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

Bayona Maldonado, L.M.

Chapter 7

Concluding remarks and future perspectives

Optimization of Extraction Parameters: obtaining a real metabolome

Since the first report on marine natural products (MNP), marine organisms have been a valuable source of bioactive chemicals due mainly to their chemical diversity and significant bioactivities (Carroll et al. 2019). The exploration of the chemical diversity of marine organisms has increased significantly thanks to the promising results achieved with the implementation of metabolomics, an approach that is based on unbiased chemical profiling that provides a holistic view of their chemical diversity as well as biological activities and ecological functions (Goulitquer et al. 2012; Paul et al. 2019). This profiling technique has allowed the discovery of new compounds that exhibit positive results in different bioassays. But even further, as shown in Chapter 2, the information obtained from the metabolomics studies could be used to unravel the putative ecological functions of many metabolites. However, the great chemical diversity exhibited by MNPs is challenging even for the metabolomics approach. The first and perhaps most difficult challenge encountered is the selection of the extraction method of the samples. Considering that the goal of metabolomics is to study the whole metabolome, it is quite clear that no extraction method could possibly deliver such an extract. The variation in polarity and hydro/lipophilicity of the different metabolites added to their interaction with different matrixes and extreme concentration range make it impossible to extract all metabolites with one single solvent. Notwithstanding this, it is possible at least to optimize conditions related to the extraction process itself that can increase its efficiency in terms of yield and number of metabolites obtained. The influence of factors such as temperature, number of extraction cycles and matrix composition on the chemical diversity of extracts is unsurprising and has been extensively studied (Heavisides et al. 2018; Johnson et al. 2017). Chapter 3 describes the study of extraction parameters such as solvent polarity, temperature, pressure and number of extraction cycles, using a pressure assisted extraction system followed by a Design of Experiment (DOE) analysis. The results showed that the combination of polar solvents like ethanol together with lower extraction temperatures yielded an extract of a giant barrel sponge with the highest chemical diversity. Although these studies provide the first steps towards the development of a protocol for extensive extraction methods, the design of a universal extraction method, particularly for marine sessile organisms, is still far from being a reality. Actually, the understanding of the complex relationship of marine organisms such as corals, algae, and sponges with their symbionts and the debate about which organism really produces many of the MNP could benefit from new extraction strategies which could provide extracts of the host and their symbionts separately.

Redwoods of the reef: Giant barrel sponge age

Marine sponges have been widely studied due to their chemical diversity and the ecological roles as part of many marine ecosystems. Among sponges, giant barrel sponges have drawn particular attention as they are conspicuous organisms in the reefs. In the Caribbean Sea, a growth model of these sponges has shown that they can live for thousands of years, being thus, among the most long-living animals on the planet. In Chapter 5, the study of the influence of the age of sponges on their metabolome showed that the level of phosphatidylcholine phospholipids is higher in older sponges. Moreover, many fatty acids in these phospholipids were found to contain bromine, which have been widely reported in giant barrel sponges. The increase of these lipids, as a constitutive part of the cell membrane, might be related to an increase in the cellular division of the sponge cells. In addition, the higher level of brominated fatty acids in the phospholipids could be related to a chemical defense of the sponge since this kind of compound has been reported to be antiviral, antibacterial and/or cytotoxic (Zhou et al. 2010). From this perspective, these specific fatty acids could play a role in the defense mechanism since giant barrel sponges might use membrane lipids as a storage of these metabolites that can be released by hydrolysis when the sponge is attacked by a predator or a pathogen. This would balance the metabolic cost of these brominated fatty acids (Thoms and Schupp 2007).

The accurate determination of the age of sponges is a difficult task because they lack characteristic markers linked to their age. This difficulty has been circumvented by developing growth models. The Caribbean giant barrel sponge (*Xestospongia muta*) was the first species of sponges for which the age was determined using a growth model (McMurray et al. 2008). Therefore, the influence of the age on the metabolome of sponges shown in Chapter 5 could contribute to the identification of markers of sponge age. Recently, growth models have been developed for other sponge species, which are not as long-lived as *X. muta* (McGrath et al. 2018; Olinger et al. 2019). Studies about whether the age of these sponges can also influence their metabolome could extend the use of chemical markers in the determination of their age. Furthermore, better quantitative information of the relationship between age and chemical production could have implications in the field of drug discovery, as some of the active metabolites may be produced only at certain stages of development (age), a well-known factor in some plants (Yoon et al. 2019). Lastly, the knowledge of age and its effect on sponges could contribute to conservation efforts, such as the creation of artificial reefs. Changes in the metabolome of sponges due to age could affect interactions between sponges and other

animals in the reef, causing differences in the biodiversity between natural and artificial reefs (Perkol-Finkel et al. 2006).

