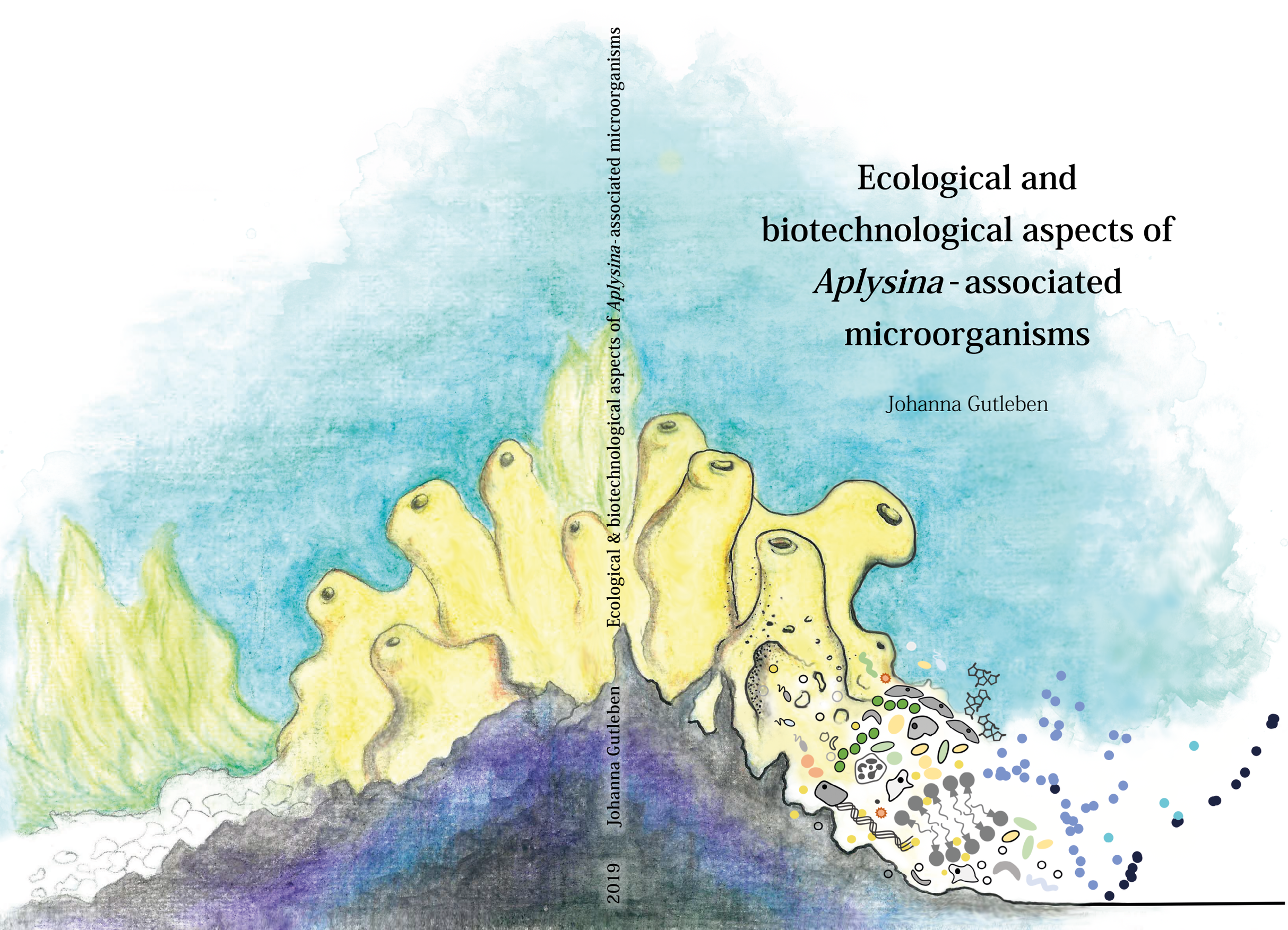
Ecological and biotechnological aspects of *Aplysina*-associated microorganisms

Johanna Gutleben



Ecological & biotechnological aspects of *Aplysina*-associated microorganisms
Johanna Gutleben
2019

Propositions

1. The infamous host-species-specificity of sponge-associated microbial communities does not apply to Caribbean *Aplysina* species.
   (This thesis)

2. We currently do not know enough about the metabolic requirements of dominant sponge-associated bacteria to enable their growth outside the sponge host.
   (This thesis)

3. The human mind is partially controlled by single-celled organisms.

4. Cancer for humans is comparable to the behavior of humanity towards the global ecosystem.

5. Scientists work on creating new reality.

6. Luck is coincidence in your favor.

Propositions belonging to the thesis, entitled:

Ecological and biotechnological aspects of *Aplysina*-associated microorganisms.

Johanna Gutleben

Wageningen, 21 November 2019

Ecological and biotechnological aspects of *Aplysina*-associated microorganisms

Johanna Gutleben

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Johanna Gutleben

Thesis

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Chapter 1

General Introduction

From Start to Sponges

First, there was nothing. Then, microbes emerged.

The origin of life is still a mystery to scientists, but certainly the first traces of microbial life on Earth appeared about 3900 million years ago (Valentine & Marshall, 2015), less than 1 billion years after the formation of the planet itself. The first organisms could have developed in any of the numerous environments where reduced organic molecules, the prerequisites for life, abounded: these include volcanoes, "primordial soups" in prehistorical puddles, or even meteorites (Martin *et al.*, 2008).

Recently, the discovery of submarine hydrothermal vents as geochemically reactive habitats supporting the life of diverse microbial communities without the input of solar energy sparked further discussions (Martin *et al.*, 2008; Dodd *et al.*, 2017). Life originally developed in anoxic conditions (Lambertz *et al.*, 2013), and the energy-releasing geochemical processes of carbon reduction found in submarine vents represent early biochemical pathways (Martin *et al.*, 2008). In these environments, anaerobic methanotrophic archaea co-occur with sulfate reducing bacteria, forming syntrophic metabolic relationships (Hoehler *et al.*, 1994; Nauhaus *et al.*, 2002; Girguis *et al.*, 2005). Such habitats might resemble the earliest microbial ecosystems on Earth. Life soon spread throughout and to the surface of ancient oceans, where sunlight represented an alternative source of energy. Dense phototrophic microbial mats formed at the shores of ancient oceans, and the emergence of photosynthesis led to an enrichment of oxygen in the atmosphere (Bolhuis *et al.*, 2014; Shih, 2015). The primary production by lithoautotrophic and phototrophic microorganisms, i.e. their unique ability to convert inorganic molecules into organic compounds, slowly established the physiological context that enabled other forms of life to evolve.

The Rise of Multicellular Eukaryotes

The eukaryotic cell emerged from within this microbial world, and multiple endosymbiotic theories for the origin of eukaryotic cells are under discussion. The most recent theories build on the concept of anaerobic syntrophy: the host, a hydrogen-dependent archaeon, phagocytized a facultative anaerobic bacterium, which was able to respire oxygen and produce hydrogen through fermentation in anoxic conditions (Martin *et al.*, 2015). The recently discovered 'Lokiarchaeota', a hydrogen-dependent archaeal candidate phylum, would fit this hypothesized description of the host, and to date represents the closest relative to eukaryotic cells in phylogenomic analyses (Spang *et al.*, 2015; Sousa *et al.*, 2016).

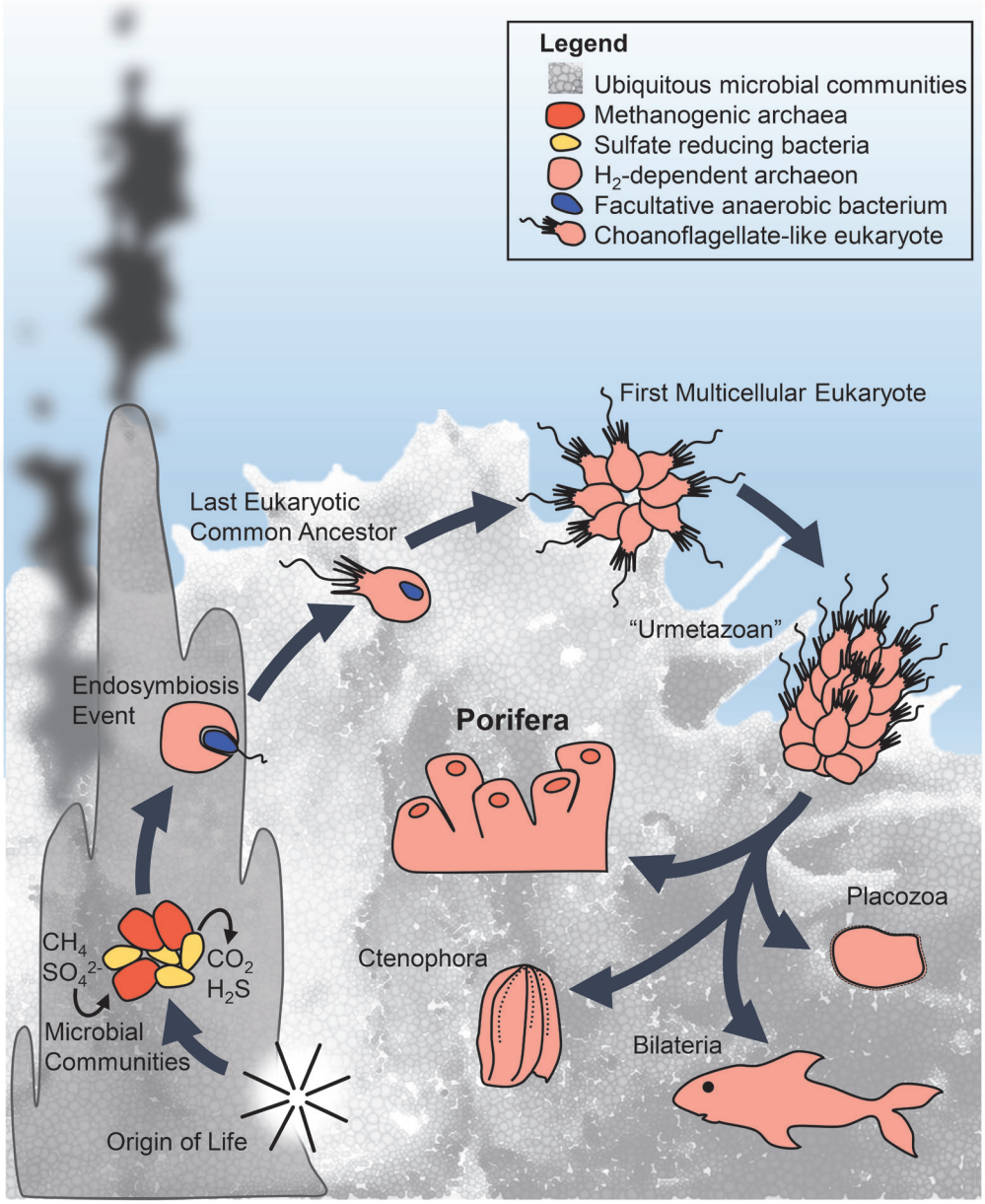


Figure 1 Schematic summary of the current consensus of hypotheses on the evolution of animals from within a microbial world. While the Origin of Life is still unresolved, the endosymbiotic event leading to the formation of eukaryotes presumably occurred in the proximity of submarine hydrothermal vents. The "Urmetazoan" likely resembled sponge larvae, and the Porifera arguably represent the oldest surviving metazoan lineage. Microorganisms played a crucial role in the evolution of animals.

From the emergence of the first simple eukaryotes until the emergence of multicellular animals (metazoa), phylogenomic analyses are only poorly informative (Burki, 2014). Relaxed molecular clock analyses estimate that within 300–600 million years after the Last Eukaryotic Common Ancestor, the first multicellular organisms emerged (Sharpe *et al.*, 2015) from an unknown ancestor within a paraphyletic assemblage of protists including choanoflagellates (Burki, 2014) (Figure 1).

The multicellular lifestyle including functional specialization and collaboration of cells proved advantageous in the majority of (microbial) ecosystems through avoidance of predation, efficient nutrient acquisition, advantages of scale and complex 3D structures and the division of labor (Szathmáry & Smith, 1995; Grosberg & Strathmann, 2007; Aguilar *et al.*, 2015). Since important traits for multicellularity such as adhesion, signaling and transcriptional regulation had already evolved in unicellular organisms including bacteria and archaea, the actual step to multicellularity has been called a 'minor' major evolutionary transition (Grosberg & Strathmann, 2007; Valentine & Marshall, 2015; Sebé-Pedrós *et al.*, 2017).

Genomic signatures, transcriptomic investigations throughout the development of an organism and the overall interpretation of literature on metazoan evolution currently suggests that the "Urmetazoan", the last common ancestor of all multicellular animals, resembles sponge larval developmental modules (Nielsen, 2008; Maldonado, 2010; Ruiz-Trillo *et al.*, 2010). Thomas Cavalier-Smith formulated the hypothesis that an archetypal animal evolved from an agglomeration of choanoflagellates, forming a presponge which settled on solid surfaces. These presponges increased feeding efficiency through larger body size, enabling them to filter more water for food, and integrating photosynthetic bacteria for additional carbon supply (Cavalier-Smith, 2017).

Additionally, choanoflagellates agglomerating to pre-placozoans might have co-existed on marine sediments, and specialized for feeding upon the sedimentary microbiota (Dufour & McLlroy, 2018). This model also implies a phagocytic and/or chemosymbiotic lifestyle, implying that microbes constitute the main source of energy, either as food or as chemically-derived energy-supplying symbionts, to the early animal.

Even though the exact mechanisms leading to the rise of metazoans remain unresolved, sponges arguably were amongst the first multicellular eukaryotes to appear and thus represent the earliest-branching taxon in the animal kingdom (Erpenbeck, 2009; Maldonado, 2010; Voigt *et al.*, 2012; Simion *et al.*, 2017). Molecular fossils and phylogenomic modelling date the origin of sponges back to ~650–540 million years ago (Yin *et al.*, 2015; Gold *et al.*, 2016), or even 750 million years ago (Pisani *et al.*, 2017), long before the Cambrian explosion of species ~542 million years ago. Thus, our last common metazoan ancestor likely was the sea sponge, more specifically, a larva of a homoscleromorph-like organism (Figure 1) (Maldonado, 2010). Early metazoans may have built upon the success of metabolic syntrophy by adopting associations and interactions with the ubiquitous microbes in their surroundings (de Bruijn, 2011). Nowadays, these associations are recognized as an important force in eukaryotic evolution, and to date, all eukaryotes associate with microorganisms (McFall-Ngai *et al.*, 2013; Garcia & Gerardo, 2014). Numerous sponge larvae have incorporated microbes to ensure vertical transmission of some of these symbionts (Usher & Ereskovsky, 2005; Sipkema *et al.*, 2015), and settle preferentially on biofilms with certain microbial composition (Maldonado, 2006; Whalan & Webster, 2014).

The Sponge Organism

Sponge larvae develop into sessile animals with a simple anatomy. The filter feeding organisms draw in water through small pores (ostia) along the outer surface (pinacoderm), and eject the filtered water from larger openings at the top (osculum) (Simpson, 2011) (Figure 2). In this way, sponges can filter up to 24 000 L of seawater per kg of sponge per day and phagocytize dissolved and particulate organic carbon including algae, bacteria and even viruses (Hadas *et al.*, 2006; Vogel, 2006; Taylor, Radax, *et al.*, 2007). This incredibly high flow rate enables sponges to concentrate nutrients suspended in the highly oligotrophic seawater and rapidly assimilate this matter into sponge biomass (De Goeij *et al.*, 2013). Metabolic waste and old cell material is expelled through the osculum.

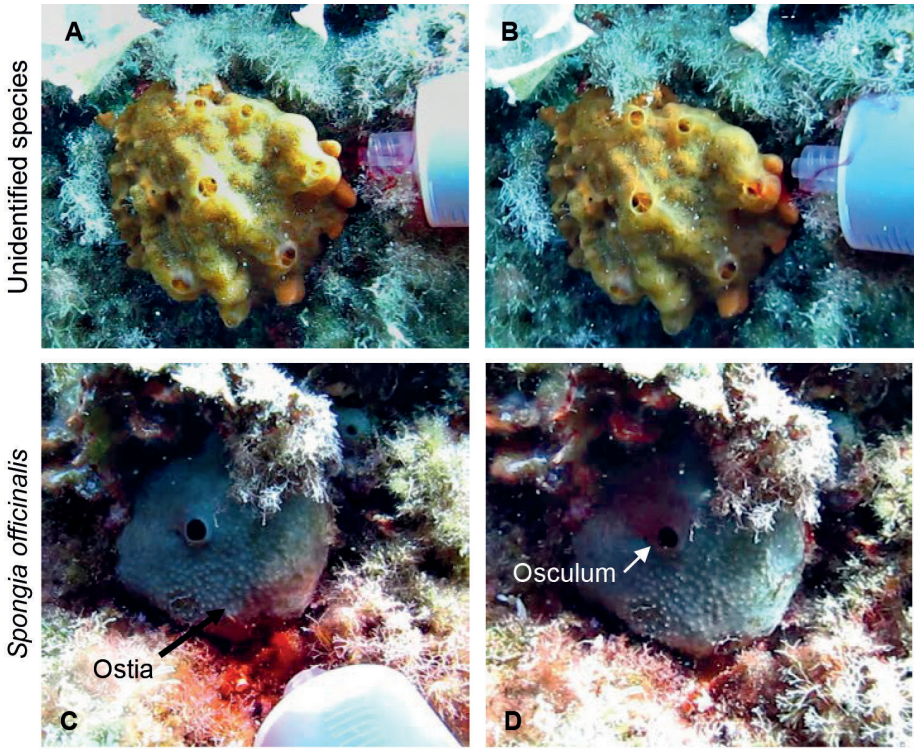


Figure 2 Visualization of sponge pumping activity in the Mediterranean Sea. Red food colorant was applied in proximity to an unidentified sponge species (A) and *Spongia officinalis* (C). The sponges drew in the colorant through the ostia (A, C) and expelled it through the osculum (B, D) after a few seconds (film by Johanna Wiedling, https://youtu.be/w5JTn_TUmc0).

Sponges lack the tight inter-cellular connections found in the epithelia of more complex animals (Simpson, 2011). Instead, the different types of sponge cells can freely change their position within the tissue (mesohyl), allowing them to adapt their body shape to take maximum advantage of changing currents for their filter-feeding lifestyle (Leys *et al.*, 2011). Amoebocytes, also called archaeocytes, are located in the mesohyl, and essentially represent the invertebrate equivalent of white blood cells. Amoebocytes can change into any other sponge cell type, and ingest food particles and microorganisms, passed on from the choanocytes (Pomponi, 2006; Simpson, 2011; Hooper & Van Soest, 2012). Choanocytes closely resemble free-living choanoflagellates. These cells are arranged outlining so-called choanocyte chambers and create the water flow through the sponge body with the propelling movement of their flagella. The flagella are surrounded by a collar of microvilli, which filter food particles and dissolved nutrients from the flowing water (Reiswig, 1971). Choanocyte chambers inside leuconoid sponges are arranged in such a way that each cubicle of water is passed through only one choanocyte chamber before expulsion in order to maximize filtering efficiency (Simpson, 2011).

The outer surface as well as lining of water channels inside the sponge (pinacoderm) is composed of a layer of pinacocytes (Figure 3). These skin cells prevent seawater from entering the sponges' inner tissue, the mesohyl. This connective tissue is a collagenous matrix containing reinforcing fibers, which shape the sponge body (Reiswig, 1974; Weisz *et al.*, 2008; Hentschel *et al.*, 2012). Various other sponge cell types live within the mesohyl such as lophocytes and collencytes (excreting the collagen matrix), sclerocytes (secreting spicules), spongocytes (secreting spongin fibers) and bacteriocytes (containing dense bacterial populations) (Simpson, 2011; Hooper & Van Soest, 2012; van Soest *et al.*, 2012). Furthermore, porocytes are specialized, hollow cells embedded within the pinacoderm that form the water inlet pores called ostia. These pores can be opened and closed by muscle cells called myocytes (Bagby, 1966). All the cells are only loosely connected to each other and can mostly move freely within the tissue, however, they form one coherent animal that can react as a whole, even though sponges do not possess a nervous system (Leys, 2015).

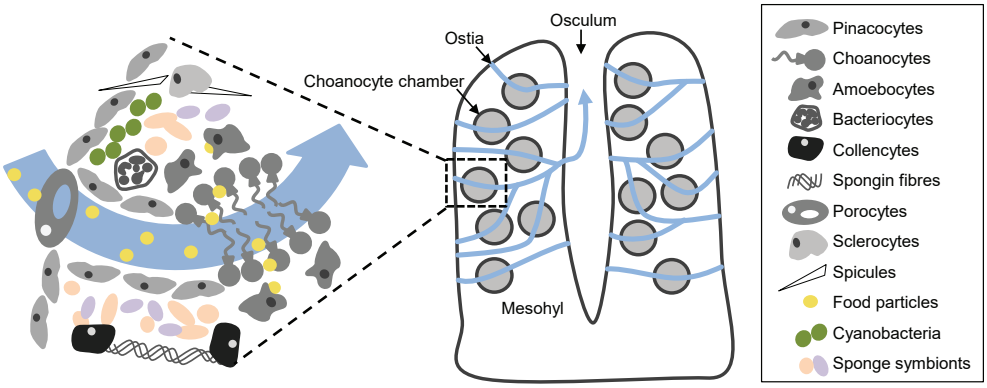


Figure 3 Cell types and body architecture of a leuconoid demosponge. Water (blue arrows) flows through inlet pores (ostia) via pinacocyte-lined water channels to choanocyte chambers, where food particles are filtered by choanocytes, and exits through the central osculum. The mesohyl is inhabited by amoebocytes that phagocytize filtered food particles, as well as by other differentiated sponge cells with functions including spicule formation or fiber production. Furthermore, the mesohyl can harbor dense microbial communities, including Cyanobacteria near the light-exposed surface of the sponge (adapted from Hentschel, 2012).

Sponges have multiple options for reproduction, which makes them noticeably different from most other animals. Since sponges lack anatomically differentiated tissue, a cut-off piece of a sponge is still a complete animal and can settle again on any suitable surface (Osinga *et al.*, 2010). Many sponges can reproduce by external or internal budding. Internally developed asexual buds are referred to as gemmules and can survive extended periods of desiccation, freezing or anoxia, before developing into adult animals under appropriate conditions (Simpson & Gilbert, 2006).

In order to sexually reproduce, sponges form gametes from somatic cells such as archaeocytes or choanocytes (Simpson, 2011). Spermatozoa fertilize the egg, which develop into a free-swimming, often flagellated larva, facilitating geographic distribution of the species (Simpson, 2011). In some sponges, symbiotic bacteria are vertically transmitted by inclusion into the larvae, illustrating the importance of the microbial associates for the sponge animal (Sipkema *et al.*, 2015). Most sponges reproduce seasonally, and timing is species-dependent (Riesgo & Maldonado, 2008). After settling on a suitable surface, sponge larvae develop an aquiferous system and start their pumping activity (Maldonado & Young, 1996; Whalan & Webster, 2014).

Poriferan Diversity

Throughout their evolutionary history, sponges conquered almost all aquatic habitats on the planet, from Antarctic deep sea trenches to tropical intertidal zones (Hooper, 2003). Sponges also made the leap from salt- to fresh water and are common inhabitants of lakes, rivers and canals all over the world (Manconi & Pronzato, 2008). Thus, sponges could be considered to belong amongst the most successful animals on Earth, and belong to the most abundant members of benthic

communities (Diaz & Klaus, 2001; van Soest *et al.*, 2012). Since the Precambrian era, the phylum Porifera diverged into calcareous sponges (class Calcarea), demosponges (class Demospongiae), glass sponges (class Hexactinellida) and the class Homoscleromorpha (van Soest *et al.*, 2019). To date, more than 9000 species are validly described in the World Porifera database (http://www.marinespecies.org/porifera/) of which more than 80% belong to the class Demospongiae (van Soest *et al.*, 2019).

