4.3. Results

In the end of the experiment only 50% and 75% of the animals survived for treatments R50T28 and R100T28, respectively. No mortality was recorded for both treatments at 15 ºC.

ANOSIM from the DGGE profiles showed there are significant differences between microbial communities retrieved from the treatments ($R_{R50T15,R100T15}$ = 0.197, P = 0.027; $R_{R100T28,R50T15}$ = 0,432, P = 0.001; $R_{R50T28,R100T28}$ = 0.528, P = 0.001; $R_{R50T28,R50T15}$ = 0.670, P = 0.002; $R_{R50T28,R50T28}$ = 0.663, P = 0.001; $R_{R100T28,R100T15}$ = 0.639, P = 0.001).

The first two axes of the PCO explained 19,8% of the variation in the data set (Figure 4.2); samples from treatment R50T28 (with the exception of one sample) are clearly clustered apart from the others. Samples of treatments at 15 ºC also cluster together.

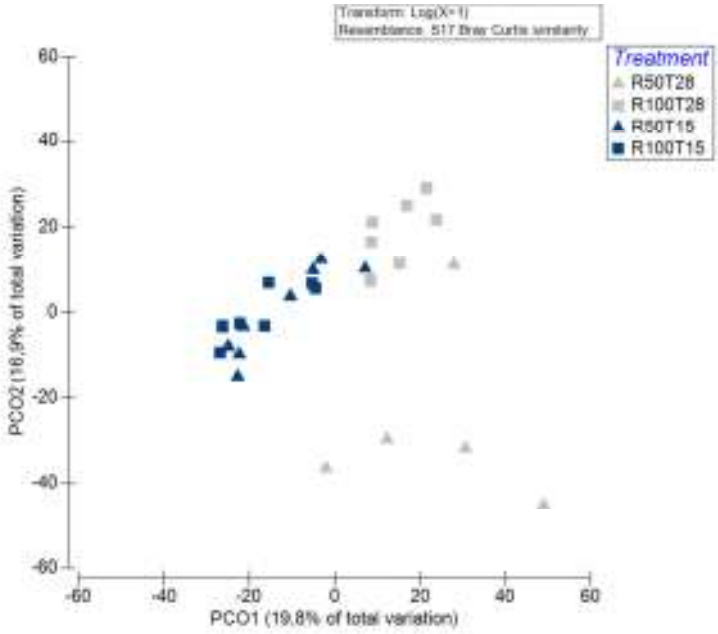

Figure 4.2. Ordination showing the first two axes of the PCO for the four treatments tested: R50T28, R100T28, R50T15, R100T15.

The sequencing effort yielded 38,383 sequences reads (R50T28 = 13,925; R100T28 = 10,656; R50T15 = 9,561 and R100T15 = 4,241), which were assigned to 370 OTUs after quality control, OTU picking, and removal of chimera. Bacterial OTUs were assigned to thirteen phyla, Actinobacteria, Bacteroidetes, Cyanobacteria, Fibrobacteres, Firmicutes, Gemmatimonadetes, GN02, Lentisphaerae, Proteobacteria, Spirochaetes, Tenericutes, Verrucomicrobia and WWE1. Twenty OTUs remained unclassified at the phylum level. In addition to this, OTUs were assigned to 22 classes, 48 orders, 66 families, 76 genera, and 20 species.

BLAST was used to find closely related organisms to all 35 most abundant (≥100 sequences) OTUs (Table 4.1, in the end of the chapter). The most abundant OTU overall was OTU-1, assigned to the phylum Proteobacteria, class Alphaproteobacteria, order Kiloniellales and represented by 5,939 sequence reads. This OTU was also the OTU dominant in R50T28 with 5,575 sequences and almost or non-existent in treatments with 100% radiation. OTU-2 is the second most abundant OTU and its presence is dominant in treatment R50T15 (40% of the most abundant sequence reads), being almost or non-existing in the other treatments. This performance is also observed for OTU-4, 7, 12 and 14 for the same treatment and for OTU-23 and 26 for treatment R100T28, with much less number of sequence reads. OTU-3, 6 and 46 are abundant in treatments with high temperature (R50T28 and R100T28).

There were also marked differences in the abundance of higher bacterial taxa among treatments (Figure 4.3). Proteobacteria is the most abundant phylum encompassing 60%, 88%, 95%, 87% of the relative abundance in R50T15, R100T15, R50T28 and R100T28

treatments, respectively. In this phylum, Alphaproteobacteria is the most abundant class, mainly in R50T28 treatment with 78% of relative abundance and 10 OTUs. In this class, order Kiloniellales goes from inexistent in normal conditions (R100T15) to dominant in extreme conditions (R50T28).

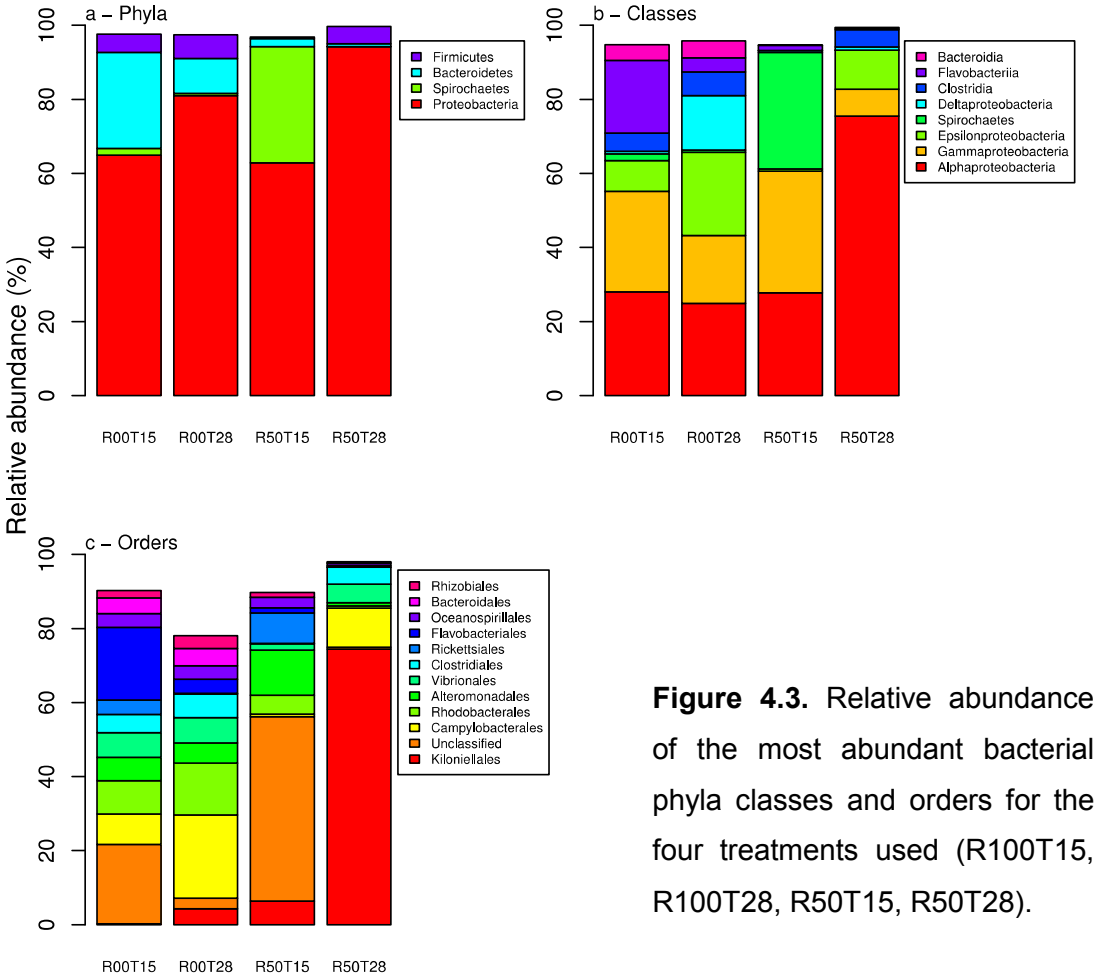

Figure 4.3. Relative abundance of the most abundant bacterial phyla classes and orders for the four treatments used (R100T15, R100T28, R50T15, R50T28).

Spirochaetes appears only in treatments at 15ºC. Class Clostridia (Firmicutes phylum) exhibit reduced relative abundance to almost non-existent in R50T15 (0.1%).

The phylogenetic tree (Figure 4.4) displays two main clusters where OTUs belonging to different classes and phyla are arranged. Each main cluster encompasses a smaller but distinct clusters consisting of OTUs from class Alphaproteobacteria, Epsilonproteobacteria or class Gammaproteobacteria.

Using PiCrust, four pathways were selected to be studied: nitrogen metabolism, terpenoid backbone biosynthesis, pyruvate metabolism and lipopolysaccharide biosynthesis (Figure 4.5). In the nitrogen metabolism the percentage of total genes is greater in treatments with

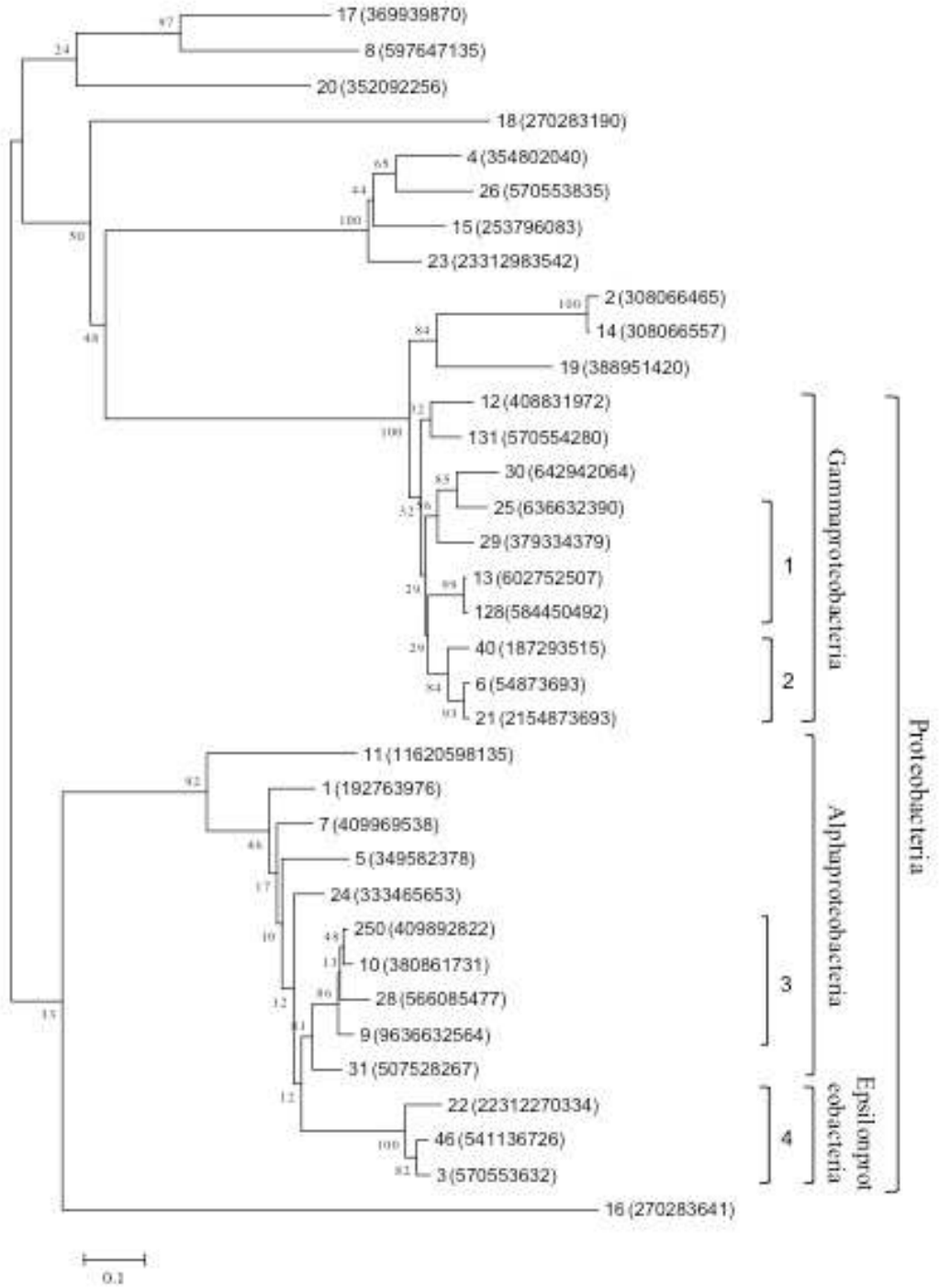

Figure 4.4. Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from the studied treatments; bootstrap values lower than 50 % were omitted. The number of each OTU is indicated with the GenBank GenInfo sequence identifiers of the bacterial sequences. Numbers 1, 2, 3 and 4 refer to orders Alteromonadales, Vibrionales, Rhodobacterales and Campylobacteriales, respectively.

higher temperature (T = 28ºC); being superior when this effect is combined with the reduction of radiation (R50T28). Terpenoid backbone biosynthesis and lipopolysaccharide

biosynthesis showed that the percentage of total genes is higher and equivalent at 100% of radiation. In pyruvate metabolism the percentage of genes is identical in the four treatments.

