Giant barrel sponges in diverse habitats: a story about the metabolome
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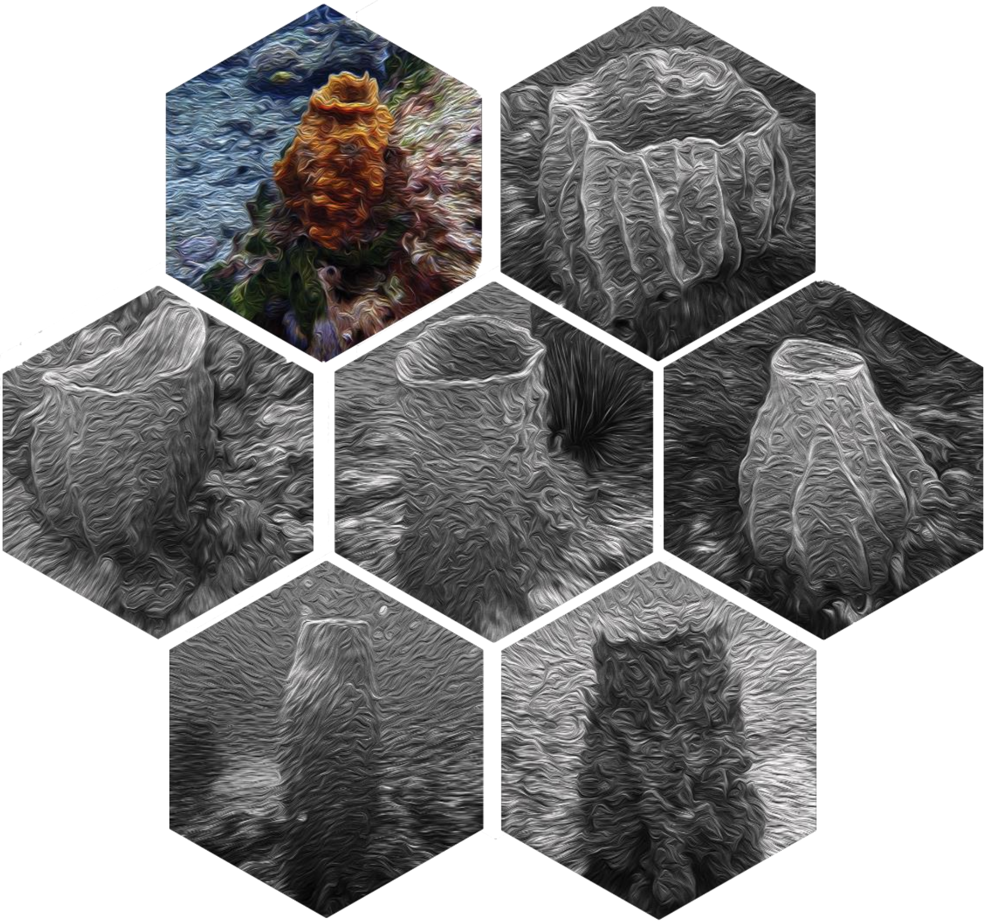

Chapter 1

General Introduction

1. Marine sponges

Oceans possess very biodiverse and characteristic ecosystems that have remained unexplored in their vast majority. However, the rapid progress of technology in recent years has allowed more and more light to be shone on these mysterious ecosystems, revealing their exciting potential. The uniqueness of marine environments, compared with terrestrial ones, is their diversity, as 32 out of 34 of the known phyla on the planet inhabit the oceans, many more than the 17 phyla found in terrestrial environments (Snelgrove 2016). This biodiversity is not limited to the number of species, which is itself impressive at more than an estimated 200,000,000 species, but more importantly, is greatly increased by the interactions among the organisms in the ecosystems (Horton et al. 2020; Mora et al. 2011; Palumbi et al. 2009). The stocks of nutrients in the oceans are very limited and highly localized. Therefore, marine organisms have necessarily needed to develop an efficient way to access and use these resources. Ecosystems, such as coral reefs, mangroves, estuaries, and seagrass beds are examples of the tight relationships between many organisms evolved over time as successful survival strategies (de Goeij et al. 2013). The fact that many of these relations are essentially mediated through chemical compounds, has led to the highly developed chemical diversity observed in marine organisms (Naman et al. 2017). This has boosted their use as a prolific source of chemicals with unique features (Blunt et al. 2018; Carroll et al. 2019, 2020; Gerwick and Moore 2012).