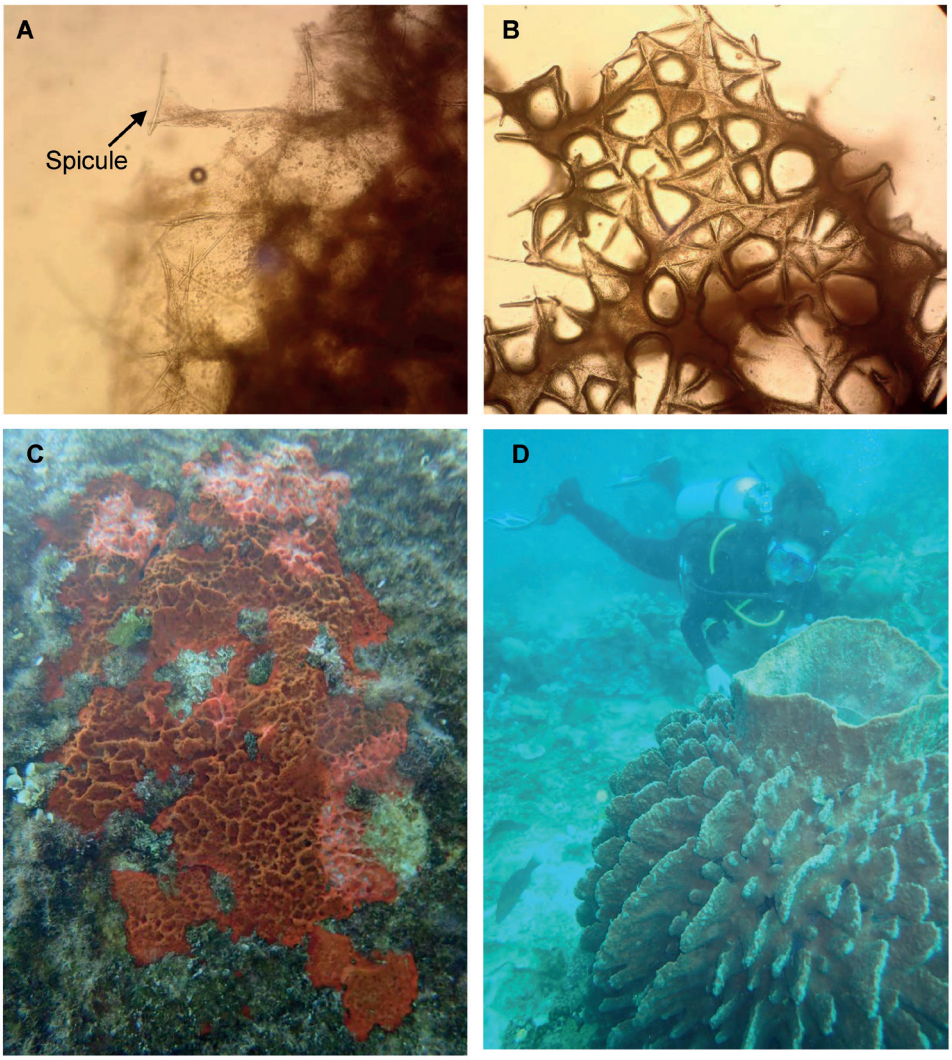


Figure 4 Examples of micro and macro sponge morphologies. 20x magnification of tissue from an unidentified Mediterranean sponge tissue viewed under the light microscope (A). 10x magnification of spongin tissue from orange encrusting sponge *Reniera fulva* (B). *Crambe crambe*, an encrusting species (C). *Xestospongia muta*, the massive barrel sponge (D).

The morphology of sponges can vary substantially, ranging from the encrusting, millimeter-thin layers of *Crambe crambe* to the meter-wide barrels of *Xestospongia muta* (Figure 4). Size as well as shape of sponges is influenced by the structure and composition of the sponge skeleton, which is mostly composed of spicules and collagenous spongin fibers (Hooper, 2003). Spicules are constructed from either calcium carbonate or silicon dioxide and come in a diverse range of sizes and shapes, thus forming the morphological base for the identification of sponge species (Cárdenas *et al.*, 2011). Additionally, sponges occur in virtually all colors, partly due to their cyanobacterial symbionts (Usher, 2008; Burgsdorf *et al.*, 2015; Slaby & Hentschel, 2017) or pigment granules located in the amoebocytes (Bergquist, 1978).

Sponge Identification

Due to their large variety and heterogeneity of body shape, size, coloration and geographical distribution, sponges are notoriously difficult to identify for non-experts. Traditionally, sponge species are identified by the combination of spicule shapes within their tissue (Uriz *et al.*, 2003). More recently, molecular techniques are applied for sponge identification, such as sequencing fragments of the molecular barcode genes encoding cytochrome oxidase subunit 1 (CO1), 28S ribosomal RNA (rRNA) or 18S rRNA. These DNA-signature sequences are collected in an extensive reference database, which aims to cover the entire poriferan phylum (https://www.spongebarcoding.org/) (Erpenbeck *et al.*, 2008, 2016; Vargas *et al.*, 2012). Even with molecular approaches, however, unambiguous identification of certain species such as Caribbean *Aplysina* species remains challenging (Schmitt *et al.*, 2005; Erpenbeck *et al.*, 2007; Cruz-Barraza *et al.*, 2012). For such sponge species, chemosystematics has been proposed as an alternative identification method, albeit with limited success (Erpenbeck & van Soest, 2007).

The Sponge Genus *Aplysina*

The international sponge research community continuously emphasizes the need to focus research on few model sponge species (Pita *et al.*, 2016) and to build on the already existing literature body in order to accelerate progress in our understanding of these complex holobiont systems (i.e., at the 1st and 2nd International Symposium of Sponge Microbiology (Taylor *et al.*, 2011). Thus, this thesis aims to support that notion by focusing on *Aplysina* species, especially *A. aerophoba*, for which genomic information on its symbiotic communities is available for validation and comparison of results. Furthermore, this work aims to expand available literature in order to contribute to and increase the tractability of the cumulated information on *Aplysina* species.

The genus *Aplysina* belongs to the Aplysinidae (Verongida Bergquist 1978) family, taxonomically placed within the Verongiida order of the Demospongiae class (Hooper & Van Soest, 2012). *Aplysina* sponges have been of special interest due to their widespread abundance in the Caribbean and the Mediterranean seas and have been used as model species for numerous studies. These sponges lack siliceous spicules. Instead, they possess a fibrous skeleton and a collagenous mesohyl matrix, which makes their bodies homogeneous, deformable and fleshy (Schmitt *et al.*, 2005; Hooper & Van Soest, 2012). They occur in various shapes (Figure 5), from spreading colonies with multiple chimneys (*A. aerophoba*), via tall, tubular vases (*A. archeri*) to creeping ropes (*A. cauliformis*) (Schmitt *et al.*,

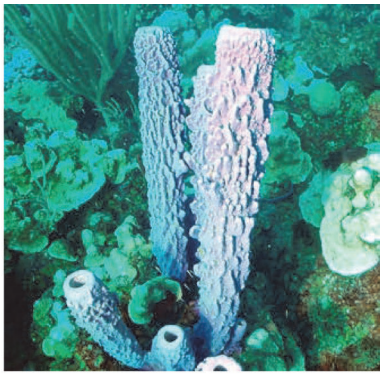
2005). *Aplysina* sponges come in a wide range of colors, and are most commonly sulfur yellow tinged with green, red or purple. Upon exposure to air or damage of the sponge tissue, the yellow sponge pigment uranidine is easily oxidized to an insoluble black material (Cimino *et al.*, 1984).

Aplysina sponges are strongly chemically defended and can accumulate brominated alkaloids in concentrations sometimes exceeding 10% of their dry weight (Thoms *et al.*, 2006). These secondary metabolites have potent antimicrobial, cytotoxic, antifouling and predator-deterring effects and are thus interesting for pharmacological applications (Pawlik *et al.*, 1995; Thoms *et al.*, 2004; Loh & Pawlik, 2014; Niemann *et al.*, 2015). To date, more than 100 chemically distinct, halogenated secondary metabolites have been described from *Aplysina* species (Lira *et al.*, 2011; Puyana *et al.*, 2015), which vary in composition and abundance between species, depth and geographic regions (Puyana *et al.*, 2015).

Currently, the biosynthetic pathways leading to these compounds remain mostly unresolved, yet more and more evidence points towards microbial symbionts being the actual producers (Sacristán-Soriano *et al.*, 2011, 2012; Öztürk *et al.*, 2013). For example, bacteria with antimicrobial activities have been isolated from *Aplysina* species (Hentschel *et al.*, 2001), and polyketide synthases, non-ribosomal peptide synthetases as well as halogenases are encoded in the genomes of the sponge microbiota (Siegl & Hentschel, 2010; Bayer *et al.*, 2013).

Several studies have investigated an emerging sponge model species, the Mediterranean *A. aerophoba*, which revealed numerous astounding insights into sponge-microbe associations. *A. aerophoba* hosts up to 6 x 10^8 bacteria per gram sponge tissue (Friedrich *et al.*, 2001). This highly stable and diverse microbiome spans more than 400 bacterial OTUs, distributed over at least 36 phyla including candidate phyla, and also encompasses diverse archaeal and eukaryotic communities (Chaib De Mares *et al.*, 2017). This sponge species can be maintained in aquaria for several months, allowing investigations on nutrient fluxes (Maldonado *et al.*, 2012), oxygen transport (Hoffmann *et al.*, 2008), secondary metabolite production dynamics (Gerçe *et al.*, 2009) and bacterial uptake (Wehrl *et al.*, 2007). Furthermore, cumulative genomic information and advanced imaging techniques have deepened our understanding of this sponge's holobiont (Bayer, Kamke, *et al.*, 2014; Burgsdorf *et al.*, 2015; Jahn *et al.*, 2016; Slaby *et al.*, 2017; Bayer *et al.*, 2018; Chaib De Mares *et al.*, 2018).

*Aplysina aerophoba**

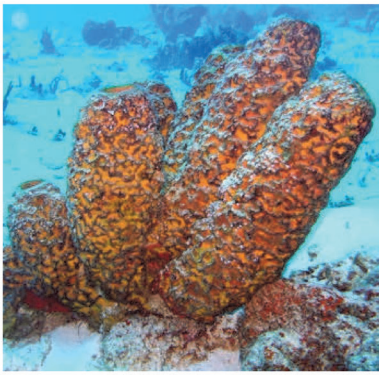


Aplysina archeri

Aplysina cauliformis

Aplysina fistularis

Aplysina fulva



Aplysina lacunosa

Figure 5 Selection of Mediterranean (*) and Caribbean *Aplysina* species investigated in this thesis. Pictures of Caribbean species were obtained from http://coralpedia.bio.warwick.ac.uk.

Ecological Roles of Sponges

Sponges are key members of aquatic ecosystems and play numerous essential ecological roles such as reef creation and substrate stabilization, bentho-pelagic coupling of nutrients as well as habitat creation for other organisms (Diaz & Klaus, 2001; Bell, 2008) (Figure 6). Sponge-associated microorganisms seem to harvest and distribute precious nutrients from the water column and distribute them in bioavailable form to other members of the reef, making sponges important players in the global cycling of numerous elements including carbon, silicon, oxygen and nitrogen (Maldonado *et al.*, 2012). For example, sponge symbionts such as members of the phyla Nitrospirae and Thaumarchaeota can convert ammonia into nitrite and nitrate at much higher rates than in marine sediments (Diaz & Klaus, 2001). The expelled nitrate is essential for the growth of phytoplankton (Capone, 2001). Since ammonia oxidation is limited by light (Ohgaki & Wantawin, 1989), the dark mesohyl of sponges, coupled with extremely high water flow rates, represents a suitable place for ammonia oxidation in light flooded coral reefs.

Due to their filtering activity and subsequent expulsion of old cells in form of detritus, sponges convert dissolved organic matter into particulate organic matter. In the postulated "sponge loop", these particles provide nutrition for detrivores such as crustaceans and polychaetes, which themselves represent valuable food sources for organisms at higher trophic levels (De Goeij *et al.*, 2013; Rix *et al.*, 2016). Thus, sponges contribute to creating and maintaining highly biodiverse reef environments in highly oligotrophic waters (Figure 6), both in warm waters as well as in energy-limited, cold water environments (Rix *et al.*, 2016). In such reef environments competition for space is fierce and predators abound, thus in order to defend themselves against predation, overgrowth and pathogens, many sponges produce an extensive arsenal of bioactive compounds (Kelman *et al.*, 2009; Pawlik, 2011; Loh & Pawlik, 2014). These structurally complex molecules with toxic, antibiotic, antiviral or anti-carcinogenic properties stir scientific and industrial interest alike, due to their potential for applications as pharmaceuticals or industrial chemicals (Belarbi *et al.*, 2003; Sipkema *et al.*, 2005; Wijffels, 2008; Laport *et al.*, 2009).

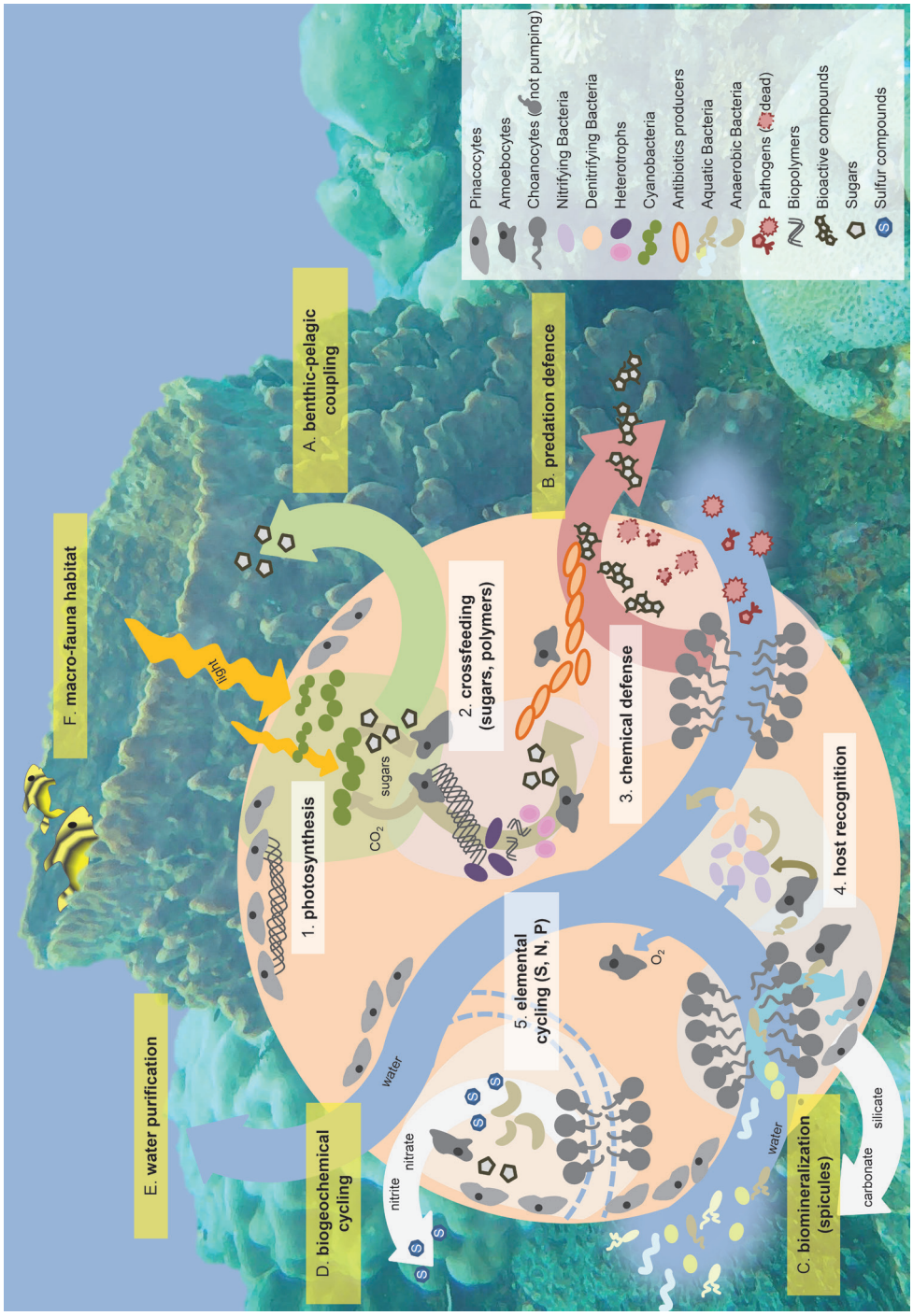

A. benthic-pelagic coupling
B. predation defence
C. biomineralization (spicules)
D. biogeochemical cycling
E. water purification
F. macro-fauna habitat
1. photosynthesis
2. crossfeeding (sugars, polymers)
3. chemical defense
4. host recognition
5. elemental cycling (S, N, P)
light
sugars
CO_2
O_2
water
nitrite
nitrate
carbonate
silicate
Pinacocytes
Amoebocytes
Choanocytes (not pumping)
Nitrifying Bacteria
Denitrifying Bacteria
Heterotrophs
Cyanobacteria
Antibiotics producers
Aquatic Bacteria
Anaerobic Bacteria
Pathogens (dead)
Biopolymers
Bioactive compounds
Sugars
Sulfur compounds

Figure 6 Diagram of selected host and microbe-mediated processes occurring within the sponge (white, 1-5), and in interaction with the marine environment (yellow, A-F). 1. Photosynthesis: Cyanobacteria and/or microalgae fix carbon and can add an important part to sponge nutrition by transferring photosynthate to the host. 2. Crossfeeding: The metabolic waste products of sponge cells or microorganisms can constitute the food source for others within the holobiont. 3. Chemical defense: certain sponge-associated microbes are renown for the production of bioactive compounds to repel incoming pathogens. 4. Host recognition: sponge-associated microbes might shield themselves from phagocytosis, or sponge-cells are actively distinguishing between food and resident microorganisms. 5. Elemental cycling: sponge-associated microbes are involved in sulfur, phosphorus and nitrogen cycling, partially in anoxic niches occurring during non-pumping periods.

A. Benthic-pelagic coupling: By depleting the water column of dissolved organic matter and converting it to particulate organic matter, sponges make dissolved water-column nutrients available to benthic fauna. B. Predation defense: Sponges rarely get preyed upon due to their strong chemical defense. C. Calcification and silicate-spicule production contribute to reef building. D. Biogeochemical cycling: Through their high pumping rates sponges are contributing to rapid rates of nutrient cycling with global impact. E. Water purification: The feeding activity of sponges eliminates large amounts of microorganisms and thus contributes to maintain a clean water column over reefs. F. Macro-fauna habitat: Due to their often massive bodies, sponges can offer shelter to other reef-inhabiting organisms. (This figure was adapted from my contribution to Steinert *et al.* 2018).

Secondary Metabolites

Sponges represent one of the most prolific sources of bioactive natural products within the marine environment, and several thousand compounds have been isolated from marine sponges (Laport *et al.*, 2009; Mehbub *et al.*, 2014). Of these, some compounds have surpassed the tedious pharmaceutical development pipeline and reached the market as treatment for acute myelogenous leukemia (Cytosar-U®) or herpes infections (Vira-A®) (Steinert *et al.*, 2018). A large bottleneck in sponge-derived drug development is the supply of sufficient material, since bioactive compounds often constitute less than 10^{-6} % of the sponge wet weight (Mendola, 2003). At least several grams are needed for preclinical drug development, not to mention the kilogram quantities needed for clinical use (Kingston, 2011). Harvesting large amounts of animals from their natural habitats, however, is ecologically unjustifiable, and sponge aquaculture is still in its infancy (Mendola, 2003; Page *et al.*, 2005; Osinga *et al.*, 2010; Leal *et al.*, 2018). Also, many compounds are structurally too complex for chemical synthesis (Yu *et al.*, 2013), and the biosynthetic pathways need to be better understood for successful heterologous expression (Mao *et al.*, 2017).

In recent years, evidence has accumulated that in many cases sponge-associated bacteria are the actual producers of these bioactive natural products (Dunlap *et al.*, 2007; Taylor, Radax, *et al.*, 2007; Piel, 2009; Uria, 2015; Indraningrat *et al.*, 2016; Lackner *et al.*, 2017). This opens new possibilities for bioprospecting, since potentially, bacteria are more readily culturable in controlled laboratory environments with the possibility to upscale fermentation processes.

Sponges and Microbes

Considering the evolutionary history of animals within the surrounding of a microbial sea (Figure 1), it is not surprising that sponges and consequently all other animals, developed and maintained interactions and associations with microbes (Zilber-Rosenberg & Rosenberg, 2008). Sponge-microbe relationships include both beneficial and harmful interactions, which range from microbes being a source of food, over parasitism and microbial pathogenesis, to commensalism and mutualism (Taylor, Radax, *et al.*, 2007). In most cases, however, the nature of the interactions remains to be elucidated in detail. At least some associations are thought to be mutually beneficial, since numerous sponge larvae are already equipped with an arsenal of species-specific microbes (Sipkema *et al.*, 2015), and despite several approaches being explored it has been impossible to create axenic sponges or sponge cell aggregates (Sipkema *et al.*, 2003; Sipkema, 2004; Richardson *et al.*, 2012; Schippers, 2013).

Sponges are acknowledged marine holobionts and can be literally full of microorganisms (Gloeckner *et al.*, 2014; Webster & Thomas, 2016). Their body architecture, which has remained essentially unchanged over the last 600 million years (Yin *et al.*, 2015), is suitable to house very dense and diverse microbial communities (Taylor, Radax, *et al.*, 2007; Hentschel *et al.*, 2012). Therefore, sponge-microbe associations are probably amongst the most ancient relationships between animals and microorganisms known to date (Taylor, Radax, *et al.*, 2007). A diverse range of associated eukaryotic and prokaryotic organisms can make up to 35% of the biomass of certain sponge species (Hentschel *et al.*, 2012), whereas other species can be virtually free of microorganisms. This division into high- and low microbial abundance (HMA and LMA) sponges generally does not follow discernible phylogenetic or geographic patterns, and only few sponge species harbor an intermediate microbial load as determined by electron microscopy and 16S rRNA gene sequencing (Bayer, Kamke, *et al.*, 2014; Gloeckner *et al.*, 2014; Erwin *et al.*, 2015).

One key characteristic of sponge-microbe associations is the sponge-species-specificity of the microbiota (Moitinho-Silva *et al.*, 2014; Steinert *et al.*, 2016; Thomas *et al.*, 2016). Recently developed molecular tools, especially high throughput amplicon sequencing of marker genes and metagenomics, facilitate research on sponge microbiomes and provide insights into the diversity, community structures or phylogenetic relationships of sponge-specific microbial community assemblages, and their potential establishment and maintenance mechanics (Hentschel *et al.*, 2012; Thomas *et al.*, 2016; Chaib De Mares *et al.*, 2017).

For instance, phylogenetic reconstructions based on near-full-length 16S rRNA gene sequences suggested that a certain proportion of the sponge-associated microbiota can be divided into 173 globally distributed sponge-specific, monophyletic sequence clusters (Simister *et al.*, 2012). Sponges were found to associate with microbes from an astounding 52 microbial phyla including Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Nitrospirae, Cyanobacteria, Gemmatimonadetes, the candidate phyla "Poribacteria", "Tectomicrobia", and Thaumarchaea (Webster & Thomas, 2016). As an example, the taxonomic affiliation of microbes dominant in Aplysinidae sponges is illustrated in Figure 7. These microbial assemblages can range from a few to thousands of genetically distinct operational taxonomic units (OTUs) per host (Webster *et al.*, 2010; Webster & Thomas, 2016), with each sponge harboring at least 13 different phyla (Thomas

et al., 2016). In comparison, the human microbiome currently encompasses around 22 microbial phyla (Pasolli *et al.*, 2019) of which four are predominant, namely Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Huttenhower *et al.*, 2012). Overall, the microbiome of the same sponge species from different oceans is more similar than the microbiome of two neighboring sponges of different species. The variability of the symbiotic communities within most sponge species is low, indicating that the sponge species restrictively select for their microbial inhabitants (Thomas *et al.*, 2016). At the functional level, community variability between sponge species is often driven by the presence or absence of phototrophic symbionts (Freeman & Thacker, 2011), or differences in abundance of genes involved in nitrogen and carbon cycling (Bayer, Moitinho-Silva, *et al.*, 2014). Thus, their phylogenetically and metabolically versatile microbiome may allow seemingly competing sponge species to exploit unique physiochemical niches within marine ecosystems (Freeman *et al.*, 2014).

