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Genomics and metabolomics in the North Atlantic deep-sea sponge *Geodia barretti*

KARIN STEFFEN



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Abstract

Steffen, K. 2022. Genomics and metabolomics in the North Atlantic deep-sea sponge *Geodia barretti*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 305. 73 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1365-8.

Sponges are among the earliest diverging taxa in the animal tree of life. They are sessile, filter-feeding animals found in marine and freshwater habitats. Many species are characterized by a close, specific and consistent association with microbes, mainly Bacteria and Archaea. This feature has been known for a long time and is suggested to be a factor contributing to the rich and diverse chemical output of the sponges. This thesis explored the effect of the habitat, specifically water mass or depth on sponges, their associated microbes, and their combined chemical output. The focal species of this thesis was the North Atlantic deep-sea high microbial abundance (HMA) demosponge *Geodia barretti*.

In **Paper I**, 16S rRNA gene amplicon sequencing and untargeted metabolomics were used to quantify variation in prokaryotic community composition and chemical output in three sponge species. Water masses structured the prokaryotic community composition in the HMA species *G. barretti* and *Stryphnus fortis*. The community composition of the low microbial abundance (LMA) sponge *Weberella bursa* was unaffected by depth. Untargeted metabolomic data was modelled by depth. This allowed for identification of individual compounds varying with depth. Among those compounds were many putative osmolytes as well as diketopiperazines. Bioactive peptides and brominated tryptophan derivatives were unaffected by depth.

In **Paper II** the diversity of the barrettide peptide family was explored in DNA sequencing data and chemical profiles across a wide selection of sponge species and *G. barretti* in particular. Five new barrettides were predicted and one sequence, barrettide C, was confirmed by solid phase peptide synthesis and co-elution with a native extract, antifouling bioassays and NMR structure elucidation. The confidence gained from sequence analysis and validating predictions lead us to suggest barrettides are a family of antifouling peptides in *G. barretti*.

In **Paper III**, a reduced representation sequencing approach was used to evaluate the Stacks de novo pipeline in HMA sponges with the help of a whole genome assembled for this purpose. With this data, gene flow and connectivity were investigated in *G. barretti* populations sampled across the North Atlantic. The de novo pipeline was found to assemble and retain many putatively microbial loci and should thus only be used with reservations in HMA sponges. However, regarding biological inferences, strong population structure was recovered despite the apparent contamination.

Keywords: demosponge, whole genome sequencing, population genetics, peptide synthesis

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Für mein P

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

- I **Steffen, K.***, Indraningrat, A.A.G*, Erngren, I., Haglöf, J., Becking, L.E., Smidt, H., Yashayaev, I., Kenchington, E., Pettersson, C., Cárdenas, P. , Sipkema, D. Go with the flow: oceanographic setting influences the prokaryotic community and metabolome in deep-sea sponges. (Manuscript submitted)

- II **Steffen, K.**, Laborde, Q., Gunasekera, S., Payne, C.D., Rosengren, K. J., Riesgo, A., Göransson, U., Cárdenas, P. Barrettides: A Peptide Family Specifically Produced by the Deep-Sea Sponge *Geodia barretti*. *Journal of Natural Products*. <https://doi.org/10.1021/acs.jnatprod.1c00938>

- III **Steffen, K.**, Arias, M. B., Proux-Wéra, E., Drewery, J., Kenchington, E., Taboada, S., Riesgo, A., Cárdenas, P. Sea for yourself: evaluating the ddRADseq Stacks de novo pipeline with a reference genome in the deep-sea sponge *Geodia barretti*. (Manuscript)

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Abbreviations

ASV Amplicon sequence variant

bp Base pairs

ddRADseq Double digest restriction-site associated DNA sequencing

DNA Deoxyribonucleic acid

ESI Electrospray ionization

HILIC Hydrophilic interaction liquid chromatography

HMA High microbial abundance

HMW High molecular weight

HTS High-throughput sequencing

HRMS High resolution mass spectrometry

LMA Low microbial abundance

NMDS Non-metric multidimensional scaling

NMR Nuclear magnetic resonance

NP Natural product

OPLS Orthogonal projection to latent structures

PacBio Pacific Biosciences

PCA Principal component analysis

PCR Polymerase chain reaction

PERMANOVA Permutational multivariate analysis of variance

RP Reversed phase chromatography

RRS Reduced representation sequencing

RiPP Ribosomally synthesized and post-translationally modified peptide

RNA Ribonucleic acid

SNP Single nucleotide polymorphism

SPPS Solid phase peptide synthesis

UPLC Ultra performance liquid chromatography

Introduction

In the thesis you are reading I describe my research in genomics and metabolomics of deep-sea sponges conducted during my doctoral education. This work centers around the North-Atlantic deep-sea demosponge species *Geodia barretti* Bowerbank, 1858 with the goal to understand what drives the species' chemical richness. To investigate this question, I applied a range of methods from collecting the specimens, via molecular biology, analytical and natural product chemistry, and organic synthesis to bioinformatics. The scale at which the chemical output of *G. barretti* was investigated ranged from geographic dimensions (North Atlantic) to and individuals and their genes and proteins. Chemical output was addressed from different perspectives, by investigating the sponge itself, its associated microbes and the effect of a sponge compound on other organisms (the bay barnacle).

To unify this diversity of methods, scales, and perspectives, I will start by introducing sponges and different aspects of these animals relevant to my thesis work. This is followed by touching upon the different laboratory and data analysis methods used, outlining similarities and characteristics. To conclude the thesis, I will then summarize the three chapters and highlight the main discoveries of this work by integrating the results across the studies in a broader context.

Sponges

Most people relate to sponges by at least one of these two representations: the cartoon character SpongeBob SquarePants or the bath sponge they might have used in the bath. But the taxonomic affiliation of sponges, that is their position in the tree of life or what kind of organism they are, is frequently confounded. Sponges are animals. For perspective, in the tree of life, the branch of multicellular animals (Metazoa), contains five monophyletic groups: Porifera, Placozoa, Ctenophora, Cnidaria, and Bilateria (Adl et al. 2019). Porifera are the sponges. Placozoa are literally called “flat animals” and are so idiosyncratic and unique that there are only three species known to science. Ctenophora are the comb jellies, they look transparent like jellyfish but do not sting. However, they feature

combs made of many small cilia (hairs) that diffract the light giving them a beautiful iridescent look (Welch et al. 2006). Cnidaria is a phylum including e.g. jellyfish, anemone, and corals. Finally, Bilateria comprise all animals you are probably thinking of. That is, animals with a bilateral symmetry in their body plan.

Now, what makes sponges animals is more complex to answer. Today, phylogenetic methods for inferring relationships rely heavily on DNA (and amino acid) sequences for scoring commonalities and differences across the homologous (comparable) sequences from different organisms (Kapli et al. 2020). Applying such methods, sponges fall in the clade of animals. If you are looking for something more tangible to coincide with the phylogenetically recovered group, the definition of Metazoa contains details on their sexual reproduction, characteristics of early embryonic development, their heterotrophic lifestyle and what constitutes their tissues (Adl et al. 2019). The reason why the definition of Metazoa may seem overly specific is that there are so many different lineages of organisms, many of which are not commonly known. Thus, being highly specific is necessary to distinguish between all of this biological diversity.

Evolution

Sponges are not just any animal. Based on both chemical biomarkers preserved in ancient rocks (Zumberge et al. 2018) and molecular phylogenetic evidence (i.e. DNA, amino acid sequences), sponges are hypothesized to be the oldest extant animals (Feuda et al. 2017; Nielsen 2019; Pisani et al. 2015, 2016; Redmond and McLysaght 2021). This evolutionary hypothesis is called “Porifera-sister” hypothesis. But there is a vivid ongoing dispute, fueled by support for the alternative “Ctenophora-sister” hypothesis (Halanych et al. 2016; Li et al. 2021; Whelan et al. 2015, 2017). Oftentimes, sponges are described as ‘primitive’ or ‘simple’ as at a glance, they seem less complex in terms of their body structures (e.g. lacking nerve cells, muscles or organs, or any kind of body symmetry). However, the perceived lack of complexity may be due to the (lack of) effort spent to study them and biases in interpretation of existing traits (Dunn et al. 2015). There are 9 468 extant sponge species described to date (de Voogd et al. 2021; Hooper and van Soest 2002). Those fall into four classes, Calcarea, Hexactinellida, Homoscleromorpha and Demospongiae. The latter class, also referred to as demosponges, is the most speciose. The three species investigated in this thesis, *Geodia barretti* Bowerbank, 1858, the focal species of my studies Fig. 1, as well as *Stryphnus fortis* (Vosmaer, 1885) and *Weberella bursa* Vosmaer, 1885 are all demosponges (de Voogd et al. 2021).



Figure 1. The deep-sea demosponge *Geodia barretti*. Left: sampling on the Swedish West coast, right: view of a Canadian sponge ground at Flemish Cap, 1581 m depth (Canadian DFO/ROPOS 2010).

Reproduction & dispersal

Sponges are generally described as sessile animals, meaning they are fixed to the ground (Hooper and van Soest 2002), a descriptor that applies to the adult sponge. Beyond that, as far as life history traits are concerned, our knowledge of sponges in general and *G. barretti* specifically is limited and fragmentary. *G. barretti* reproduces sexually and is gonochoristic (separate males and females) as inferred from gametes observed in histological sections (Koutsouveli et al. 2020b; Spetland et al. 2007). Its reproductive season extends from late spring to early autumn in the Northeast Atlantic (Koutsouveli et al. 2020a; Spetland et al. 2007), with synchronous gametogenesis at the population level (Koutsouveli et al. 2020a,b). *G. barretti* is oviparous, meaning gametes (oocytes and sperm cells) are released into the water column, where fertilization happens. However, the larvae of *G. barretti* or that of any member of the order Tetractinellida have never been observed (except for the pelagic Thoosina larvae) (Bautista-Guerrero et al. 2010; Carballo et al. 2018; Maldonado 2006). Asexual reproduction has never been observed in *G. barretti* (Spetland et al. 2007). While sperm and oocytes can disperse with the water column, the lack of nutrients in sperm and limited amount of yolk in oocytes makes long distance dispersal or extended survival unlikely (Koutsouveli et al. 2020b). This leads to the conclusion that dispersal of *G. barretti* likely happens at the larval stage via transport with the water column or demersal crawling (Maldonado 2006). We know nothing

of pelagic larval duration of *G. barretti* and very little about other sponge larvae, but it is known that their swimming capacities are limited (Lanna and Riesgo 2020; Maldonado 2006).