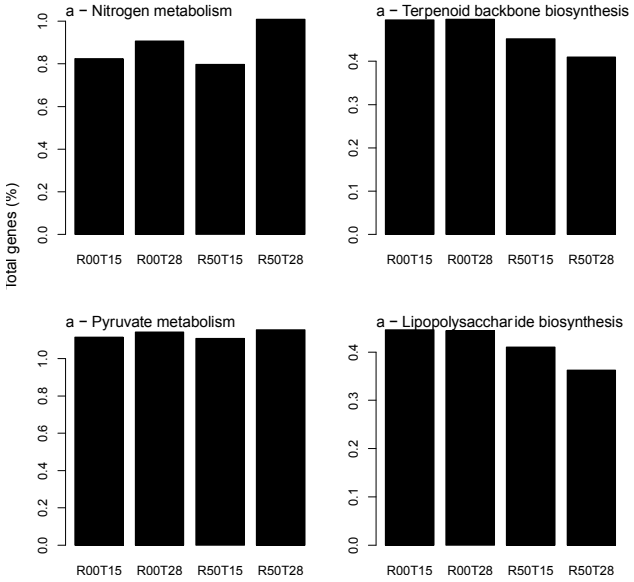

Figure 4.5. Percentage of total genes for four pathways selected to be studied in PiCrust: nitrogen metabolism, terpenoid backbone biosynthesis, pyruvate metabolism and lipopolysaccharide biosynthesis.

Within each pathway a Kegg ortholog was selected: glutamate synthase (NADPH/NADH) small chain (nitrogen metabolism), acetyl-CoA C-acetyltransferase (terpenoid backbone biosynthesis), 2-isopropylmalate synthase (pyruvate metabolism) and UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase (lipopolysaccharide biosynthesis). Figure 4.6 presents the distribution of the total genes (%) by selected orders. Glutamate synthase (NADPH/NADH) small chain and acetyl-CoA C-acetyltransferase have a higher number of total genes when compared to 2-isopropylmalate synthase and UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase. With the exception of percentage of total genes, the taxa profiles of the four treatments are very similar between the four pathways. For Kiloniellales the number of total genes increases with temperature and reduction of radiation. When the two factors are combined (R50T28) the increase of total genes is exacerbated. Contrary, the percentage of genes for Rhodobacterales, Rhizobiales, Flavobacteriales, Alteromonadales and other, decreases for the same treatment. Percentage of total genes for Alteromonadales is lower at high temperatures (T100T28 and R50T28). In treatment R100T28, acetyl-CoA C-acetyltransferase for the terpenoid backbone biosynthesis, presents higher percentage of total genes.

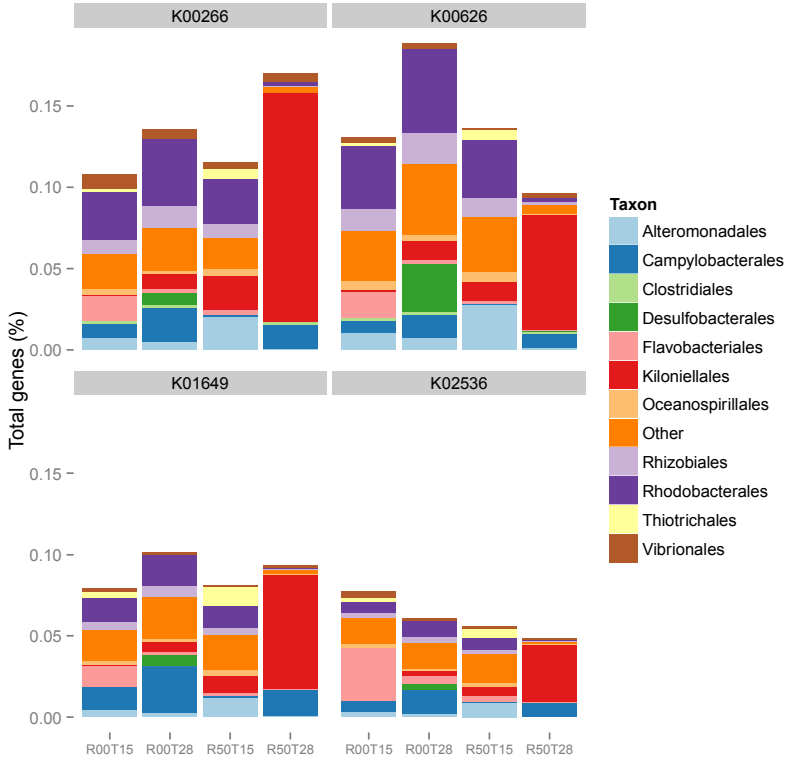

Figure 4.6. Distribution of total genes (%) by selected orders for the Kegg ortholog of each pathway. K00266 - Glutamate synthase (NADPH/NADH) small chain (nitrogen metabolism), K00626 - acetyl-CoA C-acetyltransferase (terpenoid backbone biosynthesis), K01649 - 2-isopropylmalate synthase (pyruvate metabolism), and K02536 - UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase (lipopolysaccharide biosynthesis).

4.4. Discussion

In this study it was observed that depth (simulated through a reduction in radiation) does not affect the bacterial populations existing in the snakelocks anemone *A. viridis* in temperate costal waters. This result is consistent with the knowledge that short wavelengths (ultraviolet) are absorbed rapidly and in 1 meter of depth of coastal water 63% of UVR is absorbed (http://www.britannica.com/EBchecked/topic/531121/seawater/301669/Optical-properties, accessed 08.08.2014). However, in treatments with increased temperatures (R50T28 and R100T28) differences in bacterial populations were observed, thus agreeing with previous publications (Leutenegger et al., 2007, Moya et al., 2012, Richier et al., 2008) referring to the effects of increasing seawater temperatures. Thermal stress combined (or not) with UVR has been reported to induce the disruption of

Symbiodinium - *A. viridis* symbiosis and the bleaching of the cnidarian host (Moya et al., 2012). Our data revealed that, when combining these two factors, they cause a reduction in zooxantellae (data not shown) although not enough to trigger a bleaching effect; but induce changes in the composition bacterial populations. In fact bacterial communities in R50T28 are clearly different from other treatments (Figure 4.2).

Pyrosequencing reveals that class Alphaproteobacteria growths significantly with the increase of temperature and depth. Pyrosequencing also revealed that the most abundant bacterial sequence in R50T28 belonged to *Kiloniella laminariae* gen. nov., sp. nov. (Wiese et al., 2009) first isolated from a marine macroalga. This alphaproteobacteria is a chemoheterotrophic aerobe with the potential for denitrification that only appears (≥100 sequences) in treatments where radiation was reduced to half. This information may indicate that, when changes in host-zooxantellae occur (for e.g. due to depth), bacterial populations can act to maintain the holobiont equilibrium.

Crossing this information with results obtained for important metabolic pathways in living organisms, it was observed an increase in gene expression of enzyme glutamate synthase (NADPH/NADH) small chain (Nitrogen metabolism), responsible for the synthesis of L-Glutame (non-essential amino acid and key compound in cellular metabolisms), in R50T28. Although higher at elevated temperatures, production of natural products, such as terpenes, sterols and sisqueterpenes (Terpenoid backbone biosynthesis and pyruvate metabolism) is favored at 100% of radiation; hence major production in tide pools and shallow coastal waters. Finally, environmental shifts reduces gene expression in Lipopolysaccharide biosynthesis (UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase).

4.5. Conclusions

Extreme environments affect not only the sea anemone–zooxanthellae symbiosis but also associated bacterial populations. Additionally, species-specific abundance of bacteria can change radically and may even promote the disappearance of some strains. Moreover, our study reveals that bacteria associated with *A. viridis* can indeed produce natural products (e.g., terpenes) and that this kind of biosynthesis is most active under full solar radiation and abnormally high temperatures. These observations will allow direct future studies on the production of bioactive compounds by theses bacteria.

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Table 4.1. List of most abundant OTUs (≥100 sequences) including OTU numbers, number of sequences (reads) for each treatment (R50T28, R50T15, R100T28 and R100T15) and the sum of all the reads (Total), their taxonomic affiliation, GenBank GenInfo sequence identifiers (GI) of closely related organisms identified using BLAST and sequence identity (Sq ident) of these organisms with our representative OTU sequences.

OTU	Reads					Domain	Phylum	Class	Order	Family	Genus	GI	Sq Ident
	Total	R50T28	R50T15	R00T28	R00T15								
1	5939	5575	360	4	0	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales			192763976	98.27
2	1594	0	1589	0	5	Bacteria	Spirochaetes	Spirochaetes				308066465	96.29
3	1240	277	20	886	57	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	570553632	100
46	865	505	7	341	12	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	541136726	100
6	573	345	0	226	2	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	54873693	99.77
4	502	1	495	0	6	Bacteria	Proteobacteria	Gammaproteobacteria				354802040	99.77
5	466	2	362	0	102	Bacteria	Proteobacteria	Alphaproteobacteria				349582378	87.93
7	422	0	408	0	14	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae		409969538	95.07
10	368	3	133	189	43	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		380861731	100
8	336	233	0	89	14	Bacteria	Firmicutes	Clostridia	Clostridiales	[Acidaminobacteraceae]	Fusibacter	507647136	99.61
9	330	7	63	249	11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Loktanella	636632564	100
11	307	36	5	266	0	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae	Thalassospira	620598135	99.51
13	298	21	114	159	4	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas	602752507	100
12	273	0	254	0	19	Bacteria	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae		406831972	100
250	253	7	22	218	6	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		409892822	100
14	242	0	241	0	1	Bacteria	Spirochaetes	Spirochaetes				308086557	96.52
15	211	30	0	181	0	Bacteria	Proteobacteria	Deltaproteobacteria	Spirobacillales			253796083	93.74
128	201	20	101	73	7	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas	584450492	100
26	198	0	0	198	0	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	570553835	99.08
20	195	3	3	168	21	Bacteria	Firmicutes	Clostridia	Clostridiales	[Acidaminobacteraceae]	WH1-8	352092256	100
16	189	5	0	184	0	Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales			270283641	100
17	188	12	19	151	6	Bacteria	Proteobacteria	Deltaproteobacteria	GMD14H09			369939670	100
40	171	0	70	63	38	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae		187293515	99.53
18	169	97	0	72	0	Bacteria	Firmicutes	Clostridia	Clostridiales	[Acidaminobacteraceae]	Fusibacter	270283190	99.53
19	165	4	51	102	8	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Rubritalea	388951420	96.74
21	162	37	0	120	5	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	54873693	98.14
23	138	0	0	138	0	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	Desulfofrigus	312983542	97.91
29	131	0	117	1	13	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Colwelliaceae		379334379	97.69
22	130	4	0	126	0	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae		312270334	99.51
24	130	13	2	115	0	Bacteria	Proteobacteria	Alphaproteobacteria	Kordiimonadales	Kordiimonadaceae		333465653	99.01
25	119	5	75	29	10	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	636632390	100
28	107	20	10	76	1	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Marivita	566085477	99.75
30	105	1	48	46	10	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	642942064	100
131	105	3	51	45	6	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae		570554280	100
31	100	3	8	89	0	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae		507526267	100

CHAPTER 5

Marine natural products in Cnidarians

Cnidarians as a source of new marine bioactive compounds - an overview of the last decade and future steps for bioprospecting

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Rocha J., Peixe L., Gomes N.C.M., Calado R. *Cnidarians as a source of new marine bioactive compounds - an overview of the last decade and future steps for bioprospecting*. Marine drugs, 2011. **9**(10): 1860-1886.