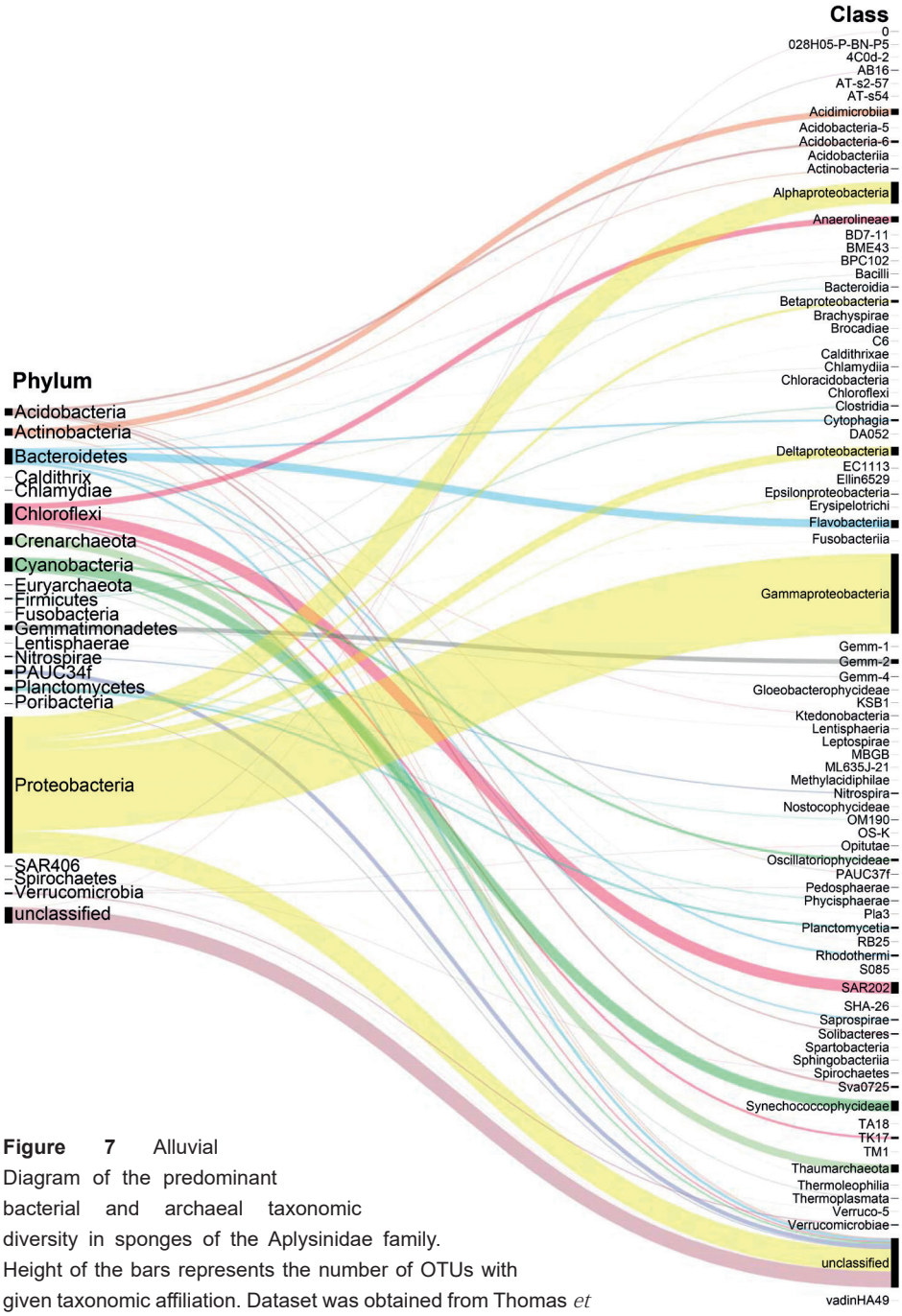


Figure 7 Alluvial Diagram of the predominant bacterial and archaeal taxonomic diversity in sponges of the Aplysinidae family. Height of the bars represents the number of OTUs with given taxonomic affiliation. Dataset was obtained from Thomas *et al.* 2016, and was subset to the top 22 phyla and their respective classes. The diagram was visualized using RAWGraphs (http://app.rawgraphs.io/).

The microbiota in the surrounding seawater and sediments are distinctly different, although few sponge-specific microbes can be found within the marine rare biosphere at relative abundances below 2% (Taylor *et al.*, 2013). Sponge-specific microbes in the marine environment seem to provide a 'seed bank' for a later uptake by sponges and their subsequent colonization. This adds to the hypothesis of 'leaky vertical transmission', suggesting that both vertical (through sponge larvae) and horizontal transmission (via the seawater) of sponge symbionts to juveniles occurs (Sipkema *et al.*, 2015).

The knowledge on symbiont functions remains limited to date which is partly due to the fact that all of the highly abundant sponge symbionts remain recalcitrant to cultivation. Recent advances in multi-omics techniques however have revealed genetic features of sponge-associated microorganisms that facilitate their symbiotic lifestyle and contribute to the sponge metabolism (Slaby *et al.*, 2017; Astudillo-García *et al.*, 2018; Bayer *et al.*, 2018; Karimi *et al.*, 2018; Kiran *et al.*, 2018; Podell *et al.*, 2019). Some of these processes are illustrated in Figure 5, 1-5, and a few selected symbiotic taxa and their functions are discussed below.

Cyanobacteria occur in at least 38 sponge genera, are often the most predominant symbionts, and can be transmitted vertically via sponge larvae (Lester *et al.*, 2009; Burgsdorf *et al.*, 2015). The cyanobacterium "*Candidatus* Synechococcus spongiarum" can provide photosynthate, fix nitrogen and shield the sponge from excessive light (Usher, 2008; Webster & Taylor, 2012; Freeman *et al.*, 2013). In some cases, Cyanobacteria also aid in chemical defense (Unson *et al.*, 1994), or produce and store polyphosphate granules (Fan Zhang *et al.*, 2015; Astudillo-García *et al.*, 2018).

Chloroflexi represent a taxonomically and physiologically diverse microbial phylum and include highly predominant symbionts of many high-microbial-abundance sponges (Schmitt *et al.*, 2011). As sponge symbionts, they might synthesize amino acids from ammonia, aid in co-factor biosynthesis and secondary metabolite production (Sacristán-Soriano *et al.*, 2011; Bayer *et al.*, 2018), while degrading sponge-derived carbohydrates (Chaib De Mares *et al.*, 2018).

Poribacteria have been discovered in association to sponges due to their particular compartmentalized cell structure (Fuerst *et al.*, 1998). The bipolar, spherical granules likely represent carbon reserves (Jahn *et al.*, 2016). Poribacteria are estimated to comprise up to one third of the bacteria in high-microbial-abundance sponges such as *Aplysina* species (Bayer, Kamke, *et al.*, 2014). They form a distant lineage related to the Planctomycetes, Verrucomicrobia and Chlamydia superphylum, and their 16S rRNA gene sequences mostly fail to amplify with widely used universal primers, which is why this lineage evaded detection until recently (Fieseler *et al.*, 2004; Kamke *et al.*, 2013). Single cell-genomics revealed their genetic ability to degrade sponge-derived polysaccharides such as chitin and glycoproteins due to the presence of uronic acid degradation pathways and sulfatases (Kamke *et al.*, 2013). Poribacteria might also perform autotrophic carbon fixation, contribute to nitrogen-cycling, produce a number of vitamins (Kamke *et al.*, 2013) and presumably metabolize propanediol inside their microcompartments (Jahn *et al.*, 2016). Metatranscriptomic evidence reported a high expression of genes encoding membrane transport-associated proteins, transposases and several proteins mediating prokaryote-eukaryote interactions (Jahn *et al.*, 2016). The very few Poribacteria detectable in the water column remain metabolically inactive, indicating that these bacteria are well adjusted to life as a sponge symbiont (Webster *et al.*, 2010; Moitinho-

Silva *et al.*, 2014). Overall, microbial symbionts of sponges are regularly found to contribute to urea metabolism (Siegl *et al.*, 2011; Su *et al.*, 2013; Bayer, Moitinho-Silva, *et al.*, 2014; Moitinho-Silva *et al.*, 2017). *In vivo* sponge metabolism measurements and metagenomics studies point out that nitrogen cycling processes [denitrification and anaerobic ammonium oxidation (anammox)] (Hoffmann *et al.*, 2009; Schläppy *et al.*, 2010), sulfur cycling [sulfate reduction and –oxidation] (Hoffmann *et al.*, 2005; Jensen *et al.*, 2017) and vitamin biosynthesis (Webster & Thomas, 2016) are mediated by microbial sponge symbionts with both heterotrophic and autotrophic lifestyles (Hoffmann *et al.*, 2009; Moitinho-Silva *et al.*, 2017; Chaib De Mares *et al.*, 2018).

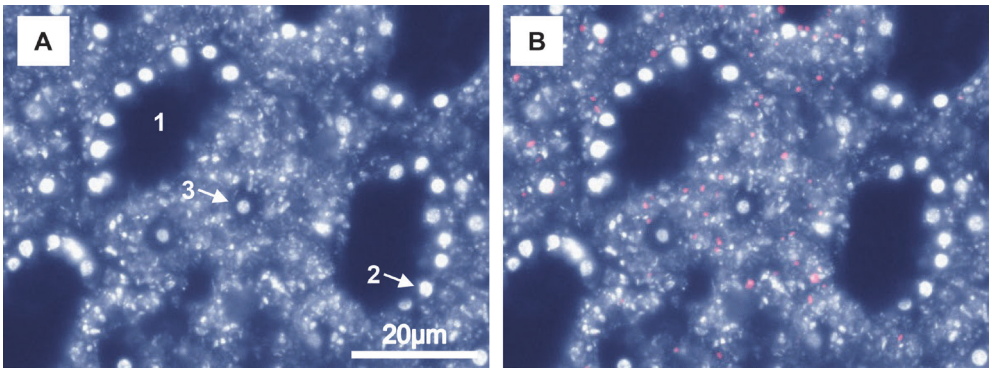


Figure 8 CARD FISH of tissue slices of *Aplysina aerophoba*, whose mesohyl tissue is packed with microbial cells. A. DAPI-staining creates fluorescence of nucleic acids under UV-exposure. 1. Choanocyte chamber. 2. Nucleus of a choanocyte. 3. Sponge cell in the mesohyl tissue, potentially amoebocyte. B. Vizualization of uncultured Tectomicrobia (red). Corresponding tissue section was overlayed with the fluorescent image of cells labelled with Tectomicrobia-specific probe and the Alexa Fluor 647 label. Picture credit: Giorgia Palladino.

While playing a crucial role in sponge metabolism, microbes living within the mesohyl (Figure 8) need to simultaneously prevent phagocytosis by their host and adapt to this particular niche. Thus, sponge-associated microorganisms encode for numerous symbiotic factors, including proteins that deal with microenvironmental stress factors such as heat- or osmotic shock and oxidative stress (Astudillo-García *et al.*, 2018). Proportionally high numbers of genes responsible for the stable insertion of mobile DNA, as a means to rapidly adapt to the host, have been reported for cyanobacterial symbionts (Burgsdorf *et al.*, 2015). Furthermore, sponge microbial metagenomes are enriched in tetratricopeptide-, ankyrin- and leucine-rich- repeat domains, involved in protein-protein interactions between pro- and eukaryotes (Thomas *et al.*, 2010; Fan *et al.*, 2012; Burgsdorf *et al.*, 2015). It has been demonstrated that the expression of such proteins can modulate phagocytosis by amoebal cells, and might represent a mechanism for symbionts to avoid digestion by their sponge host (Nguyen *et al.*, 2014; Reynolds & Thomas, 2016). The mechanisms of how sponges select and maintain their personalized microbiome remain mostly unknown to date, however, microbes might actively aid in establishing symbiosis (Tout *et al.*, 2017).

To date, knowledge on the precise interactions of many microbes within the sponge holobiont is limited due to the inherent complexity of the system and the continuous lack of model sponges and cultured sponge symbionts. While multi-omics techniques reveal more and more details on the genetic potential of sponge microbiomes, most findings remain hypothetical without experimental evidence.

Culturing the Uncultivated

Sponges are no exception when it comes to the observation that, in most ecosystems, the extant microorganisms outnumber, by orders of magnitude, those that are accessible through cultivation. This fact has been described as the "great plate-count anomaly" (Staley, 2002; Nichols, 2007). In spite of the tremendous progress in cultivation technology (Bruns *et al.*, 2002b; Zengler *et al.*, 2005; Aoi *et al.*, 2009; Ferrari & Gillings, 2009; Belanger *et al.*, 2010; Park *et al.*, 2011; Jung *et al.*, 2014; Rettedal *et al.*, 2014; Tillich *et al.*, 2014), this phenomenon has not been brought to a solution. There are numerous reasons why so many microbes remain recalcitrant to cultivation. The most basic reason is probably the lack of understanding of the (bio)chemical parameters that define the microbes' natural milieu and hence our incapability of reproducing these conditions accurately *in vitro*. Parameters that are especially difficult to mimic include the chemistry of a (micro-)habitat, interactions with other microbes and the host, or interactions of biotic and abiotic factors (Alain *et al.*, 2009).

Nevertheless, microbial cultivation represents a reliable technique to validate hypotheses derived from multi-omics studies and is crucial for the annotation of novel or unknown genes (Pham & Kim, 2012; Muller *et al.*, 2013). Furthermore, microbial metabolism and interactions can be studied in detail, and stable cultures pave the way to industrial and biotechnological exploitation of microorganisms. Bioactive metabolite-producing sponge-associated microorganisms are of special interest, since their successful cultivation could solve the compound supply issue for the pharmaceutical development pipeline. Lastly, culturing sponge-symbionts would aid greatly in studying the sponge holobiont in more detail.

Apart from conventional agar-plating techniques, many innovative isolation methods were applied to culture sponge-symbionts. These include floating filters (Sipkema *et al.*, 2011), diffusion growth chambers (Steinert *et al.*, 2014) and *in situ* cultivation devices (Jung *et al.*, 2014), the addition of several antibiotics (Versluis *et al.*, 2017) and arsenic (Keren *et al.*, 2015), or the implementation of sponge-derived physiological and genomic information (Lavy *et al.*, 2014). Such cultivation experiments typically result in large numbers of isolates, including many new species and some also produce novel secondary metabolites. Reported cultivability ranges from 0.1-14% of the total sponge-associated microbial diversity detected with molecular methods, depending on the sponge species (Webster & Hill, 2001; Li *et al.*, 2007; Muscholl-Silberhorn *et al.*, 2008; Abdelmohsen *et al.*, 2010; Santos *et al.*, 2010; Sipkema *et al.*, 2011; Öztürk *et al.*, 2013; Steinert *et al.*, 2014; Hardoim *et al.*, 2014; Lavy *et al.*, 2014; Rua *et al.*, 2014; Keren *et al.*, 2015; Esteves *et al.*, 2016; Versluis *et al.*, 2017).

Few true symbionts of sponges have been successfully isolated: Members of the alphaproteobacterial genus *Pseudovibrio* get isolated frequently (Muscholl-Silberhorn *et al.*, 2008; Santos *et al.*, 2010; Bondarev *et al.*, 2013; Lavy *et al.*, 2014; Versluis *et al.*, 2017). *Pseudovibrio* in some cases is vertically transmitted through larvae (Enticknap *et al.*, 2006), and this bacterium produces sponge-derived brominated alkaloids (Nicacio *et al.*, 2017). Furthermore, cyanobacterial *Leptolyngbya* have been isolated from several sponge species, which can sequester and store phosphorus from the seawater (Lafi *et al.*, 2005; Fan Zhang *et al.*, 2015; Konstantinou *et al.*, 2018). Also culturable Actinomycetes could be detected within the sponge tissue (Tae *et al.*, 2005), and a cultured Streptomyces strain seems to play an important role in chemical defense of its sponge host (Selvin, 2009). However, most of these culturable microorganisms are found in low relative abundances within sponges, thus, their importance in sponge-microbe symbiosis is under debate.

Several studies focused on cultivating microorganisms from the model species *A. aerophoba*, and could culture bacteria with antimicrobial (Hentschel *et al.*, 2001; Pabel *et al.*, 2003), and reductive dehalogenation capabilities (Ahn *et al.*, 2003). More recently, Actinomycetes (Abdelmohsen *et al.*, 2010), nitrite-oxidizing Nitrospira (Off *et al.*, 2010) and fungi (Naim *et al.*, 2017) have been isolated from *A. aerophoba*, however, whether these microorganisms are true symbionts is under debate. An attempt to cultivate Poribacteria resulted in the isolation of a Planctomycetes strain, which was also of low abundance within the sponge tissue (Pimentel-Elardo *et al.*, 2003). To date, no study reports obtaining any of the dominant microbial symbionts of *A. aerophoba*. Thus, the need for further attempts with alternative cultivation approaches remains high. To date, more information on the *in vivo* conditions of the mesohyl habitat have become available through physiological tests and multi-omics tools. For example, temporarily anoxic parts occur during non-pumping periods (Hoffmann *et al.*, 2005, 2008), and numerous nitrogen cycling-related genes and taxa suggest nitrogen cycling to be an important process within the sponge (Bayer *et al.*, 2008; Bayer, Moitinho-Silva, *et al.*, 2014; Slaby *et al.*, 2017; Dat *et al.*, 2018). Additionally, complex carbohydrates forming the extracellular matrix of sponge mesohyl might represent an energy-source degradable by many symbionts (Kamke *et al.*, 2013; Fiore *et al.*, 2015; Bayer *et al.*, 2018; Chaib De Mares *et al.*, 2018). Furthermore, one should consider that the doubling-time of environmental microorganisms adapted to oligotrophic conditions is often longer than the duration of many cultivation experiments (Zengler *et al.*, 2002; Alain *et al.*, 2009). Integrating such information into novel cultivation strategies provides a new edge to the continued challenge to cultivate dominant, sought-after taxa such as those belonging to the Chloroflexi, Tectomicrobia, Poribacteria, *Synechococcus* Cyanobacteria or abundant Proteobacteria.

Aims and Thesis Outline

Sponges harbor dense and diverse microbial communities. To date, many open questions remain on the taxonomic composition and functional potential of the microbiomes of numerous sponge species. The general aims of this thesis are to further explore both ecological aspects as well as the biotechnological potential of sponge-associated microorganisms. In this work, the taxonomic, phylogenetic and functional diversity of microorganisms from *Aplysina* species, especially the Mediterranean species *Aplysina aerophoba*, was further investigated.

The work described in this thesis was embedded within the European Sponge Biotechnology Project BluePharmTrain (Steinert *et al.*, 2018), which focused on *A. aerophoba* as one of four key species for intensive study towards biotechnological exploitation. *Aplysina aerophoba* emerged as promising model species within the European sponge research community due to its high production of brominated isoxazoline alkaloids (Thoms *et al.*, 2004; Lira *et al.*, 2011; Niemann *et al.*, 2015) and its dense microbiome (Chaib De Mares *et al.*, 2017), which might contain the actual producer of the bioactive compounds (Sacristán-Soriano *et al.*, 2011). Additionally, this species is highly abundant in the Mediterranean, grows at light-exposed sites between 5 and 15m depth and is thus easily accessible by SCUBA-diving (Hoffmann *et al.*, 2008).

Nonetheless, neither the sponge nor any of its dominant microbial symbionts has been sustainably cultivated under controlled conditions, highlighting that the understanding of the biological mechanisms and functions of this holobiont remains fragmented. Furthermore, the actual producers of the prominent bioactives remain to be elucidated. Thus, further cultivation-based, as well as supporting molecular studies are necessary in order to render this sponge accessible to successful biotechnological exploitation.

This thesis aims to contribute to the collective information available on *Aplysina* species by investigating inter- and intraspecies diversity of functional genes and the community composition of Poribacteria and other members of the sponge microbiome (Figure 9). Subsequently, this thesis explores how information derived from molecular methods can be applied to improve microbial cultivability, both experimentally and by reviewing available literature (Figure 9). In this way, this thesis aims to bridge ecological observations with potential biotechnological applications.

Chapter 1 introduces the sponge animal and its associated microbial communities within their evolutionary and ecological context, and serves as a reference point regarding our current understanding of the sponge-microbe system. This assemblage of organisms can be studied using culture-dependent as well as culture-independent techniques, of which both find applications within this thesis.

Sponges are known for the production of bioactive compounds, many of which are halogenated. It has been demonstrated that in many cases, the associated microbes are the actual producers of such substances. **Chapter 2** describes the diversity and phylogenetic novelty of halogenase genes in multiple sponge species of the genus *Aplysina* from the Caribbean and the Mediterranean seas. Additionally, this chapter attempts to link the discovered halogenase genes to their potential producers within the sponges' microbial community.

Some bacterial members of these highly diverse communities are particularly specific in their association to sponges, such as the candidate phylum Poribacteria, a prominent symbiont of many HMA sponge species. However, common universal primers used for molecular diversity studies fail to amplify marker genes from these microbes. Using Poribacteria-specific primers, **Chapter 3** investigates the diversity and phylogenetic distribution of Poribacteria amongst a wide range of sponge hosts collected in various geographic regions. Furthermore, this chapter investigates the intraspecific diversity of Poribacteria within different specimen of *Aplysina aerophoba*.

Poribacteria, as well as most other sponge-associated bacteria, thus far resist cultivation and consequently their biotechnological potential remains inaccessible. **Chapter 4** describes several approaches to increase the cultivability of bacteria associated to the sponge *Aplysina aerophoba*. After establishing that most bacteria remain viable after cryo-preservation, alternative cultivation setups such as a Winogradsky-column approach, a liquid-solid media approach as well as media based on multi-omic-derived information on the metabolism of Poribacteria were applied.

Chapter 5 reviews how information that can be extracted from multi-omics data can be applied for the cultivation of hitherto uncultured microorganisms. Ground-breaking examples for this approach are summarized, concluding in a proposed workflow to facilitate future omics-aided cultivation experiments. Despite advances in molecular methods, cultivating bacteria in controlled conditions remains essential since it enables detailed physiological studies and exploration of the biotechnological potential of the strains.

Chapter 6 is the general discussion, integrating the results obtained in **Chapters 2-5**, and portraying them in the light of recent work from within the field of sponge microbiology and ecology.

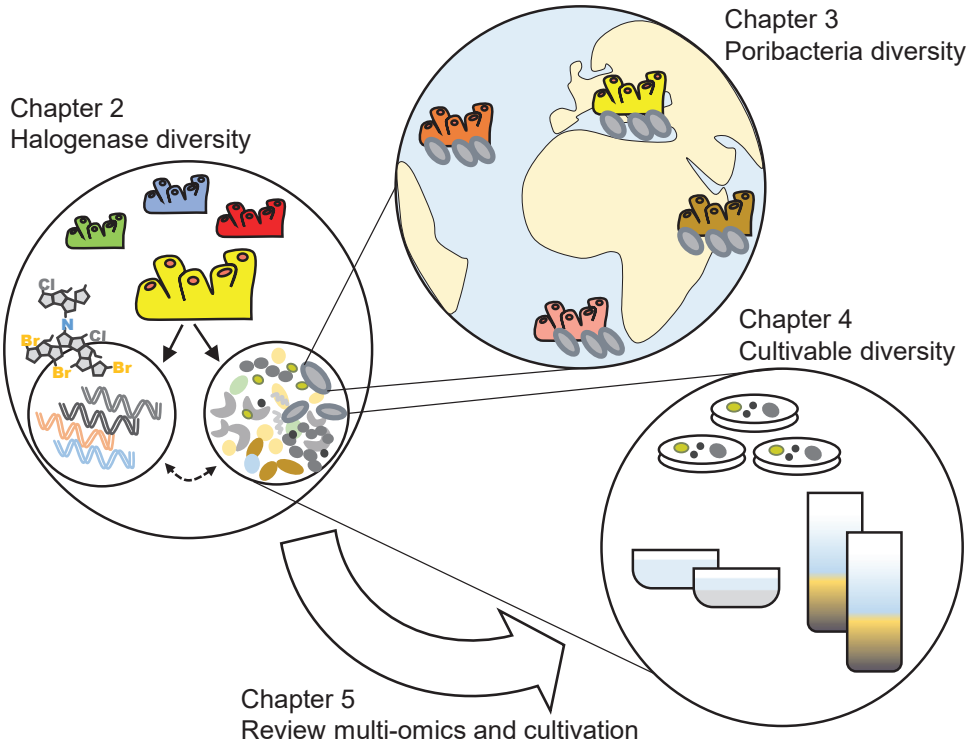
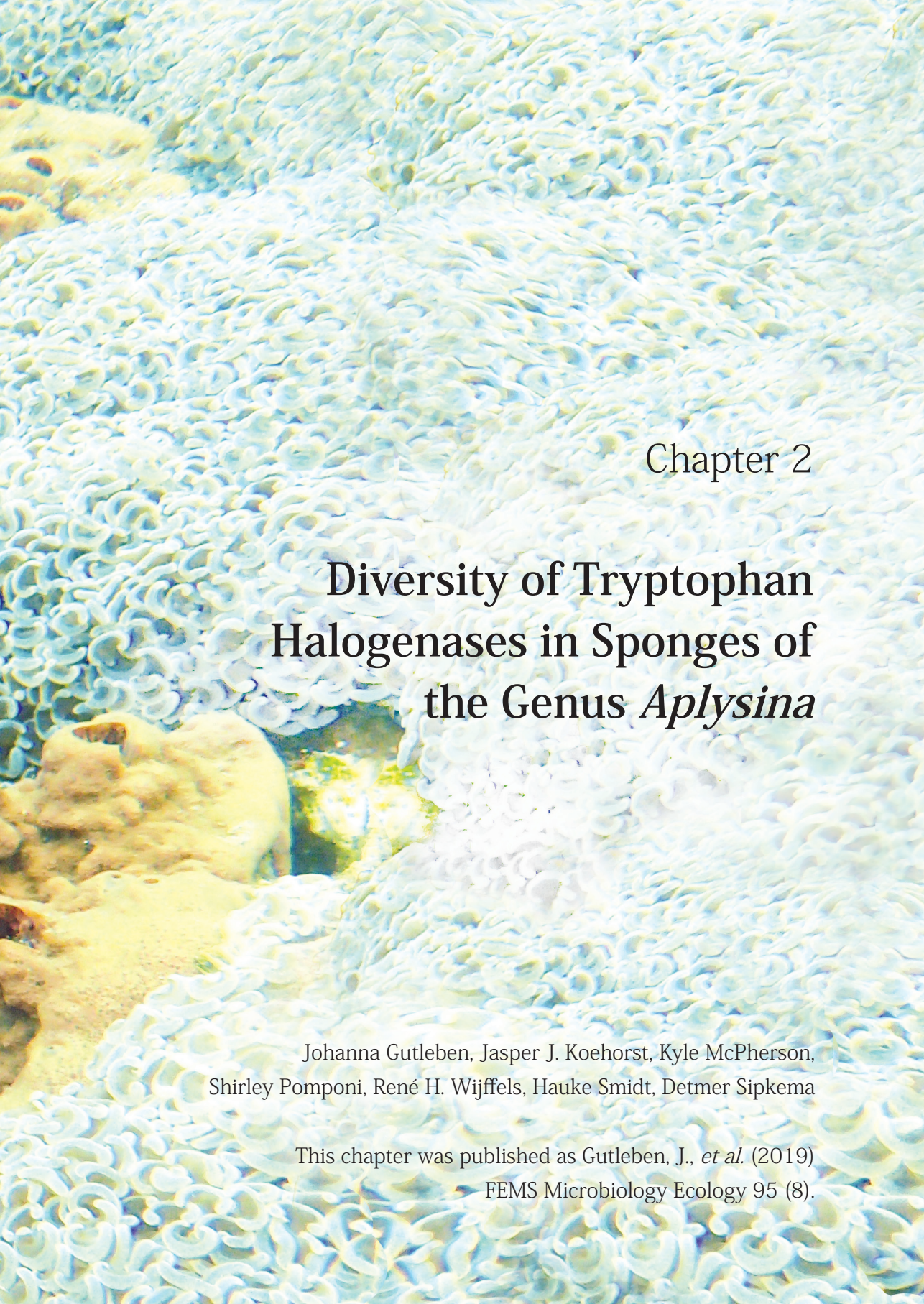


Figure 9 Outline of this thesis: The halogenated bioactive compounds found in sponges are often of microbial origin. **Chapter 2** describes the diversity and phylogenetic novelty of halogenase genes in multiple sponge species of the genus *Aplysina* and attempts to link the discovered halogenase genes to their potential producers within the sponges' microbial community. Many microorganisms within these communities, such as members of the candidate phylum Poribacteria, are particularly specific in their association to sponges. **Chapter 3** investigates the global distribution and phylogenetic diversity of Poribacteria, a prominent symbiont of many high-microbial-abundance sponge species. Most sponge-associated bacteria, including Poribacteria, resist cultivation and thus their biotechnological potential remains inaccessible. **Chapter 4** describes several approaches to increase their cultivability using alternative cultivation setups and innovative media designs based on multi-omics information available for Poribacteria. **Chapter 5** reviews the state of the art of the –omics-based cultivation approach and summarizes examples where multi-omic information has led to the cultivation of hitherto uncultivated microorganisms.