Among the many marine organisms, sponges are one of the most abundant in many ecosystems, both in number and biomass.(Bell et al. 2017; Bell and Carballo 2008; Bell and Smith 2004). Despite this, their ecological roles have not yet been as extensively studied as that of other marine animals like corals. For example, it has been reported that sponges influence the quality and quantity of substrate in coral reefs and other ecosystems (Diaz and Rützler 2001). Some sponge species, known as boring sponges, actively participate in the bioerosion process which transforms the calcareous substrate into smaller particles and while this is a natural process needed in the reef, in some cases it could affect the condition and health of corals as sponges erode their skeletons (Wulff 2006, 2016). On the other hand, the presence of some sponges in the substrate has proved to increase coral survival, especially after storms, by stabilizing the substrate and binding coral fragments (Bell 2008; Swierts, et al. 2018). In addition, sponges are believed to play a vital role in bento-pelagic coupling, as they actively participate in carbon, silicon, and nitrogen cycling, the latter with help of nitrifying bacteria symbionts (Maldonado et al. 2012; Zhang et al. 2019). Due to the strong interaction of sponges with the water column resulting from their massive water pumping, they act as a link between organisms in the water column and higher trophic levels. The relationship of sponges with other organisms can vary in very diverse ways according to the circumstances, from associations with microorganisms as an additional source of energy or chemical defense, to predation and spatial competition with other animals in the reef (Bell 2008; Webster and Taylor 2012; Wulff 2006).

Sponges are considered to be among the most ancient species. Their fossils date back to 535 million years ago that locates their first generation in the early Cambrian (Antcliffe et al. 2014; Li et al. 1998). Moreover, the species diversification of sponges has been traced to the Cambrian period. At an anatomical level, sponges are the most primitive animals, they are filter-feeding animals that use specialized flagellated cells to actively pump water through their bodies in order to absorb the nutrients from the water column (Bergquist 1978). An external layer of flattened cells (pinacocytes) called pinacoderm separates the sponge from the surrounding water. The pinacoderm has pores (ostia) that lead to chambers where flagellated cells (choanocytes) form the choanoderm. These cells are responsible for pumping the water through the sponge. The skeleton of sponges is located in the mesohyl, a layer of connective tissue between the pinacoderm and the choanoderm. This skeleton can be organic (spongin fibers), inorganic (silica or calcium carbonate) or a mix of both (Bergquist 1978). Furthermore, in the mesohyl, a specific type of cells (archeaocytes) can digest dissolved nutrients and microorganism filtered from the water column (Bergquist 1978; Van Soest et al. 2012). However, not all the microorganisms are necessarily digested, as some of them can be incorporated into the mesohyl as symbionts (Hentschel et al. 2006; Webster and Taylor 2012). The amount of symbionts such as bacteria, archaea, and fungi found in sponges can be used to classify sponges in two functionally different groups (Gloeckner et al. 2014). Sponges with bacterial counts of 10^8- 10^{10}, that is 2 to 4 times the order of magnitude than that of the microbial count of the surrounding water, are called high microbial abundance (HMA) sponges. On the other hand, those with bacterial counts of around 10^4-10^6, i.e., similar to those found in the surrounding water, are classified as low microbial abundance (LMA) sponges. These two groups differ also in their filtering capacity.