5.1. Marine ecosystems, holobionts, environmental stressors and the bioprospecting of marine natural products

Marine ecosystems harbor a substantial fraction of earths biodiversity and provide a wide range of goods and services (Burke et al., 2012, Halpern et al., 2012). Among them a wide variety of marine invertebrates from cnidarian species are key elements in the sustainability of such habitats and therefore provide indisputable benefits for the populations. Coral reefs are one of these dynamic and highly productive ecosystems providing essential services upon which millions of people depend. They are important sources of food and livelihoods, attract tourism, protect shoreline and produce compounds used in treatments for diseases (Burke et al., 2012). As plants, which have been a traditional source of compounds for the treatment of several disorders for many populations, many reef-dwelling species produce chemical compounds that have the potential to form the basis of life-saving pharmaceuticals (Burke et al., 2012, Leal et al., 2013). The empirical consume of these compounds by people living near coastal areas combined with the urgent need for new chemical diversity to fuel the drug discovery pipeline may have triggered marine bioprospecting in these regions. Tropical coral reefs are the main ecosystems explored in the bioprospecting of new natural products (Leal et al., 2012b) and are important sources of new molecules (Leal et al., 2012a). While success stories of marine derived-drugs are already a reality (Molinski et al., 2009), the true potential of marine natural products from invertebrates as future drug candidates is yet to be unraveled (Leal et al., 2012a, Rocha et al., 2011). To tangle this challenge, there are growing evidences and, thus the realization, that microorganisms associated with marine invertebrates may be the true producers of some of the natural products isolated from these animals and even be responsible for the variation of chemical diversity at species level (Newman & Cragg, 2007, Piel, 2006, Radjasa et al., 2011). The abundant and unique symbiotic microbial diversity hosted by marine invertebrates (Rohwer et al., 2002) plays a very important role in their biology and ecology, particularly in its nutrition (Agostini et al., 2012, Leal et al., 2014a), disease-resistance (Rohwer et al., 2002, Shnit-Orland & Kushmaro, 2009) and response to environmental perturbations (Ainsworth et al., 2010), as well as in the chemical mediation of interactions among marine organisms (Paul & Puglisi, 2004). The role of marine microbial symbionts in chemical defenses of marine invertebrates against both predators and competitors has already been recognized (Hay, 2009, Paul & Puglisi, 2004, Paul et al., 2011). The symbionts produce chemically diverse and biologically active secondary metabolites, such as anti-inflammatory, antibiotic, antitumor, antibacterial and antifungal compounds, whose properties are particularly

interesting for drug discovery (Molinski et al., 2009, Piel, 2004). However, as most compounds produced occur as secondary metabolites they are naturally produced in low quantities. This alone imposes critical challenges to the drug discovery pipeline, particularly during the early phases of discovery and the selection of which should advance to the next step (Li & Vederas, 2009, Radjasa et al., 2011).

The production of marine natural products at a commercial level is still a remarkable challenge. Large-scale production of a given compound can be possible either through chemical synthesis or through its extraction from the source organism. Unfortunately, the first option is not always possible, as several complex molecules are simply impossible to produce or attain production costs unaffordable for commercial applications (Proksch et al., 2003, Qian et al., 2009). The harvest of the source animal (holobiont) from the wild for the extraction of a compound is invariably an unsustainable practice and hardly a long term option (Arrieta et al., 2010). Additionally, the dependence on natural samples may still entail replicability issues (Li & Vederas, 2009) as wild marine organisms collected for bioprospecting are exposed to environmental variability (including some more extreme environmental conditions associated with global climate change), as well as changes at the community level, which may significantly affect their chemical ecology (Hay & Fenical, 1996). Individuals of the same species sampled in different geographical areas, or time frames, may not display the same symbionts and thus exhibit the same chemical composition (Aratake et al., 2012) and, therefore, fail to guarantee the supply of a target metabolite (a pitfall commonly termed “loss of the source”). This may also be a potential limitation for the initial detection of bioactive metabolites, as environmental and individual variability in the chemical composition of target organisms may bias bioprospecting (Li & Vederas, 2009). Also associated with replicability issues is the potential loss of the source through extinction of target species. This issue is particularly relevant in the oceans of today and tomorrow, as vulnerability to extinction in marine ecosystems is predicted to be higher (Burke et al., 2012) due to climate changes and other external inputs.

The production of source organism’s biomass (either *in situ* or *ex situ*) has been considered as a potential alternative to the collection of wild specimens (Leal et al., 2014b, Schippers et al., 2012). Additionally, the production of source organisms under controlled conditions may help to control ecophysiological diversity promoted by environmental interactions and maximize the production of target marine molecules. Unfortunately, the culture of most organisms has revealed to be more technically challenging and significantly more expensive than initially assumed (Mendola, 2003, Page et al., 2011).

As seen, critical challenges are being address in order to find a constant and reliable supply to fuel the marine drug discovery (Leal et al., 2014b). Although it is consensual that

the use of wild marine organisms is not the best approach, studies with these animals allow researchers to better direct their lines of investigations in drug discovery. Simple knowledge's as described in chapter 3 or more complex ones as seen in chapter 4, support future choices when addressing bioprospecting of marine natural products. In these chapters we observe that environmental or physical parameters impact the holobiont by changes on the microbial populations associated with the cnidarian host and thus affect the production of chemicals with potential to have biotechnological interest. Environmental stressors may inhibit, reduce or even enhance the production of some chemicals. They can also activate dormant genes not needed in previous conditions, or allow the incorporation of new ones enabling adaptation and evolution. This variety of possibilities can and should be driven to leverage the biotechnological potential of marine natural products as precursors of new drugs.

5.2. Cnidarians as a source of new marine bioactive compounds - an overview of the last decade and future steps for bioprospecting

5.2.1. Abstract

Marine invertebrates are rich sources of bioactive compounds and their biotechnological potential attracts scientific and economic interest worldwide. Although sponges are the foremost providers of marine bioactive compounds, cnidarians are also being studied with promising results. This diverse group of marine invertebrates includes over 11,000 species, 7500 of them belonging to the class Anthozoa. We present an overview of some of the most promising marine bioactive compounds from a therapeutic point of view isolated from cnidarians in the first decade of the 21st century. Anthozoan orders Alcyonacea and Gorgonacea exhibit by far the highest number of species yielding promising compounds. Antitumor activity has been the major area of interest in the screening of cnidarian compounds, the most promising ones being terpenoids (monoterpenoids, diterpenoids, sesquiterpenoids). We also discuss the future of bioprospecting for new marine bioactive compounds produced by cnidarians.

5.2.2. Introduction

In terms of biodiversity, marine environments are among the richest and most complex ecosystems. Harsh chemical and physical conditions in the environment have been important drivers for the production of a variety of molecules with unique structural features. These marine molecules exhibit various types of biological activities (Jain et al., 2008), with compounds of high economic interest having potential applications in the pharmaceutical and medical sectors. Although nearly 20,000 compounds have been discovered since the field of marine bioactive compound biochemistry began in the mid-1960s, only a very limited number have seen industrial application. It has been clear since marine bioprospecting began that the world's oceans and their diverse biota represent a significant resource, perhaps the greatest resource on Earth, for the discovery of new bioactive compounds. Early National Cancer Institute (NCI) programs in the USA demonstrated that marine invertebrates were a superb source of potential lead molecules. The decisive boost to this new age of bioprospecting was provided by the NCI when it was found that bioassays with marine organism extracts were far more likely to yield anticancer drugs than terrestrial sources (Fenical et al., 2009). In this way, it is not surprising that over the past 40 years major advances in the discovery of marine drugs have been recorded in clinical trials for cancer (Hill & Fenical, 2010). Apart from anticancer activity, these compounds have proven to be an abundant source of pharmacologically active agents for the production of therapeutic entities (Glaser & Mayer, 2009) against AIDS, inflammatory conditions and microbial diseases.

Marine bioactive compounds display varied potential applications, namely as molecular tools, in cosmetics, as fine chemicals, as nutraceuticals and in agrochemical industries (Fusetani, 2010).

Although only a few marine-derived products are currently on the market (e.g., Prialt® and Yondelis®), several new compounds are now in the clinical pipeline and several more are in clinical development. The few approvals so far for the commercialization of drugs from the sea have not been due to a lack of discovery of novel marine bioactive compounds, but because of the complexity of issues raised upon the development of these products (Glaser & Mayer, 2009). Faulkner (Faulkner, 1986, Faulkner, 1987, Faulkner, 1988, Faulkner, 1990, Faulkner, 1991, Faulkner, 1993, Faulkner, 1994, Faulkner, 1995, Faulkner, 1996, Faulkner, 1997, Faulkner, 1998, Faulkner, 1999, Faulkner, 2000, Faulkner, 2001, Faulkner, 2002), Blunt *et al*. (Blunt et al., 2003, Blunt et al., 2004, Blunt et al., 2005, Blunt et al., 2006, Blunt et al., 2007, Blunt et al., 2008, Blunt et al., 2009, Blunt et al., 2010, Blunt et al., 2011), and Mayer (Mayer & Gustafson, 2004, Mayer & Gustafson, 2006, Mayer & Gustafson, 2008, Mayer & Hamann, 2002, Mayer & Hamann,

2004, Mayer & Hamann, 2005, Mayer et al., 2011, Mayer et al., 2009, Mayer et al., 2007) have provided extensive reviews on the total number of marine natural products (MNPs) discovered over the last 25 years, the most promising ones being produced by marine invertebrates. Sponges (phylum Porifera) have long been recognized as the most interesting group of marine invertebrates for the discovery of new drugs (Fusetani, 2010, Newman & Cragg, 2004, Sipkema et al., 2005). However, with growing bioprospecting efforts and the screening of previously unexplored marine habitats, the biotechnological potential of other groups of marine invertebrates has also started to attract the attention of researchers. The ability of cnidarians (such as jellyfish, sea anemones and corals) to produce powerful toxins and venoms (Turk & Kem, 2009) has been well documented. However, further research has demonstrated that MNPs produced by cnidarians are more than toxins and venoms. The phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates that includes over 11,000 extant species (Daly et al., 2007). Over 3000 MNPs have been described from this phylum alone, mostly in the last decade.

In this work, we present an overview of the most promising marine bioactive compounds isolated from cnidarians in the first decade of the 21st century, which may have applications in the therapy of human diseases. The present study also discusses future perspectives for the bioprospecting of new MNPs produced by this speciose group of marine invertebrates.

5.2.3. Methodology

The most relevant peer reviewed literature published during the first decade of the 21st century covering MNPs was surveyed for the present work (Blunt et al., 2003, Blunt et al., 2004, Blunt et al., 2005, Blunt et al., 2006, Blunt et al., 2007, Blunt et al., 2008, Blunt et al., 2009, Blunt et al., 2010, Blunt et al., 2011, Faulkner, 2001, Faulkner, 2002, Mayer & Gustafson, 2004, Mayer & Gustafson, 2006, Mayer & Gustafson, 2008, Mayer & Hamann, 2004, Mayer & Hamann, 2005, Mayer et al., 2011, Mayer et al., 2009, Mayer et al., 2007). During this period alone, over 2000 molecules from cnidarians were described. In order to focus our study and address only those compounds displaying a high potential for industrial applications, we have decided to use as guidelines the values of IC_{50} (half maximal inhibitory concentration). IC_{50} is a quantitative measure, which indicates how much of a particular substance (inhibitor) is needed to inhibit a given biological process or component of a process by half. It is important to highlight that the NCI has renamed the

IC_{50} to GI_{50} (Boyd et al., 1992) in order to emphasize the correction for cell count at time zero in cancer cells; in this way, some results on this quantitative measure are now also presented under these directives. Additionally, the ED_{50} (the median dose that produces the desired effect of a drug in half the test population) was also used to identify promising marine bioactive compounds produced by cnidarians. Only the compounds displaying an $IC_{50} \leq 10.0$ µg/mL or µM (except where stated otherwise) and $ED_{50} \leq 4.0$ µg/mL were considered for the present study, as these values are commonly used in the surveyed literature to ascertain relevant bioactivity (e.g., Duh et al., 2002b, El-Gamal et al., 2005). In the few cases were neither IC_{50} nor ED_{50} values were described for a MNP in a manuscript, that compound was selected to be part of the present survey only if either the authors of that manuscript, or those citing that manuscript, clearly stated that the results recorded were highly promising for industrial applications. All species producing the compounds selected for the present work were grouped into classes and orders of phylum Cnidaria (Table 5.1) (according to the classification proposed in the World Register of Marine Species (WoRMS)) (Appeltans et al., 2010).

This approach allowed us to identify which taxonomic groups of cnidarians screened so far display the highest potential to yield new drugs or pharmacological products derived from marine bioactive compounds. Nonetheless, it is important to highlight that cnidarian species identification is a challenging task and it is possible that some of the species (or even genera) referred to in the scientific literature may not be correct (Fautin, 2011). In this way, it is of paramount importance that in future works the authors addressing marine bioactive compounds produced by cnidarians provide a detailed description on how target species have been identified.

5.2.4. Class Anthozoa

Class Anthozoa currently includes 10 orders and over 7,500 valid species (about 2/3 of all known cnidarian species) (Table 5.1). Within the Anthozoa, the order Alcyonacea (soft corals) and Gorgonacea (sea fans) are the ones which have contributed with the highest number of promising bioactive marine compounds, although other orders, such as Actiniaria (sea anemones) and Scleractinia (hard corals), have also yielded relevant compounds (Fontana et al., 1998, Meyer et al., 2009, Miyaoka et al., 2006, Strukelj et al., 2000).