Chapter 2

Diversity of Tryptophan Halogenases in Sponges of the Genus *Aplysina*

Johanna Gutleben, Jasper J. Koehorst, Kyle McPherson, Shirley Pomponi, René H. Wijffels, Hauke Smidt, Detmer Sipkema

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Abstract

Marine sponges are a prolific source of novel enzymes with promising biotechnological potential. Especially halogenases, which are key enzymes in the biosynthesis of brominated and chlorinated secondary metabolites, possess interesting properties towards the production of pharmaceuticals that are often halogenated. In this study we used a PCR-based screening to simultaneously examine and compare the richness and diversity of putative tryptophan halogenase protein sequences and bacterial community structures of six *Aplysina* species from the Mediterranean and Caribbean seas. At the phylum level bacterial community composition was similar amongst all investigated species and predominated by Actinobacteria, Chloroflexi, Cyanobacteria, Gemmatimonadetes, and Proteobacteria. We detected four phylogenetically diverse clades of putative tryptophan halogenase protein sequences, which were only distantly related to previously reported halogenases. The Mediterranean species *A. aerophoba* harbored unique halogenase sequences, of which the most predominant was related to a sponge-associated *Psychrobacter*-derived sequence. In contrast, the Caribbean species shared numerous novel halogenase sequence variants and exhibited a highly similar bacterial community composition at the OTU level. Correlations of relative abundances of halogenases with those of bacterial taxa suggest that prominent sponge symbiotic bacteria including Chloroflexi and Actinobacteria are putative producers of the detected enzymes and may thus contribute to the chemical defense of their host.

Introduction

Bioactive Compounds and the Role of Halogenase Enzymes

The need to discover novel drug candidates is high on the policy agenda due to the ongoing emergence of multi-drug resistant microbial strains (O'Neill, 2016). The call for a better supply of new drugs against a large range of infectious diseases points towards novel natural products as a yet inexhaustible source of bioactivity. Especially the marine environment proved to be a particularly rich resource for novel bioactive compounds, and many of them are halogenated (Gribble, 2015). Carbon-halogen bonds lead to an increase in thermal and oxidative stability as well as increased permeability through biological membranes compared to their non-halogenated counterparts (Smith *et al.*, 2017). Halogenated molecules exhibit a wide range of biological activities and may have antifungal, antibacterial, antiviral, anticancer, and/or anti-inflammatory properties (Butler & Sandy, 2009; Gribble, 2015). Thus, many pharmaceutical and agrochemical compounds as well as polymers are halogenated (Bolton *et al.*, 2011; Lu *et al.*, 2012; Jeschke, 2013), such as the commercially important antibiotics chloramphenicol, vancomycin and teicoplanin (Van Pée & Zehner, 2003). In nature, halogens including chloride, bromide, fluoride or iodide get attached to organic molecular scaffolds by halogenase enzymes, which have been detected in fungi, bacteria and algae from terrestrial and marine environments (Atashgahi *et al.*, 2018; Latham *et al.*, 2018). While marine enzymes preferentially halogenate with bromide (Neubauer *et al.*, 2018), chlorinated compounds are regularly detected in terrestrial sources (Latham *et al.*, 2018). To date, more than 5000 naturally produced halogenated compounds (Gribble, 2015) and six independently evolved halogenase enzyme families have been identified (Xu & Wang, 2016). These enzyme families, namely cofactor-free haloperoxidase, vanadium-dependent haloperoxidase, heme iron-

dependent haloperoxidase, non-heme iron-dependent halogenase, flavin-dependent halogenase, and S-adenosyl-L-methionine-dependent halogenase, differ in their cofactor dependence, sequence homology and mechanistic features (Xu & Wang, 2016). The most extensively characterized halogenases are flavin (FADH2)-dependent halogenases (FDHs) (Van Pée & Patallo, 2006; Zhao *et al.*, 2015). In contrast to haloperoxidases, FDHs often halogenate regioselectively, and are often part of secondary metabolite gene clusters encoding for non-ribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) producing halogenated molecules (Walsh *et al.*, 2001; Van Pée & Unversucht, 2003; Dorrestein *et al.*, 2005; Van Pée & Patallo, 2006). Regioselective halogenation under mild reaction conditions without harmful waste generation renders FDHs promising tools for biocatalysis of halogenated compounds (Smith *et al.*, 2013; Grüschow *et al.*, 2015; Shepherd *et al.*, 2015; Menon *et al.*, 2016; Weichold *et al.*, 2016; Latham *et al.*, 2018). The amino acid sequences of FDHs contain a highly conserved flavin binding site (GxGxxG) near the N-terminus, which binds the cofactor in order to create the reactive halogen, as well as a motif with two tryptophan residues (WxWxIP), which presumably prevents the binding of a substrate close to the flavin (Van Pée & Zehner, 2003; Van Pée & Patallo, 2006). These motifs can be detected in almost every FDH described today, and serve as identification marker for novel FDH genes (Bayer *et al.*, 2013). FDHs can be distinguished into three classes depending on their preferred substrate moieties: phenols, pyrroles or tryptophan (Murphy, 2006; Van Pée & Patallo, 2006). In most cases, the exact substrates for halogenases encoded within biosynthetic gene clusters remain to be identified, however, many of the bacterial FDHs characterized to date halogenate a range of tryptophan-derivatives and other aromatic substrates (Payne *et al.*, 2013; Frese *et al.*, 2014; Shepherd *et al.*, 2015). These flavin-dependent tryptophan halogenases (THs) exhibit a broad substrate tolerance for other electron-rich organic scaffolds, although these are halogenated with lower reaction efficiencies in *in vitro* activity tests (Weichold *et al.*, 2016; Agarwal *et al.*, 2017). Since halotryptophans occur fairly frequently in natural products (Smith *et al.*, 2017), we focussed our efforts on THs in this study. Most flavin-dependent THs described to date require the activity of an additional enzyme, a flavin reductase, to provide the reduced flavin cofactor, and are thus two-component systems (Agarwal *et al.*, 2017). Single-component halogenating enzymes possess both domains for flavin-reduction and halogenation. To date, only two single-component halogenating enzymes have been reported from marine bacteria but they remain poorly characterized (Agarwal & Moore, 2014; Agarwal *et al.*, 2014).

Sponges as Source of Novel Halogenase Sequence Variants

Sponges are the most prolific marine invertebrates for the discovery of novel bioactive compounds (Blunt *et al.*, 2009; Hu *et al.*, 2011; Mehbub *et al.*, 2014; Rocha-Martin *et al.*, 2014; Sipkema, 2017; Carroll *et al.*, 2019) since many of them depend on a chemical arsenal to defend themselves against diseases, competitors and predators (Thoms *et al.*, 2006; Taylor, Radax, *et al.*, 2007; Pawlik, 2011). These ancient, filter feeding animals harbour dense and diverse microbial communities including members of the bacterial phyla Actinobacteria, Acidobacteria, Bacteriodetes, Chloroflexi, Cyanobacteria, Planctomycetes, Proteobacteria, Nitrospira, Poribacteria, Tectomicrobia, Verrucomicrobia, as well as archaea and numerous microeukaryotes (Taylor, Radax, *et al.*, 2007; Webster & Taylor, 2012; Thomas *et al.*, 2016; Chaib De Mares *et al.*, 2017). Some of these largely uncultured bacteria can produce highly potent bioactive natural products, many of which are halogenated (Gribble, 2010; Smith *et al.*, 2017). Amongst marine sponges, the Demosponge genus

Aplysina represents a morphologically diverse group of species challenging to identify due to their lack of a mineral skeleton (Zea *et al.*, 2014), as well as a high degree of sequence conservation in molecular marker genes (Erpenbeck *et al.*, 2007; Cruz-Barraza *et al.*, 2012). Chemotaxonomy was suggested as additional phylogenetic marker (Erpenbeck & van Soest, 2007), since *Aplysina* species are strongly chemically defended and especially renowned for the production of more than 100 halogenated natural products (Lira *et al.*, 2011; Loh & Pawlik, 2014). Such metabolites can make up to 12% of the sponge dry weight (Turon *et al.*, 2000; Thoms *et al.*, 2006).

A survey of natural products from Caribbean *Aplysina* species suggested that the brominated alkaloids were sponge-derived rather than microbiome-derived, since metabolite profiles were highly correlated to sponge morphotype rather than location and depth (Puyana *et al.*, 2015). It should be noted, however, that this study did not assess microbial composition, and hence, an essential or auxiliary role of the microbiome in metabolite production cannot be excluded. Halogenated compounds have been reported to be located in spherulous cells of *Aplysina aerophoba*, suggesting production by the sponge, or a complex symbiotic pattern with microorganisms involved at different levels of the biotransformation pathway (Turon *et al.*, 2000). Significant correlations of the relative abundances of a member of the Chloroflexi, a deltaproteobacterium and an unidentified bacterial OTU with the concentrations of three alkaloids (aerophobin-1, aplysinamisin-1 and isofistularin-3) in *A. aerophoba* indicated that bacteria were correlated to the production of brominated alkaloids (Sacristán-Soriano *et al.* 2011, 2016). This finding corroborates observations that the majority of halogenating enzymes has been described from algae, fungi and bacteria (Xu & Wang 2016). Halogenated natural products or the corresponding biosynthetic gene clusters could previously be associated to specific bacterial symbionts in other marine sponges (Unson *et al.*, 1994; Flatt *et al.*, 2005; Ridley *et al.*, 2005; Hochmuth & Piel, 2009; Della Sala *et al.*, 2013; Öztürk *et al.*, 2013; Smith *et al.*, 2017), but to the best of our knowledge this remains unresolved for the metabolites of *Aplysina* species, both from the Mediterranean and Caribbean Sea (Puyana *et al.*, 2015; Sacristán-Soriano *et al.*, 2016). Currently, only dehalogenation mechanisms could be directly linked to the microbial community of *A. aerophoba* (Ahn *et al.*, 2003). However, the conserved nature of key enzymes encoded in biosynthetic gene clusters allows the design of degenerate PCR primers, thus facilitating the screening and discovery of novel sequence variants of these enzymes from environmental DNA (Hornung *et al.*, 2007; Kennedy *et al.*, 2008; Borchert *et al.*, 2016). Accordingly, PCR-based surveys have led to the identification of numerous putative flavin-dependent halogenase encoding genes from different environmental samples (Erol *et al.*, 2017), multiple marine sponges (Bayer *et al.*, 2013; Öztürk *et al.*, 2013) and cultivated Actinomycetes strains (Hornung *et al.*, 2007; Gao & Huang, 2009; Liao *et al.*, 2016).

Aim

With this study we aimed to investigate the phylogenetic diversity and distribution of flavin-dependent tryptophan halogenase protein sequence variants within six species of the marine sponge genus *Aplysina*. By using 16S rRNA and TH gene amplicon sequencing we aimed to determine the resident sponge bacterial community of different *Aplysina* species and explore potential links between the microbial populations and the associated halogenase sequences. This will increase our understanding of the putative producers of halogenated secondary metabolites in sponges.

Materials and Methods

Sponge Collection

Three individuals each of the Caribbean sponge species *Aplysina archeri* (AAr), *Aplysina cauliformis* (ACa), *Aplysina fistularis* (AFi), *Aplysina fulva* (AFu) and *Aplysina lacunosa* (ALa) were collected around Bonaire by SCUBA diving at depths between one and ten meters on 8 and 9 October 2012 (Table 1). A sampling permit was given to Detmer Sipkema by the government of Bonaire. Sampled sponge individuals grew at least 1 m apart from each other. The sponge species were identified in the field by Dr. Shirley Pomponi. After sampling, the sponge fragments were rinsed three times with sterile artificial seawater (ASW, 33 g/L Reef Crystals, Blacksburg, VA, USA), cut into 1 cm^3 pieces and stored in RNAlater (Sigma Aldrich) at -20°C. Additionally, three individuals of the Mediterranean sponge species *Aplysina aerophoba* (AAe) were collected by SCUBA diving at Cala Montgo, Spain (N 42.114, E 3.168), on 19 October 2010 and 15 January 2012. The collection of *A. aerophoba* samples was conducted in strict accordance with Spanish and European regulations within the rules of the Spanish National Research Council with the approval of the Directorate of Research of the Spanish Government. After sampling, the sponges were transported to the laboratory and rinsed three times with sterile artificial seawater (ASW) before grinding with a sterilized mortar and pestle. To obtain a homogenous cell suspension, two volumes of ASW were added. Cell suspensions were aliquoted and mixed with sterile glycerol in ASW for a final concentration of 17% glycerol. Samples were frozen at -20°C and stored at -80°C until DNA extraction (Sipkema *et al.*, 2011).

DNA Isolation and PCR Amplification of Tryptophan Halogenase Genes

DNA was extracted using the FastDNA Spinkit for Soil (MP Biochemicals, Santa Ana, CA, USA) according to manufacturer's instructions with the following modification for the first step: instead of using 500 mg of soil, 750 µL of cryopreserved *Aplysina aerophoba* cell suspension was centrifuged at 14,000 g for 10 min and the pellet was used for the extraction. For the Caribbean sponge species, 500 mg of wet-weight sponge tissue was rinsed in sterile ASW, cut into small pieces, and used for DNA extraction.

A PCR-based method was used to screen the sponge samples for the presence and identity of potential flavin-dependent tryptophan halogenase genes. In a preliminary screening among previously reported primers, the degenerate halogenase gene targeted primers SZ002 and SZ005 (Zehner *et al.*, 2005) were found the most suitable to amplify an approximately 500 bp DNA fragment. Barcoded PCR amplicons (1 specific barcode for each sample) were obtained through a two-step PCR reaction.

For the first PCR reaction, the halogenase primers (SZ002 and SZ005) included linker sequences (341F and 806R): 341F-SZ002 5'-CMTAYGGGRBGCASCAG-TCGGYGTSGGCGARGCGACCRTCCC-3' and 806R-SZ005 5'-GGACTACNNGGGTATCTAAT-GCCGGAGCAGTCGAYGAASAGGTC-3'. The linker sequences represent the binding regions for the barcoding primers in the second PCR. As we routinely use barcoded 16S rRNA gene targeted primers for prokaryotic community composition analysis, we employed those for barcoding of the less routinely used TH gene-targeted primers. Thus, the more widely used barcoding primers could be applied to barcode halogenase gene amplicons.

The first PCR amplification was performed in a volume of 50 µL using 10 µL 5x GoTaq buffer, 2 µL 10 mM dNTP mixture, 0.5 µL 5 U/µL GoTaq DNA polymerase (Promega, Madison, WI, USA), 3 µL 10 µM solution of both primer 341F-SZ002 and primer 806R-SZ005, 22.5 µL nuclease-free water and 1 µL template DNA (10-20ng/µL) for each of the samples listed in Table 1. PCR conditions were: initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 s), annealing (60°C for 40 s), elongation (72°C for 50 s), and a final extension (72°C for 5 min). Amplification products were visualized on a 1.25% (w/v) agarose gel and purified using the Millipore DNA Gel Extraction Kit (Millipore, Billerica, MA, USA). A second barcoding PCR was performed as described above, except that a pyrosequencing adapter A (CCATCTCATCCCTGCGTGTCTCCGACTCAG) and 18 different barcodes of 10 nucleotides length connected to the 341F linker sequence were used as forward primer and pyrosequencing adapter B (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) connected to the 806R linker sequence as reverse primer. Furthermore, the number of amplification cycles was reduced to 15. PCR products were visualized on a 1% (w/v) agarose gel, and the bands of PCR products were excised from the gel and purified as described above. The amplified fragments with adapter and barcodes were quantified using a Qubit™ fluorometer (Invitrogen) and mixed in approximately equal concentrations (4×10^5 copies μL^{-1}) to ensure equal representation of each sample in the pool. A 454-sequencing run was performed on a GS FLX Standard PicoTiterPlate (70×75) using a GS FLX pyrosequencing system according to the manufacturer's instructions (Roche, Mannheim, Germany) at the Technical University of Copenhagen. Pyrosequencing data of halogenase genes were deposited at the NCBI Sequence Read Archive under sample accession numbers SRR7853828 to SRR7853845.

PCR Amplification of 16S rRNA Genes

Barcoded amplicons of bacterial 16S rRNA genes for all sponge samples were amplified from the extracted DNA. PCR reactions were performed in a volume of 100 µL containing 20 µL High Fidelity Buffer (ThermoFisher Scientific, Waltham, MA, USA), 2.5 µL 10 µM 338R-I, 2.5 µL 10 µM 338R-II reverse primer (Daims *et al.*, 1999), 2 µL 10 mM dNTP mixture, 1 µL 2 U/µL Phusion Hot start II DNA polymerase, 65 µL nuclease free water. 5 µL 27F-DegS forward primer (van den Bogert *et al.*, 2011) with Titanium adapter A and a sample-specific barcode (8 nt) (Hamady *et al.*, 2008) attached to the 5'- end as well as 2 µL template DNA (10-20 ng/µL) were added to each reaction. Amplification conditions were: initial denaturation (98°C for 30 s), followed by 30 cycles of denaturation (98°C for 10 s), annealing (56°C for 20 s), elongation (72°C for 20 s), and a final extension (72°C for 10 min). Amplification products were visualized, purified, pooled and sequenced as described above. Pyrosequencing data of 16S rRNA genes were deposited at the NCBI Sequence Read Archive under sample accession numbers SRR7853935 to SRR7853950.

Halogenase Gene Amplicon Data Analysis

Halogenase gene pyrosequencing data were demultiplexed using QIIME version 1.9.0 (Caporaso *et al.*, 2010). Sequences that i) were shorter than 200 bp or longer than 1000 bp, ii) contained more than one mismatch in the forward or reverse primer sequences, iii) contained ambiguous bases or iii) were represented with less than three reads were removed. Chimeric sequences were detected using the usearch61 algorithm (Edgar, 2010) and removed. Two samples (AAe2 and AFi3) did not pass quality criteria of the 16S rRNA gene data analysis and were thus also removed from the halogenase gene analyses. The remaining sequences were translated into the three forward open reading frames using the transeq algorithm (Blankenberg *et al.*, 2007) as implemented in Galaxy (Afgan *et al.*, 2016), and ORFs containing stop codons were removed using customized Bash and R scripts (https://github.com/mibwurrepo/Gutleben_et.al_Halogenases_Aplysinas). Sequences were clustered at 95% amino acid sequence identity based on the average protein sequence identity in genomes of the same bacterial species (Rodriguez-R & Konstantinidis, 2014; Chaib De Mares *et al.*, 2018) using the uclust algorithm (Edgar, 2010). The most abundant sequence per cluster was retained as representative sequence.

For identification of putative flavin-dependent TH sequences a reference database was created, by subsetting the UniProt/SwissProt database (Bairoch, 2002; Bateman *et al.*, 2017) to 5427 "halogenase" protein entries, since similarity searches to smaller databases return more sensitive results (Jagtap *et al.*, 2013; Pearson, 2014). Of these, 75 were manually annotated and reviewed entries (SwissProt). Representative amino acid sequences were aligned (blastp) against i) the entire UniProt/SwissProt database (release 2018_02) and ii) the halogenase database using the Diamond alignment tool (Buchfink *et al.*, 2014). Protein families (Pfam) were assigned using the InterProScan pipeline 5.17 (Quevillon *et al.*, 2005) based on an evalue cutoff of 10-6.

Amino acid sequences that aligned significantly (e-value <0.001 (Pearson 2014)) against an entry in the halogenase database were retained for phylogenetic analyses. Sequences were aligned using the ClustalW algorithm (Larkin *et al.*, 2007), together with the most closely related database entries, four reference sequences (tryptophan 5-halogenase PyrH (*Streptomyces rugosporus*, A4D0H5), flavin-dependent TH RebH (*Lechevalieria aerocolonigenes*, Q8KHZ8), flavin-dependent TH PrnA (*Pseudomonas fluorescens*, P95480), halogenase ClaH (*Streptomyces uncialis*, G3K6J6)), putative TH protein sequences previously found in the sponge *C. crambe* (Öztürk *et al.*, 2013) and the outgroup sequences NADH-dependent flavin oxidoreductase BaiH (*Clostridium scindens,* P32370) and NADPH-flavin oxidoreductase Frp (*Vibrio harveyi*, Q56691). The halogenase sequences from *A. aerophoba* obtained by Bayer *et al.* (2013) could not be included in this analysis since they covered a different region of the gene.

The resulting alignment was manually refined and trimmed to the amplified regions excluding the primers using Jalview (Waterhouse *et al.*, 2009). A maximum likelihood phylogenetic tree was calculated using RaXML HPC (Stamatakis, 2006) under the PROTGAMMAWAG substitution model, and 100 bootstrap replicates were used to evaluate clusters. The best-scoring tree was visualized using iTol (Letunic & Bork, 2016).
R (version 3.4.3) (Sasaki *et al.*, 2005) was used for diversity analyses of amino acid sequences identified as putative tryptophan halogenases. For visualization and interpretation, relative abundance information was used for interpretation and was visualized using the ggplot2 v.2.2.1

package (Wickham, 2016b). Weighted UniFrac dissimilarities (Lozupone *et al.*, 2011) were calculated and ordinated using Principal Coordinates Analysis as implemented in the phyloseq package (McMurdie & Holmes, 2013). Phyloseq and the microbiome package (Lahti *et al.*, 2017) were used for calculating observed richness and Shannon index diversity. Faith's phylogenetic diversity was calculated using the package picante (Kembel *et al.*, 2010). Venn diagrams were calculated and visualized using online tool jvenn (Bourtzis *et al.*, 1996). Caribbean core halogenases were defined as being present in at least one sample from all the Caribbean species and identified using jvenn.

16S rRNA Gene Amplicon Data Analysis

Bacterial 16S rRNA gene pyrosequencing data was analyzed using mothur v.1.39.5 (P. D. Schloss *et al.*, 2009) by following the 454 Standard operating procedure (https://www.mothur.org/wiki/454_SOP). In brief, sequences were demultiplexed, denoised, and sequences with i) more than two mismatches in the primers, ii) more than one mismatch in the barcode, iii) more than 8 homopolymer were discarded (trim.flows and trim.seqs commands). Reads were reduced to unique sequences (unique.seqs) and aligned to the SILVA SSU 128 database (Quast *et al.*, 2013) (align.seqs: flip=t). Aligned reads were kept (screen.seqs: optimize=start-end, criteria=98, minlength=250), and empty alignment columns were removed (filter.seqs: vertical=T, trump=.). Read counts for sequences that were within ≥99% sequence similarity to a more abundant sequence were merged (pre.cluster: diffs=2). Chimeric sequences were detected with Vsearch (chimera.vsearch) (Rognes *et al.*, 2016) and removed (remove.seqs). Taxonomy was assigned using the SILVA SSU 128 database (classify.seqs: cutoff=80) (Wang *et al.*, 2007). Sequences that were not classified at Domain level as well as chloroplast sequences were removed (remove.lineage). Uncorrected pairwise distances between aligned sequences were calculated (dist.seqs: cutoff=0.15), OTUs were generated based on 97% sequence identity (cluster: method=opti, cutoff=0.03), and files were converted to .shared format (make.shared: list=, group=,). Taxonomy was assigned to OTUs (classify.otu: list=, name=, taxonomy=, label=0.03), and representative sequences for each OTU were picked (get.oturep: phylip=, list=, fasta=, label=0.03, sorted=size). Further OTU table processing was done with Bash and R scripts (https://github.com/mibwurrepo/Gutleben_et.al_Halogenases_Aplysinas). Relative abundance information was used for visualization and interpretation. Calculation of community metrics and UniFrac dissimilarities were performed as described above.