Aside from these filter -feeding mechanisms, further generalizations on sponges are remarkably complicated due to their great diversity (Bergquist 1978). In the oceans, they are found in many habitats including mangroves, coral reefs, polar regions and deep oceans (Van Soest et al. 2012). A classification based on morphology is also difficult because of the great plasticity they exhibit when adapting to local or environmental conditions (De Vos et al. 1991). So far sponges have been grouped into four classes. The Demospongiae class accounts for above 80% of the living sponge species, and are characterized by a skeleton formed of spongin fibers sometimes complemented with siliceous structures; Hexactinellida, have a siliceous skeleton and are also known as glass sponges; Calcarea, known as calcareous sponges present calcium carbonate spicules in their skeleton; Homoscleromorpha, which was taken out of the Demospongiae class, and is the most recently accepted class (Bergquist 1978; Gazave et al. 2012).

2. Chemical components of sponges: a promising source of bioactive natural products

Sponges have resulted of great interest as a source of bioactive natural compounds from the very beginning of marine organism research (Ebada and Proksch 2012). To date, over 10,000 new metabolites have been reported to be isolated from sponges and in the past decade (2000 – 2010), compounds isolated from marine sponges in particular account for approximately 30% of all compounds isolated from marine organisms (Figure 1.1a). Although this percentage has been decreasing as the number of marine microorganism studies increased, sponges still maintain their status as an abundant source of new chemicals, contributing an average of 200 new compounds every year (Figure 1.1b) (Blunt et al. 2017; Carroll et al. 2019, 2020). Among the sponge phyla, Porifera is considered to be one of the most prolific in terms of the chemical diversity of their metabolites (Paul et al. 2019). From a chemical perspective, sponge metabolites display a wide diversity, revealed not simply by the existence of structural analogues but by the immense variety of chemical structures, which includes terpenoids, alkaloids, peptides, polyketides, steroids, quinones, and fatty acids (Figure 1.1c). Among the chemicals isolated from sponges, terpenoids and alkaloids are undoubtedly the predominant secondary metabolites groups. These two families corresponded to over 60% of all the metabolites isolated from sponges between 2014 and 2018 (Blunt et al. 2016, 2017, 2018; Carroll et al. 2019, 2020).

Sponge metabolites have been reported to display interesting responses to a broad range of bioassays for cytotoxicity, antiviral, antifungal, anti-inflammatory, and antifouling activities among others (Belarbi et al. 2003; Keyzers and Davies-Coleman 2005; Proksch et al. 2010; Sipkema et al. 2005). The presence of these metabolites with strong and diverse activities might be the consequence of the numerous ecological interactions that sponges experience in their natural environment (Perdicaris et al. 2013). Cytotoxic compounds, for example could work to keep predators at bay or as a way to increase their competitiveness in the struggle for space with other sessile organisms (Ye et al. 2015). Interestingly, more than 800 antibiotics together with several antiviral compounds have been reported from sponges, probably having a role in the defense system against pathogens present in the water (Laport et al. 2009; Sagar et al. 2010). In addition, the production of compounds that inhibit the formation of biofilms to prevent fouling can be attributed to an important defense mechanism of sponges to maintain their capacity to filter water (Stowe et al. 2011).

Figure 1.1: A: Percentage of compounds isolated from marine sponges over the total reported from marine organisms between 2001 and 2018. B: Number of new compounds reported from marine sponges between 2001 and 2018. C: Occurrence of secondary metabolites groups isolated from marine sponges between 2014 and 2018.

Marine natural products (MNP) research locations have been concentrated mainly along the continental coastlines due to the technical challenges represented by deep sea exploration. The discovery of MNP's from sponges in particular, is spread all over the world in a remarkable contrast with other marine phyla, due to their widespread distribution (Carroll et al. 2020). For example, the phylum Porifera is the most prolific source of MNP in all climate zones (tropical, subtropical, temperate, and polar), accounting for between 25 and 35% of all compounds reported in each climate zone (Carroll et al. 2020). Sponges have adapted to a large range of environmental conditions all over the world having developed a large arsenal of secondary metabolites as a result of this adaptation (Becerro et al. 2003). The combination of these circumstances, that is, their wide distribution in such distinct environmental conditions could provide a plausible explanation for the enormous variety of sponge MNPs.