Table 5.1. Classes and orders in the phylum Cnidaria followed in this paper.

Phylum	Class	Order	
Cnidaria (≈11,287 species)	Anthozoa (≈7500 species)	Actiniaria	Zoanthidea
		Antipatharia	Alcyonacea
		Ceriantharia	Gorgonacea
		Corallimorpharia	Helioporacea
		Scleractinia	Pennatulacea
	Cubozoa (≈36 species)	Carybdeida	Chirodropida
	Hydrozoa (≈3500 species)	Anthoathecata	Limnomedusae
		Leptothecata	Narcomedusae
		Siphonophorae	Trachymedusae
		Actinulida	
	Polypodiozoa (1 species)	Polypodiidea	
	Scyphozoa (≈200 species)	Coronatae	Semaeostomeae
		Rhizostomeae	
	Staurozoa (≈50 species)	Stauromedusae	

5.2.4.1. Order Alcyonacea (Soft Corals)

Soft corals are generally brightly colored and rich in nutritionally important substances. However, the incidence of predation in the majority of these organisms is low due to the toxic compounds they produce to deter predators (Hooper & Davies-Coleman, 1995). Several biosynthetic studies have been carried out on the metabolites of soft corals (Bhakuni & Rawat, 2005) and some of those compounds have already shown to have great potential for the development of new pharmaceuticals and antifoulants. Table 5.2 summarizes the most promising compounds from order Alcyonacea (class Anthozoa) described in the present review.

Soft corals are rich sources of secondary metabolites such as diterpenes, sesquiterpenes, furanoditerpenes, terpenoids, capnellenes and steroids (e.g., *Lobophytum*, *Sinularia* (Figure 5.1A), *Sarcophyton* (Konig & Wright, 1998) (Figure 5.1C), *Capnella* (Chang et al., 2008), *Dendronephthya* (Grote et al., 2008)), that have shown to display HIV-inhibitory (Rashid et al., 2000), cytotoxic (Duh & Hou, 1996, Su et al., 2006), anti-inflammatory (Norton & Kazlauskas, 1980, Williams & Faulkner, 1996), anticancer (Li et al., 2005,

Weinheimer et al., 1977) and antimicrobial activity (Aceret et al., 1998), as well as cardiac and vascular responses (Aceret et al., 1996). Soft corals of the family Nephtheidae are known for their content of sesquiterpenes and particularly capnellenes (Blunt et al., 2010). Some sesquiterpenes isolated from *Capnella imbricate* (Chang et al., 2008, Kaisin et al., 1985, Kaisin et al., 1974, Sheikh et al., 1976) showed anti-inflammatory activity and a dihydroxycapnellene (capnell-9(12)-ene-8β,10α-diol) from *Dendronephthya rubeola* demonstrated a good antiproliferative activity against murine fibroblasts cell line (L-929, GI_{50} 6.8 μM/L) and a good cytotoxicity against cancer cell lines implicated in human leukemia (K-562, IC_{50} 0.7 μM) and human cervix carcinoma (HeLa, IC_{50} 7.6 μM) (Grote et al., 2008). Capnell-9(12)-ene-8β,10α-diol strongly inhibits the interaction of the oncogenic transcription factor Myc with its partner protein Max (Hermeking, 2003, Peukert et al., 1997), making it a therapeutically interesting compound in oncology (Grote et al., 2008).

Table 5.2. Most promising compounds studied in the last decade from cnidarian species in order Alcyonacea (soft corals), class Anthozoa.

Family and Species	Drug Class	Compound	Chemistry	Country	Ref.
Alcyoniidae					
Klyxum simplex	Anti-inflammatory	Simplexin E	Diterpenoid	TAIW	(Wu et al., 2009)
Klyxum simplex	Antitumor	Klysimplexin B and H	Diterpenoid	TAIW	(Chen et al., 2009)
Lobophytum sp.	Antitumor	Lobophytene	Diterpenoid	VN	(Nguyen et al., 2010)
Lobophytum sp.	Anti-HIV	Lobohedleolide	Diterpenoid	PHL	(Rashid et al., 2000)
Lobophytum sp.	Anti-HIV	(7*Z*)-lobohedleolide,	Diterpenoid	PHL	(Rashid et al., 2000)
Lobophytum sp.	Anti-HIV	17-dimethylamino lobohedleolide	Diterpenoid	PHL	(Rashid et al., 2000)
Lobophytum crassum	Anti-inflammatory	Crassumolides A and C	Terpenoid	TAIW	(Chao et al., 2008)
Lobophytum cristagalli	Antitumor	Cembranolide diterpene	Diterpenoid	RSC	(Coval et al., 1996)
Lobophytum durum	Anti-inflammatory	Durumolides A–C	Terpenoid	TAIW	(Cheng et al., 2008)
Lobophytum durum	Anti-inflammatory	Durumhemiketalolide A–C	Cembranoid	TAIW	(Cheng et al., 2009b)
Sarcophyton crassocaule	Antitumor	Crassocolides H–M	Cembranoid	TAIW	(Huang et al., 2009)
Sinularia sp.	Antiulcer	Sinulide	Spermine		(Fusetani, 1990)
Sinularia sp.	Antimicrobial	Lipids	Polyketide	RUS	(Dmitrenok et al., 2003)
Sinularia flexibilis	Antitumor	Flexilarin D	Cembranoid	TAIW	(Lin et al., 2009b)
Sinularia flexibilis	Antifoulant	11-episinulariolide	Diterpenoid	AUS	(Michalek & Bowden, 1997)
Sinularia gibberosa	Anti-inflammatory	Gibberoketosterol	Steroid	TAIW	(Ahmed et al., 2006)
Sinularia querciformis	Anti-inflammatory	Querciformolide C	Terpenoid	TAIW	(Lu et al., 2008)
Clavulariidae					
Clavularia sp.	Nervous system	Stolonidiol	Diterpenoid	JPN	(Yabe et al., 2000)
Clavularia koellikeri	Antitumor	Cembrane-type diterpenoid	Diterpenoid	JPN	(Iwashima et al., 2000)
Clavularia viridis	Antitumor	Claviridic acid	Prostanoid	TAIW	(Lin et al., 2008)
Clavularia viridis	Antitumor	Clavulones	Prostanoid	TAIW	(Lin et al., 2008)
Clavularia viridis	Antitumor	Claviridenone	Prostanoid	TAIW	(Duh et al., 2002b)

Table 5.2. *Cont.*

Clavularia viridis	Antitumor	Halogenated prostanoids	Prostanoid	JPN	(Watanabe et al., 2001)
Clavularia viridis	Antitumor	Bromovulone III	Prostanoid	TAIW	(Chiang et al., 2005, Shen et al., 2004)
Clavularia viridis	Antitumor	Yonarasterols	Steroid	JPN	(Iwashima et al., 2001)
Clavularia viridis	Antitumor	Stoloniferone E	Steroid	TAIW	(Duh et al., 2002b)
Telesto riisei	Antitumor	Punaglandins	Prostaglandin	USA	(Verbitski et al., 2004)
Nephtheidae					
Dendronephthya sp.	Antifoulant	Isogosterones A–D	Steroid	JPN	(Tomono et al., 1999)
Dendronephthya rubeola	Antitumour	Capnell-9(12)-ene-8β,10α-diol	Sesquiterpenoid	DE	(Grote et al., 2008, Hermeking, 2003, Peukert et al., 1997)
Nephthea chabroli	Antitumor	Chabranol	Terpenoid	TAIW	(Cheng et al., 2009c)
Nephthea erecta	Anti-inflammatory	Ergostanoids 1 and 3	Ergostanoid	TAIW	(Cheng et al., 2009a)
Xeniidae					
Asterospicularia laurae	Antitumor	Asterolaurin A	Diterpenoid	TAIW	(Lin et al., 2009a)
Cespitularia hypotentaculata	Antitumor	Cespitularin C	Diterpenoid	TAIW	(Duh et al., 2002a)
Xenia novaebritanniae	Antibacterial	Xeniolide I	Diterpenoid	ISR	(Bishara et al., 2006)
Xenia plicata	Antitumor	Blumiolide C	Diterpenoid	TAIW	(El-Gamal et al., 2005)

AUS: Australia; DE: Germany; ISR: Israel; JPN: Japan; PHL: Philippines; RSC: Republic of Seychelles; RUS: Russia; TAIW: Taiwan; VN: Vietnam.

Nephthea chabroli also produces a nor-sisquiterpene compound, chabranol, which displays moderate cytotoxicity against P-388 (mouse lymphocytic leukemia cells) with an ED_{50} 1.81 µg/mL (Cheng et al., 2009c). *Nephthea erecta* produces two proteins in mediated inflammatory responses, the oxygenated ergostanoids 1 and 3. These compounds at a concentration of 10 µM significantly reduced the levels of the iNOS (inducible nitric oxide synthase) (45.8 ± 9.9 and 33.6 ± 20.6%, respectively) and COX-2 (cyclooxygenase-2) protein (68.1 ± 2.3 and 10.3 ± 6.2%, respectively), when compared with the control cells stimulated with lipopolysaccharides (LPS) (Cheng et al., 2009a).

Species in the genus *Xenia* (family Xeniidae) (Figure 5.1B) are a rich source of diterpenoids. Xeniolides I, isolated from *Xenia novaebrittanniae* demonstrated antibacterial activity at a concentration of 1.25 mg/mL in *Escherichia coli* ATCC and *Bacillus subtilis* (Bishara et al., 2006). Blumiolide C, a diterpenoid from the *Xenia blumi* (presently accepted as *Xenia plicata*), exhibited potent cytotoxicity against mouse lymphocytic leukemia (P-388, ED_{50} 0.2 µg/mL) and human colon adenocarcinoma (HT-29, ED_{50} 0.5 µg/mL) cells (El-Gamal et al., 2005).

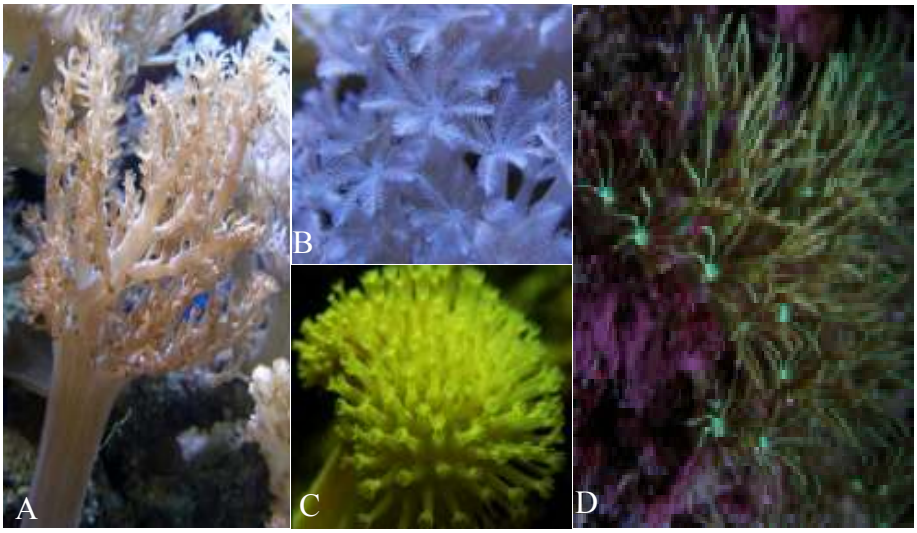

Figure 5.1. Some cnidarians addressed in this review (all images by Ricardo Calado). (**A**) *Sinularia* sp.; (**B**) *Xenia* sp.; (**C**) *Sarcophyton* sp.; (**D**) *Briareum* sp.