Core taxa were defined as being present in at least one sample from all analyzed sponge species, or from all Caribbean sponge species (Caribbean core), and were identified using jvenn. Correlations between relative halogenase and bacterial abundances were expressed as Spearman coefficients for all taxa and all halogenase genes, as well as for the Caribbean core taxa and core halogenase genes. Only coefficients $>\pm 0.5$ and with $p<0.05$ were considered significant, and only taxa and halogenases shared by all Caribbean species were included in this analysis to maximize statistical power. Heatmaps were generated using the pheatmap v1.0.8 package (Kolde, 2012b). Analyses are available as R Markdown (https://github.com/mibwurrepo/Gutleben_et.al_Halogenases_Aplysinas).

Results

Identification of Putative Tryptophan Halogenases

To capture the diversity of PCR-amplified halogenase genes in *Aplysina* species, 454-pyrosequencing was performed. A total of 37 374 DNA reads, representing 3653 unique protein sequences was obtained from TH gene targeted amplicon sequencing. After clustering the unique sequences at 95% amino acid sequence identity, 1918 protein sequence clusters were retained with a maximum of 109 sequences per cluster. Detailed results per sample are given in Table 1.

Out of the representative (most abundant) sequences of these 1918 clusters, 1654 (86.24%) resulted in a hit against the entire UniProt protein database. However, only 40 sequences aligned significantly (e-value <0.001 (Pearson, 2014)) against two flavin-dependent TH entries present in the UniProt database with low sequence identities (<45% amino acid identity) and low bitscores (mean=56). Thus, the 1918 sequences were blasted against a manually curated "halogenase" protein database containing the halogenase protein sequence subset (5427 entries) from UniProt. In total, 86 sequences (4.5%) resulted in a significant (e-value <0.001) hit against one of 16 entries from the "halogenase" database with high amino acid sequence similarities (32.9%-100%) and high bitscores (mean=173 (Table 2)) and were thus identified as putative TH protein sequence fragments. Of these 86 sequences, 25 had 67-80% amino acid sequence identity to a TH from marine gammaproteobacterium HTCC2080, an abundant oligotrophic marine microorganism belonging to the NOR5/OM60 clade (Cho & Giovannoni, 2004; Thrash *et al.*, 2010). Another 19 sequences matched most closely (82-100%) to a TH fragment from *Psychrobacter* sp. D8, a gammaproteobacterium isolated from the sponge *Crambe crambe* (Öztürk *et al.*, 2013). The closest database match of another 15 sequences, although with lower (36-51%) amino acid sequence identity, was a TH from the cyanobacterium *Calothrix* sp. NIES-2100 (Hirose *et al.*, 2017).

Table 1 Metadata, sample information and results of 16S rRNA gene as well as halogenase gene sequencing of the *Aplysina* species analyzed in this study. THs: tryptophan halogenases. (*) Samples did not pass quality criteria and were excluded from the analyses. Putative halogenase sequences were clustered at the amino acid sequence level.

Sample ID	Species	Sea	Lati-tude	Longi-tude	Depth [m]	Temp.	Preservation	16S rRNA genes			Halogenases	
								Reads	OTUs (97%)	Phyla	Reads	Putative THs (95%)
AAe1	*Aplysina aerophoba*	Mediterranean	42.114	3.168	8	*NA*	Cryopreservation	8707	268	17	2813	10
AAe2*	*Aplysina aerophoba*	Mediterranean	42.114	3.168	8	*NA*	Cryopreservation	*NA*	*NA*	*NA*	*NA*	*NA*
AAe3	*Aplysina aerophoba*	Mediterranean	42.115	3.168	12	*NA*	Cryopreservation	6032	241	19	1716	12
AAr1	*Aplysina archeri*	Caribbean	12.160	-68.283	10	28°C	RNALater	9258	312	17	844	8
AAr2	*Aplysina archeri*	Caribbean	12.160	-68.283	10	28°C	RNALater	9786	359	16	2927	18
AAr3	*Aplysina archeri*	Caribbean	12.160	-68.283	10	28°C	RNALater	5397	361	16	1631	17
ACa1	*Aplysina cauliformis*	Caribbean	12.026	-68.251	16	29°C	RNALater	11664	429	18	907	15
ACa2	*Aplysina cauliformis*	Caribbean	12.026	-68.251	16	29°C	RNALater	5523	341	18	1218	20
ACa3	*Aplysina cauliformis*	Caribbean	12.026	-68.251	16	29°C	RNALater	3890	292	14	1210	17
AFi1	*Aplysina fistularis*	Caribbean	12.094	-68.232	1	30°C	RNALater	4789	223	16	658	11
AFi2	*Aplysina fistularis*	Caribbean	12.094	-68.232	1	30°C	RNALater	8429	181	17	688	11
AFi3*	*Aplysina fistularis*	Caribbean	12.094	-68.232	1	30°C	RNALater	*NA*	*NA*	*NA*	*NA*	*NA*
AFu1	*Aplysina fulva*	Caribbean	12.160	-68.283	10	28°C	RNALater	9995	304	18	5324	12
AFu2	*Aplysina fulva*	Caribbean	12.160	-68.283	10	28°C	RNALater	9988	295	17	2110	11
AFu3	*Aplysina fulva*	Caribbean	12.160	-68.283	10	28°C	RNALater	10140	424	17	4308	14
ALa1	*Aplysina lacunosa*	Caribbean	12.160	-68.283	10	28°C	RNALater	4896	314	16	2360	15
ALa2	*Aplysina lacunosa*	Caribbean	12.160	-68.283	10	28°C	RNALater	7198	459	18	6976	22
ALa3	*Aplysina lacunosa*	Caribbean	12.160	-68.283	10	28°C	RNALater	6450	282	15	4497	18

Additionally, the 86 protein sequences with significant "halogenase" database hits were also investigated for protein domains using InterProScan (Finn *et al.*, 2017). This confirmed for 84 sequences that they belong to the "Tryptophan halogenase" protein family (PF04820) and all contained a flavin-TH domain (IPR006905). Only two short protein sequences (S1691 and S1792), which were identified as TH by blastp, did not contain a detectable domain.

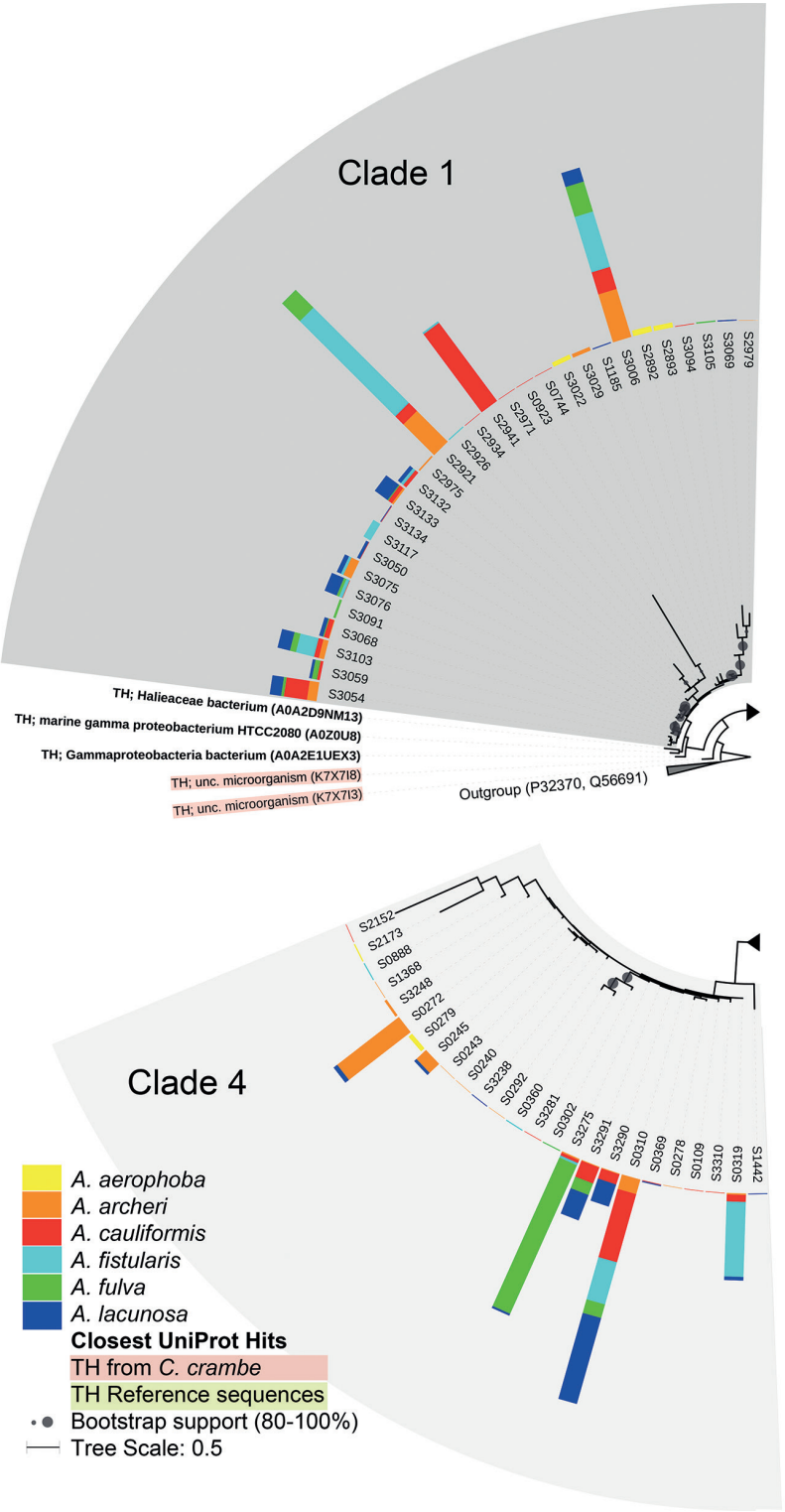
Phylogenetic Analyses of Putative Tryptophan Halogenases

Phylogenetic analyses of the 86 identified putative TH protein sequences resulted in four distinct clades, and only two sequences (S1874 and S2870) did not cluster within these four clades (Figure 1). Except for *A. aerophoba*, all analyzed sponge species contained halogenases from all four phylogenetic clades. The largest clade (Clade 1) contained 30 sequences, followed by Clade 4 (25 sequences), Clade 3 (19 sequences) and Clade 2 (10 sequences).

Only one species-specific halogenase clade could be identified, namely an *A. lacunosa*-specific subclade within Clade 2 (7 out of 10 sequences, Figure 1). The sequences within this subclade were all most closely related to a TH from a Dehalococcoidales bacterium (A0A2E9M8J4). Within Clade 3 (19 sequences), the halogenase most abundant (88%) in *A. aerophoba* (S1779) was closely related to a putative halogenase sequence (K7XJ2) previously detected in Mediterranean *C. crambe* as well as to a halogenase from *Psychrobacter* sp. D8 isolated from the same sponge (Öztürk *et al.*, 2013). Especially Clade 4 (25 sequences) was only distantly related to previously reported halogenases from Verrucomicrobia and Cyanobacteria and contained mainly sequences from Caribbean *Aplysina* spp., indicating a large number of novel halogenases within these sponges and their microbiota. The well-characterized flavin-dependent TH gene products RebH (*L. aerocolonigenes*, Q8KHZ8 (Yeh *et al.*, 2007)) and PrnA (*P. fluorescens*, P95480 (Dong *et al.*, 2005)) clustered outside the sponge-derived clades and were closely related to only one not abundant (<1%) sequence from *A. aerophoba* (S2870).

Table 2 Hit table (blastp) of sequences aligned against the manually curated halogenase protein database. *TH: Tryptophan halogenase, THf: Tryptophan halogenase (Fragment). %ID is given as % amino acid sequence identity.

No. of sequences	Closest match UniProt accession	Protein name	Organism	%ID min.	%ID max.	Bitscore avg.	Publication
25	A0Z0U8	TH	marine Gammaproteobacterium HTCC2080	67.3	80.4	223	(Thrash *et al.*, 2010)
19	K7W8V2	THf	*Psychrobacter* sp. D8	82.1	100	194	(Öztürk *et al.*, 2013)
15	A0A1Z4GVL5	TH	*Calothrix* sp. NIES-2100	36.2	51.2	116	(Hirose *et al.*, 2017)
7	A0A2E9M8J4	TH	Dehalococcoidales bacterium	55.9	76.2	169	(Tully *et al.*, 2018)
5	A0A1Z4LA92	TH	*Nostoc linckia* NIES-25	38.2	39.9	118	(Tully *et al.*, 2018)
3	A0A2D9NM13	TH	Halieaceae bacterium	71.2	83.9	172	(Tully *et al.*, 2018)
3	A0A2E6EA89	TH	*Woeseia* sp.	75.5	76.2	245	(Bagnoud *et al.*, 2015)
1	A0A0F2QFJ7	TH	Hyphomonadaceae bacterium BRH_c29	67.7	67.7	84	(Bagnoud *et al.*, 2015)
1	A0A0F5Q235	TH	*Devosia psychrophila*	36.2	36.2	93	(Lepp *et al.*, 2015)
1	A0A0J7XXQ8	TH	*Novosphingobium barchaimii* LL02	32.9	32.9	46	(Pearce *et al.*, 2015)
1	A0A0M4LWL3	TH	*Altererythrobacter epoxidivorans*	42.6	42.6	48	(Li *et al.*, 2016)
1	A0A1M6AX62	TH	*Rubritalea squalenifaciens* DSM 18772	37.9	37.9	100	(Varghese, 2016)
1	A0A2D9ICU6	TH	*Citromicrobium* sp.	45.5	45.5	41	(Tully *et al.*, 2018)
1	A0A2E1UEX3	TH	Gammaproteobacteria bacterium	80.7	80.7	200	(Tully *et al.*, 2018)
1	Q9RPF9	TH	*Myxococcus fulvus*	53.9	53.9	182	(Hammer *et al.*, 1999)
1	T1WAM0	TH	uncultured organism	39.5	39.5	48	(Nyyssönen *et al.*, 2013)


Clade 1
S2979
S3069
S3105
S3094
S2893
S2892
S3006
S1185
S3029
S3022
S0744
S0923
S2971
S2941
S2934
S2926
S2921
S2975
S3132
S3133
S3134
S3117
S3050
S3075
S3076
S3091
S3068
S3103
S3059
S3054
TH; Halieaceae bacterium (A0A2D9NM13)
TH; marine gamma proteobacterium HTCC2080 (A0Z0U8)
TH; Gammaproteobacteria bacterium (A0A2E1UEX3)
TH; unc. microorganism (K7X7I8)
TH; unc. microorganism (K7X7I3)
Outgroup (P32370, Q56691)
Clade 4
S2152
S2173
S0888
S1368
S3248
S0272
S0279
S0245
S0243
S0240
S3238
S0292
S0360
S3281
S0302
S3275
S3291
S3290
S0310
S0369
S0278
S0109
S3310
S0319
S1442
A. aerophoba
A. archeri
A. cauliformis
A. fistularis
A. fulva
A. lacunosa
Closest UniProt Hits
TH from C. crambe
TH Reference sequences
Bootstrap support (80-100%)
Tree Scale: 0.5

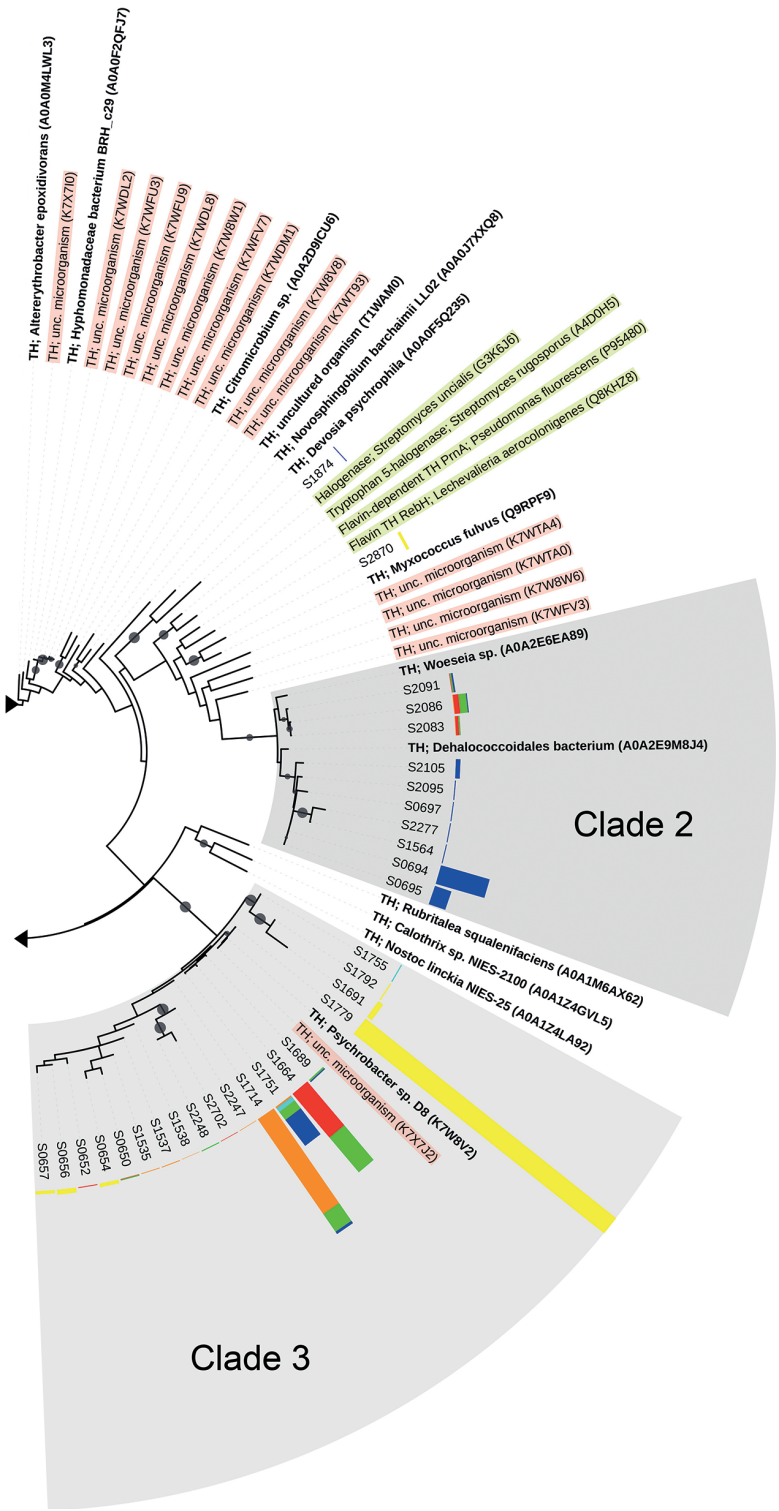


Figure 1 Caption ►

Figure 1 Maximum Likelihood Phylogeny of putative tryptophan halogenase (TH) protein sequences from six *Aplysina* species. Sequences obtained in this study are labelled with S. The tree was constructed from 247 amino acid positions. Two NADH-dependent flavin oxidoreductases (P32370 from *Clostridium scindens* and Q56691 from *Vibrio harveyi*) were used as outgroup. Four well-studied TH obtained from UniProt (green) and putative TH from the sponge *Crambe crambe* (pink) are included as reference sequences. Bars indicate relative abundance of the sequences in the sponge species, with the outer rim of the grey shading indicating 88%. Bootstrap values >80% are indicated by grey circles at the branch points. Sequences in bold refer to the closest relatives from the "halogenase" database. UniProt sequence accession numbers are given inside brackets. Tree scale corresponds to the mean expected number of amino acid substitutions per site.

While the Caribbean species shared many halogenases, it is noteworthy that sequences from the Mediterranean *A. aerophoba* were closely related to Caribbean halogenase sequences, albeit in no case identical. Furthermore it could be observed that some halogenases were highly abundant in only one sponge species such as S2941 in *A. cauliformis* or S3275 in *A. fulva*, while others such as S0310 or S3006 were detected in similar abundances in all Caribbean species.

Bacterial and Halogenase Diversity

A total of 122 142 high-quality bacterial 16S rRNA gene sequences were obtained from all sponge samples. These sequences clustered into 1993 OTUs at 97% sequence similarity. Bacterial composition at phylum level was similar for all *Aplysina* species, with Acidobacteria, Actinobacteria, Chloroflexi, Cyanobacteria, Gemmatimonadetes, SBR1093 and Proteobacteria representing the most predominant out of 20 detected bacterial phyla (Supplementary Figure S1). The 50 overall most abundant OTUs cumulatively accounted for a relative abundance between 59% (ALa2) and 90% (AFi2) in individual samples (Supplementary Figure S2). Each sponge species exhibited a large number of unique OTUs, while 39 OTUs were shared between all species. These core OTUs comprised between 6% and 45% relative abundance in the sponge samples and contained seawater-derived clades, such as Chloroflexi SAR202 (Morris *et al.*, 2004), as well as putative sponge-symbiotic bacteria such as Rhodospirillales (Karimi *et al.*, 2018), Acidobacteria and Nitrospira (Schmitt, Tsai, *et al.*, 2012).

All Caribbean *Aplysina* species shared 85 OTUs, amongst which were a predominant sponge-associated member of the Cyanobacteria (Otu0001, 1-67% relative abundance in individual samples), an unclassified bacterial OTU (Otu0010, 0.6%-8.2% relative abundance), a member of the actinobacterial OM1 clade (Otu0007, 0.6%-8.2% relative abundance) and an SBR1093 OTU (Otu0019, 0.5%-4.3% relative abundance). In total, 37 of the 50 overall most abundant OTUs were shared by all of the Caribbean species. Due to the large overlap in bacterial community structure of the Caribbean species, only *A. fistularis* clearly separated from the other Caribbean *Aplysina* spp. due to the high relative abundance of the cyanobacterial Otu0001 (>63% relative abundance). In addition, the Mediterranean *A. aerophoba* could be clearly separated from the other species in ordination plots (Figure 2 E).

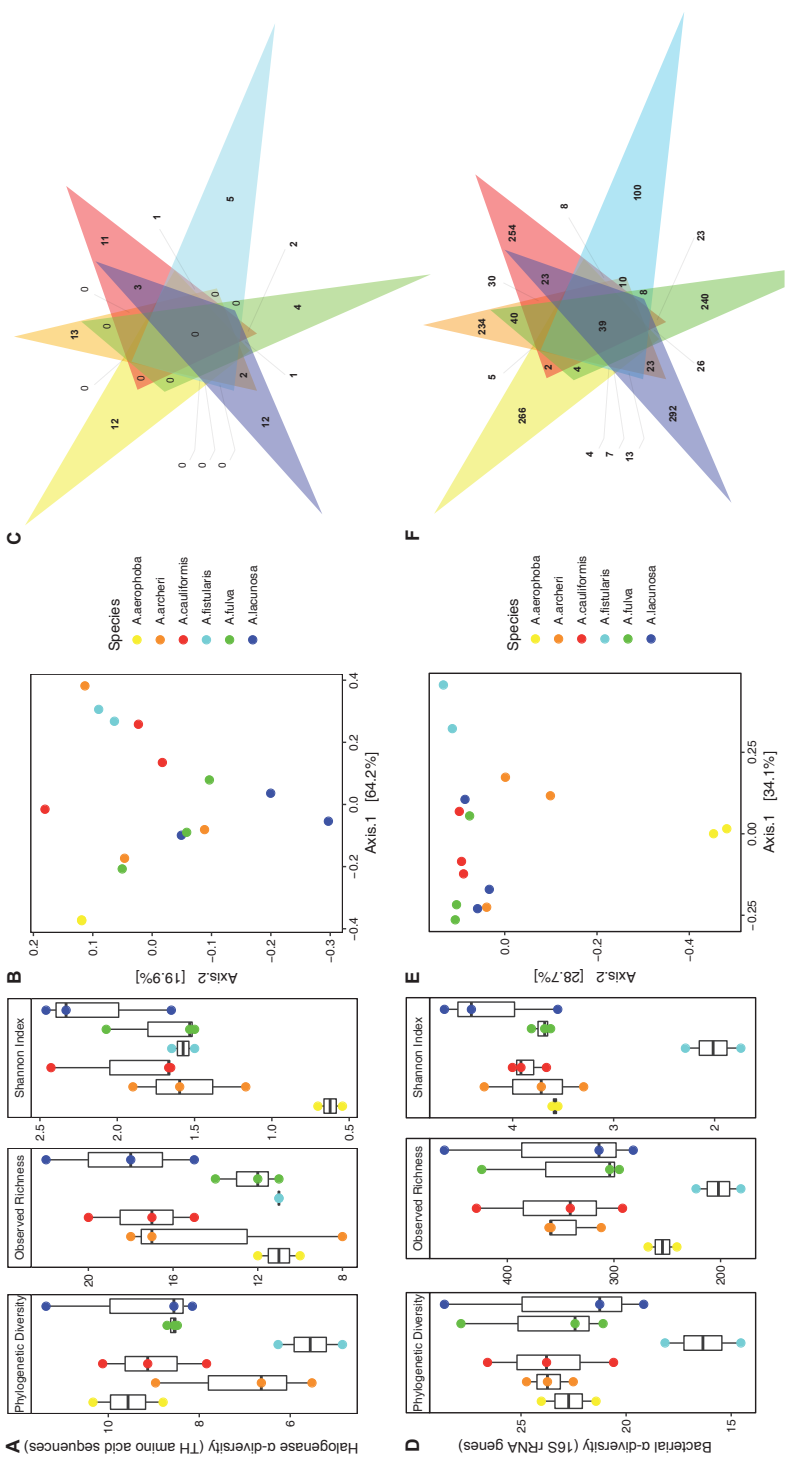


Figure 2 Halogenase and bacterial 16S rRNA gene alpha diversity indices (A,D). PCoA analysis of weighted UniFrac distances on relative abundance information of tryptophan halogenase (TH) amino acid sequences (B) and 16S rRNA genes (E). A. *aerophoba* replicates are almost identical and thus overlap in plot B. Venn diagrams of shared and unique TH sequences (C) and 16S rRNA genes (F) per sponge species.