Ecology

G. barretti is widespread, inhabiting the continental shelves of the North Atlantic and even coastal waters and fjords in Sweden and Norway, with a documented bathymetric distribution range of 30 to 2000 m (Cárdenas and Rapp 2013, 2015; Cárdenas et al. 2013). Its geographic range also extends into more southern waters like the Cantabrian Sea and the Mediterranean (Cárdenas et al. 2013; Cristobo et al. 2019; Sánchez et al. 2017). Sponges generally can be found growing individually on hard substrate, with *G. barretti* frequently found in large aggregations called sponge grounds (Klitgaard and Tendal 2004; Kutti et al. 2013; Murillo et al. 2012).

In these North Atlantic sponge grounds, *G. barretti* and other key species create a biogenic habitat providing diverse ecosystem functions and services. By providing substrate, they act as reservoir for biodiversity including commercially exploited fish (Beazley et al. 2013; Kenchington et al. 2013; Klitgaard 1995). They contribute to benthopelagic coupling through recycling of dissolved organic matter into more bioavailable particulate organic matter in a process called the “sponge-loop” (Bart et al. 2021; de Goeij et al. 2013; Kutti et al. 2013; Maier et al. 2020). In a similar fashion, sponges contribute to the global biogeochemical cycling of nitrogen and silicon (Maldonado et al. 2020; Maldonado et al. 2021; Rooks et al. 2020). Many of these studies were performed on *G. barretti* and that, together with its propensity to generate dense habitats, led to the species being designated a key sponge ground species.

Today, sponge grounds are threatened due to anthropogenic stressors, mainly bottom contact fishing (Klitgaard and Tendal 2004; Morrison et al. 2020; OSPAR 2017), but also deep-sea mining or oil and gas extraction in some places (Taboada et al. 2018). The United Nations General Assembly Resolution 61/105 (Paragraph 90) (UNGA 2007) highlighted the need for protection of these vulnerable ecosystems, and is resonated in FAO (FAO 2009) and OSPAR (OSPAR 2017) guidelines and assessments, as well as national management decisions (Boletín Oficial del Estado 2011; Gougeon 2020; Kutti et al. 2013; McIntyre et al. 2016).

The number of individuals and also the effective population size in these sponge grounds can be quite large. However, comparison between studies is difficult due to various assessment methods such as video transects

(Kutti et al. 2013) or trawls with different gear and different units of reporting (individuals or biomass per area) (Klitgaard and Tendal 2004; Murillo et al. 2012). For *G. barretti*, population densities have been estimated to 0.3 indiv/m² across an area of 13 by 4 km (1.56×10^7 individuals, corresponding to 4 kg/m²) by analysis of still images from video transects in a Norwegian marine protected area (Kutti et al. 2013). Converting the numbers of total weight after 30 min bottom trawl at 3 knots at the Flemish cap results in population densities estimates somewhat lower (0.0768 kg/m²) (Murillo et al. 2012). To bridge gaps in knowledge of the species distribution and inform future sampling and management decisions, habitat suitability modelling has filled in the gaps of potential sponge grounds in the North Atlantic revealing potentially extensive, connected habitats (Howell et al. 2016; Knudby et al. 2013; Murillo et al. 2018; Roberts et al. 2021). It remains unclear how sponges would be able to maintain such large connected habitats as modelling of larval dispersal and hydrodynamic constraints showed limited dispersal potential (Culwick et al. 2020; Gary et al. 2020; Lanna and Riesgo 2020; Wang et al. 2021). We need to keep in mind these many unknowns about sponge dispersal abilities and sponge ground connectivity, when assessing results from methods new to the field. It may be helpful to consider for instance historic evidence of sponge grounds (Murillo et al. 2016).

Connectivity or gene flow between individuals of a population can be measured directly by using molecular markers (genes, microsatellites, SNPs) (Pérez-Portela and Riesgo 2018). However, apart from three studies (Brown et al. 2017; Busch et al. 2021; Taboada et al. 2018), gene flow has exclusively been investigated in shallow sponge populations. Looking across many other marine invertebrate species, it seems that population connectivity differs markedly between shallow and deep-sea habitats and organisms. A common pattern is populations being more connected in the deep sea (Taylor and Roterman 2017) as compared to typically isolated shallow populations (Pérez-Portela and Riesgo 2018). Thus, the findings from shallow water sponges cannot readily be extended to the deep sea. Additionally, differences in the methodology of measuring gene flow can have an impact on the population genetic inference (Hodel et al. 2017). Typically, sponges have been genotyped for microsatellites which are short repetitive genetic loci variable enough even among close relatives (Bell et al. 2014; Blanquer et al. 2009; Blanquer and Uriz 2010; Chaves-Fonnegra et al. 2015; Dailianis et al. 2011; Duran et al. 2004; Giles et al. 2015; Griffiths et al. 2021, 2020; Guardiola et al. 2012; Padua et al. 2018; Pérez-Portela et al. 2015; Riesgo et al. 2016; Riesgo et al. 2019; Taboada et al. 2018). These microsatellite studies, typically including about six to 14 loci, are technically already multilocus studies. However, they are beginning to be replaced by reduced representation sequencing

(RRS) methods such as ddRADseq which generates even more unlinked loci data (Brown et al. 2017; Busch et al. 2021; Kelly et al. 2021; Leiva et al. 2019). To summarize, **there is no study investigating large-scale connectivity in deep-sea sponges to date.**

Sponge-associated microbes

Sponges were recognized to be colonized by a plethora of microbes in the publications of the first electron micrographs of their mesohyl (their body/“tissue”) (Vacelet 1975; Vacelet and Donadey 1977). These sponge-associated microbes are frequently called symbionts, which is understood in the widest possible sense, i.e. “associated”, without any connotation about the nature of the association (de Bary 1879; Taylor et al. 2007). The sponge and its associated microbes are frequently referred to as “holobiont” (Zilber-Rosenberg and Rosenberg 2008). This term aims to highlight the importance and the many ways microbes contribute to the sponge. It also allows to address the both parts, which is useful as it is frequently not possible to determine whether an observed result or phenotype is caused by the sponge, a symbiont or both. However, this term is currently also used to imply a number of very specific evolutionary assumptions (Bordenstein and Theis 2015) which seem unwarranted (Douglas and Werren 2016; Hammer et al. 2019).

Some sponges contain more bacteria than others lending them the name bacteriosponge (Reiswig 1981). The observation has cemented into a widely recognized dichotomy, denoted by the two categories ‘HMA’ high microbial abundance and ‘LMA’ low microbial abundance sponges (Gloeckner et al. 2014; Hentschel et al. 2003). The respective HMA and LMA microbiota are stable and host specific (Erwin et al. 2012). As HMA sponge microbiota are also richer and characterized by different prokaryotic phyla, the HMA/LMA status of a sponge can be predicted by the taxonomic composition of the microbiota (Moitinho-Silva et al. 2017b). From a global perspective, many prokaryotic phyla are associated with sponges (Moitinho-Silva et al. 2017a; Thomas et al. 2016) and to find the prokaryotes consistently associated, a ‘core microbiota’ concept is sometimes utilized (Schmitt et al. 2012). Another approach to find essential symbionts is the investigation of sponge specific prokaryotic lineages (Hentschel et al. 2002; Simister et al. 2012) with various cut-offs for presence across samples being used in individual studies.

The sponge microbiome research field has generated a large body of knowledge that is too wide to be summarized within the scope of this thesis (the interested reader is referred to Taylor et al. 2007; Freeman et al. 2021). A sizeable fraction of studies are descriptions of the microbial

community composition of individual sponge species, often in connection to sampling across an environmental gradient or variable (Pita et al. 2018). Generally, many of these studies conclude that the sponge microbiota is remarkably stable across the parameter investigated. Examples of such studies are the investigation of sponge microbiota across seasons or time (Erwin et al. 2012; Freeman et al. 2021; Simister et al. 2013), salinity (Glasl et al. 2018), temperature or warming (Erwin et al. 2012; López-Legentil et al. 2010; Pita et al. 2013a; Strand et al. 2017), irradiance (Erwin et al. 2012), latitude or geographic distance (Luter et al. 2012; Pita et al. 2013b), sediment plumes (Luter et al. 2012), transplantation to another habitat (Cárdenas et al. 2014), or depth (Steinert et al. 2016). When some of these studies do report a change, it is in diseased, fatally stressed states or near death situations (e.g. López-Legentil et al. 2010; Webster et al. 2008; Luter et al. 2012) or a sign of cryptic speciation (Kelly et al. 2021). Changes in the microbiota are more frequently reported when investigating individual taxa (e.g. Chloroflexi (Burgsdorf et al. 2014) or ammonia oxidizing archaea (Weigel and Erwin 2017)). This body of literature suggests that large-scale deviations from typical microbiota are rare and it thus seems that once acquired, the community is resilient to a wide range of stressors. Generally, not all sponges react the same way to environmental variation. In addition, experimental methodology or level of replication sometimes limit the detection of changes. Regardless of all these local or global sequencing efforts (Moitinho-Silva et al. 2017a; Thomas et al. 2016), **to date, there is no clearly defined study investigating sponge microbiota across water mass boundaries approaching the entire bathymetric range.**

The aim of these microbiota surveys is to identify taxa changing across an environmental gradient, so that hypotheses can be made about the nature of the contribution of those taxa to the sponge. In addition, ocean warming and/or acidification limits of the sponge may be important for habitat suitability modelling, but in those cases, the microbiota is not necessarily the most suited response variable to measure. Alternatives to these ecological or experimental correlation approaches are emerging. For instance, in large-scale metagenomics the functional repertoire and capabilities of sponge symbionts can be directly assessed from their metagenome assembled genomes (MAGs) (Robbins et al. 2021; Slaby et al. 2017).

A topic of interest and recent new insights is the acquisition of the sponge microbiota. Horizontal (from the environment) and/or vertical (inheritance) processes are accepted modes of acquisition (Koutsouveli et al. 2020b; Oliveira et al. 2020; Sipkema et al. 2015; Taylor et al. 2007; Webster et al. 2010). There is solid evidence that some microbes are inherited vertically (Busch et al. 2020b; Enticknap et al. 2006; Koutsouveli et al.

2020a), but recent work has challenged the generalized view of specificity and reliability of vertical processes (Björk et al. 2019; Cleary et al. 2019).