Polyoxygenated cembranoids, crassocolides H–M from *Sarcophyton crassocaule*, demonstrated cytotoxicity against cancer cell lines of human medulloblastoma (Daoy cells) where crassocolides I and M were found to be more active (IC_{50} 0.8 and 1.1 µg/mL, respectively). Crassocolide H was also found to inhibit the growth of human oral epidermoid carcinoma (KB) cells (IC_{50} 5.3 µg/mL) and crassocolide L active against human cervical epitheloid carcinoma (HeLa) cells (IC_{50} 8.0 µg/mL) (Huang et al., 2009).
Another example of a potential new therapeutic anticancer agent is a cembranolide diterpene from *Lobophytum cristagalli*, which has shown a potent inhibitory activity (IC_{50} 0.15 µM) (Coval et al., 1996) over farnesyl protein transferase (FPT, an important protein in signal transduction and regulation of cell differentiation and proliferation (Nakao & Fusetani, 2007)). This type of FPT inhibition enhanced interest in this group of metabolites (Konig & Wright, 1998). Other species of this genus also showed cembranolide diterpenes (lobophytene) with significant cytotoxic activity against human lung adenocarcinoma (A549) and human colon adenocarcinoma (HT-29) cell lines (Nguyen et al., 2010). *Lobophytum durum* and *Lobophytum crassum* produce durumolides A–C (Cheng et al., 2008), durumhemiketalolide A–C (Cheng et al., 2009b) and crassumolides A and C (Chao et al., 2008), with anti-inflammatory effects. They have been shown to inhibit up-regulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated murine macrophage cells at IC_{50} < 10 µM (Chao et al., 2008, Cheng et al., 2008). The diterpenoids, lobohedleolide, (7*Z*)-lobohedleolide, and 17-dimethylaminolobohedleolide, were isolated from the aqueous extract of *Lobophytum* species and exhibited moderate HIV-inhibitory

activity (IC_{50} approximately 7–10 µg/mL) in a cell-based *in vitro* anti-HIV assay (Rashid et al., 2000).

Klyxum simplex produces diterpene compounds, such as simplexin E, which at a concentration of 10 µM was found to considerably reduce the levels of iNOS and COX-2 proteins to 4.8 ± 1.8% and 37.7 ± 4.7%, respectively. These results have shown that this compound significantly inhibits the accumulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells (Wu et al., 2009). This species also produces two diterpene compounds, klysimplexins B and H, exhibiting moderate cytotoxicity towards human carcinoma cell lines. Klysimplexin B exhibits cytotoxicity toward human hepatocellular carcinoma (Hep G2 and Hep 3B), human breast carcinoma (MDA-MB-231 and MCF-7), human lung carcinoma (A549) and human gingival carcinoma (Ca9-22) cell lines with IC_{50}'s of 3.0, 3.6, 6.9, 3.0, 2.0, and 1.8 µg/mL, respectively. Metabolite klysimplexin H demonstrated cytotoxicity (IC_{50}'s 5.6, 6.9, 4.4, 5.6, 2.8 and 6.1 µg/mL) toward human hepatocellular carcinoma (Hep G2 and Hep 3B), human breast carcinoma (MDA-MB-231 and MCF-7), human lung carcinoma (A549) and human gingival carcinoma (Ca9-22) cell lines, respectively (Chen et al., 2009).

In *Sinularia* sp. (Figure 5.1A), a tetraprenylated spermine derivative has been isolated - sinulamide - which revealed an H,K-ATPase inhibitory activity. H,K-ATPase is a gastric proton pump of stomach and is the enzyme primarily responsible for the acidification of the stomach contents. Its inhibition is a very common clinical intervention used in diseases including dyspepsia, peptic ulcer, and gastroesophageal reflux (GORD/GERD). Sinulide is a potential antiulcer drug, as it inhibits production of gastric acid by H,K-ATPase (IC_{50} 5.5 µM) (Fusetani, 1990). Although it has been synthesized (Sata et al., 1999), no clinical trials seem to have been reported. The steroid gibberoketosterol (Ahmed et al., 2006), isolated from *Sinularia gibberosa*, and the diterpenoid querciformolide C (Lu et al., 2008) from *Sinularia querciformis*, showed significant inhibition of the up-regulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated murine macrophages at concentration <10 µM (Ahmed et al., 2006, Lu et al., 2008). *Paralemnalia thyrsoides* showed significant inhibition of pro-inflammatory iNOS protein expression (70% at IC_{50} 10 µM) (Huang et al., 2006). *Sinularia* species produce significant molecules: lipids from *Sinularia grandilobata* and another unspecified species of *Sinularia* possesses antibacterial and antifungal activity (Dmitrenok et al., 2003). The diterpene 11-episinulariolide from *Sinularia flexibilis* is an interesting antifoulant exhibiting strong algacidal properties (Michalek & Bowden, 1997). This species also produces cembrenoids, named flexilarins, which evidence cytotoxic activity in cancer cell lines. Flexilarin D exhibited potent cytotoxicity in human hepatocarcinoma (Hep2) cells with IC_{50} 0.07 µg/mL, and moderate cytotoxic activity against human cervical epitheloid carcinoma

(HeLa, IC_{50} 0.41 µg/mL), human medulloblastoma (Daoy, 1.24 µg/mL) and human breast carcinoma (MCF-7, 1.24 µg/mL) cell lines (Lin et al., 2009b).

Antifouling agents from natural sources are of increasing interest since the International Maritime Organization (IMO) banned the use of certain antifouling agents, such as tri-*n*-butyltin (TBT), due to the ecological impacts of these biocides in the marine environment. Several studies have demonstrated that soft corals can yield large quantities of promising antifouling metabolites (Coll, 1992, Maida et al., 2006). In fact, 17.95% of potential antifouling natural compounds are from cnidarians (e.g., soft coral) (Chambers et al., 2006). One of the most promising natural antifouling agent identified so far is an isogosterone isolated from an unspecified *Dendronephthya* (Tomono et al., 1999).

The genus *Clavularia* contains secondary metabolites with unique structures and remarkable biological activities. Some of the species in this genus produce prostanoids (icosanoids) (Duh et al., 2002b, Iwashima et al., 2002, Shen et al., 2004, Watanabe et al., 1996, Watanabe et al., 2001), steroids (Iwashima et al., 2001) and diterpenoids (Iwashima et al., 2000, Kusumi et al., 1992). The bioactive marine diterpene, stolonidiol, isolated from an unidentified *Clavularia*, showed potent choline acetyltransferase (ChAT) inducible activity in primary cultured basal forebrain cells and clonal septal SN49 cells, suggesting that it may act as a potent neurotrophic factor-like agent on the cholinergic nervous system (Yabe et al., 2000). Cholinergic neurons in the basal forebrain innervate the cortex and hippocampus, and their function may be closely related to cognitive function and memory. The degeneration of neuronal cells in this brain region is considered to be responsible for several types of dementia including Alzheimer's disease. One of the neurotransmitters, acetylcholine, is synthesized from acetyl coenzyme A and choline by the action of ChAT. Therefore, induction of ChAT activity in cholinergic neurons may improve the cognitive function in diseases exhibiting cholinergic deficits (Bartus et al., 1982, Davies & Maloney, 1976, Whitehouse et al., 1982).

Prostanoids (claviridic acid) isolated from *Clavularia viridis* exhibited potent inhibitory effects on phytohemagglutinin-induced proliferation of peripheral blood mononuclear cells (PBMC, 5 µg/mL), as well as significant cytotoxic activity against human gastric cancer cells (AGS, IC_{50} 1.73–7.78 µg/mL) (Lin et al., 2008). Claviridenone extracts also showed potent cytotoxicity against mouse lymphocytic leukemia (P-388) and human colon adenocarcinoma (HT-29), and exceptionally potent cytotoxicty against human lung adenocarcinoma (A549) cells, with ED_{50} between 0.52 pg/mL and 1.22 µg/mL (Duh et al., 2002b). Halogenated prostanoids also showed cytotoxic activity against human T lymphocyte leukemia cells (MOLT-4, IC_{50} 0.52 µg/mL), human colorectal adenocarcinoma (DLD-1, IC_{50} 0.6 µg/mL) and human diploid lung fibroblast (IMR-90, IC_{50} 4.5 µg/mL) cells (Watanabe et al., 2001). The cyclopentenone prostanoid, bromovulone III - a promising

marine natural compound for treatment of prostate, colon and hepatocellular carcinoma - showed anti-tumor activity against human prostate (PC-3) and human colon (HT29) cancer cells at an IC_{50} of 0.5 μM (Shen et al., 2004), and induced apoptotic signaling in a sequential manner in Hep3B cells (Chiang et al., 2005). In the case of prostate cancer cells, this compound displayed an anti-tumor activity 30 to 100 times more effective than cyclopentenone prostaglandins (known to suppress tumor cell growth and to induce apoptosis in prostate cancer cells), by causing a rapid redistribution and clustering of Fas (member of the tumor necrosis factor (TNF) receptor superfamily). Apoptotic stimulation of Fas by specific ligand or antibodies causes the formation of a membrane-associated complex comprising Fas clustering) in PC-3 cells (Chiang et al., 2006). *C. viridis* also produces steroids that show cytotoxic activity against human colorectal adenocarcinoma (DLD-1, $0.02 < IC_{50} < 50$ μg/mL) and also against human T lymphocyte leukemia cells (MOLT-4, $0.01 < IC_{50} < 10$ μg/mL), in the case of yonarasterols (Iwashima et al., 2001). Stoloniferone additionally displayed potent cytotoxicity against mouse lymphocytic leukemia (P-388), human colon adenocarcinoma (HT-29) and human lung adenocarcinoma (A549) cells (Duh et al., 2002b). This species produces several compounds with anti-tumor activity in different types of human tumors, although more *in vitro* studies are needed to determine which compound are potential anticancer agents. *Clavularia koellikeri* produces diterpenoids as secondary metabolites, which display cytotoxic activity against human colorectal adenocarcinoma (DLD-1, IC_{50} 4.2 μg/mL) and strong growth inhibition against human T lymphocyte leukemia cells (MOLT-4, IC_{50} 0.9 μg/mL) (Iwashima et al., 2000).

In the genus *Cespitularia*, several interesting diterpenes of cembrane and neodolabellane skeletons have been identified. In *Cespitularia hypotentaculata* (family Xeniidae) a significant production of diterpenoids was detected. Cespitularin C exhibited potent cytotoxicity against mouse lymphocytic leukemia (P-388, ED_{50} 0.01 μg/mL) and human lung adenocarcinoma (A549, ED_{50} 0.12 μg/mL) cells, while cespitularin E exhibited potent cytotoxicity against human lung adenocarcinoma (A549, ED_{50} 0.034 μg/mL) cell cultures (Duh et al., 2002a). A less active diterpene, Asterolaurin A, from *Asterospicularia laurae* (a species from the same family) exhibited cytotoxicity against human hepatocellular carcinoma (HepG2) cells with an IC_{50} 8.9 μM (Lin et al., 2009a).

Telesto riisei produces punaglandins, highly functional cyclopentadienone and cyclopentenone prostaglandins. Cyclopentenone prostaglandins have unique antineoplastic activity and are potent growth inhibitors in a variety of cultured cells. These punaglandins have been shown to inhibit P53 accumulation (a tumor suppressor protein) and ubiquitin isopeptidase activity (IC_{50} between 0.04 and 0.37 μM) (enzyme involved in protein degradation system) *in vitro* and *in vivo* (Verbitski et al., 2004). Since these

proteasome inhibitors exhibit higher antiproliferative effects than other prostaglandins (Tsukamoto & Yokosawa, 2010), they may represent a new class of potent cancer therapeutics.

5.2.4.2. Order Gorgonacea (Sea Fans)

Gorgonians are a well-known source of compounds exhibiting significant biological activity (Chai et al., 2010). Table 5.3 summarizes the most promising compounds from order Gorgonacea (class Anthozoa) described in the present review. Studies on *Isis hippuris* have resulted in the isolation of a series of novel metabolites such as sesquiterpenes (Sheu et al., 2000), steroids (González et al., 2001), *A*-nor-hippuristanol (Sheu et al., 2004) and isishippuric acid B (Sheu et al., 2004). These compounds exhibit potent cytotoxicity against cancer cell lines of human hepatocellular carcinoma (HepG2 and Hep3B, IC_{50} 0.08–4.64 μg/mL and 0.10–1.46 μg/mL, respectively) (Chao et al., 2005, Sheu et al., 2004), human breast carcinoma (MCF-7, IC_{50} 0.20–4.54 μg/mL and MDA-MB-231, IC_{50} 0.13–2.64 μg/mL) (Chao et al., 2005), mouse lymphocytic leukemia (P-388), human lung adenocarcinoma (A549), and human colon adenocarcinoma (HT-29) with ED_{50} values less than 0.1 μg/mL (González et al., 2001, Sheu et al., 2004) and IC_{50} of 0.1 μg/mL (Sheu et al., 2000).

Table 5.3. Most promising compounds studied in the last decade from cnidarian species in order Gorgonacea (sea fans), class Anthozoa.