In order to investigate connections between the halogenase diversity and the sponge-associated bacterial diversity, alpha and beta diversity indices were calculated for the 86 putative halogenase sequences and the trends were compared to the diversity indices calculated for the bacterial 16S rRNA gene sequences retrieved from the *Aplysina* samples (Figure 2 and Supplementary Figure S3). Phylogenetic Diversity, Observed Richness and Shannon Diversity indices for halogenases and bacterial 16S rRNA gene sequences followed a similar trend for all species (Figure 2 A, D), however, correlations of diversity indices were only significant for Observed Richness (Supplementary Figure S3). Overall, a high phylogenetic diversity of both bacterial OTUs and halogenase sequences was detected for *A. aerophoba*, *A. cauliformis* and *A. lacunosa*, while *A. fistularis* exhibited the least phylogenetically diverse gene repertoire (Figure 2 A).

The observed richness of *A. aerophoba* for both halogenases and bacterial 16S rRNA gene sequences was lower than for the Caribbean species, however the phylogenetic diversity of both measured variables was high, indicating a large phylogenetic breadth. These differences were not statistically significant (anova padj>0.05). Beta diversity analyses showed no discernible species separation as well as a large spread in ordination space for the Caribbean species, while the *A. aerophoba* samples clustered apart from the other species and extremely close to each other, indicating an almost identical halogenase profile as well as bacterial 16S rRNA gene profile (Figure 2, E). Overall, the first two axes explained 84% of variation in the halogenase dataset, and 63% in the 16S rRNA gene dataset, where all Caribbean species spread along the first axis, separating only *A. aerophoba* along the second axis (Figure 2 E). This analysis revealed an overlap in the halogenase composition of the Caribbean sponges, who shared four halogenase sequences (S3006, S0310, S3103, S3275). Additionally, each sponge species harbored between four (*A. fulva*) and 13 (*A. archeri*) species-specific halogenase sequences (Figure 2 C).

Co-correlation of Bacterial 16S rRNA Gene and Halogenase Relative Abundances

Since diversity calculations indicated an interrelation between halogenase and bacterial 16S rRNA gene diversity, Spearman correlations on the relative abundances of halogenase amino acid sequences with bacterial 16S rRNA genes were calculated for the 50 most predominant bacterial OTUs. All 50 most predominant OTUs exhibited significant ($p<0.05$, Spearman $r > \pm 0.5$) correlations with two or more of the 86 putative halogenases (Supplementary Figure S4). It should be noted, however, that the large number of unique and distinct halogenases as well as bacterial OTUs derived from the Mediterranean species *A. aerophoba* caused a prominent block of strong correlations (Supplementary Figure S4), and these results should thus be interpreted carefully. Therefore, we further evaluated only the relative abundance co-correlations of bacterial 16S rRNA genes and halogenases that were shared between all Caribbean species (Figure 3).

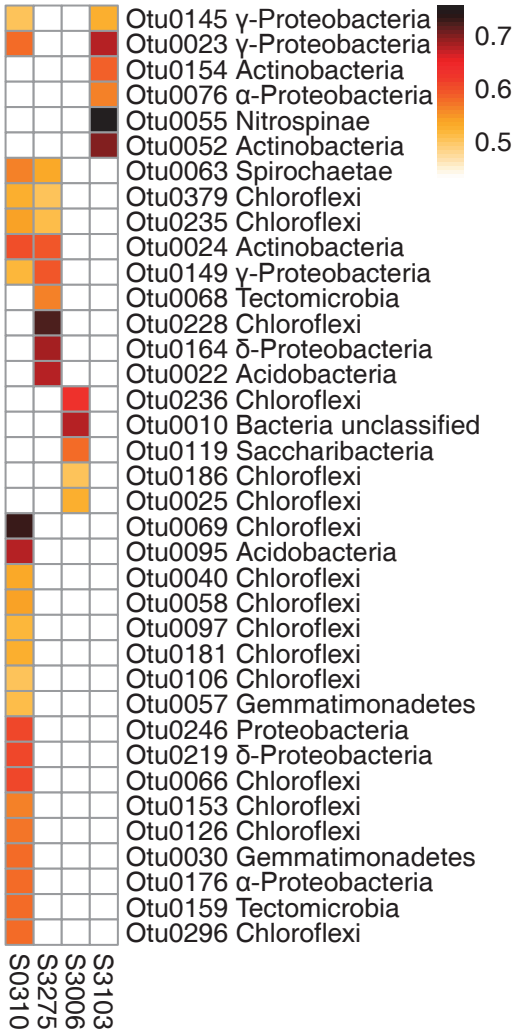


Figure 3 Heatmap displaying significant (Spearman r>0.5, p<0.05) co-occurance correlations of tryptophan halogenases (columns) and bacterial 16S rRNA gene OTUs (rows) shared between all Caribbean *Aplysina* species. OTU IDs and phyla are given to identify bacterial taxa. Columns and rows are clustered by Euclidian distance. Colors indicate correlation coefficient ranging from 0.5 (orange) to 1 (black).

Of the 85 shared bacterial OTUs, 37 were significantly (p<0.05, Spearman r>0.5) positively correlated with the four shared putative halogenases. The shared bacterial OTUs constituted between 28% and 80% (mean=46%) of the total relative abundance per sample, while the shared halogenases represent between 10% and 71% (mean=30%) relative abundance. The majority of

the correlated OTUs were affiliated to the phyla Chloroflexi (16 OTUs), Proteobacteria (8 OTUs, Alpha-, Delta- and Gammaproteobacteria), Actinobacteria (3 OTUs) and Tectomicrobia (2 OTUs) (Figure 3), all of which are renown for the production of halogenating enzymes (Bayer *et al.*, 2013, 2018; Öztürk *et al.*, 2013; Liao *et al.*, 2016; Smith *et al.*, 2017). For three halogenases, one specific bacterial OTU was found especially highly correlated (r>0.72), suggesting this distinct bacterial taxon as potential halogenase producer: The highly predominant halogenases S0310 and S3275, both phylogenetically associated to clade 4, were suggested to be produced by the Chloroflexi Otu0069, member of the sponge-associated lineage TK10 (Schmitt *et al.*, 2011; Burgsdorf *et al.*, 2014), and an unclassified Chloroflexi Otu0228 with 98% 16S rRNA gene sequence identity to a sponge-associated member of the Chloroflexi (GenBank FJ481334, (Montalvo & Hill, 2011)), respectively. The less abundant halogenase S3103, phylogenetically placed in clade 1, was most highly correlated to an unclassified Nitrospinae Otu0055, displaying 97% 16S rRNA gene sequence identity to an uncultured sponge-associated bacterium (GenBank FJ900348, (Kamke *et al.*, 2010)). Despite this analysis being of only predictive nature, the co-occurrence of certain halogenases with specific bacterial taxa can narrow down the range for searching potential microbial producers of these proteins.

Discussion

The marine environment is a prolific source of novel enzymes with potential industrial applications. To screen marine samples for such enzymes, metagenomics approaches are promising, whereby all extracted DNA is investigated with DNA sequence-based methods (Hugenholtz & Tyson, 2008; Kennedy *et al.*, 2008; Vakhlu *et al.*, 2008; Wilson & Piel, 2013; Barone *et al.*, 2014; Loureiro *et al.*, 2018). For genes where suitable PCR primers can be designed, a PCR-based screening approach is especially useful to rapidly explore diversity of the gene of interest in a larger number of environmental samples. Such approaches have previously been applied for the discovery of novel natural product biosynthesis genes (Zhao *et al.*, 2008; Milshteyn *et al.*, 2014; Amos *et al.*, 2015; Müller *et al.*, 2015; Borchert *et al.*, 2016), lipases (Wang *et al.*, 2010) or alcohol dehydrogenases (Itoh *et al.*, 2014), to just name a few examples from a broad range of functional genes discovered in environmental samples (Kotik, 2009). In this study, the PCR-based screening approach resulted in the discovery of a large phylogenetic breadth of previously undescribed, putative halogenase protein fragments, which may be involved in the biosynthesis of numerous brominated natural products found in Mediterranean and Caribbean *Aplysina* species.

Sponges Harbor Numerous Novel Putative Tryptophan Halogenases

Out of all detected protein sequences clustered at 95% amino acid sequence identity, 86.24% had a match in the entire UniProt database, hinting at still a large number of unknown protein sequences in marine sponges. Additionally, only 86 out of 1918 sequences could successfully be identified as putative halogenases. This could indicate a low specificity of the primers applied, which is a regularly observed problem for primers targeting functional genes in microbial communities (Mohamed *et al.*, 2010; Pereyra *et al.*, 2010; Gaby & Buckley, 2012; Bonilla-Rosso *et al.*, 2016) due to relatively low degrees of sequence conservation in comparison to the 16S rRNA gene.

On the other hand, the number (86) of putative THs is similar for sponges and other environmental samples such as freshwater, marine and soil environments or cultivated Actinomycetes strains (18 (Öztürk *et al.*, 2013), 36 (Bayer *et al.*, 2013), 38 (Erol *et al.*, 2017), 103 (Hornung *et al.*, 2007), 254 (Neubauer *et al.*, 2018)). The low sequence identities (mean=44%), and the match to only few (16) "halogenase" entries from the UniProt database, further indicate a large, yet untapped resource of halogenases in marine sponges. Only one sequence (S2870 in *A. aerophoba*) with low abundance (<1%) was related with <44% amino acid sequence similarity to two well-charaterized flavin-dependent THs, PrnA and RebH (Figure 1). PrnA is encoded in the biosynthetic gene cluster for the production of the antifungal compound pyrrolnitrin in *Pseudomonas fluorescens* (Harris *et al.*, 1985), wheras RebH is part of the biosynthetic gene cluster for the anticancer compound rebeccamycin in *Lechevalieria aerocolonigenes* (Onaka *et al.*, 2003).

Due to low sequence identies to well-studied halogenases, the putative THs could not be confidentially assigned to any of the known structural classes of halogenated compounds. These enzymes probably represent novel types, which halogenate different structural moieties compared to previously reported halogenases. Such a TH with an unusual substrate preference has been discovered in the metagenome of the sponge symbiont *Candidatus* Entotheonella serta (Smith *et al.*, 2017). This halogenase displays only between five and 27% amino acid sequence identity to the halogenases from this study (data not shown) and represents another recent example of the large, yet untapped genetic resources for novel halogenases in marine sponges. Our phylogenetic analyses resulted in four distinct clades, potentially separating functionally divergent groups of halogenases. These findings expand on previously reported results for *A. aerophoba* (Bayer *et al.*, 2013) and *C. crambe* (Öztürk *et al.*, 2013) that showed three distinct sponge-specific clades of halogenases. Each analyzed sponge species harbored sequences from all four clades, hinting at a potentially highly diverse spectrum of halogenated molecules that can be synthesized within each sponge. Rua *et al.* (2018) hypothesized that larger microbiome diversity influences the potential of bioactive compound production in sponges. In our study we found that a high phylogenetic diversity and richness of microbial taxa corresponded to a high diversity of halogenases. The Mediterranean *A. aerophoba* harbored a bacterial community distinct from that of the Caribbean species, which is consistent with previous investigations (Thomas *et al.*, 2016; Chaib De Mares *et al.*, 2017), and while its halogenases were closely related to Caribbean ones, they were in no case identical.

All except for two of the detected novel putative halogenases as well as their closest relatives exhibited active binding sites for flavin and L-tryptophan. Since these enzymes are known to tolerate a wide range of organic scaffolds (Agarwal *et al.*, 2017) and since all FDHs known to date can also function as brominases (Xu & Wang, 2016) they are potentially halogenating completely unknown organic substrates. Thus, these enzymes may contribute to the production of the more than 100 halogenated natural products reported from *Aplysina* species (Turon *et al.*, 2000; Thoms *et al.*, 2006; Lira *et al.*, 2011; Loh & Pawlik, 2014; Puyana *et al.*, 2015). However, further studies are necessary to unravel the exact functions of these enzymes as no closely related enzymes have been functionally characterized (Figure 1).

Prominent Sponge Symbionts are Potential Halogenase Producers

In an attempt to predict the potential bacterial producers of the detected halogenase genes, we correlated the relative abundances of the four 16S rRNA gene OTUs and the 85 putative halogenases shared by the Caribbean *Aplysina* species. We hypothesized that the shared halogenases are produced by bacteria that can be found amongst the shared bacterial taxa in the different sponge species. Furthermore, an increased relative abundance of the producers is expected to be reflected in an increased number of detected halogenase genes. Two halogenases (S0310 and S3275) were the most highly correlated with sponge-associated Chloroflexi (Otu0069 and Otu0228 respectively, Figure 3). These clades include predominant sponge symbionts with the genomic repertoire for chemical defense (Slaby *et al.*, 2017; Bayer *et al.*, 2018). Chloroflexi were previously found to contain halogenases (Bayer *et al.*, 2013) and were linked to the production of brominated compounds in *A. aerophoba* (Sacristán-Soriano *et al.*, 2011, 2016). We furthermore detected strong correlation of a halogenase sequence to a Nitrospinae OTU, however, little is known about this taxon in sponges to date. Nonetheless, Nitrospinae are closely related to the candidate phylum Tectomicrobia, which occurs in *Aplysina* species (Chaib De Mares *et al.*, 2018) and of which some members are renowned for their large secondary metabolism gene repertoire (Wilson *et al.*, 2014; Smith *et al.*, 2017).

One highly predominant cyanobacterial OTU was present in all Caribbean species and was not significantly correlated to halogenases in this study, adding to the hypothesis that the role of Cyanobacteria in sponge-microbe symbioses of high microbial abundance sponges might be mainly related to nutrient production rather than chemical defense (Freeman *et al.*, 2013; Burgsdorf *et al.*, 2015). In low microbial abundance sponges, however, the production of brominated metabolites has been linked to cyanobacterial symbionts (Unson *et al.*, 1994; Flatt *et al.*, 2005). Thus, our results support previous findings and for the first time indicate potential microbial producers of brominated compounds found in Caribbean *Aplysina* species. However, further studies, including comparative and functional genomics of sponge symbionts are necessary to reliably link halogenated compound production to their microbial producers.

Conclusion

The plethora of previously undescribed putative flavin-dependent THs from the metagenomic DNA of Mediterranean and Caribbean *Aplysina* species unraveled here indicates a large potential for the discovery of novel halogenating enzymes from these marine invertebrates and their associated microbiomes. The separation into four phylogenetically distinct clades of halogenase protein sequences indicates that multiple classes of organic scaffolds may be halogenated by sponge-associated microbes. High bacterial diversity was in most cases indicative of a high halogenase diversity, and while the Caribbean species shared many halogenases as well as bacterial OTUs, the Mediterranean *A. aerophoba* could be clearly distinguished. Based on co-occurrence, three prominent bacterial sponge symbionts belonging to the Chloroflexi and Nitrospinae were identified as potential sources of abundant halogenases. These results may thus contribute to explaining the origin of the numerous halogenated compounds discovered in *Aplysina* species.

Acknowledgements

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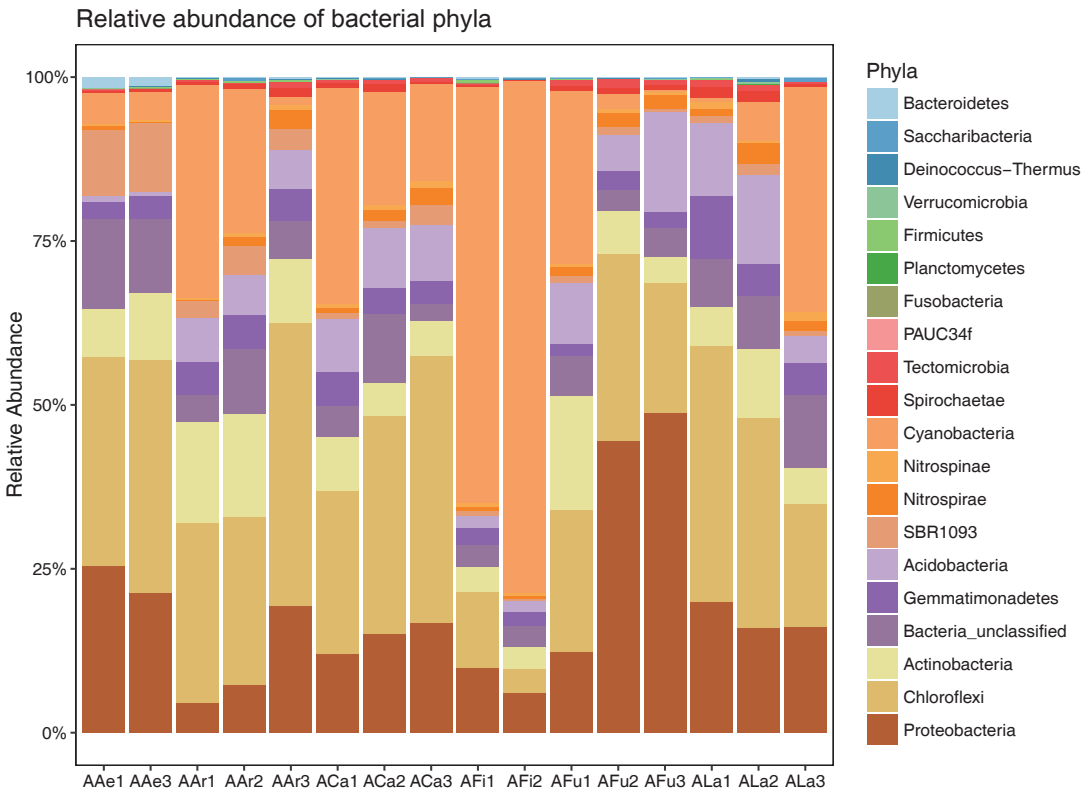
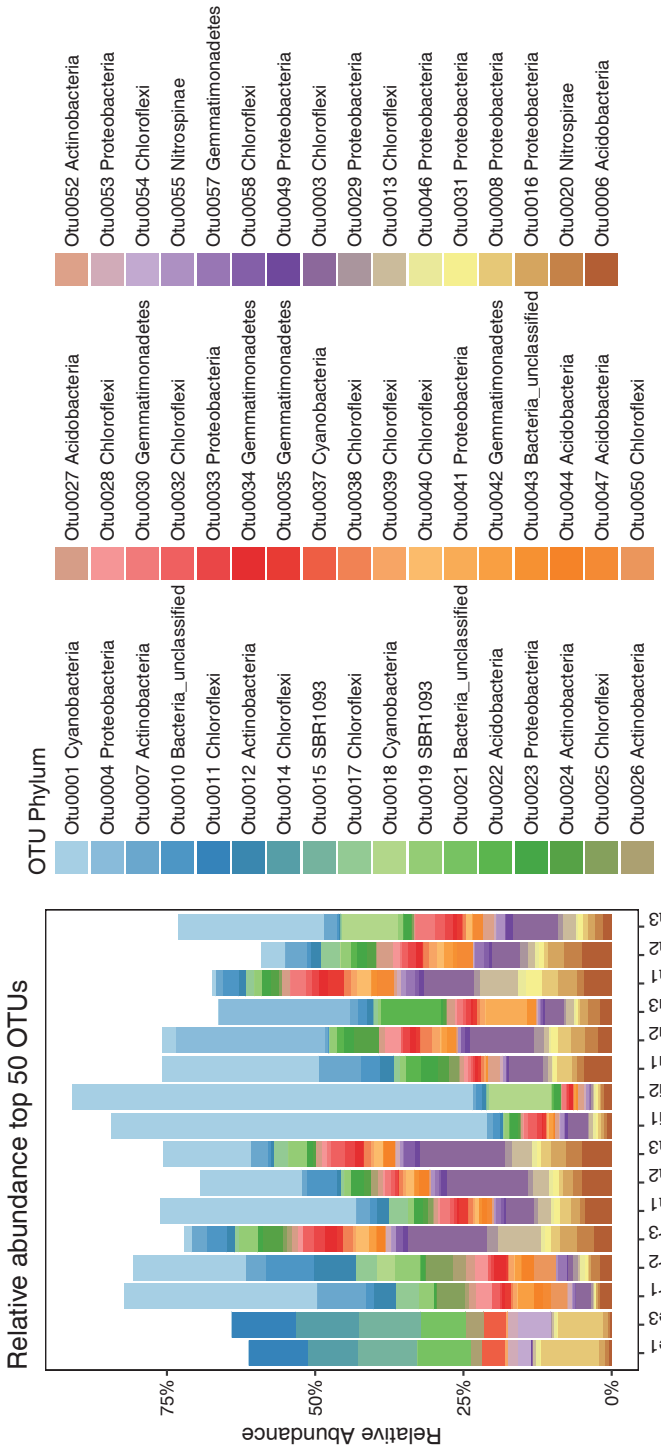
We thank María J. Uriz from the Centre d'Estudis Avançats de Blanes (CEAB), CSIC, Blanes, Spain for providing support for sample collection and access to the laboratories for prompt processing, Søren Sørensen and Waleed Abu Al-Soud with the sequencing of the halogenase amplicons and Sudarshan Shetty and Georg Steinert for help with the data analysis.