Cryptic species and metabolomics

As a whole, the identification and classification of marine organisms, particularly sponges, still remain an exceedingly difficult task largely because of the ambiguity of their morphologic characteristics which eventually leads to misclassifications and underestimation of the ocean biodiversity. Although the introduction of genetic markers has made it possible to discover cryptic species in many marine taxa, including sponges, the accurate taxonomical classification lags behind (Xavier et al. 2010). Therefore, the possibility of finding cryptic species (two or more different species which are classified as a single species) is quite high. In fact, conserving biodiversity cannot be completed without accurate taxonomical information as well as the knowledge about community stability and population size of the members of an ecosystem (Bickford et al. 2007). Likewise, the commercial use and biotechnological applications of marine sponges, such as the discovery of new bioactive compounds, could be undermined by the utilization of erroneously classified organisms.

The conventional classification of giant barrel sponges species in the past was reviewed in recent studies, and as a result *X. muta* and *X. testudinaria* were divided into nine putative species (genetic groups) (Swierts 2019). Therefore, the differentiation of some of these species from a metabolic perspective was comprehensively studied in Chapter 6, and to a lesser extent in Chapter 5. Chapter 6 shows that the genetic groups present in one location in the Indo-Pacific can be clearly distinguished by their metabolic fingerprint. The difference between genetic groups was mainly attributed to *lyso*-phospholipids that contain phosphatidylcholine. The level of this kind of compound in sponges has been reported to be related to environmental factors as will be discussed in the next section, as well as the reproductive cycle. Most sponges are dioecious, and giant barrel sponges reproduce by mass spawning events in which eggs and sperm are released by different individuals into the environment, a process which thus relies upon the synchronicity of the release of gamete cells. However, spawning events of giant barrel sponges have been observed in different seasons both in the Caribbean and in the Indo-Pacific (Fromont and Bergquist 1994; McMurray et al. 2008; Ritson-Williams et al. 2005) and systematic studies of *X. muta* spawning events also showed some variability at a seasonal, lunar, and temporal scale (Neely and Butler 2020). In this sense, quantitative and qualitative changes in *lyso*- phospholipids contents could indicate that the genetic groups of giant barrel sponges were at different periods in their reproductive

cycle which could result in reproductive isolation of the genetic groups. This would agree with the presence of separate species of giant barrel sponges.

In addition, the differences in the chemical production among the genetic groups of giant barrel sponges can also change the way they interact with other organisms. Previous reports have established that giant barrel sponges display variability in their chemical defenses, implying that the metabolome of sponges of the same population can differ substantially according to their exposure to predators (Chanas and Pawlik 1997; Loh and Pawlik 2014). These observations could be also be related to differences in the metabolic composition of each genetic group since individuals of one or more genetic groups may have a different chemical component in their defense system. This was particular the case in the Caribbean fire sponge, *Tedania ignis,* where the differences in starfish predation of individuals that were supposed to belong to the same species prompted genetic and morphological studies that resulted in the distinction between *T. ignis* and the new species *Tedania klausi*. Lastly, the presence of cryptic species could exacerbate the problem of supply for the discovery of new metabolites as a source of new drugs. For solutions such as mariculture, the selection of the right species for the culture is crucial to secure the production of the active metabolites that are needed. Different species could produce the compound in smaller amounts or eventually produce none at all, resulting in a dead end for this biotechnological approach to get a sustainable supply.

Environmental factors can change the metabolome

As filter feeding organisms, marine sponges have developed an elaborate system to interact with their environment. Therefore, changes in abiotic factors such as light, temperature, pH, salinity, and nutrient concentration can influence the production of secondary metabolites in marine sponges. In Chapters 4, 5 and 6 the effect of different environmental conditions was evaluated to determine their effect on the metabolome of giant barrel sponges. From a global perspective, Chapter 4 shows the differences in the chemical profile of giant barrel sponges collected in two locations in the Caribbean Sea and two in the Indo-Pacific region. The location can be perceived as a complex factor, as multiple conditions such as temperature, pH, predatory stress, water currents, and nutrients might differ. Therefore, the metabolic production observed in each location is the result of the interaction between all these conditions. At the same time, establishing general trends is difficult, as shown for example by the activity of some of the tested extracts against *Staphylococcus aureus*. No correlation was found between the locations and activity of the samples indicating that the production of

active compounds might be triggered either by environmental conditions occurring at a smaller geographical scale or by biotic factors.