Regardless of the purpose and results of the microbial surveys, it is important to note that currently the most common method of assessing sponge symbionts is 16S rRNA gene amplicon sequencing. This method only detects prokaryotes, i.e. Bacteria and Archaea, thus systematically excluding microeukaryotes (He et al. 2014; Naim et al. 2017; Rodríguez-Marconi et al. 2015) and viruses (Jahn et al. 2019; Pascelli et al. 2020). Even among the Bacteria, sponge-specific lineages such as the *Candidatus* Poribacteria are known to poorly amplify and thus be underrepresented in 16S rRNA gene sequencing surveys (Podell et al. 2019). This resulting relative abundance data is compositional but oftentimes portrayed as quantitative, which can lead to errors (Morton et al. 2019). In the HMA sponge *G. barretti*, up to 47% of the total sponge volume is occupied by sponge-associated microbes and the extracellular matrix (Leys et al. 2018), see Fig. 2. Its microbiome has been studied for composition and disease states (Luter et al. 2017) as well as from a functional point of view (Radax et al. 2012a,b).

Sponge-derived chemistry

Sponges are widely recognized as a prolific source of natural products (NPs) (Carroll et al. 2020; Ebada and Proksch 2012; Galitz et al. 2021; Kornprobst 2014; Leal et al. 2012), which are compounds produced by organisms. NPs are of interest as drug leads, for agrochemical or industrial purposes. The search for novel NPs is called bioprospecting. Sometimes, NPs are classified into “primary” and “secondary” or “specialized” metabolites but these definitions are difficult to clearly and consistently delineate (Kossel 1891). Lipids and sterols, compounds typically regarded as primary metabolites due to their role in cell membranes, are considered to be of sponge origin (Djerassi and Silva 1991; Kornprobst 2014; Silva et al. 1991) and the sterol biosynthetic capabilities of sponges have been shown (Gold et al. 2016). However, for all other compounds, it remains unknown whether they are produced by the sponge or an associated microbe. In *G. barretti*, several NP compound classes have been studied (reviewed in Bohlin et al. 2017). For the compounds subsequently mentioned as “identified”, their identity was confirmed by MS/MS spectra and retention time of a reference compound or their structure fully elucidated by NMR. Otherwise the compound or feature identity was “annotated” (Sumner et al. 2007).

- The sterols ergosta-5,24(28)-dien-3- β -ol (24-methylenecholesterol), stigmasta-5,24(28)-dien-3- β -ol (fucosterol), 24,28-dehydroaplysterol

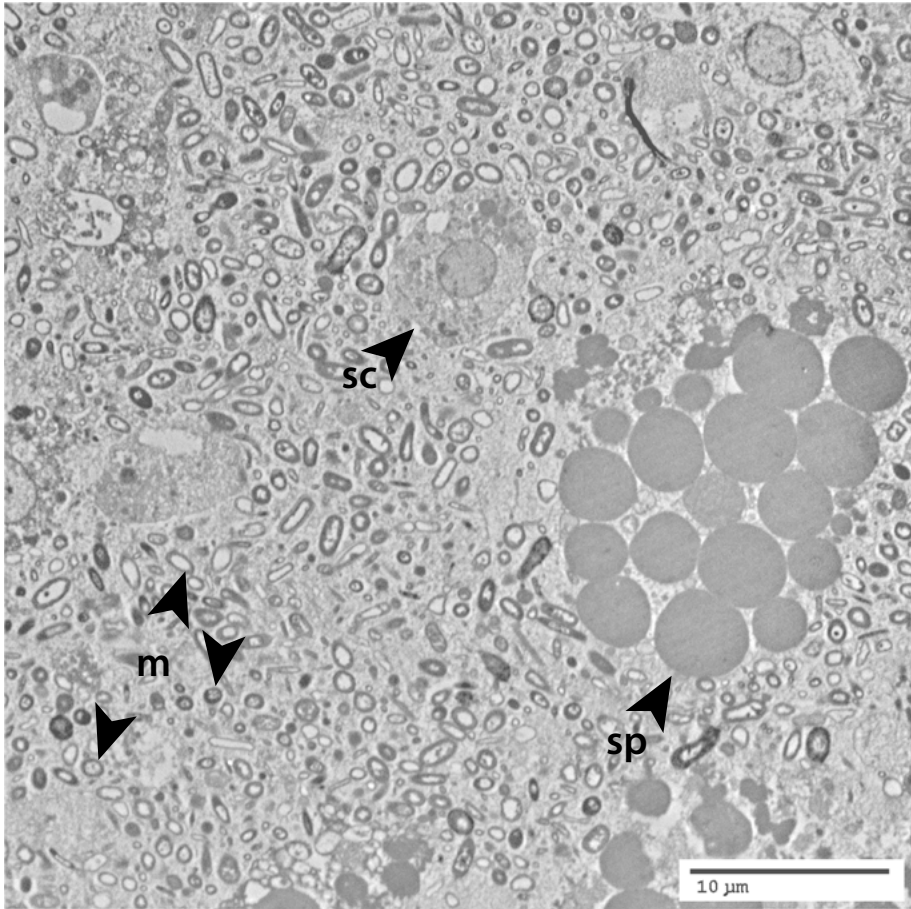


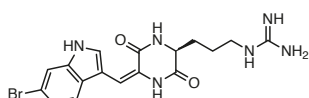
Figure 2. Transmission electron micrograph of *G. barretti* mesohyl showing a few sponge cells, sc (including a sphaerulous cell, sp) and many microbial cells, m. Picture courtesy of Vasiliki Koutsouveli NHM/GEOMAR, and Ana Riesgo, NHM/MNCN.

were identified, as well as an additional 6 sterols annotated by their mass spectra (Hougaard et al. 1991b).

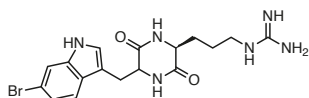
- Fatty acids identified in *G. barretti* include four long chain fatty acids composed of 26, 27, and 28 carbon atoms (C₂₆ 5,9, C₂₇ 5,9 branched and unbranched, C₂₈ 5,9) (Thiel et al. 2002). More recently, the fatty acid composition was assessed with LC-MS annotating 532 lipids, among which 155 triacylglycerides, 96 free fatty acids, 62 oxylipins, 61 phosphatidylcholines, and 26 sphingolipids and glycosphingolipids (Koutsouveli et al. 2021).
- The quaternary ammonium compounds glycine betaine, β -alanine betaine, γ -aminobutyric acid betaine, and the tetramethylammonium ion have been identified from *G. barretti* by Hougaard et al. (1991a). Choline, phosphocholine, glycerophosphocholine, carnitine, and acetylcarnitine were identified and choline sulfate and 2-methylbutyrylcarnitine annotated by Erngren et al. (2021) respectively.
- Nucleosides, N-methylated nucleosides and (modified) nucleobases/purines identified include adenosine, inosine, 3-methylcytidine, 3-methyl-2'-deoxycytidine, 3-methyl-2'-deoxyuridine (Lidgren et al. 1988), and uridine (Erngren et al. 2021).
- Two peptides, the barrettides A and B composed of 31 amino acid residues and containing two intramolecular disulfide bonds were identified (Carstens et al. 2015).
- Particularly brominated Trp-Arg 2,5-diketopiperazines (DKPs) appear characteristic for *G. barretti*. These DKPs are represented by baretin (Lidgren et al. 1986), 8,9-dihydrobaretin (Sjögren et al. 2004), 8,9-dihydro-8-hydroxybaretin (Sölter 2004), bromobenzisoxalone baretin (Hedner et al. 2006), and geobarrettins A and B (Di et al. 2018). Additionally, one unbrominated 2,5-DKP cyclo(Pro-Arg) was identified (Sölter 2004).
- The tryptophan derivatives or indoles 6-bromoconicamin, 6-bromo-8-hydroxyconicamin (Olsen et al. 2016), geobarrettin C and L-6-bromohypaphorine (Di et al. 2018) have been identified.
- Other compounds identified include herbipolin (Ackermann and List 1957a), histamine (Ackermann and List 1957b), arsenobetaine **Paper I**, taurine (Hougaard et al. 1991a). The lack of a reference compound for uranidine (Cimino et al. 1984) hinders confirmation of the compound via LC-MS.

This richness in compounds containing tryptophan moieties is intriguing as it is an essential amino acid and not found in *G. barretti* in its free form (Hougaard et al. 1991b). Apart from isolation and structure elucidation of individual compounds, addressing natural product diversity in a com-

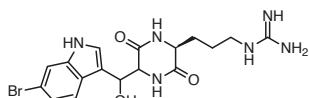
**2,5-diketopiperazines
(barettings: Trp-Arg)**



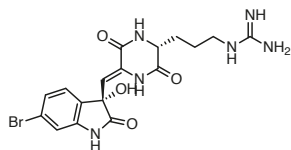
m/z 419.0826 [M+H]⁺ baretin
(*E:Z*; 87:13) (Trp-L-Arg, Trp-D-Arg; 81:19)



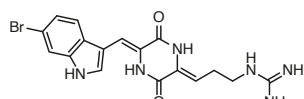
m/z 421.0982 [M+H]⁺ 8,9-dihydrobaretin



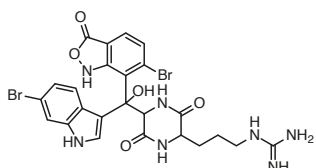
m/z 437.0937 [M+H]⁺ 8,9-dihydro-8-hydroxy-baretin



m/z 451.0728 [M+H]⁺ geobaretin A

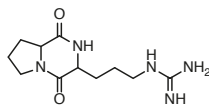


m/z 417.0675 [M+H]⁺ geobaretin B



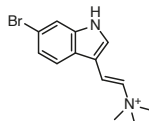
m/z 648.0200 [M+H]⁺ bromobenzisoxalone baretin

**2,5-diketopiperazines
(Pro-Arg)**

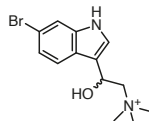


m/z 254.16 [M+H]⁺ Cl-4 (L-Arg-D-Pro)
verpacamide A (L-Arg-L-Pro)

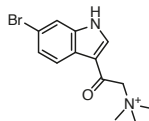
**(Brominated) tryptophan
derivatives, indoles**



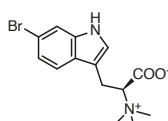
m/z 279.0491 [M]⁺ 6-bromoconicamin



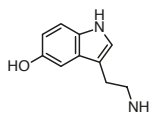
m/z 297.0586 [M]⁺ 6-bromo-8-hydroxy-conicamin



m/z 295.0440 [M]⁺ geobaretin C



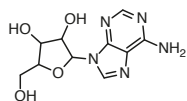
m/z 325.0550 [M+H]⁺ L-6-bromohypaphorine



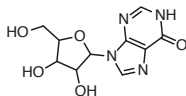
m/z 176.0950 [M+H]⁺ serotonin*

Figure 3. Compounds isolated from *G. barretti*.