Despite the potential of sponges as a great source of bioactive compounds, the number of drugs sourced from them that have been approved for commercialization is very low. So far, only three sponge-derived drugs have been approved by the Food and Drug Administration (FDA) (Gerwick and Moore 2012; Newman and Cragg 2016). The low success rate in the output of drugs is mainly due to the lack of a stable supply of sources required for the development of a new drug. Several solutions have been proposed to overcome this problem, such as aquaculture, mariculture, cultivation of the microsymbionts, and chemical synthesis (Belarbi et al. 2003; Gomes et al. 2016; Sipkema, Osinga, et al. 2005). Moreover, understanding the driving factors involved in the production of active compounds remains too unclear to contribute to the application of some of these solutions.

3. Giant barrel sponges: conspicuous members of the reef

Giant barrel sponges are sponges belonging to the genus *Xestospongia* (order Haplosclerida: class Demospongiae) (Horton et al. 2020; Van Soest et al. 2012) and three extant species, *Xestospongia bergquistia, Xestospongia muta*, and *Xestospongia testudinaria*. Sponges from any of these species have a characteristic barrel-like shape and are large in size, exceeding 1 m in height (Van Soest et al. 2012). *Xestospongia bergquistia* is endemic to Australian reefs, a region in which *X. testudinaria* is also present. These two species can be distinguished by structural differences in their skeleton (Fromont 1991). On the other hand, morphological differences between *X. muta* and *X. testudinaria* are very subtle but are very subtle and it seems that the main difference between these two species comes from their geographical location (Setiawan et al. 2016). While *X. muta* is present in the Caribbean Sea from Florida down to South American coasts, *X. testudinaria* is present in the Indo-Pacific region from the Red Sea, the east coast of Africa up to China and New Caledonia (Van Soest et al. 2020a,b). Moreover, recent studies have shown that the previous classification in two species (*X. muta* and *X. testudinaria*) is an oversimplification of a much more complex situation species-wise, as they include several genetic groups that can be differentiated through certain mitochondrial and nuclear DNA markers (Swierts et al. 2013, 2017). Interestingly, when using this new genetic classification, some groups were found to be genetically more closely related to groups on the other side of the world than to those co-existing in the same location (Swierts et al. 2017).

Giant barrel sponges are present in a wide range of environments. As mentioned, their geographical location is widespread, being found in the Caribbean Sea and the Indo-Pacific region. This is very unusual for sponges because due to the limited traveling capacity of their larvae, many sponge species are usually endemic to specific regions (Van Soest et al. 2012). Furthermore, giant barrel sponges inhabit a very wide depth gradient, from 10 to beyond 100 m depth (Morrow et al. 2016; Olson and Gao 2013). This broad distribution suggests that these sponges have the ability to adapt to the very diverse environmental conditions existing throughout such depth gradients, including temperature, light, pressure, nutrients, and predatory stress. Unsurprisingly, according to the previous reports, both *X. muta* and *X. testudinaria*, present large variations in their morphology, and it is possible to find individuals with a very smooth surface, with digitate or lamellate structures and with different shapes and sizes (Figure 1.2) (Kerr and Kelly-Borges 1993; Swierts et al. 2013).