In Chapter 5, another complex factor, depth, was evaluated with sponges collected in the Caribbean Sea. It was determined that of the two genetic groups studied, only sponges belonging to genetic group 7 showed a change in their metabolome along the depth gradient. Depth is also considered a complex factor due to variations in conditions experienced by the sponges at different depths. Further aspects related to environment are reported in Chapter 6 which describes the effects of pH and sea surface temperature (SST) of *Xestospongia testudinaria* in Indonesia. Once again, among the three studied genetic groups (groups 1, 2 and 3) only the metabolome of group 1 displayed changes due to differences in SST, while none showed significant variations in the metabolome as a result of differences in pH. Interestingly, in both studies the response of different genetic groups to similar gradients in environmental conditions was different. Even though the classification into nine genetic groups could imply that there are at least nine different species of giant barrel sponges (Swierts 2019), these species are still very closely related. The fact that they respond differently to changes in their environment could indicate that they have different levels of adaptability when exposed to similar conditions. Among others, this could have implications on the survival of these sponges, particularly nowadays, as ocean environments are experiencing extreme changes related to global events such as global warming and ocean acidification. From a conservational perspective, this could mean that one or more of the genetic groups of giant barrel sponges could be less fit to endure the new environmental conditions and these groups could decline or even disappear. This prospect is more worrying if we take into account that the different genetic groups of giant barrel sponges were discovered only a few years ago, and similar cases could be occurring for several other sponge species. Consequently, the lack of adaptation of some of these cryptic species could result in a largely unnoticed loss of biodiversity.

Another aspect that is noteworthy is that the microbiomes of sponges in general, and giant barrel sponges in particular, have been reported to change their composition depending on environmental factors such as geographical location, depth, temperature and season. It is reasonable to anticipate that these changes in the microbiome will be reflected in changes in the metabolome, since the analyzed extracts correspond to the holobiont. However, it is also possible that modifications in the metabolome of the holobiont are not strictly related to the composition of the microbiome but can also respond to the activation of different metabolic pathways in the microorganism associated with the sponge. Extensive studies into the

interactions between sponges and their microsymbionts are therefore needed to gain insight into the production of metabolites and its regulation.

Future perspectives

The use of metabolomics to explore the ecological relationships of marine organisms and as an incentive for the discovery of new molecules from marine sources has provided a holistic perspective to these fields. This interest has been enhanced by the continuous development of technical and bioinformatic tools for metabolomics analysis that has allowed a deeper understanding of the metabolism of marine organism. At present, metabolomics provides only a snapshot, an instantaneous image of the metabolism of an organism at the moment of its collection. However, natural processes are extremely dynamic, and even more so in marine environments, therefore a method that continually monitors the metabolome in small time lapses would provide valuable information about the metabolism of marine organism and the ecological interactions mediated through chemical compounds, which is especially significant in sessile organisms such as sponges. In addition, in the last few years, the debate about which organism produces the secondary metabolites commonly isolated from marine invertebrates is favoring the associated microorganisms. This has increased the interest in the metabolites produced by microorganisms. Yet, while they have proved to be a prolific source of new compounds, in many cases the chemical space occupied by the metabolites isolated from associated microorganisms is very different from the one occupied by metabolites from the holobiont. This thesis explores the changes related to environmental conditions in the metabolome of giant barrel sponges as holobionts. Studies conducted on the composition of the microbiome in similar conditions have also shown that they are similarly affected (Lesser et al. 2016; Montalvo and Hill 2011; Swierts et al. 2018; Villegas-Plazas et al. 2019). However, the intersection between these situations has not really been evaluated. Understanding the correlation between the changes in the microbiome and the metabolome would be a good starting point to unravel the complexity of the networks that connect the sponge cells with the sponge microsymbionts through chemical compounds. The development of technologies such as mass spectrometry imaging and single cell metabolomics, constitute a step in that direction, showing that specific metabolites are accumulated in the sponge cells while others are mainly in the microsymbionts. However, the question of the relocation of compounds that can be used as precursors from the microsymbiont to the sponge cells or vice-versa remains unknown.