Family and Species	Drug Class	Compound	Chemistry	Country	Ref.
Briareidae					
Briareum excavate	Anti-inflammatory	Briaexcavatin E	Diterpenoid	TAIW	(Sung et al., 2006)
Briareum excavate	Antitumor	Briaexcavatolides L and P	Diterpenoid	TAIW	(Sung et al., 2001)
Briareum asbestinum	Antimalarial	Briarellin D, K and L	Diterpenoid	PAN, USA	(Ospina et al., 2003)
Ellisellidae					
Junceella fragilis	Anti-inflammatory	Frajunolides B and C	Terpenoid	TAIW	(Shen et al., 2007)
Junceella juncea	Antifoulant	Juncin ZII	Diterpenoid	TAIW	(Qi et al., 2009)
Gorgoniidae					
Leptogorgia setácea	Antifoulant	Homarine	Pyridine	GEO	(Targett et al., 1983)
Leptogorgia virgulata	Antifoulant	Homarine	Pyridine	GEO	(Targett et al., 1983)
Leptogorgia virgulata	Antifoulant	Pukalide	Diterpenoid	USA	(Gerhart et al., 1988)
Leptogorgia virgulata	Antifoulant	Epoxypukalide	Diterpenoid	USA	(Gerhart et al., 1988)
Pseudopterogorgia sp.	Antitumor	Secosterols	Sterol	USA	(He et al., 1995)
Pseudopterogorgia sp.	Anti-inflammatory	Secosterols	Sterol	USA	(He et al., 1995)
Pseudopterogorgia acerosa	Antitumor	Bis(pseudopterane) amine	Dialkylamine	BHS	(Kate et al., 2009)
Pseudopterogorgia bipinnata	Antituberculosis	Bipinnapterolide B	Terpenoid	USA	(Ospina et al., 2007)
Pseudopterogorgia bipinnata	Antimalarial	Caucanolide A and D	Diterpenoid	COL, PAN, USA	(Ospina et al., 2005)
Pseudopterogorgia elisabethae	Antimicrobial	Pseudopterosin X	Diterpenoid	USA	(Ata et al., 2004)
Pseudopterogorgia elisabethae	Antituberculosis	Ileabethoxazole	Diterpenoid	USA	(Rodríguez et al., 2006)

Table 5.3. *Cont.*

Pseudopterogorgia elisabethae	Antituberculosis	Homopseudopteroxazole	Diterpenoid	USA	(Rodriguez & Rodriguez, 2003)
Pseudopterogorgia elisabethae	Antituberculosis	Caribenols A and B	Terpenoid	USA	(Wei et al., 2007)
Pseudopterogorgia elisabethae	Antituberculosis	Elisapterosin B	Diterpenoid	USA	(Rodriguez et al., 2000)
Pseudopterogorgia elisabethae	Antimalarial	Aberrarone	Diterpenoid	COL	(Rodríguez et al., 2009b)
Pseudopterogorgia kallos	Antimalarial	Bielschowskysin	Diterpenoid	PAN, USA	(Marrero et al., 2004)
Pseudopterogorgia kallos	Antitumor	Bielschowskysin	Diterpenoid	PAN, USA	(Marrero et al., 2004)
Pseudopterogorgia rígida	Antimicrobial	Curcuphenol	Terpenoid	USA	(McEnroe & Fenical, 1978)
Isididae					
Isis hippuris	Antitumor	Suberosenol B	Terpenoid	TAIW	(Sheu et al., 2000)
Isis hippuris	Antitumor	Polyoxygenated steroids	Steroid	IND	(Chao et al., 2005, González et al., 2001)
Isis hippuris	Antitumor	A –nor-hippuristanol	Steroid	TAIW	(Sheu et al., 2004)
Isis hippuris	Antitumor	Isishippuric acid B	Steroid	TAIW	(Sheu et al., 2004)
Plexauridae					
Eunicea sp.	Antimalarial	Sesquiterpenoids	Sesquiterpenoid	COL, PAN, USA	(Garzón et al., 2005)
Eunicea fusca	Anti-inflammatory	Fuscisides	Diterpenoid	USA	(Shin & Fenical, 1991)
Euplexaura flava	Anti-inflammatory	Butenolide	Lipid	JPN	(Kikuchi et al., 1982)

ND: Not Determined; BHS: Bahamas; COL: Colombia; GEO: Georgia; IND: Indonesia; PAN: Panama; TAIW: Taiwan; USA: United States of America.

Species from the genus *Pseudopterogorgia* are a rich source of unusual biologically active diterpenoids, sesquiterpenes, and polyhydroxylated steroids, which exhibit diverse structures (Fenical, 1987, Ospina et al., 2007, Rodríguez, 1995). A sample of the organic extract of *Pseudopterogorgia bipinnata* was included in an initial screening carried out as part of an effort in the discovery of new antimalarial agents. This extract was found to be active in inhibiting the growth of *Plasmodium falciparum* (a protozoan parasite responsible for the most severe forms of malaria). Caucanolide A and D demonstrated significant *in vitro* antiplasmodial activity against chloroquine-resistant *P. falciparum* W2 (IC_{50} 17 µg/mL and IC_{50} 15 µg/mL, respectively) (Ospina et al., 2005). Three secosterols isolated from an unidentified gorgonian from genus *Pseudopterogorgia* inhibited human protein kinase C (PKC) α, βI, βII, γ, δ, ε, η, and ζ, with IC_{50} values in the range 12–50 µM (He et al., 1995). PKC is a key player in cellular signal transduction and has been implicated in cancer, cardiovascular and renal disorders, immunosuppression, and autoimmune diseases such as rheumatoid arthritis (Nakao & Fusetani, 2007). Semisynthetic derivatives also showed a similar activity (He et al., 1995). Promising antimicrobial substances were also reported from *Pseudopterogorgia rigida* (e.g., curcuphenol) (McEnroe & Fenical, 1978) and from *Pseudopterogorgia elisabethae* (e.g., pseudopterosin X and Y) (Ata et al., 2004). Ileabethoxazole, homopseudopteroxazole, caribenols A and B and elisapterosin B from *P. elisabethae* and bipinnapterolide B from *P. bipinnata* inhibit *Mycobacterium tuberculosis*

$H_{37}Rv$ at a concentration of 12.5 µg/mL (Rodriguez & Rodriguez, 2003, Rodriguez et al., 2000) (for elisapterosin B and homopseudopteroxazole) and at a concentration range of 128–64 µg/mL (Marrero et al., 2006, Rodríguez et al., 2006, Wei et al., 2007) (for other compounds). In fact, the inhibition of *M. tuberculosis* $H_{37}Rv$ is within the ranges recorded for rifampin (Rodríguez et al., 2006). *P. elisabethae* and *P. bipinnata* also produce antituberculosis compounds. Bielschowskysin, a naturally occurring diterpene isolated from *Pseudopterogorgia kallos* (Marrero et al., 2004) and aberrarone isolated from *P. elisabethae* (Rodriguez et al., 2009b) exhibited antiplasmodial activity (IC_{50} 10 µg/mL) when tested against *P. falciparum*. The first compound was also found to display strong and specific *in vitro* cytotoxicity against the EKVX non-small cell lung cancer (GI_{50} < 0.01 µM) and CAKI-1 renal cancer (GI_{50} 0.51 µM) (Marrero et al., 2004). Bis(pseudopterane) amine from *Pseudopterogorgia acerosa* was found to exhibit selective activity against HCT116 (IC_{50} 4 µM) cell lines (Kate et al., 2009).

Fuscosides, originally isolated from *Eunicea fusca* (Shin & Fenical, 1991), selectively and irreversibly inhibited leukotriene synthesis. Leukotrienes are molecules of the immune system that contribute to inflammation in asthma and allergic rhinitis and its production is usually related to histamine release (Martelletti et al., 1989). Pharmacological studies indicated that fuscoside B inhibits the conversion of arachidonic acid (AA) to leukotriene B_4 and C_4 (LTB_4 and LTC_4) (Jacobson & Jacobs, 1992, Shin & Fenical, 1991) by inhibiting the 5-Lipoxygenase (5-LO), in the case of LTB_4 with an IC_{50} of 18 µM (Jacobson & Jacobs, 1992). These selective inhibitors of lipoxygenase isoforms can be useful as pharmacological agents, as nutraceuticals or as molecular tools (Nakao & Fusetani, 2007). Sesquiterpenoids metabolites isolated from *Eunicea* sp. display antiplasmodial activity against the malaria parasite *P. falciparum* W2 (chloroquine-resistant) strain, with IC_{50} values ranging from 10 to 18 µg/mL (Garzón et al., 2005).

The gorgonian *Junceella fragilis* produces secondary metabolites, frajunolides B and C, with anti-inflammatory effects towards superoxide anion generation and elastase release by human neutrophils, with an IC_{50} > 10 µg/mL (Shen et al., 2007). When properly stimulated, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion ($O_2^{\bullet-}$), a precursor of other reactive oxygen species (ROS), granule proteases, and bioactive lipids (Lacy & Eitzen, 2008, Nathan, 2006). The production of the superoxide anion is linked to the killing of invading microorganisms, but it can also directly or indirectly damage surrounding tissues. On the other hand, neutrophil elastase is a major secreted product of stimulated neutrophils and a major contributor to the destruction of tissue in chronic inflammatory disease (Pham, 2006). The anti-inflammatory butenolide lipide (Boukouvalas & Loach, 2008) from the gorgonian *Euplexaura flava* (Kikuchi et al., 1982) can

be currently synthesized, opening the possibility of advancing into a new level of anti-inflammatory pharmaceuticals.

Some of the most interesting compounds identified so far in the on-going search for new anti-fouling agents have been recorded in the order Gorgonacea. Good examples of such compounds are juncin ZII from *Junceella juncea* (Qi et al., 2009), homarine from *Leptogorgia virgulata* and *Leptogorgia setacea* (Targett et al., 1983), pukalide and epoxypukalide recorded so far only from *L. virgulata* (Gerhart et al., 1988).

Species of genus *Briareum* (family Briareidae) (Figure 5.1D) (which commonly exhibit an incrusting appearance rather than the fan-like shape of many gorgonians) are widely abundant in Indo-Pacific and Caribean coral reefs. These organisms have been recognized as a valuable source of bioactive compounds with novel structural features. Briarane-related natural products are a good example of such promising compounds due to their structural complexity and biological activity (Sung et al., 2005, Sung et al., 2002). Briaexcavatin E, from *Briareum excavata* (Nutting 1911), also occasionally referred to as *Briarium excavatum*, inhibited human neutrophil elastase (HNE) release with an IC_{50} between 5 and 10 μM (Sung et al., 2006). Briaexcavatolides L and P, diterpenoids from the same species exhibited significant cytotoxicity against mouse lymphocytic leukemia (P-388) tumor cells with ED_{50} of 0.5 (Sung et al., 2001) and 0.9 μg/mL (Wu et al., 2001), respectively. Diterpenoids produced from *Briareum polyanthes* (presently accepted as *Briareum asbestinum*), namely Briarellin D, K and L, exhibited antimalarial activity against *P. falciparum* with an IC_{50} between 9 and 15 μg/mL (Ospina et al., 2003).

5.2.4.3. Other Orders

Sea anemones (order Actiniaria) are a rich source of biologically active proteins and polypeptides. Several cytolytic toxins, neuropeptides and protease inhibitors have been identified from them (Strukelj et al., 2000). In addition to several equinatoxins, potent cytolytic proteins and an inhibitor of papain-like cysteine proteinases (equistatin), were isolated from the sea anemone *Actinia equina* (Lenarcic et al., 1997). Equistatin has been shown to be a very potent inhibitor of papain and a specific inhibitor of the aspartic proteinase cathepsin D (Lenarcic & Turk, 1999). While papain-like cysteine proteases have been implicated in various diseases of the central nervous system, such as brain tumors, Alzheimer's disease, stroke, cerebral lesions, neurological autoimmune diseases and certain forms of epilepsy (Brömme & Petanceska, 2002), aspartic proteinase cathepsin D is involved in the pathogenesis of breast cancer (Wolf et al., 2003) and possibly Alzheimer's disease (NCBI, 2013).

Cycloaplysinopsin C, a bis(indole) alkaloid isolated from *Tubastrea* sp. (order Scleractinia), was found to inhibit growth of two strains of *P. falciparum*, one chloroquine-

sensitive (F32/Tanzania) and other chloroquine-resistant (FcB1/Colombia) with IC_{50} 1.48 and 1.2 μg/mL, respectively (Meyer et al., 2009). Cladocorans A and B, isolated from *Cladocora caespitosa* (order Scleractinia) (Fontana et al., 1998), are marine sesterterpenoids which possess a γ-hydroxybutenolide moiety, which is thought to be responsible for the biological activity of these compounds. The potent anti-inflammatory activity of these natural metabolites was attributed to the inhibition of secretory phospholipase A_2 ($sPLA_2$, IC_{50} 0.8–1.9 μM). Given the general role of inflammation in diseases that include bronchial asthma and rheumatoid arthritis, identifying and developing potent inhibitors of sPLA2 continues to be of great importance for the pharmaceutical industry, with this type of metabolite being of paramount importance for future research (Miyaoka et al., 2006).