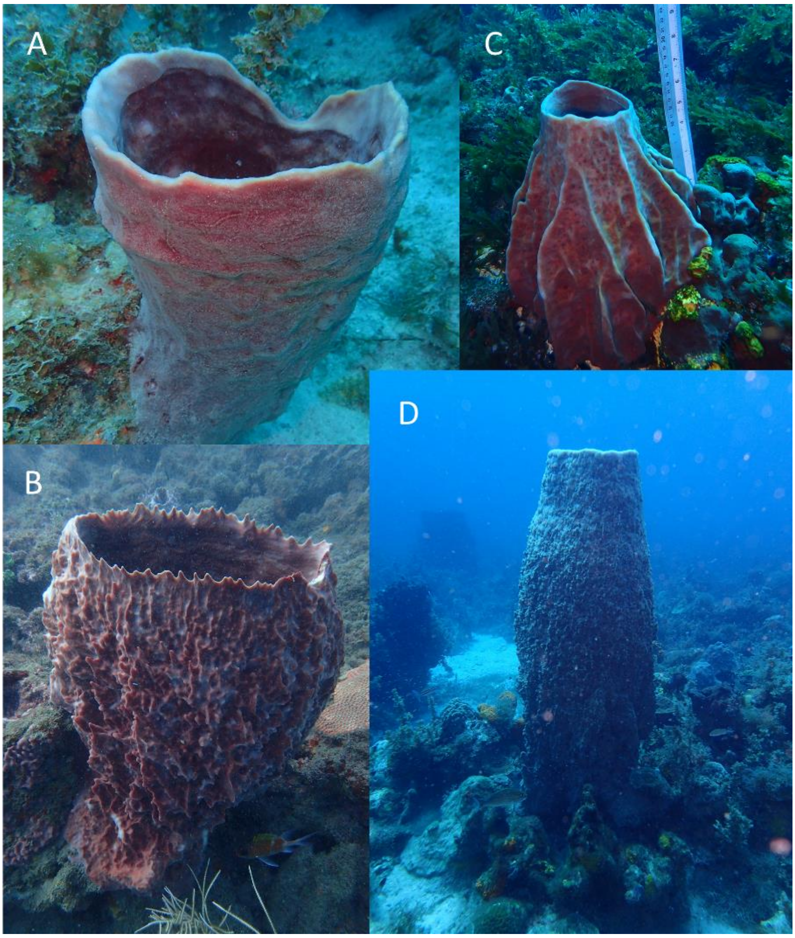

Figure 1.2: Pictures showing different morphologies (A: smooth and B: Digitate) and sizes (C and D) of giant barrel sponges (Photos by Nicole J. de Voogd and Esther van der Ent).

Besides environmental conditions, variations among sponges could also occur with age. *Xestospongia muta* are known as the redwood of the reef, due to their longevity and size (McMurray et al. 2008, 2010). Using measurements such as height, base diameter, and osculum diameter, McMurry and co-workers (McMurray et al. 2008) were able to create a model to calculate the age of *X. muta* in the Caribbean. Using this model, a specimen found in Curaçao was determined to be about 2300 years old, indicating that *X. muta* is one of the oldest animals alive (Van Soest et al. 2012). Similar studies conducted in the Indo-Pacific on *X. testudinaria* revealed that they can grow to similar sizes as their Caribbean counterparts in a shorter time-lapse, which means that sponges in the Indo-Pacific of the same size as those in the Caribbean can be estimated to be much younger (McGrath et al. 2018).