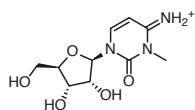
Nucleosides



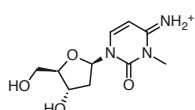
m/z 268.1040 [M+H]⁺
adenosine



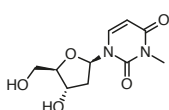
m/z 269.0880 [M+H]⁺
inosine



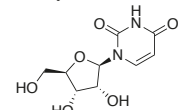
3-methylcytidine (3mCyd)



3-methyl-2'-deoxycytidine
(3mdCyd)

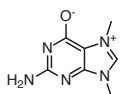


3-methyl-2'-deoxyuridine
(3mdUrd)

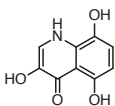


m/z 152.034 [Fragment]⁺
uridine

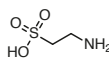
Miscellaneous



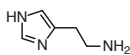
m/z 180.0880 [M+H]⁺
herbipoline



m/z 194.0448 [M+H]⁺
uranidine*



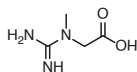
m/z 126.0219 [M+H]⁺
taurine



m/z 112.0869 [M+H]⁺
histamine



m/z 179.0048 [M+H]⁺
arsenobetaine



m/z 132.0768 [M+H]⁺
creatine*

Quaternary ammonium compounds

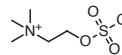
m/z 74.0970 [M]⁺
tetramethylammonium ion



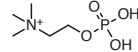
m/z 104.107 [M]⁺
choline



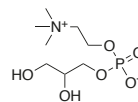
m/z 184.0638 [M+H]⁺
choline sulfate*



m/z 184.0733 [M]⁺
phosphocholine



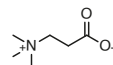
m/z 258.110 [M]⁺
glycerophosphocholine



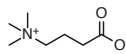
m/z 118.0863 [M+H]⁺
glycine betaine



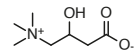
m/z 132.1019 [M+H]⁺
 β -alanine betaine



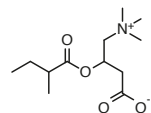
m/z 146 [M+H]⁺
 γ -aminobutyric acid betain



m/z 162.1125 [M+H]⁺
carnitine



m/z 246.1700 [M+H]⁺
2-methylbutyrylcarnitine*



m/z 204.123 [M+H]⁺
acetylcarnitine

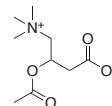


Figure 4. Compounds isolated from *G. barretti*. *compounds annotated, not confirmed with reference compounds.

prehensive manner through metabolomics has recently been validated in *G. barretti* (Erngren et al. 2021).

To illustrate structural diversity and similarities of these *G. barretti* compounds, a selection is displayed in Fig. 3 and Fig. 4, arranged for structural similarity. Many of these compounds have been tested for bioactivities (frequently as a requirement for publication of a novel NP):

- Antifouling activity was reported for baretin, 8,9-dihydrobaretin (Sjögren et al. 2004), bromobenzisoxalone baretin (Hedner et al. 2006), and barrettides A and B (Carstens et al. 2015).
- Acetylcholinesterase inhibition was reported for baretin, 8,9-dihydrobaretin, and 6-bromoconicamin (Olsen et al. 2016).
- Anti-inflammatory activity was reported for baretin (Lind et al. 2013), geobarrettins B and C (Di et al. 2018).
- Selective 5-HT (serotonin) receptor binding was reported for baretin and 8,9-dihydrobaretin (Hedner et al. 2006).
- Beyond these more commonly tested activities, baretin was found to be an antioxidant (Lind et al. 2013) and L-6-bromohypaphorine an agonist of the human $\alpha 7$ nicotinic acetylcholine receptor (Kasheverov et al. 2015).

These bioactivities are interesting from a pharmaceutical perspective, even though they might not be the compounds' function in nature. The compounds may be produced to perform very different activities within the sponge, among its symbionts or outside of it. Regardless of the true function, the multitude of bioactivities in many sponge-derived NPs has translated into four approved drugs (and one derivative) and three more compounds in clinical trials. Cytarabine (Cytosar-U®), a nucleoside derived from *Tectitethya crypta*, Gemcitabine (*generic*), a fluorinated derivative of cytarabine, and Panobinostat (Farydak®) a hydroxamic acid from *Psammaphysilla* sp. are cytostatika. Another compound Eribulin Mesylate (Halaven®), a macrolide from *Halichondria okadai*, is an antineoplastic. All four compounds are prescribed to treat cancer. Another antiviral drug, the nucleoside Vidarabine (Arasena A®) derived from *Tectitethya crypta*, has been discontinued in favour of other drugs. In phase II trials is a polyketide (Plocabulin (PM184)) from *Lithoplocamiamia lithistoides* for solid tumours and in phase I are two antibody drug conjugates, one based on eribulin (MORAb-202) and one based on hemisterlin (STRO-002), both for different cancer forms (Mayer and Pierce 2021).

Historically, localization of a compound in sponge cells (or cell fractions) as opposed to bacteria (or bacterial cell fractions) was stated as proof of animal origin (Roué et al. 2010; Ternon et al. 2016; Thompson et al. 1983; Turon et al. 2000). However, the intracellular presence of biosynthetic

bacteria challenges the universal validity of this line of evidence (Simmons et al. 2008; Tianero et al. 2019). Instead, many studies have elucidated the microbial producer of the NP of interest (El Maddah et al. 2019; Mohanty et al. 2020b; Podell et al. 2020; Rust et al. 2020; Storey et al. 2020; Tianero et al. 2019; Unson et al. 1994; Wilson et al. 2014). From a NP chemistry point of view, this route could be considered favourable, as it would allow cloning of genes, heterologous expression and production of the compounds of interest in larger quantities. Thereby, difficulties arising from growing microbial cultures, sponge aquaculture (Conkling et al. 2019; Gutleben et al. 2020) and NP isolation could be circumvented. Discovering biosynthetic gene clusters could also feed into models to discover more such genes in the future (Blin et al. 2021; Kautsar et al. 2019).

A number of scenarios to explain the presence of NP in sponges are conceivable. Compounds can indeed be produced by the sponge, by a microbial symbiont, by both, or they can be taken up from the environment, i.e. through diet (Morita and Schmidt 2018). Compared to bacteria, fungi and plants, animals stand out with their paucity of NP and even toxins first believed to be of animal origin, but subsequently found to derive from their diet (Morita and Schmidt 2018; Torres et al. 2020; Torres and Schmidt 2019). In this context we highlight that **for none of the *G. barretti* compounds (Fig. 3 and 4) has a definitive producer been established.**

Methods

Across the projects summarized later in this thesis, many experiments can be described as “high-throughput” or “big data” studies. The term loosely refers to large quantities of data that require specialized analysis tools. These are 16S rRNA gene sequencing data and untargeted metabolomics data in **Paper I**, transcriptome sequencing data in **Paper II**, and whole genome and ddRADseq data in **Paper III**. While I have not been personally involved in the production and processing of the 16S rRNA gene sequencing, I have analyzed the pipeline output. Equally, I have not participated in the sequencing of transcriptomes but have made use of data kindly provided by collaborators or available online. Subsequently, I will describe what it entails to generate and work with high-throughput data.

Genomics, metabolomics and “big data” production

(Untargeted) Metabolomics

The metabolome is the realm of small molecules (metabolites), typically within 50-1500 Da (Engskog et al. 2016), and metabolomics is the study of those molecules. In **Paper I**, metabolites were extracted from lyophilized sponge sample. Once dry, the sample is very brittle and can be manually ground to a fine powder with a spatula in a glass vial. This breaks open cells and membranes sufficiently, and the metabolites can be dissolved by addition and incubation in a suitable solvent. After spinning down remaining undissolved debris, which in the case of sponges includes the spicules, the supernatant that can be transferred to a new vial contains the metabolites. The solvent can be removed by evaporation or lyophilization and the dry metabolite extract can be stored at -80°C until analysis. As the mesohyl contains both sponge cells and all its associated microbes, the metabolome contains sponge and microbe metabolites alike.

Importantly, this sample preparation is straightforward, which is important. Downstream metabolomics data analysis is extremely sensitive to even small changes and the fewer steps involved, the less noise will be introduced in the resulting data (Engskog et al. 2016). Metabolomics experiments can include lots of samples, so swift and repeatable methods are paramount. Key aspects are randomization of samples throughout all steps, preparing a homogeneous bulk of every consumable used, e.g. using the same batch of glass vials, the same batch of extraction solvent, and weighing in exactly the same amount of starting material (Engskog et al. 2016).

I will skip the technicalities of UPLC-HRMS data acquisition and state that the method used in **Paper I** measures individual ionized molecules (the ‘HRMS’ part) as they elute from a chromatographic column, separating the molecules according to polarity (the ‘UPLC’ part) (Theodoridis et al. 2012). Unsurprisingly, there are many molecules in an extract of sponge sample and so the result is a large data file with all ions recorded at any given time (very narrow discrete intervals). These raw data files can be in a proprietary format and need to be converted and processed to produce the usable data.

High-throughput sequencing

The field of molecular biology, although by name dealing with many molecules, has a strong proclivity for DNA as molecule of interest (according to my biased experience). From DNA, we can derive several ‘-omes’. The genome is the heritable information of an organism. When DNA is actively used, short sections are reproduced, i.e. transcribed into

RNA which constitutes the transcriptome. Similar to the metabolome, to extract DNA, the cells within the sponge choanosome are broken open (this time more carefully and with more steps), then the DNA can be isolated. As for the metabolome, the resulting DNA is a mixture of sponge, microbial, organellar and environmental DNA (or RNA, depending on extraction method) (Mariani et al. 2019). The other techniques applied in this thesis, 16S rRNA gene sequencing and ddRADseq (Baird et al. 2008; Peterson et al. 2012), introduce additional laboratory processing steps but all start off with genomic DNA. The steps between DNA extraction and sequencing are called sequencing “library preparation”, with the main aspects of them being (1) to ensure the right (sized) DNA fragments are present, and (2) adding various adapters and modifications to the DNA fragments depending on the sequencing platform and experimental design (Goodwin et al. 2016). At the end of the laboratory workflow typically remains an Eppendorf tube with a few hundred microliters of clear liquid containing the DNA to be shipped to the sequencing facility. If everything goes well, the sequencing facility will send a download link for the data a couple of weeks later.