5.2.5. Class Hydrozoa

Class Hydrozoa includes seven orders and nearly 3,500 valid species (Table 5.1), some of which are solitary, some of which are colonial. Among the most emblematic species are probably hydroids and the Portuguese man-o-war (*Physalia physalis*). Despite the large number of species in class Hydrozoa, only a few of them have yielded interesting MNPs in the last decade.

Immune escape plays an important role in cancer progression and, although not completely understood, it has been proposed that indoleamine 2,3-dioxygenase (IDO) plays a central role in evasion of T-cell-mediated immune rejection (Muller et al., 2005a). IDO catalyzes the oxidative cleavage of the 2,3 bond of tryptophan, which is the first and rate-limiting step in the kynurenine pathway of tryptophan catabolism in mammalian cells (Grohmann et al., 2003). The polyketides annulins A, B, and C, purified from the marine hydroid *Garveia annulata* (order Anthoathecata), potently inhibited IDO *in vitro* (K_i 0.12–0.69 μM) (Pereira et al., 2006). These annulins are more powerful than most tryptophan analogues known to be IDO inhibitors. These compounds are active at concentrations higher than ~10 μM and therefore more effective than 1-methyltryptophan (K_i 6.6 μM), one of the most potent IDO inhibitors currently available (Muller et al., 2005b). Solandelactones C, D, and G are cyclopropyl oxylipins isolated from the hydroid *Solanderia secunda* (order Anthoathecata) and exhibit moderate inhibitory activity against farnesyl protein transferase (FPT, 69, 89, and 61% inhibition, respectively) at a concentration of 100 μg/mL (Seo et al., 1996). Note that FPT is associated with cell differentiation and proliferation and its inhibition may be a target for novel anticancer agents (as already referred above for the soft coral *L. cristagalli*).

5.2.6. Class Scyphozoa

Approximately 200 species are currently classified in three orders in class Scyphozoa (Table 5.1). However, in the last decade, only a single MNP purified from the mesoglea of the jellyfish *Aurelia aurita* (order Semaeostomeae) was considered to be promising enough to be included in the present work. This compound is a novel endogenous antibacterial peptide, aurelin, which exhibited activity against Gram-positive and Gram-negative bacteria. As an example, aurelin displayed an IC_{50} of 7.7 µg/mL for *Esherichia coli* (Gram negative bacteria) (Ovchinnikova et al., 2006).

5.2.7. Other Classes

The classes Staurozoa, Cubozoa and Polypodiozoa are the least speciose in the phylum Cnidaria (Table 5.1). This fact may explain the current lack of data on secondary metabolites produced by these organisms. It is possible that with growing bioprospecting new MNPs may be revealed once these cnidarian species are screened. Cubozoa (box jellies), for example, produce some of the most harmful cnidarian toxins for humans (Brinkman & Burnell, 2009).

5.2.8. Exploring the Unexplored and Being Creative: Future Perspectives for the Bioprospecting of Cnidarians

For several years, the bioprospecting of cnidarians was commonly limited to habitats that could be readily sampled by researchers, such as shallow coral reefs and the intertidal region. However, with improvements in SCUBA gear, researchers are now able to dive deeper and longer, allowing them to collect a wider range of cnidarian species for the screening of MNPs. The growing efforts to explore Earth's last frontier, the deep sea, made it possible to start bioprospecting several unique marine ecosystems that had remained either previously unrecorded or inaccessible to researchers (Synnes, 2007). New cnidarian species (some of them belonging to new genera and probably even to new families) (e.g., Moura et al., 2007, Rodriguez et al., 2009a) are currently being sampled from the deep sea. These findings suggest that many new species are yet to be discovered along deep continental margins and open good perspectives for the discovery of new MNPs with ongoing surveys of deep sea fauna. Cnidarians are known to colonize unique deep sea biotopes, namely chemosynthetic sites (such as hydrothermal vents, cold seeps and whale falls (Fautin, 2009)), as well as seamounts (Clark et al., 2006).

Some of these organisms are endemic to these habitats and display remarkable adaptations to extreme environments (e.g., chemosynthetic sea anemones) (Rodríguez & Daly, 2010). These species are certainly interesting candidates for the discovery of new MNPs (Skropeta, 2008). However, some of these remarkable biotopes, namely deep sea coral reefs, are already facing serious threats to their conservation (Clark et al., 2006) and thus, the bioprospecting of these and other endangered habitats must be carefully addressed (Kingston, 2011, Synnes, 2007).

Another interesting source of cnidarian species for bioprospecting is the marine aquarium industry. Over 200 species of hard and soft corals, along with several other anemone, zoanthid and corallimorph species, are harvested every year from coral reefs to supply the marine aquarium trade (Wabnitz et al., 2003). However, researchers using these organisms in the bioprospecting of new MNPs must be aware that it is not commonly possible to get reliable information on either the place of origin or the scientific name of most traded specimens. With the advent of high-throughput screening (HTS) (White, 2000), it will be possible to rapidly survey these organisms for interesting MNPs, although HTS of natural sources may present several challenges (see Koehn & Carter, 2005, Li & Vederas, 2009). If necessary, additional biomass of target organisms producing interesting MNPs can be achieved using inexpensive techniques (Sella & Benayahu, 2010, Shafir et al., 2006) and eliminate problems commonly faced by researchers screening marine organisms for MNPs - the loss of the source and reproducibility (Li & Vederas, 2009).

The discovery of a new compound commonly requires only small amounts of biomass. However the production of these compounds at a scale large enough to fulfill commercial applications is still nearly impossible (Qian et al., 2010). In theory, large-scale production of bioactive compounds can be achieved by chemical synthesis or through extraction from marine animals, either harvested from the sea or maricultured. The existence of ecophysiological diversity (e.g., differences between individuals often due to differences in environmental interactions) can interfere with the production of MNPs and must be carefully addressed in future efforts for large-scale production of these compounds. The harvest of target animals from the wild for the production of chemical compounds is commonly an unsustainable solution, while mariculture has proven to be more technically challenging and expensive than previously assumed (Mendola, 2003). In other considerations, chemical synthesis is not yet developed to synthesize complex molecules at the kilogram scale and, in cases where this may already be technically possible, most of the compounds cannot be synthesized at a price affordable for commercial applications (Qian et al., 2010). Potential solutions for such bottlenecks may be the use of diverted

total synthesis (Paterson & Anderson, 2005) and/or metabolic engineering (Khosla & Keasling, 2003).

There is growing evidence that microbes associated with marine invertebrates may be the true producers of some of the bioactive compounds isolated from these animals (Qian et al., 2010). Whether this is the case of bioactive compounds currently assumed to be produced by cnidarians remains unanswered (Piel, 2004, Piel, 2009). If so, we face another constraint for the commercial use of these compounds, as the culture of symbiotic microorganisms is generally not possible using classic/standardized methodologies.

5.2.9. Conclusions

The intense pressure to find and develop more profitable molecules for all sorts of industries continues to fuel the bioprospecting of marine invertebrates. Although the phylum Cnidaria is not the most significantly bioprospected at present, this review shows that some cnidarian species are promising sources of marine bioactive compounds of medical, economic and scientific interest. Green fluorescent protein (GFP), GPF-like proteins, red fluorescent and orange fluorescent protein (OPF) are good examples of biotechnological metabolites currently employed as molecular biomarkers. They were first purified from a fluorescent hydrozoan medusa (Shimomura et al., 1962) and since then have been recorded in other cnidarian species (Ai et al., 2006, Chan et al., 2006, Goulding et al., 2008, Ip et al., 2007, Schnitzler et al., 2008, Tu et al., 2003).

In the present survey, only about 0.31% of extant cnidarian species are represented, with class Anthozoa displaying by far the highest number of promising MNPs (Figure 5.2). This result is probably due to the fact that this class is the most speciose in the phylum (Table 5.1). Additionally, many anthozoans occupy marine habitats which can be readily accessed for the collection of biomass (e.g., coral reefs and intertidal regions), which facilitates bioprospecting. Of all the compounds presented in this review, 84% were detected in cnidarians collected from tropical waters (mostly from Southeast Asia and the Caribbean Sea) and the remaining 16% were recorded from species mostly occupying temperate waters (e.g., European countries and Japan).

Antitumor drugs are the main area of interest in the screening of MNPs from cnidarians (41%, Figure 5.3). This is not surprising, as the major financial effort for the screening of new marine compounds is made in cancer research (Pomponi, 2001). Terpenoids (terpenoid, diterpenoid, sesquiterpenoid, sesterterpenoid, cembranoid) (Blunt & Munro, 2008) (Figure 5.4) are the main chemistry group within the MNPs analyzed in this survey.

Even though most pharmaceutical industries abandoned their natural product-based discovery programs over a decade ago, the lack of new compounds in their pipelines in some strategic areas (e.g., antibiotics) suggests that renewed interest in this field is imminent. The establishment of small biotech companies can play a decisive role in the initial discovery of promising marine bioactive compounds, as these enterprises will work closely together with academics and governmental agencies performing the initial steps in the discovery of new MNPs. Collaboration between private companies and public institutions can be of paramount importance for financial support in the discovery process.

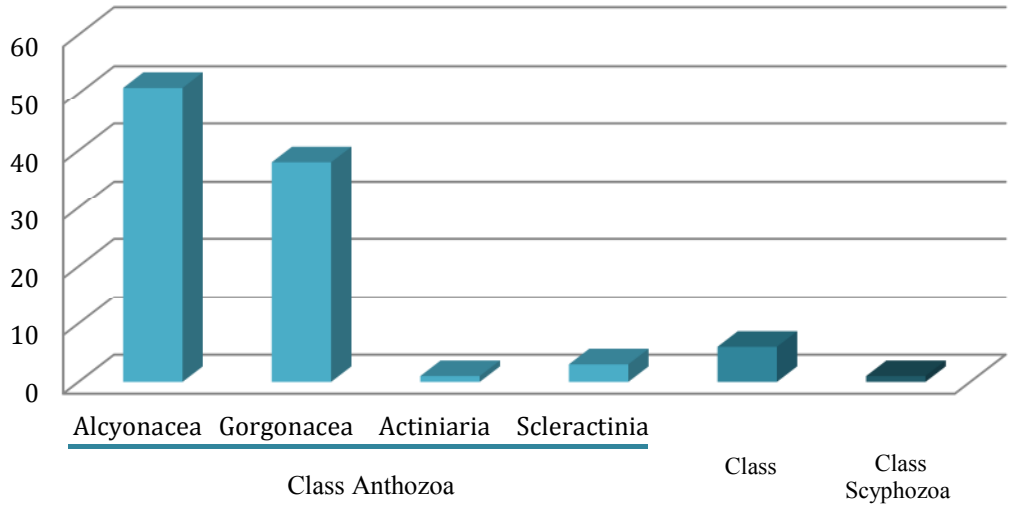

Figure 5.2. Marine bioactive compounds with high biotechnological potential studied from the phylum Cnidaria in the last decade.

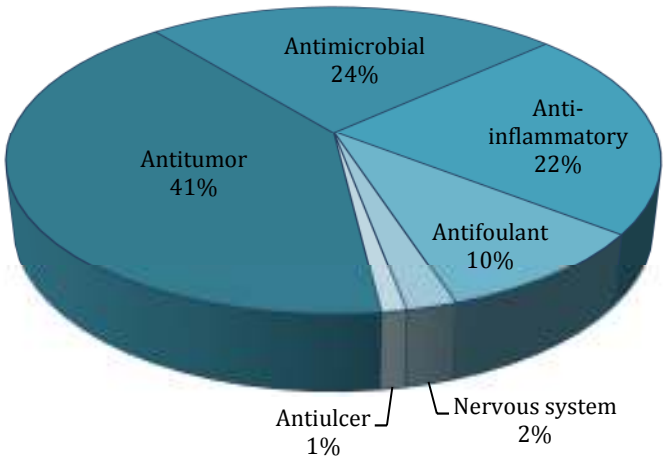

Figure 5.3. Distribution in drug classes of marine bioactive compounds with high biotechnological potential studied from cnidarian species in the last decade.

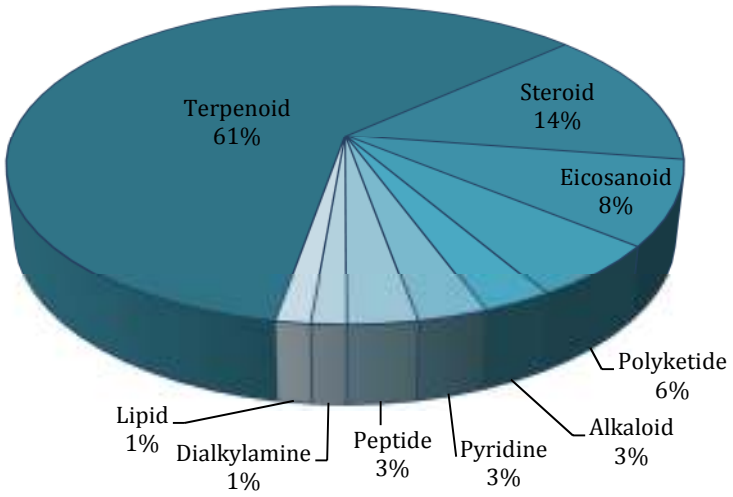

Figure 5.4. Distribution of chemistry classes of marine bioactive compounds with high biotechnological potential studied from cnidarian species in the last decade.