Several studies in the Caribbean have shown *X. muta* to be one of the most abundant animals in the coral reef, covering up to 13 % of the substrate (McMurray et al. 2010; Zea 1993). Although studies on *X. testudinaria* are not as extensive as in the Caribbean, its presence has been reported in reefs throughout the Indo-Pacific (McGrath et al. 2018; Swierts et al. 2013, 2017). Besides, due to their bulky size, giant barrel sponges are very important organisms in the bento-pelagic coupling as they can overturn a 30 m deep water column in only a few days (McMurray et al. 2014). Their large size, particularly their exceptional height, results in a different interaction with the water column compared to that of other benthic organisms as the interference with water currents around them can create microenvironments for nearby organisms (Bell 2008). Furthermore, giant barrel sponges can provide shelter for other organisms in the reef, e.g. corals (Hammerman and García-Hernández 2017; Swiert et al. 2018). Among the large variety of organisms that establish a symbiotic relationship with giant barrel sponges, microsymbionts are considered to be the most influential. These sponges, classified as HMA, host millions of microorganisms in their mesohyl (Gloeckner et al. 2014). These microorganisms have been thought to play an important role in the adaptation and survival of the sponges, though their detailed functions are yet little known (Fan et al. 2012; Moitinho-Silva et al. 2017; Papale et al. 2020). Several bacteria phyla, such as proteobacteria, poribacteria, actinobacteria, and cyanobacteria have been reported to be regularly present in *Xestospongia* spp. microbiome (Fiore et al. 2013; Polónia et al. 2017). However, their relative abundance and the presence of other minor phyla are greatly influenced by environmental and biological factors (Montalvo and Hill 2011; Morrow et al. 2016; Swierts et al. 2018; Villegas-Plazas et al. 2019).

Xestospongia sponges have shown a great potential to produce a variety of chemicals. More than 300 new compounds have been reported so far from the genus including alkaloids, terpenoids, steroids, quinones, and fatty acids (Zhou et al. 2010). Along with the chemical diversity, many of the compounds have shown significant bioactivities such as cytotoxicity as well as antiviral, antibacterial, and anti-inflammatory properties. Particularly, giant barrel sponges have been characterized for the production of sterols and brominated fatty acids.

Among the sterols, some include unusual functional groups such as cyclopropane and branched side chains. In the past, the sterol content in *X. muta* was used as a chemotaxonomy marker to establish the presence of three chemotypes that could be related to the presence of cryptic species among individuals of this sponge (Kerr and Kelly-Borges 1993). Additionally, the presence of acetylenic groups and very long fatty acids have been identified as characteristic of these sponges (Zhou et al. 2015).

4. Metabolomics: a way to gain an insight into the overall metabolome of organisms

Metabolomics is the most recently developed 'omics' technology. Its aim it to obtain a comprehensive overview of metabolome present in biological samples (tissues, organs or biofluids) under a given set of conditions (Kim et al. 2010). One of the biggest challenges in metabolomics studies is how to deal with the great chemical diversity exhibited by small molecules in the samples. In practice, all the metabolites have very different physical and chemical characteristics which makes impossible for one single analytical technique to cover all of them (Dunn and Ellis 2005). Therefore, various analytical platforms must be employed in metabolomics studies in order to widen the coverage of the metabolome. Undoubtedly, liquid chromatography hyphenated to a mass spectrometry detector (LC-MS) and nuclear magnetic resonance (NMR) are recognized as the most inclusive analytical platforms in metabolomics studies (Emwas et al. 2019; Goulitquer et al. 2012; Markley et al. 2017). Naturally, each of them has several advantages and some limitations. Therefore, to achieve the goal of metabolomics, i.e., to obtain a comprehensive understanding of the chemical diversity present in an organism, the best option is a combination of both techniques.

The (sponge) metabolome is composed not only of regularly synthesized compounds but also of those that are induced as a response to a wide range of internal or external factors. Thus, the metabolome is not static, but highly susceptible to the change of environmental, biological, or genetic factors (Bundy et al. 2008; Shulaev et al. 2008). These qualitative and/or quantitative alterations, could affect metabolic levels which in turn could induce changes in genomic and proteomic expression or silencing (Reverter et al. 2016; 2018; Saito and Matsuda 2010). In the case of marine organisms, temperature, salinity, light intensity, pressure, and predatory stress are among the many environmental factors that can induce changes in the metabolome, However, the factor which could be by far the most influential, is the interaction with co-existing organisms. For example, as an HMA sponge, *Xestospongia*'s metabolome is composed not only of the metabolites produced by the sponge *per se*, but also by those that result from the interaction of their metabolism with that of associated microorganisms. Thus, modifications in the microbiome of the sponges might be reflected in alterations in the metabolome of the holobiont.