In the three chapters, various kinds of sequencing data (“reads”) generated on both Illumina and PacBio platforms were used (Goodwin et al. 2016). Without going too much into detail, Illumina sequencing generally returns short DNA reads (150-350 bp) whereas PacBio has the potential to produce much longer reads (~20 kbp and over) (Goodwin et al. 2016; Wenger et al. 2019). Both platforms can produce several millions of reads from the DNA fragments, although Illumina typically produces more reads. The output of DNA sequencing is a simple text file containing these hundreds of millions of reads, i.e. text sequences made up of the letters A, C, G, T (and N) corresponding to the four nucleotides and typically also a complementary line of letters and symbols to indicate the quality (confidence) of the nucleotide in the DNA sequence.

Genomics, metabolomics and “big data”/multivariate analyses

(Untargeted) Metabolomics

Starting from raw data coming off the different instruments, I think of the downstream *in silico* work in two phases: data processing and data analysis. Data processing entails checkpoints, programs and decisions to be taken to produce the final data set. Data analysis entails interrogation of this final data set. Depending on your experimental design, this could be performing correlation tests or other statistics, ordination, or multivariate analyses.

For metabolomics, the R (R Core Team 2016) packages XCMS and CAMERA (Kuhl et al. 2012; Smith et al. 2006) were used to translate the raw mass spectra into (“anonymous”) numerical data tables. Typically, these data tables contain the signal (area under the curve, in arbitrary units, or “intensity”) for every feature (m/z with its retention time) across all samples. When using XCMS, a number of decisions have to be taken, defining e.g. peak width, a method for retention time deviation for features/peak alignment across the samples, and what is discarded as noise. Setting these parameters requires knowledge about the sensitivity and resolution of instrumentation used and the effects of changing parameters should be evaluated. Proper estimation and evaluation of the effects of all these decisions is not trivial or intuitive. In **Paper I**, given the lack of prior truly comparable work (see Erngren et al. 2021, published since), we conducted UPLC-HRMS experiments both with positive and negative ionization mode, and two different chromatographic columns (RP and HILIC), for a total of four of these data tables to be analyzed. During processing, low confidence features were removed by calculating the coefficient of variation (CV) across so-called ‘quality control samples’ for every feature and eliminating features with a CV >30%. The quality control samples were also used as a benchmark for evaluating different data transformations. Once all steps of the processing are completed, the data table(s) can be used for multivariate analyses such as PCA and OPLS. Features that stood out in our analyses were manually identified (m/z), validated with additional experiments and then used in correlation analyses.

High-throughput sequencing

For DNA sequencing, data processing starts with quality control and potential mitigation of flaws in the raw data, followed by the use of standard pipelines (16S rRNA gene sequencing pipelines in **Paper I**, Trinity (Bryant et al. 2017) in **Paper II**, Stacks (Catchen et al. 2013; Rochette and Catchen 2017) in **Paper III**) or testing and evaluating various individual strategies (whole genome assembly, see methods in **Paper III**), depending on the experiment. Many times, these assembly methods require access to a high performance computing infrastructure. After assembling the reads, contamination removal, taxonomic annotation or the like may be performed. The final data set is typically an assembly (genome, transcriptome) or derived data matrices (vcf file for ddRADseq, OTU table for 16S rRNA sequencing). At this stage, the genome or transcriptome can be annotated, interrogated for the presence or differences in abundance of genes of interest, correlating these results to phenotypes, or performing various ecologic and/or statistical analyses.

In my experience, the bioinformatics field developing algorithms and programs for DNA sequencing data analysis is dynamic and the vast majority are freely available open-source tools. Although there are also some open-source tools and packages for metabolomics analyses, too (MZmine (Pluskal et al. 2010), XCMS (Smith et al. 2006), CAMERA (Kuhl et al. 2012), ROPLS (Thévenot et al. 2015), the Galaxy platform (Afgan et al. 2018)) but there are also considerable hurdles with proprietary data formats and instrument vendor software. As an example, Waters MS^E cannot be readily transformed into MS/MS data for GNPS networking. Data deposition, sharing and reusing seems more commonplace in the DNA sequencing community than the metabolomics community. For example, sequence repositories like GenBank or UniProt/Swiss-Prot are free and publicly available, while chemical compound databases are frequently not freely accessible and not necessarily up-to-date (comprehensively reviewed in Sorokina and Steinbeck 2020).

As in untargeted metabolomics, many times we cannot directly foresee the consequences of choosing one program over another or how different settings affect our biological conclusions. However, there is ample evidence that these decisions have a large impact on the results (Ioannidis 2005; Markova-Raina and Petrov 2011). Crucially, with these big data approaches and correlations, we generate hypotheses. These hypotheses need to be tested in a properly designed follow-up experiment. Generally, coming to the same conclusions by using different methods and/or different data can give more confidence in an analysis. More fundamentally, we need to evaluate whether the results fit with our first principles, i.e. whether they are sound from a biological perspective.

Sources of error

Errors can occur across all in silico and lab work in science. When performing the lab work, contamination in metabolomic experiments can arise from foreign chemicals being present on lab ware or in reagents, and contamination in DNA sequencing experiments could stem from foreign DNA (e.g. human, skin bacteria, other organisms studied in the lab). In untargeted metabolomics, the choice of extraction solvent defines which compound classes will go into solution. In both kinds of experiments, samples can be mixed up, batch effects can be a source of error (Gilad and Mizrahi-Man 2015), and randomization can help to avoid it (Meir-mans 2015). Specific to DNA sequencing are errors arising from DNA polymerase inaccuracies involved in almost all protocols, or the “universal” primers used in 16S rRNA gene amplicon sequencing which are known to selectively exclude taxa (Podell et al. 2019).

During metabolomics data acquisition, the overall mass spectrometric signal can drift, which means results systematically degrade (increase or decrease) over time thus gradually affecting every sample more. Processes like adduct formation, fragmentation, generation of differently charged ions, isotopes, dimerization (and combinations thereof) in the ion source lead to multiple detection of the same compound (Cech and Enke 2001). Matrix effects such as ion suppression can affect detection of individual analytes. DNA sequencing errors are platform specific, e.g. Illumina clusters getting out of sync, or reading through adapters/primers, or PacBio sequencing producing random indels. Some of these are easily detected and mitigated: primer or adapter sequences can be removed *in silico*, and low quality base calls can be trimmed (Andrews 2010; Bolger et al. 2014). Sequencing the same molecule repeatedly drastically decreases error rates in PacBio sequencing (Wenger et al. 2019).

During processing, we transform and recode the original data. Any processing program and tool confers its own bias or can contain bugs (Markova-Raina and Petrov 2011). In addition, given the huge quantity of data collected, multiple testing can lead to false positive results or distorted statistics (Ioannidis 2005) and different analyses and their outputs can be inappropriate or misinterpreted (Elhaik 2021; Lawson et al. 2018; Meirmans 2015; Morton et al. 2019).

Beyond the high-throughput experiments, a number of stand-alone methods were used during the work in this thesis. These methods include three instances of field work (scientific cruises), solid phase peptide synthesis and oxidative folding, HPLC-MS and HPLC-UV for crude peptide purification, and NMR data acquisition.

Thesis aims

Understanding factors affecting the richness and diversity of NPs in *Geodia barretti* by

- assessing variations in the chemical output of the sponge and its microbes as a function of habitat depth (**Paper I**)
- assessing and linking variations of the microbiota and the metabolome (**Paper I**)
- investigating the diversity and distribution of barrettides (**Paper II**)
- investigating the extent and impact of population structure/gene flow for future decisions for bioprospecting (**Paper III**)

Developing and providing molecular resources for deep-sea sponges, specifically *G. barretti* by

- acquiring the metabolome (**Paper I**)
- assessing sponge-associated microbial communities (**Paper I**)
- investigating the genetic underpinning of sponge-derived peptides (**Paper II**)
- generating a whole genome assembly (**Paper III**)
- validating population genetic methods (**Paper III**)

By understanding large-scale patterns influencing sponges and their microbial symbionts, meaningful plans not only for bioprospecting but also marine stewardship and conservation efforts can be made. Questions addressed in the present thesis were mainly investigated in the boreal deep-sea demosponge *Geodia barretti* but several of the findings have more general validity for sponge grounds and beyond.

Study summaries

Summary of Study I

Go with the flow: oceanographic setting influences the prokaryotic community and metabolome in deep-sea sponges

Steffen, K.*, Indraningrat, A.A.G*, Erngren, I., Haglöf, J., Becking, L.E., Smidt, H., Yashayaev, I., Kenchington, E., Pettersson, C., Cárdenas, P. , Sijkema, D.

shared first authorship shared last authorship

Goal We aimed to investigate the relationship between depth and the sponge-associated microbiota and metabolomes. We sampled 52 specimens (20 of *Geodia barretti*, 15 of *Stryphnus fortis*, and 17 of *Weberella bursa*) originating from the same region of the Davis Strait between Canada and Greenland ranging from depths between 244 m and 1476 m. Oceanographic parameters (water masses) for the region were established from temperature and salinity measurements. Microbiota were surveyed by 16S rRNA gene amplicon sequencing and the metabolome was based on metabolic features (m/z) acquired via UPLC-HRMS.

Key findings Sponge-associated prokaryotic communities had a stable composition at phylum level across samples of the same species (and even across HMA sponges) which is in accordance with the literature (Pita et al. 2018). Interestingly, the HMA sponges *G. barretti* and *S. fortis* shared 316 ASVs, which amounts to 75% and 68% of their total ASVs respectively. At the same time, “individual prokaryotes” as approximated by unique ASVs varied by depth. In the high microbial abundance (HMA) sponges *G. barretti* and *S. fortis*, microbial composition differed significantly above and below 1000 m and these groups are recovered in clustering analyses. Over a third (35.5% by number, 45% by average relative abundance) and a fifth (21% by both ways of counting) of all microbial ASVs correlated with depth (increasing or decreasing) in *G. barretti* and *S. fortis*, respectively. In the LMA species *W. bursa*, microbes varied as well but to a lesser extent and without statistical significance across water masses. Overall metabolomes varied with depth as inferred from multivariate modelling. Identification of individual compounds underlying this variation with depth revealed several potentially osmoregulating

compounds, which seems plausible with regards to the changes in salinity across depth. Interestingly, several members of the barettins, bioactive 2,5-diketopiperazines associated with *G. barretti* also varied with depth. Through correlation analyses of microbial relative abundance and relative signal of bioactive compounds, we present future testable hypotheses about the prokaryote(s) producing barettins in *G. barretti*.