On the other side, crude extracts and pure compounds produced by academic laboratories may be screened by diverse bioassays as a part of broader collaboration programs, nationally and internationally, with private biotech companies. One challenge for universities is to devise mechanisms that protect intellectual property and simultaneously encourage partnerships with the private sector, by recognizing that the chances of a major commercial pay-off are small if drug discovery is pursued by a single institution (Hill & Fenical, 2010).

The commercial use of some promising marine bioactive compounds isolated from cnidarians may be several years away. New compounds other than toxins and venoms produced by members of this highly diverse group of marine invertebrates may be discovered in the quest for new marine products.

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CHAPTER 6

Conclusions and future perspectives

6.1. Conclusions and future perspectives

Interactions between microorganisms and invertebrates are common in the marine environment. However, most of their ecological functions are still enigmatic. Current literature describes endobiotic bacteria in various eukaryotic host organisms such as protozoans, sponges, cnidarians, annelids, echinoderms, and ascidians (Althoff et al., 1998, Burnett & McKenzie, 1997, Cary et al., 1997, Deming & Colwell, 1982, Schuett et al., 2007, Schuett et al., 2005). Nonetheless, the key questions concerning the phylogenetic relationship of host-microbial symbionts, their ecological and physiological function, their secondary metabolites, chemical structure and the effects of these compounds are poorly understood (Schuett et al., 2007). Cnidarians were the dominant marine organisms late in the Precambrian period and they remain important components of marine ecological communities today

Sea anemones, namely those hosting endosymbiotic photosynthetic dinoflagellates, are recognized to be important sentinel species (Winston & Heffernan, 1999). These organisms may help researchers to monitor potential environmental shifts in temperate coastal waters triggered by global climate changes. Extreme bleaching events of *Anemonia* in the Mediterranean under abnormally warm water conditions (Leutenegger et al., 2007) are a good example on the suitability of these anthozoans as sentinel species and an undeniable sign of ongoing global climate changes. Nonetheless there is still a deficiency of studies on the natural dynamics (either temporal and/or spatial) of the microbial biota associated with sea anemones impairing researchers to understand how potential shifts in the marine environment may affect the function of these symbioses.

In light of the hologenome theory (Rosenberg et al., 2007), these anemones should be considered as holobionts (Margulis & Fester, 1991), a complex symbiosis between the cnidarian animal, its photosynthetic microalgae (e.g., zooxanthellae) and its complex community of associated microorganisms (other than microalgae) that play a key role on the overall health of the cnidarian host. Consequently, it is also important to monitor potential shifts in bacteria associated with these sea anemones to understand how environmental disturbances may shape anemone individuals and populations.

The studies presented in this thesis provide detailed information on bacterial communities associating with the snakelocks anemone *Anemonia viridis* and fluctuations occurring in the bacterial biota of this species induced by seasonal and spatial variation, as well as physical stressors (temperature and radiation).

Studies addressing bleaching events of *A. viridis* occurring in the wild were also performed in the frame of the present thesis. However, preliminary results from PCR-DGGE and pyrosequencing were not conclusive and these and additional experiments

must be performed to consolidate the preliminary data that evidenced dramatic shifts in the microbial biota of bleached hosts.

Research on marine cnidarians experienced a significant advance over the last decades with the intensification of bioprospecting of marine invertebrates for drug discovery, as well as other biotechnological applications (Leal et al., 2012, Rocha et al., 2011). Alongside with this new trend, there are growing evidences that microbes associated with marine invertebrates may be the true producers of such bioactive compounds or, at least, be partially involved in the process of biosynthesis of some of these molecules (Shnit-Orland & Kushmaro, 2009). Several of these compounds are secondary metabolites produced by symbiotic microorganisms in chemical mediation and/or defense of interaction among marine microorganisms. Hence marine invertebrate natural product symbioses present a fascinating subject for basic biological and biochemical research. They also present an opportunity to develop resources in ways that circumvent environmental problems. Cultivating bacterial symbionts, understanding the regulation of compound production and optimizing biosynthesis *ex symbio* eliminates problems, such as the collection of wild specimens and can potentially improve the yield and thus the economical profits of production. However, cultivation of microbial symbionts is not a trivial issue. The major challenge in microbial ecology today dwells with the lack of representation, among laboratory strains, of microorganisms prevalent in natural environments. It is therefore necessary to develop more insightful methods for making these organisms amenable to laboratory investigation.

In the future, approaches using classical microbiological methods should be addressed with the purpose of cultivating symbiotic bacterial organisms that may produce bioactive compounds previously considered to have their origin on the invertebrate host. The objective may or may not be the isolation of bacterial strains once production of natural products may result from populations *quorum sensing*.

It is also needed to expand our thinking beyond pure cultures (that grow rapidly to high cell densities) to include consortia and growth conditions that more closely mimic those experienced *in situ* (Haygood et al., 2000). By doing so, it can be possible provide the *in vitro* growth of a larger percentage of cultivable microorganisms and the production of some natural substances, which so far could not be synthetized in the laboratory.

6.2. References

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Supplementary Information (Chapter 2)

Optimization of preservation and processing of sea anemones for microbial community analysis using molecular tools

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DNA quantification

DNA quantification was assessed using Qubit™ dsDNA HS Assay Kits (Invitrogen, Molecular Probes, Life Technologies Corporation, Eugene, Oregon, USA) for Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA). Results are shown in table Supplementary Table ST1.

Supplementary Table ST1. Average (± standard deviation) of DNA quantification for all experimental procedures (fresh samples non-homogenized (Fr_NH); fresh samples processed with homogenizer (Fr_H); fresh samples processed with mortar & pestle (Fr_MP); samples frozen with liquid nitrogen and non-homogenized (LN_NH); samples frozen with liquid nitrogen followed by processing with homogenizer (LN_H); samples frozen with liquid nitrogen followed by processing with mortar & pestle (LN_MP); samples frozen in -80 ºC and non-homogenized (F-80_NH); samples frozen in -80 ºC followed by processing with homogenizer (F-80_H); and samples frozen in -80 ºC followed by processing with mortar & pestle (F-80_MP)).

Experimental Procedure	DNA (µg/ml) Average±s.d.
Fr_NH	67,3±6,2
Fr_H	92,8±17,5
Fr_MP	67,7±6,1
LN_NH	67,0±11,0
LN_H	94,1±14,7
LN_MP	84,7±8,8
F-80_NH	32,0±8,6
F-80_H	53,6±1,7
F-80_MP	11,9±2,0

Supplementary Table ST2. Statistical values for the variation explained by individual axes of the PCO represented in the Figure 2.4.

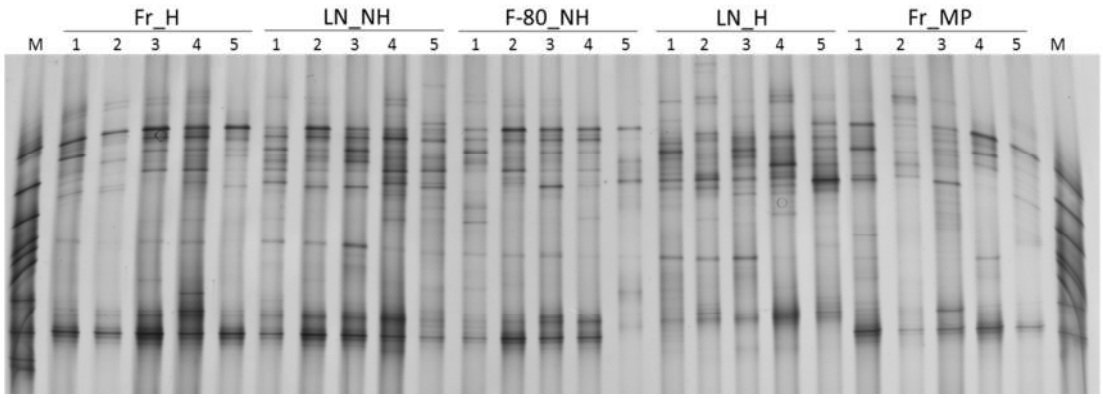
Variation explained by individual axes

Axis	Eigenvalue	Individual%	Cumulative%
1	12601,000	49,850	49,850
2	3853,800	15,250	65,100
3	2286,200	9,040	74,140
4	2009,300	7,950	82,090
5	1652,000	6,540	88,630
6	1275,100	5,040	93,670
7	699,900	2,770	96,440
8	567,760	2,250	98,690
9	448,950	1,780	100,470
10	146,960	0,580	101,050
11	82,649	0,330	101,370
12	27,037	0,110	101,480
13	-126,500	-0,500	100,980
14	-247,830	-0,980	100,000

Supplementary Table ST3. Statistical values for the principal coordinates of the PCO represented in the Figure 2.4.

Principal Coordinates

Sample	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8	Axis 9	Axis 10	Axis 11	Axis 12	Axis 13	xis 14
Fr_H1	-25,089	0,416	-0,115	10,028	-2,025	-14,750	-1,008	-1,867	-3,931	4,508	2,943	1,881	3,020	-6,531
Fr_H2	-28,831	4,577	-7,351	2,512	5,001	1,822	-3,208	12,835	-3,942	-4,109	3,680	-0,398	-4,870	-1,205
Fr_H3	-18,006	-2,016	-9,776	5,824	-7,203	-0,095	-7,204	-6,657	2,682	0,978	0,953	-4,047	1,591	-1,104
Fr_H4	0,390	-1,159	-10,551	16,065	0,599	14,382	-11,500	-2,745	3,594	-3,570	-2,718	2,008	1,545	-2,133
Fr_H5	-32,288	8,001	-8,301	-1,547	0,251	1,744	2,106	-0,677	6,312	6,764	-2,554	0,618	-5,887	2,541
LN_H1	38,185	20,450	-3,151	-16,250	-2,880	11,170	-1,090	-10,235	-9,268	0,721	1,862	0,403	-2,122	-1,200
LN_H2	39,916	4,277	2,658	2,785	14,251	-1,418	-8,932	7,949	-2,421	4,128	0,253	-0,253	3,138	7,637
LN_H3	39,736	23,564	-13,447	-8,992	-4,619	-4,610	8,162	6,873	9,686	-1,257	-0,154	-0,117	2,536	-4,022
LN_H4	33,435	-23,556	-16,601	9,484	-10,151	-12,705	6,125	-0,328	-7,623	-1,668	-2,701	-0,053	-1,952	2,526
LN_H5	31,683	-3,638	19,922	13,996	19,694	-5,130	4,558	-6,959	5,329	-1,763	0,781	-0,494	-3,289	-2,360
Fr_MP1	0,624	8,747	31,376	-3,083	-19,353	-3,448	-5,907	5,222	-1,691	-0,564	-3,007	-0,331	-0,949	-1,932
Fr_MP2	0,858	-37,093	2,091	-25,411	-2,004	-3,165	-5,635	-1,659	6,677	-1,019	1,996	0,929	-0,025	1,217
Fr_MP3	-4,430	-22,523	7,468	4,120	-1,492	21,535	12,666	5,018	-2,183	2,626	0,341	-0,483	2,360	-2,059
Fr_MP4	-31,062	14,696	6,406	6,458	-9,473	-0,586	7,439	-5,027	2,167	-3,304	2,294	0,800	2,137	9,649
Fr_MP5	-45,121	5,257	-0,628	-15,989	19,401	-4,745	3,428	-1,743	-5,387	-2,471	-3,970	-0,464	2,766	-1,025



Supplementary Figure S1. Full-length denaturing gradient gel electrophoresis (DGGE) of the cropped image in Figure 2.3. DGGE based analysis of bacterial community composition in the snakelocks anemone *Anemonia viridis*. The DGGE gel presented compares community fingerprints of 16S rRNA gene fragments amplified from DNA for: fresh samples processed with homogenizer (Fr_H); samples frozen with liquid nitrogen and processed without homogenization (non-homogenized) (LN_NH); samples frozen at -80ºC and processed without homogenization (non-homogenized) (F-80_NH); samples frozen with liquid nitrogen followed by processing with homogenizer (LN_H); fresh samples processed with mortar & pestle (Fr_MP); and marker (M). Numbers represent the samples of each procedure. Experimental procedures LN_NH and F-80_NH were cropped from Figure 2.3 to allow presentation of community fingerprints for the three experimental procedures displaying the highest H' values.