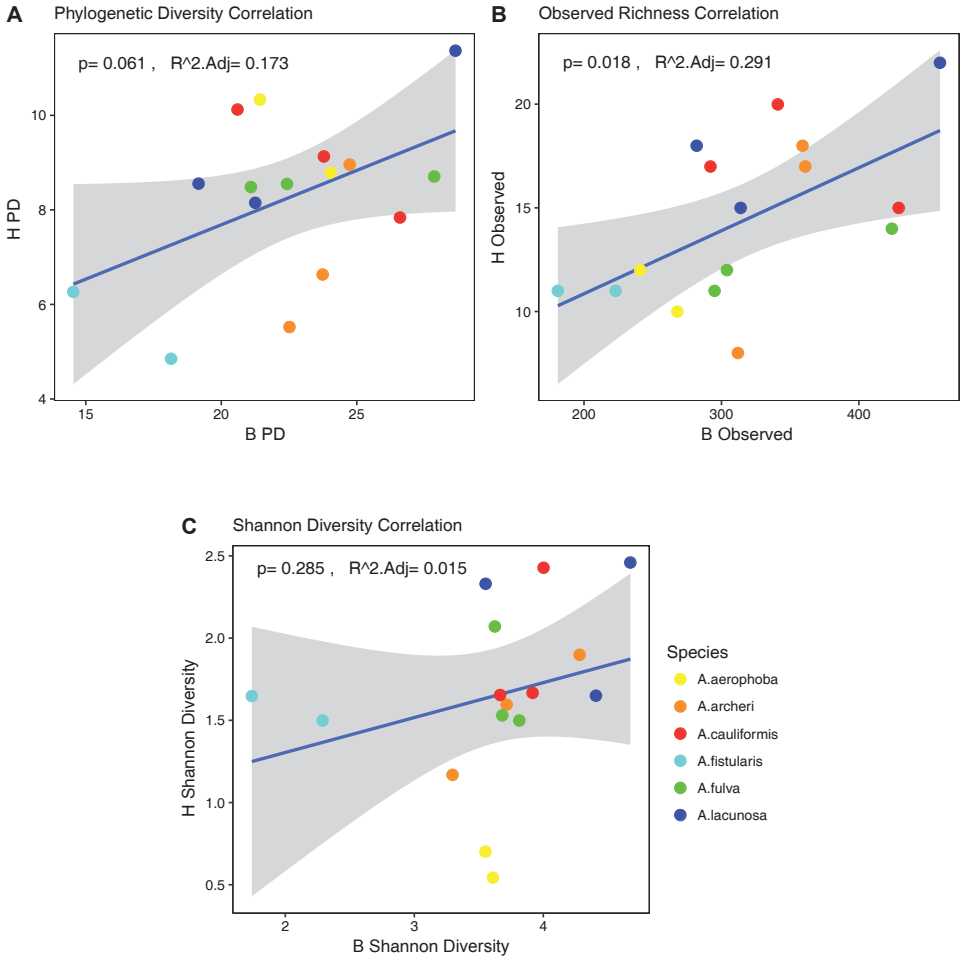
Supplementary Information



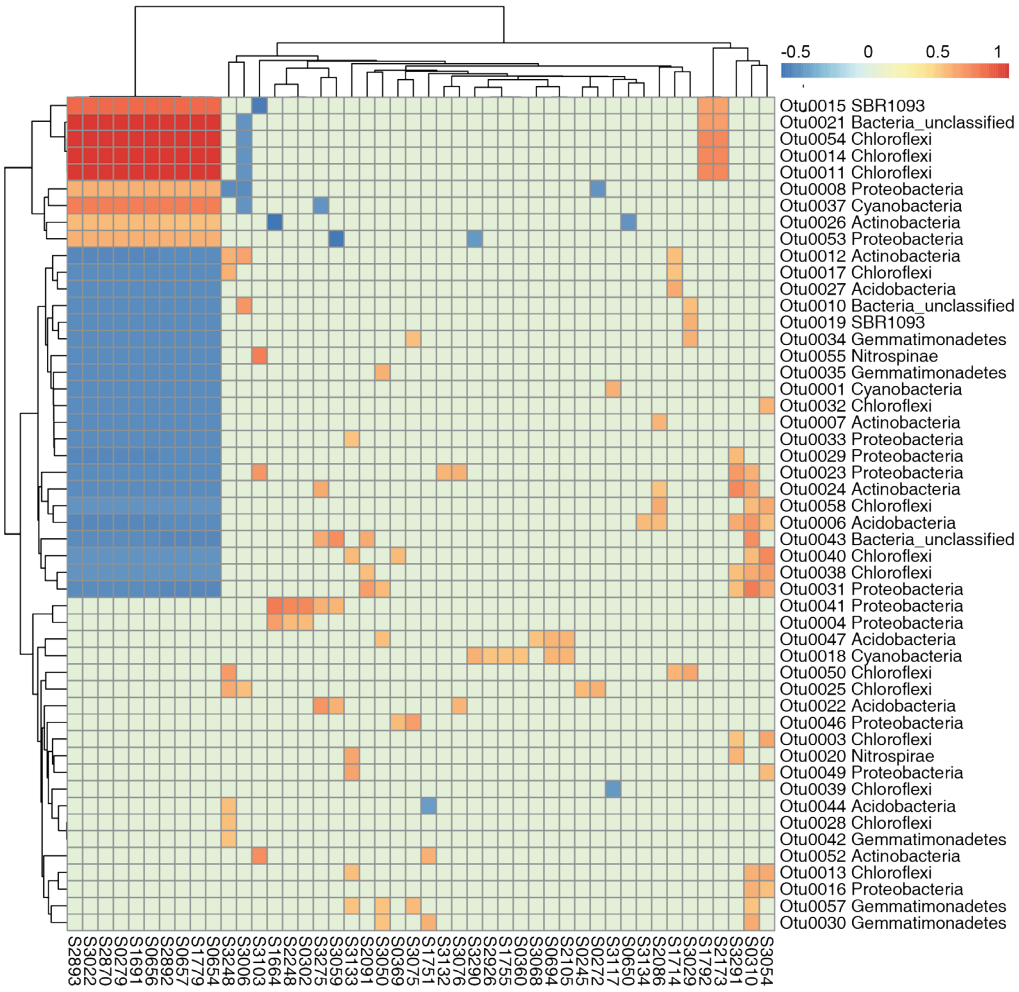
Supplementary Figure S1 Relative abundance of bacterial phyla in *Aplysina* species.



Supplementary Figure S2 Relative abundance of the overall 50 most abundant 16S rRNA gene OTUs in *Aplysina* species.



Supplementary Figure S3 Linear correlations between diversity metrics calculated for bacterial (B) 16S rRNA genes (x-axis) and halogenases (H) (y-axis).



Supplementary Figure S4 Heatmap showing significant (Spearman r>±0.5, p<0.05) correlation of relative halogenase abundances (columns) with the relative abundance of the overall 50 most prevalent bacterial OTUs (rows). Columns and rows are clustered by Euclidian distance.

Chapter 3

Coexistence of Poribacterial Phylotypes among Geographically Widespread and Phylogenetically Divergent Sponge Hosts

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Abstract

Marine sponges are benthic 'filter-feeding' invertebrates that can host dense and diverse bacterial, archaeal, and eukaryotic communities. Due to the finding of several genes encoding symbiosis factors, such as adhesins, ankyrin repeats and tetratricopeptide repeats, the candidate phylum Poribacteria is considered as a promising model microorganism for studying the origin of host-symbiont interactions in sponges. However, relatively little is known about its global diversity and phylogenetic distribution among different sponge hosts. Therefore, in the present study we investigated phylogenetic relationships among poribacterial phylotypes and generated a phylogenetic network to examine the distribution and intraspecific diversity of the phylotypes between phylogenetically divergent host-sponges at a global scale. For this study 361 poribacterial 16S rRNA gene sequences obtained by Sanger sequencing from 15 different countries and 8 marine regions were gathered. We could demonstrate that the candidate phylum Poribacteria is composed of diverse phylotypes, which are distributed among a wide range of phylogenetically divergent sponge hosts. The current phylogenetic analyses found neither conclusive evidence for co-speciation with its hosts, nor biogeographical correlation. Moreover, we identified a novel poribacterial clade, which might represent a link between the previously established four Poribacteria clades.

Introduction

Marine and freshwater sponges (Porifera) are benthic 'filter-feeding' invertebrates that form symbiotic relationships with a wide range of bacterial, archaeal, and eukaryotic microorganisms (Hentschel *et al.*, 2012). To date, 41 prokaryotic phyla (including candidate phyla) were found associated with marine sponges, of which many enriched phyla form part of sponge-specific 16S ribosomal RNA (rRNA) gene sequence clusters (Taylor, Thacker, *et al.*, 2007; Simister *et al.*, 2012; Thomas *et al.*, 2016). These sponge-associated prokaryotic assemblages, especially the abundant host-specific bacterial members, tend to remain stable over time and geographical distances (Erwin *et al.*, 2012; Pita *et al.*, 2013; Hardoim & Costa, 2014; Esteves *et al.*, 2016). Another remarkable feature of the complex sponge-microbe relationships is the categorization of sponge species into either 'high microbial abundance' (HMA) or 'low microbial abundance' (LMA) sponges, based on electron microscopy (Vacelet & Donadey, 1977; Reiswig, 1981; Hentschel *et al.*, 2003, 2006; Gloeckner *et al.*, 2014). HMA sponges in particular harbor phyla such as Acidobacteria, Chloroflexi, Cyanobacteria, Proteobacteria, and the candidate phylum Poribacteria, with characteristically higher abundances as compared to LMA sponges (Noyer *et al.*, 2010; Abdelmohsen *et al.*, 2014; Bayer, Kamke, *et al.*, 2014; Moitinho-Silva *et al.*, 2014).

The sponge-associated bacterial candidate phylum Poribacteria (referred to as Poribacteria hereafter) was first described by Fieseler *et al.* (2004) as being present in several Mediterranean sponges, recognizable by its distinct feature of cell compartmentalization (Kamke *et al.*, 2014; Jahn *et al.*, 2016). Besides the evidence that Poribacteria are highly enriched in marine sponges (Lafi *et al.*, 2009; Hentschel *et al.*, 2012), poribacterial sequences were also retrieved from various seawater samples, although only in low abundances (Pham *et al.*, 2008; Webster *et al.*, 2010; Taylor *et al.*, 2013; Moitinho-Silva *et al.*, 2014). Quantitative analyses and deep sequencing revealed that poribacterial 16S rRNA gene sequences are abundant in several HMA sponges (Schmitt, Hentschel,

et al., 2012; Moitinho-Silva *et al.*, 2014; Steinert *et al.*, 2016), and may act as a potential indicator taxon for HMA sponges (Bayer, Kamke, *et al.*, 2014). Molecular tools enable the accumulation of knowledge on the lifestyle of this phylum, despite the absence of cultured representatives. For instance, single cell genomics revealed a mixotrophic lifestyle with the genetic potential to fix CO_2 through the Wood-Ljungdahl pathway (Siegl *et al.*, 2011) and to degrade uronic acids and a wide range of carbohydrates (Kamke *et al.*, 2013). Additionally, halogenase genes (Bayer *et al.*, 2013), as well as genes associated with the uptake and utilization of dissolved organic phosphorous compounds (Kamke *et al.*, 2014) were detected. Furthermore, the close association of Poribacteria with sponges seems to be supported by several genes encoding symbiosis factors such as adhesins, ankyrin repeats and tetratricopeptide repeats (Siegl *et al.*, 2011). Considering all these genomic features, Poribacteria represent a promising model for studying the origin of host-symbiont interactions within the animal kingdom.

However, in contrast to the recent progress made in elucidating the genomic aspects that make Poribacteria a potential key symbiont phylum in marine sponges, still relatively little is known about its global diversity and phylogenetic distribution among different sponge hosts. The Poribacteria represent a monophyletic lineage within the Bacteria domain in proximity to the Planctomycetes-Verrucomicrobia-Chlamydiae (PVC) superphylum (Kamke *et al.*, 2014). Previous phylogenetic studies, either including only a single sponge species from two closely connected locations (Hardoim *et al.*, 2013), or sampling from a phylogenetically diverse range of sponges from four globally distributed geographic locations (Lafi *et al.*, 2009), revealed the presence of four poribacterial clades. Based on this small number of studies, those four phylogenetically divergent clades can be found within a single sponge species as well as across geographically and phylogenetically separated sponge specimens. However, it remains unclear whether the entire phylogenetic diversity of this presumably cosmopolitan bacterial phylum has been resolved sufficiently. Concerning the global poribacterial diversity, a recent short sequence clustering approach (i.e., IMNGS) found 151 putative species (clustered at 3% sequence dissimilarity) and 38 genera (5% sequence dissimilarity) belonging to Poribacteria to be present in the available 16S rRNA gene amplicon datasets obtained from the NCBI Sequence Read Archive (Lagkouvardos *et al.*, 2016). Unfortunately, comparisons between 'similarity-based' clustering and phylogenetic inferences are only possible to a limited extent because sequence evolution models are neglected in clustering algorithms. Moreover, without considering the geographic origin of the sponge hosts and due to rigorous sequence filtering, only a few source sponge species were taken into account in the study of Lagkouvardos *et al.* (2016). Finally, the intraspecific poribacterial diversity within different sponge specimens of the same species has yet to be addressed in 16S rRNA gene studies.

For the present study we obtained 146 novel poribacterial 16S rRNA gene sequences from four sponge taxa (*Aplysina aerophoba*, *Penares sollasi*, *Rhabdastrella globostellata*, and a *Theonella* sp.) collected in Spain and Indonesia using Sanger sequencing of PCR-amplified 16S rRNA gene fragments covering the V3-V6 region. These sequences were aligned with 215 archived poribacterial sequences from 15 recent studies, making a total of 361 sequences accumulated from 23 phylogenetically diverse sponge hosts, which were collected from eight different marine regions. With this dataset we aimed to investigate phylogenetic relationships and diversity among Poribacteria phylotypes by phylogenetic tree and network construction. Phylogenetic tree construction was used to place existing and new poribacterial 16S rRNA gene sequences

into the framework of the four phylogenetic groups that were established by Lafi *et al.* (2009). Phylotype networks were used to address biogeographic distribution of poribacterial 16S rRNA gene phylotypes amongst sponge taxa and potential co-existence with their respective hosts at a global scale. Finally, we used the 361 sequences and next-generation-sequencing data from the sponge microbiome project (Moitinho-Silva *et al.*, 2017) to estimate the richness of the candidate phylum Poribacteria.

Materials and Methods

In 2015, single individuals from three different sponge species (i.e., *P. sollasi*, *R. globostellata*, and *Theonella* sp.) were collected by SCUBA diving from different shallow locations: Batu Angus (1.50N, 125.25E), Pulau Putus (1.52N, 125.28E) and Tanjung Nanas (1.46N, 125.23E) in Lembeh Strait, North Sulawesi, Indonesia. In addition, in 2014 three *Aplysina aerophoba* specimens were collected in Cala Montgo (42.11N, 3.17E), Spain, by SCUBA diving. Tissue preparation prior to DNA extraction was performed as previously described in Sipkema *et al.*, (2011).

Genomic DNA was extracted from tissue of all sponge specimens following the standard protocol from the FastDNA Spin Kit for Soil (Qbiogene, CA, USA). Partial 16S rRNA genes were amplified from all samples by PCR using the Poribacteria-specific primers POR389f (5'-ACG ATG CGA CGC CGC GTG-3') and POR1130r (5'-GGC TCG TCA CCA GCG GTC-3') (Fieseler *et al.*, 2004). PCR amplification was performed in a volume of 50 µL containing 1 µL DNA template (~15 ng), 2 µL dNTP mix (final concentration 200 µM), 3 µL $MgCl_2$ (1.5 mM), 3 µL forward primer (0.5 µM), 3 µL (0.5 µM) reverse primer, 10 µL 5 x GoTaq buffer (Promega, WI, USA), 0.5 µL GoTaq polymerase (Promega, WI, USA) and 27.5 µL dH_20. PCR was performed using the following conditions: initial denaturation of 98°C for 5 min, followed by 30 cycles of denaturation at 98°C for 20 s, annealing at 68°C for 20 s, elongation at 72°C for 45 s, and a final elongation at 72°C for 5 min. After amplification, the PCR products were examined on a 1% agarose gel and purified using the GeneJET PCR purification kit (Fermentas, Inc. ON, Canada).

Purified PCR products from each reaction were cloned into a pGEM-T Easy Vector (Promega, WI, USA) following the manufacturer's protocol. Subsequent Sanger-sequencing was performed by GATC Biotech (Konstanz, DE). Forward and reverse raw sequence reads were automatically trimmed at both ends (error probability limit 0.05%) and then aligned to create consensus sequences in Geneious v.4.8.3 (Kearse *et al.*, 2012). Consensus sequences were converted to Fasta format and deposited in the NCBI GenBank under accession numbers KX519551 to KX519697.

In order to create a Poribacteria-specific ARB database all available Poribacteria sequences (n = 187) were downloaded from the SSU r123 SILVA database (https://www.arb-silva.de/search/ - sequence entry Poribacteria) as a globally SINA aligned ARB file (Pruesse *et al.*, 2007, 2012). In addition, the newly generated sequences (n = 146) were also aligned using the SINA online alignment service (https://www.arb-silva.de/aligner/ - variability profile: Bacteria) and merged with the downloaded Poribacteria sequences from the SILVA database in ARB (Ludwig *et al.*, 2004). Finally, the newly created sequences were used for an NCBI BLAST search against the Nucleotide Collection (nr / nt & megablast), and additional poribacterial sequences (n = 45) were downloaded, aligned by SINA

and merged with the Poribacteria ARB database. Further information about the poribacterial 16S rRNA gene sequences downloaded from the SILVA and NCBI databases can be found in Supporting Information Table S2.

Prior to phylogenetic reconstructions the final alignment (n = 379 sequences) was manually checked with the ARB alignment viewer and 18 sequences, either with non-sufficient overlap to the full alignment or a too low SINA alignment quality value (< 75%), were rejected prior to analyses, resulting in a final alignment of 361 poribacterial sequences. Two alignments were created, one with an outgroup sequence for the phylogenetic tree and another one without outgroups for the phylogenetic network. Both alignments were analyzed using the Gblocks Server to eliminate poorly aligned positions and divergent regions using standard parameters and only allowing smaller final blocks as a less stringent option (Talavera & Castresana, 2007). Maximum likelihood (ML) analysis was conducted using RAxML v.8.2.7 (rapid bootstrap, 1000 replicates, GTR substitution model of evolution) (Stamatakis, 2014). We chose nine sequences of sponge-associated Chloroflexi and Verrucomicrobia as outgroup since these bacterial phyla represent close relatives to the candidate phylum Poribacteria (for further details see Supporting Information Data S1 and Fig. 1). For the diversity and phylogenetic network analyses the non-outgroup Gblocks alignment was first clustered into OTUs ranging from unique to 95% (0.05) sequence similarity using mothur v.1.36.1 (P. D. Schloss *et al.*, 2009). The phylogenetic network for the unique OTUs was constructed using the TCS method in PopART v1.7 (Leigh & Bryant, 2015). A genetic distance matrix was calculated for the non-outgroup Gblocks alignment using ClustalOmega (McWilliam *et al.*, 2013). The taxonomy of the deblurred sponge microbiome project dataset (Moitinho-Silva *et al.*, 2017) was updated using mothur and the SILVA128 full-length references and sequences (https://www.mothur.org/wiki/Silva_reference_files). The software R v.3.4.2 was used to create a poribacterial specific subset of the sub-OTU abundance table (R Development Core Team, 2016). The Rarefaction analysis and diversity estimations (Chao1, ACE, Shannon) were performed as implemented in mothur.

In order to identify the newly added six sponge specimens, for five specimens the 5' end of the mitochondrial cytochrome oxidase subunit 1 (COI) was sequenced using the degenerate primers: jgLCO1490 (5'-TIT CIA CIA AYC AYA ARG AYA TTG G-3') and jgHCO2198 (5'-TAI ACY TCI GGR TGI CCR AAR AAY CA3') (Geller *et al.*, 2013). PCR amplification was performed in the same volume and composition as described above for the partial 16S rRNA genes, following the PCR protocol as described in Geller *et al.* (2013). However, due to amplification issues of the COI region, for one sponge sample the 28S rRNA gene C1–D1 fragment was amplified with primers 28S-C2F (5'-GAA AAG AAC TTT GRA RAG AGA GT-3') and 28S-D2R (5'-TCC GTG TTT CAA GAC GGG-3') described in Erpenbeck *et al.* (2007) following the included PCR protocol. After amplification, PCR products were examined and purified from a 1% agarose gel using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA) and cloned into a pGEM-T Easy Vector (Promega, WI, USA) following the manufacturer's protocol. Sanger sequencing was performed by GATC Biotech (Konstanz, DE). Electropherograms were processed with Geneious as described above, subsequently aligned, and stored as consensus if applicable. The three closest matches were identified by BLAST searches (https://blast.ncbi.nlm.nih.gov) against the nucleotide collection (nr/nt). Sequences were deposited at the NCBI GenBank under accession numbers KY625183-KY625188.

Results and Discussion

Sponge Host Taxonomy

The five sponge samples collected for this study were identified by Nicole J. de Voogd based on classical taxonomic identification and by molecular barcoding based on mitochondrial cytochrome oxidase subunit 1 (COI) or 28S rRNA gene sequence as: *A. aerophoba*, *P. sollasi*, *R. globostellata,* and *Theonella* sp. (Supporting Information Table S1). Overall, two Porifera classes are represented among the sponge species analyzed in this study: a) Demospongiae, which comprises 22 sponge taxa, and b) Homoscleromorpha, which includes only *Plakortis* sp. as sponge taxon belonging to this class. These 23 sponge host taxa are distributed among 17 sampling locations from 11 countries and eight marine regions (Table 1).

Poribacterial Diversity

In total, 361 poribacterial 16S rRNA gene sequences were used for all following analyses (Supporting Information Data S1). The final alignment consisted of 215 sequences retrieved from the SILVA and NCBI databases and 146 novel sequences, which were generated Sanger sequencing of PCR-amplified 16S rRNA gene fragments from the six sponge individuals described above using Poribacteria-specific primers targeting the V3-V6 region. Hence, we increased the total number of available poribacterial 16S rRNA gene sequences generated by Sanger sequencing by 40%.

To estimate the richness and diversity within the Poribacteria, the 361 sequences were clustered using different similarity thresholds (i.e., unique, 99%, 97% and 95% sequence identities) (Table 2, Supporting Information Data S2 and Data S3). The apparent trend of increasing diversity at higher Operational Taxonomic Unit (OTU) similarity (Table 2) was already reported previously for one single sponge species, *Aplysina fulva*, in two geographically closely located sponge populations from Brazil (Hardoim *et al.*, 2013), however, here we observed the same pattern in 23 phylogenetically divergent and globally distributed sponge taxa. With 172 unique sequences and 63 OTUs at 99% similarity threshold the current analysis yielded values that fall within the range of the 151 molecular species (at ~3% similarity) reported in the IMNGS cluster analysis using 2308 16S rRNA gene amplicon reads obtained from the NCBI SRA database (Lagkouvardos *et al.*, 2016). Finally, the Poribacteria-only subset of the sponge microbiome project (i.e., 1654 out of 3835 samples, comprising 204 microhabitats that consist of different sponge taxa, seawater, sediment, and biofilm) yielded 106 poribacterial sub-operational-taxonomic-units (i.e., sub-OTUs) and similar ACE and Chao richness estimates (n = 106) due to the high read coverage (Table 2). The relatively low poribacterial sub-OTU richness, despite the great sampling depth, could be explained by the short amplicon fragments (100 nucleotides) and low intragenomic heterogeneity of the targeted V4 region (Sun *et al.*, 2013), compared to the longer 16S rRNA fragments utilized in both the IMNGS (> 310 nucleotides) and the present study (598 before and 295 nucleotides after Gblocks selection, comprising the variable regions V4 to V6). In addition, taxon-specific conservation states of different 16S rRNA gene regions (Stackebrandt, 1994; Claesson *et al.*, 2010) and different taxonomic training sets (Werner *et al.*, 2012) can influence the taxonomic classification of prokaryotic 16S rRNA gene sequences, and therefore also the overall observed richness. Since the discussed species richness estimates were obtained by three different methodological approaches, these results should only be compared cautiously.

Table 1 Sample information depicting host identities, biogeographical sample origin, the originating water bodies / marine regions and the number of poribacterial sequences obtained per reference.