Significance Finding such clear structure in the microbiota is noteworthy as sponge microbiota are typically described as stable (Erwin et al. 2012; Kutti et al. 2013; Luter et al. 2017; Pita et al. 2018). In parallel to the present study, Busch et al. (2020a) conducted similar work on sponge microbiota sampled at different depths around a sea mount and find an effect of seabed bathymetry on sponge-associated microbes, too. Few other studies have addressed deep-sea sponge-associated microbes in general but not necessarily in a comparable manner (Díez-Vives et al. 2020; Kennedy et al. 2014; Steinert et al. 2020). Untargeted metabolomics is a method rarely employed in sponges (Bayona et al. 2020; Mohanty et al. 2020a,b; Reverter et al. 2016, 2018; Ternon et al. 2017; Villegas-Plazas et al. 2019), which is somewhat surprising given their renown chemical diversity (Blunt et al. 2018; Kornprobst 2014; Leal et al. 2012). What can be gained from metabolomics is information useful for supporting phylogenetic or taxonomic hypotheses (Galitz et al. 2021), but also insight into chemical ecology of the animal (Carstens et al. 2015; Hedner et al. 2008; Pawlik et al. 1995; Proksch 1994; Sjögren et al. 2006; Slaby et al. 2017).

Summary of Study II

Barrettides: A Peptide Family Specifically Produced by the Deep-Sea Sponge *Geodia barretti*

Steffen, K., Laborde, Q., Gunasekera, S., Payne, C.D., Rosengren, K. J., Riesgo, A., Göransson, U., Cárdenas, P.

Goal This study aimed to discover new bioactive peptides with similarity to barrettides A and B from the deep-sea demosponge *G. barretti*, and to investigate the distribution of barrettides within the phylum. Peptide diversity and distribution were investigated through high-throughput sequencing data mining from sponges in general and with a particular emphasis on *G. barretti*. To evaluate predictions of new peptides from transcriptomes, barrettide C was produced by solid phase peptide synthesis (SPPS) and oxidative folding, and investigated with LC-MS, NMR, and an antifouling bioassay.

Key findings We discovered five additional barrettide sequences from high-throughput sequencing data from sponges (70 transcriptomes, one metatranscriptome, one genome). We followed up the predictions by finding ions (m/z) corresponding to two of the new predictions (barrettides C and D) in previously acquired chemical profiles of *G. barretti*. We validated the identity of barrettide C by co-injection of synthetic peptide and native extract. NMR spectroscopy and a bioassay highlighted the similarity of barrettide C in structure and function to barrettides A and B. Neither transcriptomes nor chemical profiles of other sponges, even close relatives of *G. barretti*, contained barrettide sequences or ions. By investigating the transcripts and finding sequences translating to barrettides in the genome, we hypothesize that the sponge is the producer of the barrettides.

Significance Despite much research on sponge natural product chemistry, definitive proof on sponges producing any of the bioactive natural products derived from them is lacking. Our work here not only illustrated the ease of peptide discovery from pre-existing DNA sequencing data. It also showcased in depth investigation of transcripts and a range of conclusions that could be drawn from it. Errors in our reasoning, contamination, or future new insights may invalidate our hypothesis about the sponge producer of the barrettides. The ultimate proof would be a localization assay such as fluorescence in situ hybridization (FISH) with a probe against the barrettide core nucleotide sequence (O'Connor 2008).

Summary of Study III

Sea for yourself: evaluating the ddRADseq de novo pipeline with a reference genome in the deep-sea sponge *Geodia barretti*

Steffen, K., Arias, M. B., Proux-Wéra, E., Drewery, J., Kenchington, E., Taboada, S., Riesgo, A., Cárdenas, P.

Goal This study aimed to investigate two current issues in sponge population genetics and connectivity: (I) to establish whether total DNA based methods like ddRADseq can be applied to sponges, and (II) to elucidate population connectivity across large geographic scales and dispersal barriers (water masses). These goals were addressed by generating a whole genome de novo assembly and ddRADseq population level SNP data of 163 individuals of the deep-sea demosponge *G. barretti* sampled across the North Atlantic.

Key findings Regarding the technical aspect (I), we found that 96.5% of all de novo assembled loci and 34% of the final loci produced by the de novo pipeline were not validated by the genome. This highlights the potentially large fraction of microbial loci in the ddRADseq data. It also shows that current data filtering methods cannot replace a reference genome for ground truthing anonymous loci in complex systems such as sponges. Interestingly, the population genetic inferences based on the data did not reveal drastic differences. We found that sponge population structure and connectivity (II) is strongly affected by water masses. Populations along northern European coasts and shelves, the Barents Sea, and even the Davis Strait (northern Canada) connected by the North Atlantic current and AMOC were genetically highly similar. Populations from greater depths (Rosemary Bank) or geographically far (Spain) and thus beyond the influence of this current system were genetically clearly different.

Significance With regards to RRS/ddRADseq being adopted by the sponge research community (Brown et al. 2017; Busch et al. 2021; Leiva et al. 2019) it is paramount to ensure validity of the approach. But, this question is not only applicable to sponges but also to other marine invertebrates with high amounts of associated microbes such as corals (Takata et al. 2021; Titus and Daly 2018). We show that albeit highly contaminated, results appeared approximately faithful, a finding that might reflect the consistent association of certain microbes with the sponge, or the stability of the tested analysis methods to noise. However, while sponge microbiota are typically described as stable, this notion might weaken with more extensive sampling of different habitats (Björk et al.

2019; Busch et al. 2020a; Díez-Vives et al. 2020; Meirmans 2015; Pante et al. 2015; Steinert et al. 2020). Hence, caution is still warranted when applying ddRADseq or similar RRS strategies in highly complex systems.

Conclusions and future perspectives

Deep-sea sponges are chronically undersampled. For example the sponge microbiome project (Moitinho-Silva et al. 2017a; Thomas et al. 2016) which surveyed the sponge-associated microbial communities across 3569 sponge samples only contains 99 samples collected below 200 m (2.7%), 11 of those below 800 m depth. For comparison, **Paper I** and **Paper III** of this thesis combined amount to 189 samples collected below 200 m, 63 of which collected below 800 m (Figure 5). Yet, in the grand scheme of things, this is still greatly insufficient and inappropriate as it excludes the vast majority of the sea floor and thus potential habitats of deep-sea sponges (Figure 6).

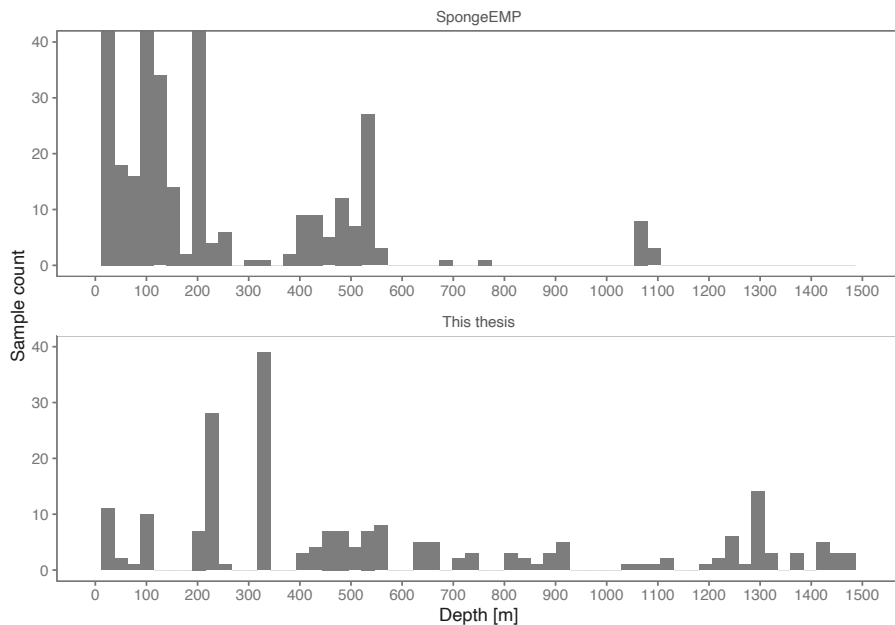


Figure 5. Number of samples binned by depth from the sponge microbiome project 'SpongeEMP' (Moitinho-Silva et al., 2017) and this thesis. Shallow bins of SpongeEMP are truncated.

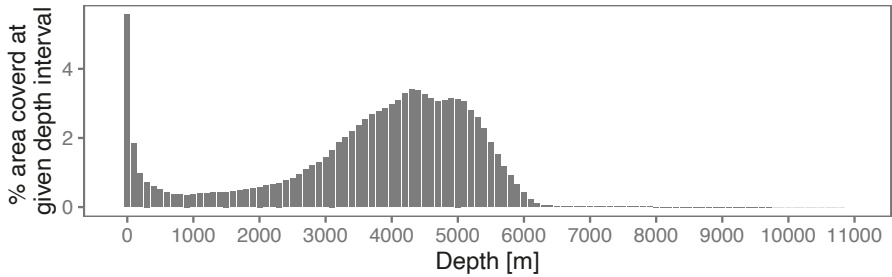


Figure 6. Global underwater area coverage by depth derived from NOAA ETOPO1 Global Relief Model (Amante and Eakins 2009).

Water masses impact the sponge and its microbes

Conceptually, depth is a proxy for (combinations of) other environmental parameters. In shallow water depth gradients, expected environmental changes include (but are not limited to) temperature decreases across the thermocline and decrease in irradiance, whereas in larger depth ranges, hydrostatic pressure (Gerringer et al. 2017; Ritchie et al. 2018; Winnikoff et al. 2021; Yancey et al. 2002) and water masses (dispersal barriers or corridors, characteristic nutrient or oxygen composition) (Murillo et al. 2018; Roberts et al. 2021) may be the important environmental variables. In this context, oceanographic expertise is indispensable to assess the current situation (e.g. Fig. 7, **Paper I**). However, as depth is easy to measure, it is frequently used as an environmental parameter in marine biology studies.

Regarding research investigating “depth effects”, it is important to note that there are general terminological inconsistencies as to what is referred to as the “deep sea”, and “deep”. A number of cut-offs can be found in the literature such as the depths below 200 m coinciding with the continental shelf break and the beginning of the twilight zone where photosynthetic life can no longer sustain itself (Levinton 2017; Ramirez-Llodra et al. 2010). Another cut-off is 1000 m depth, the beginning of the aphotic zone (Ramirez-Llodra et al. 2010). Within the deep sea, specific biogeographic regions can be delimited such as the lower bathyal (801-3500 m) and the abyssal (3501-6500 m) zones (Watling et al. 2013).