The study of marine natural products, whether undertaken using a traditional or metabolomics approach, has focused mostly on two fields: the discovery of new compounds with a potential biological activity, or chemical ecology investigations into the role of a certain compound. However, synergistic efforts to study sponge secondary metabolites in general between these two fields have been scarce, and even more so in the case of giant barrel sponges. The holistic overview of the chemical space provided by the metabolomics approach can be used to detect new potential active compounds and at the same time provide indications of the ecological and/or biological drivers that lead to the production of these compounds, seamlessly combining both fields in one collaborative effort.

5. Outline

The chemistry of marine sponges has been widely studied over the past 60 years leading to the discovery of a large number of bioactive compounds. However, the driving factors that control the production of these compounds remain mostly unknown. A deeper understanding of the factors that potentially modify the metabolome of sponges would improve their reliability as sources of bioactive compounds. Furthermore, the rapid change experimented by marine ecosystems in the past decades has made the need for this understanding more imperative as the prediction of the extent of the alterations these changes in the environmental conditions have on biological processes has become critical. Metabolomics, with its capacity to provide a comprehensive overview of the metabolome, can contribute to an increased insight into the biological process while assisting in the discovery of new active compounds.

Using multiplatform-based metabolomics analyses, this thesis will focus on the following research questions: 1) How do extraction parameters influence the chemical diversity of extracts for metabolomics studies? 2) Which environmental factors cause variation in the metabolome of giant barrel sponges? 3) Do aging processes cause changes in the metabolome of giant barrel sponges? 4) Can genetic groups of giant barrels sponges also be distinguished by their metabolome?

This thesis aims to unravel the influence of environmental conditions and genetic background on the chemical production of giant barrel sponges using a metabolomics approach. **Chapter 2** provides an extensive literature review of metabolomics studies of marine organisms. It includes an overview of every step involved in a metabolomics study from the collection of the sample until the identification of compounds. Several examples of applications of metabolomics to the study of marine organisms are shown, and future perspectives of the field are discussed. **Chapter 3** illustrates the effects of extraction conditions (temperature, pressure, number of cycles, and solvent polarity) on the metabolic diversity of *Xestospongia* extracts using a pressurized extraction system. The objective is to optimize the extraction conditions in order to obtain extracts with a composition that accurately reflects the chemical content of the sample, a most important requirement for metabolomics studies. **Chapter 4** shows the differences in the chemical composition of giant barrel sponges collected in four geographical locations, two in the Caribbean Sea (Martinique and Curaçao) and two in the Indo-Pacific region (Tanzania and Taiwan) using LC-MS and NMR based metabolomics. It also explores the relationship between the variation in chemical composition of sponges collected in different locations and their biological activity against *Staphylococcus aureus* and *Escherichia coli*. **Chapter 5** presents the changes in the metabolome of giant barrel sponges belonging to two genetic groups, associated with the age of sponges. It also reveals a differential change in the metabolome of these two genetic groups along a depth gradient (from 7 to 42 m), showing that even very genetically close sponges can react differently to changes in environmental conditions. The changes in the metabolome are established using LC-MS based metabolomics together with molecular networking. **Chapter 6** describes how different genetic groups of giant barrel sponges can be distinguished not only by their genetic markers, but also by their metabolome. This is exemplified by the results of a NMR- and LCMS-based metabolomics study conducted on sponges belonging to three genetic groups collected in the Spermonde archipelago, SW Sulawesi, Indonesia. In addition, changes in the metabolome due to environmental conditions such as pH and temperature, are reported. **Chapter 7** summarizes the main findings of this thesis regarding the chemical production of giant barrel sponges and presents general conclusions and future perspectives about the research in this field.