In addition, the notions of “shallow” and “deep” depend on study authors and sample distribution, with fairly shallow depth gradient sampling strategies as in e.g. 9-28 m (Villegas-Plazas et al. 2019), 10-90 m (Olson and Gao 2013), 10-90 m (Morrow et al. 2016), compared to specifically deep-sea sampling as in e.g. 4012-4460 m (Taboada et al. 2018), 748-1500 m (Kennedy et al. 2014), 15-960 m (Reveillaud et al. 2014), 580-2184 m (Busch et al. 2020a), 303-1522 m (Busch et al. 2021), 472-

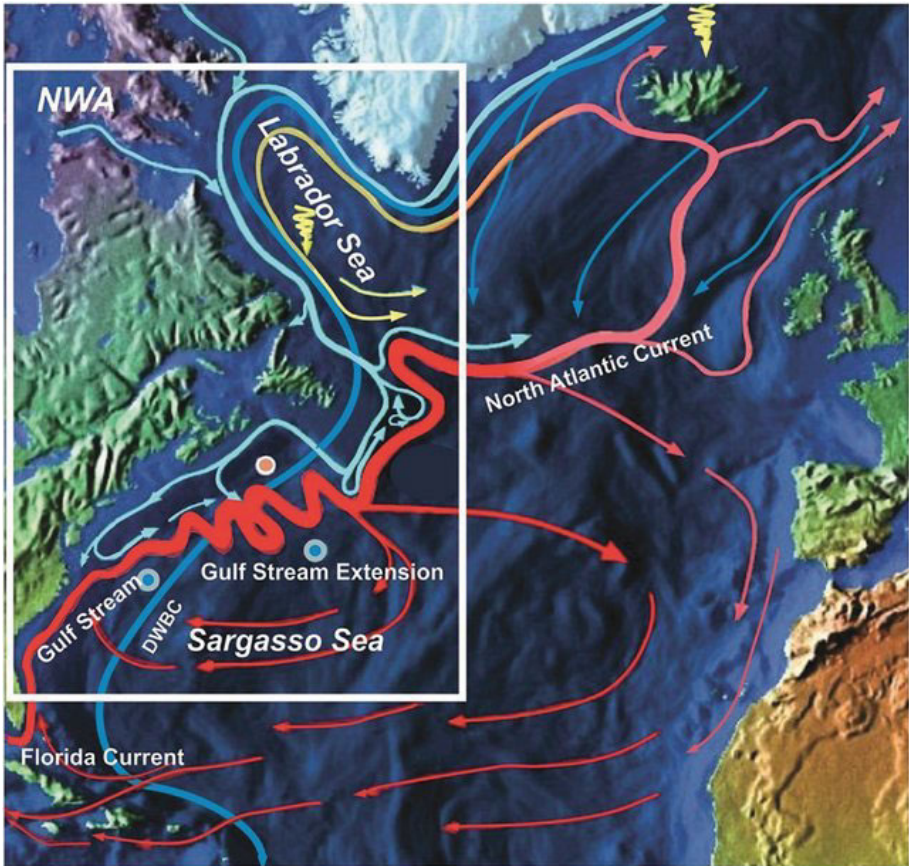


Figure 7. The North West Atlantic (NWA) current system. DWBC Deep Western Boundary Current. Figure courtesy of Igor Yashayaev, DFO.

4106 m (Steinert et al. 2020), 244-1476 m (**Paper I**), and 28-1560 m (**Paper III**).

The effect of water masses applies directly to the sponges. Preliminary results of population genetic inference in **Paper III** indicated that *G. barretti* populations were connected via the AMOC (North Atlantic current, Irminger current, Labrador Sea Water) (Bower et al. 2002). **Paper III** was the first ocean-scale study of deep-sea sponge connectivity. At this scale, we showed that deep-sea sponge grounds were much more connected via current systems than their shallow counterparts (Pérez-Portela and Riesgo 2018). It also highlighted the importance of oceanographic analyses or depth as a proxy, as one population (Scotland, Rosemary Bank) collected from even deeper waters and one population poorly connected to the main current (Spain, Le Danois Bank) (Bower et al. 2002) potentially show decreased gene flow with the remaining populations. However, a number of questions remain, mainly relating the sponge larvae and how they would reach the water column to be transported with the currents. The sponge ground physical structure slows down flows (Culwick et al. 2020) and for true long distance dispersal, the larvae need to swim upwards into the water column (Gary et al. 2020), a behavior that can be observed for some larvae (Lanna and Riesgo 2020).

Water masses also influence the sponge microbiota. Early studies on deep-sea sponge-associated microbiota include isolation and cultivation of individual deep-sea microbes (Brück et al. 2008; Romanenko et al. 2008; Romanenko et al. 2005) or estimating differences in microbial diversity by denaturing gradient gel electrophoresis and FISH (Brück et al. 2010; Meyer and Kuever 2008). High-throughput sequencing approaches include a 16S rRNA gene amplicon survey reporting similar bacterial communities comparing shallow and deep specimens of a HMA species (*Stelletta normani*), as well as shared and individual components of microbiota across LMA sponge (*Lissodendoryx diversichela*, *Poecillastra compressa*, *Inflatella pellicula*) (Kennedy et al. 2014). In addition, ammonia oxidizing Thaumarchaea increased markedly (Kennedy et al. 2014), a finding corroborated by metatranscriptomics, CARD-FISH and qPCR, and 16S rRNA gene amplicon sequencing in several species including *G. barretti* (Jackson et al. 2013; Radax et al. 2012a,b). Overall, this work demonstrated that tenets like the stability and host specificity of sponge microbiota also hold in deep-sea sponges. However, recent work has added that seafloor geomorphology (a sea mount) and oceanic fronts can influence seawater and sometimes in sponge microbiota (Busch et al. 2020a; Reveillaud et al. 2014; Steinert et al. 2020). Indeed, given our understanding of sponge microbiota acquisition by horizontal and vertical processes (Björk et al. 2019; Koutsouveli et al. 2020a), the environment, i.e. the water column around the maturing sponge should be an important predictor for

microbial (dis-)similarity. Despite being generally portrayed as the largest habitat on earth (Levinton 2017; Ramirez-Llodra et al. 2010), the ocean is structured vertically by different water masses (e.g. in the North West Atlantic Yashayaev and Loder 2017; Yashayaev 2007; Rhein et al. 2017) which affects water microbial composition (Frank et al. 2016; Galand et al. 2010; Müller et al. 2018). The results from **Paper I** thus confirm those observations, and extend them to the sponge microbiota. By a sampling approximating a natural experiment across biologically relevant environmental gradients such as the Irminger current and Labrador Sea Water, significant changes can even be observed in HMA sponge microbiota.

In contrast, across the whole sponge EMP data set (Moitinho-Silva et al. 2017a), the water temperature and host type (HMA or LMA) seemed to structure the sponge microbiota (Lurgi et al. 2019). This indicates that confounding factors and insufficient sampling (see Fig. 5,6) may bias our understanding of the sponge microbiota dynamics. However, greater sampling efforts and improvements of analytical methods may reduce that bias in the future (Martino et al. 2021).

A surprising finding in **Paper I** is that the microbiota of *G. barretti* (420 ASVs) and *S. fortis* (461 ASVs) had 316 ASVs in common. Generally, the sponge microbiota are described as host specific and can even be indicators of cryptic speciation (Kelly et al. 2021). Indeed, the large fraction of shared ASVs in **Paper I** could be a misconception based on the amplified 16S rRNA gene region not containing enough variation to discriminate between different prokaryotic strains. However, *G. barretti* and *S. fortis* often live in close proximity and horizontal processes could be the dominating effect structuring their microbiota (Björk et al. 2019; Cleary et al. 2019). This could add to the stronger recovery of water mass related changes in these two HMA sponges.

Sponge natural products and their producers

Most of the *G. barretti* NPs described to date are small molecules (e.g. Fig. 3 and 4). Among these NPs, the compound class 2,5-DKP is typically attributed to bacteria (Abbamondi et al. 2014; Borgman et al. 2019), although this has not been investigated for any of the barettings. The richness in microbes present in *G. barretti* (**Paper I**) makes it difficult to hypothesize or designate a microbial producer. However, to enable future targeted investigations following the suggested microbial origin we provided testable hypotheses about putative producer(s) of the barettings by correlating variations in baretting signals with microbial relative abundance. In contrast to the barettings, other small molecules with structural

similarity to tryptophan as well as the peptides barrettides A, B and C did not show variation with depth and thus similar suggestions are not possible (**Paper I**, **Paper II**).

Natural product research has traditionally focused on small molecules (up to 1500 Da), thereby excluding most peptides. Especially from a pharmaceutical viewpoint, peptides were considered unsuitable drug leads due to inherent properties such as serum instability and low oral bioavailability (and violating several aspects of Lipinski’s rule of 5). However, that view is rapidly changing, as among the drugs generating the largest revenue are many “new modalities” or biologicals, including peptides (Lau and Dunn 2018; Valeur et al. 2017). Many of those peptides are analogues of human peptides, but with serendipity and great effort, also peptide natural products have become FDA approved drugs (e.g. enalapril derived from a viper, bivalirudin derived from leeches, exenatide derived from the Gila monster, ziconotide derived from a cone snail) (Lau and Dunn 2018). In this context, finding five new (bioactive) peptides in **Paper II** is a contribution to the developing field and literature of peptide NPs. One aspect about the discovery making our results remarkable is that we found the amino acid sequences encoded in the transcriptomes and the genome of *G. barretti*. This allowed us to investigate the architecture of the genes applying the framework for ribosomally synthesized and post-translationally modified peptides (RiPPs) (Arnison et al. 2013) and hypothesize that the true producer is the sponge. This hypothesis could be tested with a probe designed to bind to the nucleotide sequence, which would reveal the localization of the gene producing the peptide. Unequivocally attributing this natural product to the sponge would be an important contribution towards elucidating animal natural product potential. Thus far, most compounds are found to be acquired through diet or derived from a symbiotic producer (Morita and Schmidt 2018; Torres and Schmidt 2019), but few genuine other examples exist (Torres et al. 2020).

High microbial abundance sponges are a challenging study system

The properties that make sponges interesting from a scientific point of view also make them really hard to work with. The abundance of microbes, especially in HMA sponges such as *G. barretti*, complicates all laboratory methods based on DNA extracted from sponges for several reasons.

- i) When extracting high molecular weight (HMW) DNA, we observed DNA degradation that was challenging to mitigate. As we started from freshly collected, flash-frozen samples, our hypothesis was that upon thawing and tissue lysis, restriction endonucleases from sponge-associated microbes start degrading the DNA. Abundant presence of such enzymes was reported from annotated MAGs in another sponge (Slaby et al. 2017). We found that macerating and lysing tissue in high concentrations of EDTA mitigated degradation.
- ii) Additionally, the overwhelming fraction of total DNA was of microbial origin and thus contamination for the DNA sequencing of this thesis (Mariani et al. 2019). These challenges were showcased in the overwhelming majority (96.6%) of de novo assembled loci not mapping against the reference genome (**Paper III**). Likewise, the sequencing effort as well as exploration of different assembly strategies and thus computational resources necessary for the whole genome assembly (**Paper III**) was largely due to contamination. Different assembly strategies (genomic, metagenomic, long-read, short read, hybrid) yielded very different genome lengths and the metagenomic assembly with metaSPAdes for instance required >1TB memory (**Paper III**).
- iii) The sponge chemistry also affected DNA quality. Many sponge compounds are known to bind DNA thereby directly inhibiting DNA amplification and sequencing (**Paper III**). The richness and uniqueness in compounds also poses a challenge for metabolomics due to the vast majority of features being unannotated and unknown (**Paper I**).
- iv) Sponges are underrepresented in DNA sequence databases. To date, only a handful of sponge genomes are deposited in public databases. The lack of reference sequences was obvious in **Paper III** (Fig. S5, S6) where trying to taxonomically assign ddRADseq loci only yielded 13 or 14 “eukaryotic” loci. This equally posed a challenge for the whole genome assembly as verification of the sponge origin of assembled contigs relied on general eukaryotic reference databases (and *G. barretti* transcriptomes) instead of more specific sponge genomes.

Future perspectives

Intraspecies variation in microbial composition in *G. barretti* and *S. fortis* in **Paper I**, as well as the large fraction of shared microbes among these two species, opens the floor to investigations of community assembly processes. For example, for microbes taken up from the environment, is it the

sponge that selects its microbes or a neutral succession process or combinations thereof? Can we replicate these findings (microbiota changes across water mass boundaries) in other comparable localities with different species? In a wider sense, it also poses the question as to whether both typical sponge and environmental sampling in sponge microbiota studies are sufficient.

The producer of the barrettides **Paper II** should be unambiguously identified by establishing the localization of the barrettide producing gene. Fluorescence in situ hybridization assays, (fluorescent probes hybridizing with barrettide RNA) could be used to investigate that question. Alternatively, should metagenomic data become available, it would be interesting to search for barrettide sequences and other biosynthetic genes (cyclodipeptide synthases) in metagenome assembled genomes (MAGs).

The data from **Paper III** offers great opportunities to further explore population genetic analyses in a complex system and a number of additional questions could be addressed with it. i) Repeating the analysis with much less filtering, ultimately producing a more sparse data set (vcf file) but with more individuals. As we have shown in the results thus far, even stringent filtering was unable to sufficiently remove microbial loci. Maybe this filtering did more harm than good, given that in doing so, most of the data was excluded (Hodel et al. 2017; Titus and Daly 2018). We are working on downstream processing the data in a more lenient fashion following Cerca et al. (2021). In addition, ii) we initially focused on the popular pipeline Stacks (Catchen et al. 2013; Rochette and Catchen 2017), as it is currently being adopted by the sponge population genetic community (Busch et al. 2021; Kelly et al. 2021; Leiva et al. 2019). However, it would be interesting to evaluate whether alternative approaches better suit this unique study system. There are a number of alternative pipelines that could be evaluated against Stacks, such as GATK (Auwera et al. 2013), DiscoSnp-RAD (Gauthier et al. 2020), ipyrad (Eaton and Overcast 2020), SAMtools (Li and Durbin 2009), dDocent (Puritz et al. 2014) with free-Bayes genotype caller (Garrison and Marth 2012), or ANGSD producing population genetic inference from genotype likelihoods instead of fixed genotypes (Korneliussen et al. 2014). Finally, iii) with a genome annotation, it would be interesting to investigate particular loci under selection and thereby discover particular deep-sea adaptations.

One challenge of **Paper III** was the lack of biological knowledge and observations to validate our findings. If taken at face value, the results of high connectivity could imply well-dispersing larvae with long planktonic larval duration in *G. barretti* which we should be able to observe in nature. Alternatively, we should consider other demographic histories that could produce similar population genetic inference such as range expansions or

recolonization events (Slatkin 1993; Taylor and Roterman 2017). Historic evidence of sponge grounds can help infer demographic past (Murillo et al. 2016), or different putative scenarios could be modeled and evaluated, e.g. with DILS (Fraïsse et al. 2021), or fastsimcoal (Excoffier and Foll 2011).

Svensk sammanfattning

Under min doktorandutbildning har jag forskat på svampdjur. Svampdjur är “systrar” till alla andra djur, dvs att de var först med att avvika från alla andra djur under evolutionen. Det kan vara lite svårt att få grepp om vad svampdjur egentligen är, för man påträffar dem ju väldigt sällan. Du kanske känner till den tecknade seriefiguren SvampBob Fyrkant eller så har du kanske sett eller använt en tvättsvamp någon gång? Tvättsvampen är skelettet av vissa svampdjur, efter att man har tagit bort all v vнад omkring.

Det finns över 9000 svampdjursarter, och jag har mestadels arbetat med en enda art *Geodia barretti* eller fotbollssvamp(-djur). Inuti svampdjurens kropp finns det väldigt många mikrober, mestadels bakterier och arkéer. Dessa bakterier anses ligga bakom produktionen av många så kallade kemiska naturprodukter. Detta är molekyler byggda från olika organismer, med syften vi inte känner till. Dessa naturprodukter finner framför allt användning inom läkemedelsforskning då svampdjur är välkända för sin rikedom av olika molekyler.

Det finns många saker vi inte förstår kring dessa naturprodukter, t.ex. producerar samma svampdjursart samma molekyler oavsett vilken miljö de lever i? Har svampdjuren alltid samma mikrober i sig, oavsett vilken miljö de lever i? Kan vi kanske få fram vilken av alla de hundratal mikrober som ligger bakom en viss molekyl?

Jag har försökt reda ut dessa frågor med hjälp av modern molekylärbio-
logi, kemi och massor av dataanalys. Jag har jämfört enstaka basut-
byten i arvmassan från många svampdjursprov från hela nordatlanten
och kommit fram till att alla djur som inte lever djupare än 1000 m är
väldigt lika varandra. Vi tror att detta beror på Golfströmmen, en stor
havsström som hjälper till med spridningen av svampdjurens könsceller
och larver. Däremot lever de svampdjur som befinner sig djupare än 1000
m isolerat. Det betyder att de med tiden har hunnit bli mer annorlunda
jämfört med de “grundare” individerna. Det betyder också att vi måste
skydda deras miljö särskilt, då de är unika.

Under en annan studie har jag jämfört mikrober och kemi mellan
djupa och grunda svampdjursindivider. Jag hittade tydliga skillnader,
fast på en väldigt liten skala. I en art byts över en tredjedel av alla

mikrober ut mellan grunda och djupa individer. Likaså upptäckte vi att vissa av deras naturprodukter inte fanns i de djupa individerna. Eftersom både mikrober och kemin varierar kunde vi undersöka vilka av mikroberna som skulle kunna stå för produktionen av en grupp av naturprodukter så kallade "barrettiner". Men dessa hypoteser behöver undersökas mer noggrant innan vet vi säkert. Nu har vi i alla fall inte flera hundra, utan bara ett fåtal kandidater att koncentrera oss på.

Jag har även utgått från två andra tidigare kända naturprodukter och letat upp dem i många olika svampdjurs arvs massa. På så sätt har jag kunnat hitta fem nya av dessa så kallade peptider (barrettider). Det verkar rimligt att anta att det är svampdjuren själva som producerar dessa peptider, inte mikroberna. Helt säkra kan vi dock ännu inte vara.

Sammanlagt har jag därmed beskrivit skillnader inom själva svampdjuren, deras mikrober och deras kemi särskilt med hänsyn till djupet på havsbotten där de lever. Detta kan vara till nytta för att skydda dem, men också för att upptäcka nya naturprodukter för framtida läkemedel. Att antingen dyka djupt i havet eller djupt i data kan alltså vara en genväg till nya läkemedel.

Acknowledgements

I wish to express my deepest gratitude to **Paco**, my main supervisor throughout my doctoral studies. With this position, you have given me the chance to grow as researcher and as person. You have been (and still are) a fantastic role model: perseverant, patient and kind, always open to new ideas and new collaborations. You let me explore and roam wherever I wanted, and at the same time, you were there to discuss and support me when I needed you. Thank you so so much. I would like to thank my co-supervisor **Ulf**. Despite your schedule, you have taken the time to sit down with me in front of an HPLC or mass spectrometer. You showed me how to use instruments or interpret the results, and if things didn't look right, you fixed the instrument. You have been a source of experience and your enthusiasm about my results made my days. Thank you for that. Also, thanks to my third supervisor, **Anders**.

I've been very fortunate to not be alone in my research projects but instead having great collaborators by my side. **Detmer**, it never felt like you were far away. You were there to help me figure out the overwhelming amount of results and ideas we discussed in the innumerable versions of our manuscript (Paper I). You took the time and patiently answered all my questions until I had understood them. You care about the people around you and it shows. Thank you. **Indra**, thank you for giving me the freedom and time to learn and develop our analyses, ideas and manuscript with your data, despite being under enormous pressure yourself. I appreciated that a lot. **Ida**, I cannot say how thankful I am to have worked with you. Your knowledge and skills made metabolomics feel straightforward and feasible, contrary to most things in science. Your expertise and what you taught me has been invaluable throughout much of my doctoral work. **Jakob** thank you for listening and being someone to rely on. **Igor**, your contributions have been indispensable and I have really appreciated how easygoing and approachable you are. I have learnt a lot, thanks for always taking the time. **Ellen** thank you for all your kind words of encouragement. **Quentin**, thanks to your enthusiasm and skills have I been able to complete my peptide synthesis project. Whenever I needed to find a reagent, try a new reaction, use a new instrument (or needed support when they broke down or I forgot how to use them after a hiatus from labwork) you were there in the blink of an eye. Although you say that you are new to peptide chemistry, it is easy to see you have

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Due to format constraints, supplementary tables are deposited in a Box folder, maintained until the end of March 2022.

<https://uppsala.box.com/s/ei07i4ncc9uev6htunst9ax2i58yu9ns>

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