Host sponges	Locations	Marine regions	# Seqs	Reference
Aplysina aerophoba, Penares sollasi, Theonella sp., Rhabdastrella globostellata	Cala Montgo (Spain), Batu Angus (Indonesia), Tajung Nanas (Indonesia), Pulau Putus (Indonesia)	Mediterranean Sea, Western Pacific Ocean	89, 16, 27, 15	present study
Aplysina cauliformis	Lee Stocking Island (Bahamas), Carrie Bow Cay (Belize)	Caribbean Sea	5	Olson, Thacker & Gochfeld 2014
Aplysina fulva	Caboclo Island & Tartaruga Beach (Brazil)	South Atlantic Ocean	101	Hardoim et al. 2013
Aplysina aerophoba	Coast of Rovinj (Croatia)	Mediterranean Sea	1	Bayer et al. 2013
Vaceletia crypta	Yonge Reef (Australia)	South Pacific Ocean	2	Karlinska-Batres & Wörheide 2013
Astroclera willeyana	Yonge Reef (Australia)	South Pacific Ocean	1	Karlinska-Batres & Wörheide 2013
Aplysina aerophoba	Coast of Rovinj (Croatia)	Mediterranean Sea	6	Kamke et al. 2013
Ircinia varibilis	Tossa de Mar (Spain)	Mediterranean Sea	1	Erwin et al. 2012
Xestospongia testudinaria	Manado Bay (Indonesia)	Western Pacific Ocean	1	Montalvo & Hill 2011
Ircinia strobilina	Conch Reef (USA)	Gulf of Mexico	8	Mohamed et al. 2010
Geodia barretti	Korsfjord (Norway)	North Sea	29	Hoffmann et al. 2009 & Jensen et al. 2016
Rhabdastrella globostellata, *Pseudoceratina clavata*, *Aplysina lacunosa*, *Theonella swinhoei*, *Aplysina aerophoba*, *Aplysina cavernicola*, *Aplysina fistularis*, *Aplysina lacunosa*, *Plakortis* sp., *Ircinia* sp., *Theonella swinhoei*, *Xestospongia muta*	Ngetngod Reef (Palau), Little San Salvador Island (Bahamas), San Diego (USA), Banyuls sur Mer (France)	Caribbean Sea, Eastern & Western Pacific Ocean, Mediterranean Sea	54	Lafi et al. 2009
Aplysina aerophoba	Coast of Rovinj (Croatia)	Mediterranean Sea	1	Siegel et al. 2011
Plakortis sp., *Agelas dilatata*	Little San Salvador Island (Bahamas)	Caribbean Sea	9	Taylor et al. 2007
Aplysina aerophoba	Banyuls sur Mer (France)	Mediterranean Sea	1	Fieseler et al. 2006
Aplysina aerophoba, Aplysina lacunosa, Aplysina fistularis, Aplysina insularis, Smenospongia aurea	Little San Salvador Island (Bahamas), Banyuls sur Mer (France)	Caribbean Sea, Mediterranean Sea	10	Fieseler et al. 2004

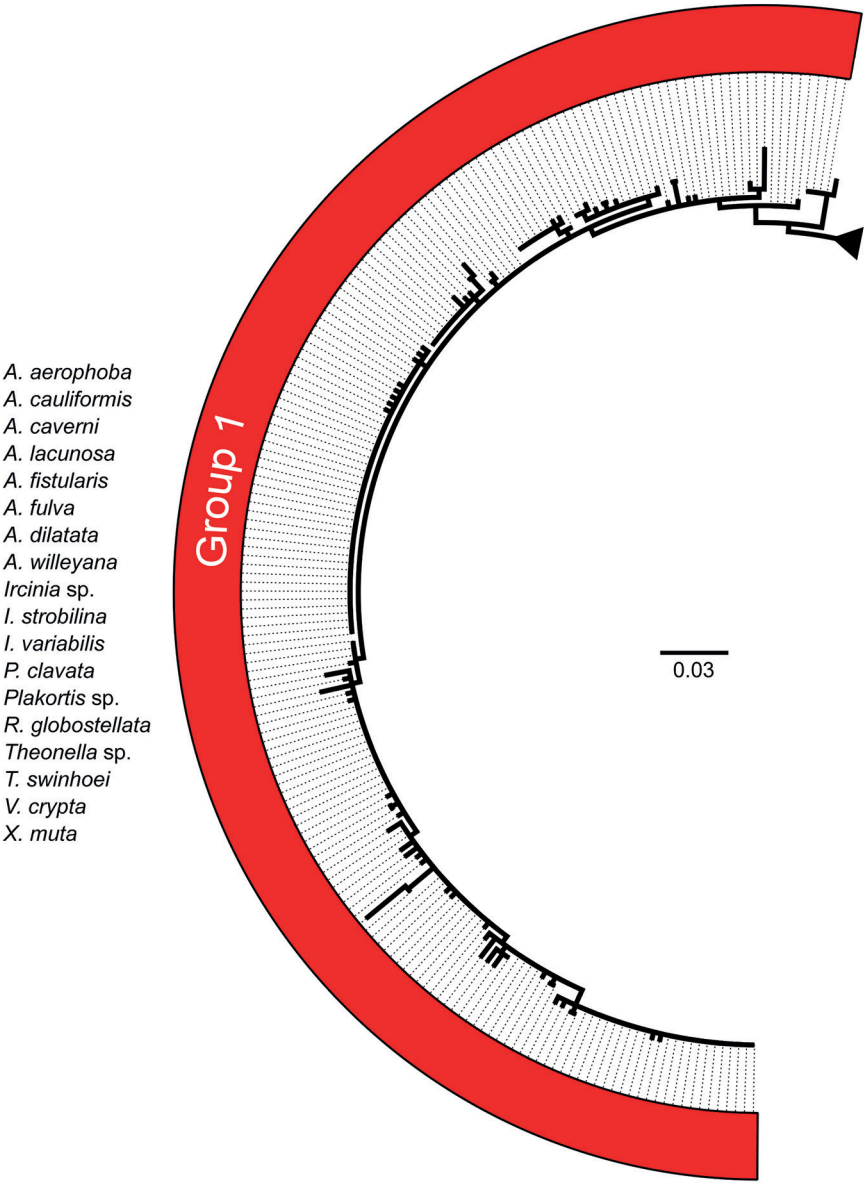
Table 2 Alpha diversity predictions estimated within mothur by the algorithms ACE, Chao1, and Shannon for different OTU clustering cut-offs ranging from unique to 0.05 sequence dissimilarity. Further indicated are the number of sequences (nseqs), Good's coverage and the number of observed OTUs (Sobs).

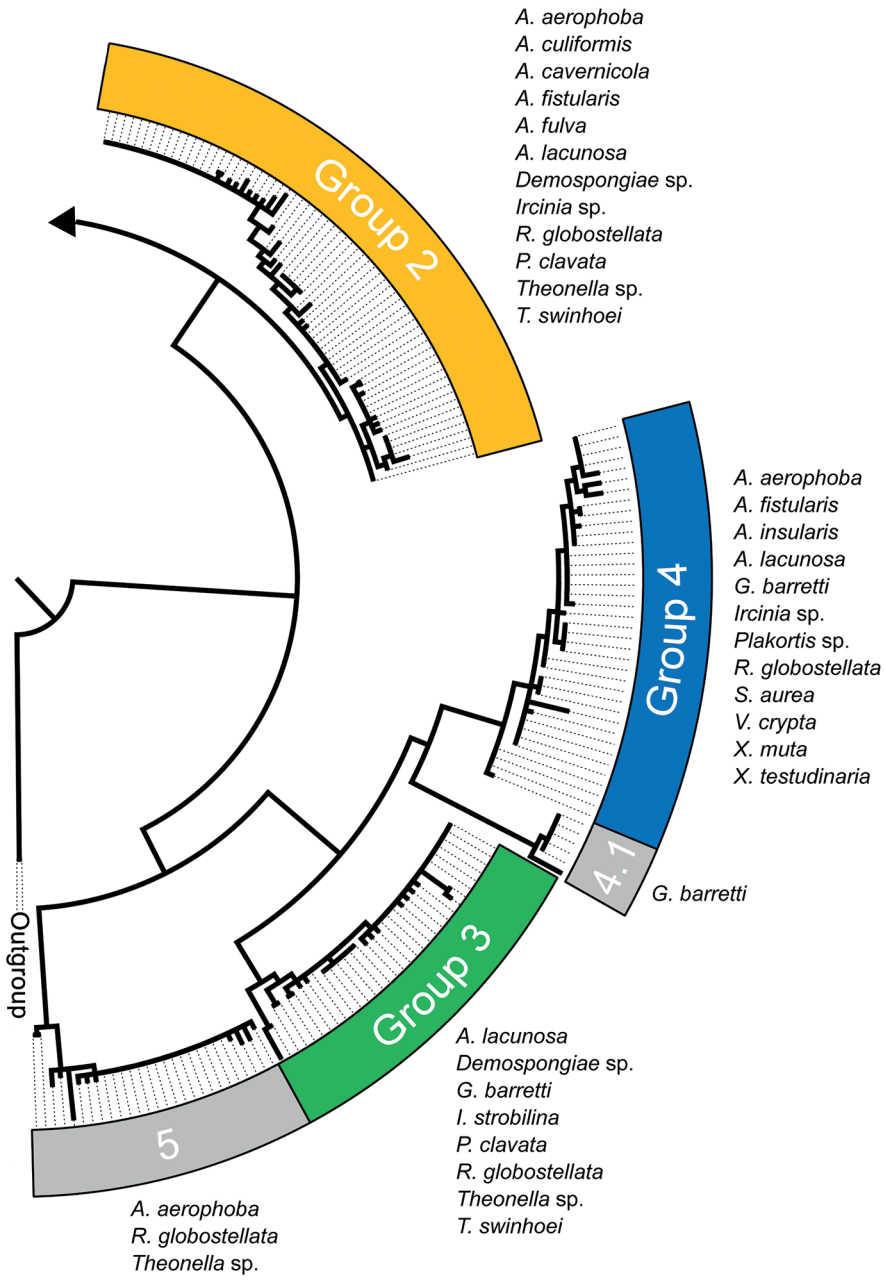
OTUs	nSeqs	Coverage	Sobs	ACE	Chao1	Shannon
EMP	1106040	1	106	106	106	2.78
unique	361	0.651	172	914.8	609.5	4.58
0.01	361	0.914	63	174.7	114.7	3.41
0.03	361	0.994	11	12.6	11.3	1.50
0.05	361	0.997	7	7.9	7.0	1.00

Poribacterial Phylogeny

The phylogenetic tree is based on poribacterial 16S rRNA gene sequence data collected by various studies from 2004 to 2016 (Table 1) and has been constructed from 361 sequences and nine outgroup sequences. To the best of our knowledge, the present phylogenetic tree (Fig. 1 and Supporting Information Fig. S2) utilizes all currently available Sanger sequences labeled as Poribacteria from the SILVA and NCBI databases. In order to identify the four poribacterial clades or groups established by Lafi *et al.* (2009) we used the poribacterial 16S rRNA gene sequences from that study as markers to identify the corresponding groups in our current phylogenetic tree and network (Fig. 1 and Supporting Information Fig. S3A). Even with the applied conservative sequence trimming prior to phylogenetic reconstruction, the phylogenetic analysis confirmed the existence of the four poribacterial clades (Fig. 1 and Supporting Information Fig. S2 and Data S1) (Lafi *et al.*, 2009; Hardoim *et al.*, 2013). Moreover, in addition to the already established four clades the resulting tree yielded a novel fifth poribacterial clade and a potential sister clade, which is closely related to the clade designated as group 4 (Lafi *et al.*, 2009). Most abundant within the final alignment were sequences belonging to group 1, which forms a large clade of 192 sequences with a low degree of sequence divergence (avg. 98.7% similarity) followed by group 2 containing 65 sequences (avg. 98.4% similarity) (Fig. 1 and Supporting Information Data S1 and Table S3). The clades that represent group 1 and group 2 are phylogenetically close (avg. 96.5% similarity) and have also been treated as only one clade previously (Hardoim *et al.*, 2013). In contrast, the remaining clades contain fewer sequences (33 in group 3, 44 in group 4 and 27 in group 5) and exhibit a higher degree of sequence divergence between the clades, ranging from 88.5% to 93.4% sequence similarity, which was also confirmed previously (Hardoim *et al.*, 2013). The newly discovered clade group 5 (Fig. 1) represents a deep-branching clade placed between groups 1-2 and groups 3-4. The sequences of group 5 (avg. 99% similarity) belong solely to Mediterranean (*A. aerophoba*) and Indonesian sponges (*R. globostellata* and *Theonella* sp.) obtained in the current study. In theory, this new group of poribacterial sequences could have remained undetected so far because (1) other studies used restriction enzymes to select clones with distinct patterns for sequencing or a different set of primer pairs (Hoffmann *et al.*, 2009; Lafi *et al.*, 2009; Jensen *et al.*, 2017), and / or (2) this study sampled the poribacterial 16S rRNA gene clone libraries in more depth than previously (e.g. this study increased the available poribacterial sequences gathered from *A. aerophoba* from previously 16 to 105). Lafi *et al.* (2009) did not consider clone sequences at > 97% similarity, however, that does not explain how group 5 escaped previous investigations since the associated sequences show a high degree of divergence to all other groups (<90% sequence similarity).

Figure 1 Maximum Likelihood phylogenetic tree containing downloaded sequences and novel sequenced clones. Sequences highlighted as 'Group 1' to 'Group 4' belong to the four poribacterial clades defined by Lafi *et al.* (2009). Nine sequences (Verrucomicrobia: AB297805, DQ302104; Chloroflexi: FJ481356, HE985107, JX280213, J280333, JQ844360, JQ844361, KF286181) were used as outgroup.




A. aerophoba
A. culiformis
A. cavernicola
A. fistularis
A. fulva
A. lacunosa
Demospongiae sp.
Ircinia sp.
R. globostellata
P. clavata
Theonella sp.
T. swinhoei
Group 2
A. aerophoba
A. fistularis
A. insularis
A. lacunosa
G. barretti
Ircinia sp.
Plakortis sp.
R. globostellata
S. aurea
V. crypta
X. muta
X. testudinaria
Group 4
4.1
G. barretti
Outgroup
Group 3
A. lacunosa
Demospongiae sp.
G. barretti
I. strobilina
P. clavata
R. globostellata
Theonella sp.
T. swinhoei
5
A. aerophoba
R. globostellata
Theonella sp.

There is no evidence for host-specificity of the different poribacterial clades, as already indicated previously (Lafi *et al.*, 2009). In fact, *R. globostellata* derived sequences are present in all groups, including the new group 5 (Fig. 1). Nevertheless, some poribacterial groups seem to be absent in certain sponge hosts and this could hint at a certain degree of host-specificity. For example, group 1 and group 2 contain sequences from all sampled sponge hosts except *Geodia barretti*, *Smenospongia aurea*, and *Xestospongia testudinaria*. Although the two latter sponges were only represented by a single poribacterial sequence, *G. barretti* was well sampled with 29 sequences (Table 1). The clade exclusivity might be due to the fact that in the study on *G. barretti* by Hoffmann *et al.* (2009) a different primer pair was used that only co-amplified poribacterial sequences (Jensen *et al.*, 2017). The presumptive differential amplification due to different primers might explain the new potential sister clade group 4.1 which appeared here for the first time (Fig. 1). In this respect, it is interesting to note that Fieseler *et al.* (2004) hypothesized the presence of yet unrecognized poribacterial subgroups, or non-specific binding to non-target prokaryotes, due to deviating FISH cohybridization signals using Poribacteria specific probes.

Global Distribution of Poribacteria and Host-Intraspecific Diversity

In order to examine the poribacterial phylotype distribution among or within sponge hosts and marine regions we performed a phylogenetic network analysis by applying the network construction algorithm of Templeton, Crandall and Sing (1992) (TCS). The TCS algorithm is suitable for inferring population genealogies when sequence divergences are low and non-bifurcating phylogenetic information at a population level can be incorporated coherently and comprehensively visualized (Templeton *et al.*, 1992; Clement *et al.*, 2002; Leigh & Bryant, 2015). Hence, phylogenetic networks enable the visualization of genealogical relationships at an intraspecific level and allow inferences about the biogeography and evolutionary history of populations.

The resulting network depicts the intra-population history of poribacterial 16S rRNA gene phylotypes, which were clustered into unique OTUs (i.e., without clustering based on similarity thresholds - Supporting Information Data S3), and colored by (A) sponge hosts, (B) marine region, and (C) individual *A. aerophoba* specimens (Fig. 2). This network consists of 172 unique phylotypes that resulted from 361 aligned sequences, whereof 46 phylotypes are shared between two or more of the 23 sampled sponge taxa (Supporting Information Data S3). The four main groups are genetically well separated, which is in accordance with the phylogenetic tree (Fig. 1). Furthermore, the network analysis confirmed the placement of the novel group 5 in between the groups 1 & 2 and 3 & 4. The group labels in Fig. 2A were derived by visualizing the position of the group-specific sequences within the network (Supporting Information Fig. S3A), which are based on the initial assignment by Lafi *et al.* (2009).

Poribacterial phylotypes showed little evidence of co-speciation with sponge hosts. Many of the major poribacterial groups and even certain phylotypes were found to be shared between different sponge hosts (Fig. 2A). However, the presence of large sequence clusters associated with only a few sponge-species, i.e. *A. aerophoba*, *A. fulva*, and *P. sollasi* (Fig. 2A and see OTU001-005 in Supporting Information Data S3), implied some degree of host-specificity among certain phylotypes. A clear relationship between phylotype distribution and geographical origin was not observed (Fig. 2B). Most striking was the fact that there was no obvious correlation between group and geographic

connectivity, which means that groups exhibit phylotypes from marine regions that are only distantly connected (e.g., South Atlantic and South Pacific Ocean) or not connected at all (Mediterranean Sea and Eastern Pacific Ocean) (Fig. 2B). However, some minor degree of geographic relatedness could be observed. For instance, group 3 was missing from Mediterranean related phylotypes, and South Atlantic Ocean Poribacteria are so far only present in group 1 and 2. Furthermore, it is noteworthy that the sequences derived from the *A. aerophoba* individuals sampled in our study represent almost exclusively edges of the phylogenetic network, which is in stark contrast to poribacterial sequences from Indonesian sponges that are positioned as ancestral and connecting sequences between the clusters in the phylogenetic network (Supporting Information Fig. S3B). This might indicate that due to the geologically late formation of the Mediterranean Sea (Garcia-Castellanos *et al.*, 2009), the colonization of the primarily Mediterranean species *A. aerophoba* happened only recently in the population history of the poribacterial phylum.

The lack of co-speciation and cosmopolitan distribution has been already observed in other putative sponge symbiotic bacteria, such as "*Candidatus* Synechococcus spongiarum" (Hentschel *et al.*, 2006). In addition, the widespread distribution across hosts and marine regions supports the hypothesis that poribacterial cells are mainly horizontally transmitted by environmental acquisition (Hardoim *et al.*, 2013), even though vertical transmission has also been observed (Sharp *et al.*, 2007). For instance, several studies found evidence for the presence of poribacterial 16S rRNA gene sequences in seawater and sediments (e.g., Taylor *et al.*, 2013; Bayer *et al.*, 2014; Thomas *et al.*, 2016), further supporting the hypothesis that seawater might act as a seed bank for sponge-associated prokaryotes. Correspondingly, poribacterial cells seem to be not active while dwelling in the seawater (Moitinho-Silva *et al.*, 2014). Most likely Poribacteria can be acquired via vertical and horizontal transmission, as already observed for other sponge-associated prokaryotes (Sipkema *et al.*, 2015).

Finally, we compared 16S rRNA gene sequences from three *A. aerophoba* specimens (Aa16, 17, and 23) to investigate intraspecific Poribacteria distribution. Here we found poribacterial sequences from five distinctly related phylotype clusters (labeled as C1 to C5) belonging to group 1, 2 and 5 (Fig. 2C). All three *A. aerophoba* individuals harbored poribacterial sequences from four of the five phylotype clusters (C1-C4), while only one specimen (Aa23) exhibited a minor cluster in group 2 (C5) that also shared similar and / or closely related sequences with *A. cauliformis*, *A. fulva* and *R. globostellata* (compared with hosts in Fig. 2A). Therefore, at least for *A. aerophoba*, several phylogenetically divergent poribacterial phylotypes co-exist within individual sponge specimens.

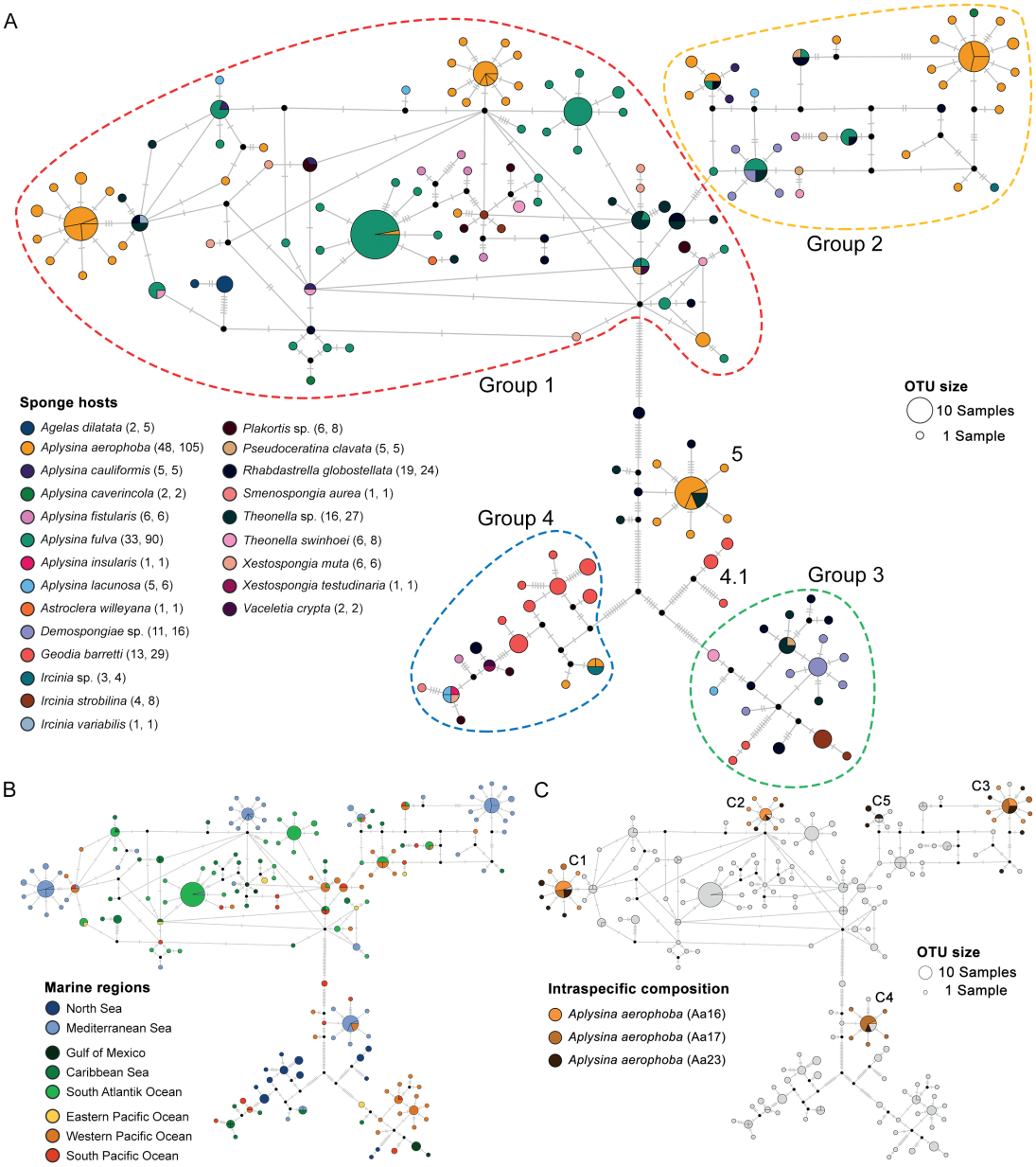


Figure 2 Phylogenetic networks based on unique poribacterial OTUs. The different sizes of the nodes represent the total count of OTU samples. Grey hatch marks indicate the number of nucleotide substitutions between nodes. Black nodes depict theoretical ancestral sequences. A) OTUs are coloured based on sponge host-identity. Dotted lines define groups according to the clades defined in Figure 1. The legend shows all investigated sponge species and in brackets the number of unique and total OTUs respectively. B) Phylogenetic network coloured by water bodies of the biogeographical sample origin. C) Intraspecific phylogenetic network of three *A. aerophoba* individuals. The labels C1 - C5 indicate almost-exclusively *A. aerophoba* specific, distinctly related phylotype clusters.

Conclusion

In the present study we could demonstrate that the candidate bacterial phylum Poribacteria is composed of a diverse range of phylotypes, which are distributed among a wide range of phylogenetically divergent sponge hosts. The distribution of poribacterial phylotypes seems unrelated to the phylogeny of the sponge hosts. Multiple poribacterial phylotypes can co-occur within the same sponge host, which was especially evident in the deeply sampled *Aplysina* spp. sponges. We identified a novel poribacterial clade (group 5), which might form an evolutionary link between the known four Poribacteria clades. By compiling all available poribacterial sequences generated by Sanger sequencing we could provide a more complete overview and fill gaps in the phylogenetic diversity and population history of Poribacteria. The comparison of richness estimates resulting from different methodological approaches indicated that poribacterial species richness remains undersampled. Moreover, investigating other sponge species using the primers applied by Hoffmann *et al.* (2009) could potentially fill additional gaps in the phylogenetic history of Poribacteria. With a large number of bacterial 16S rRNA gene sequences it is often challenging to visualize biogeographic relationships and gene evolution at a population level with bifurcating phylogenetic trees. Hitherto, the presented phylogenetic network can visualize poribacterial phylotypes being shared over a range of sponge hosts and different geographic locations. This adds to the hypothesis that the phylum Poribacteria represents a diverse cosmopolitan sponge symbiont with global propagation.

Acknowledgements

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Supplementary Information

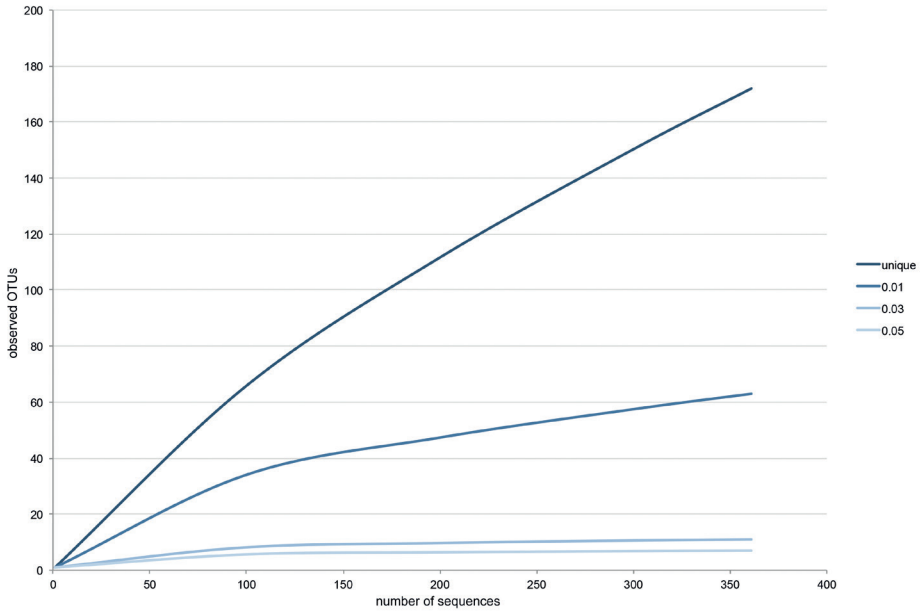

Figure S1 Rarefaction analysis curves as performed by mothur for different OTU clustering cut-offs ranging from unique to 0.05 sequence dissimilarity.

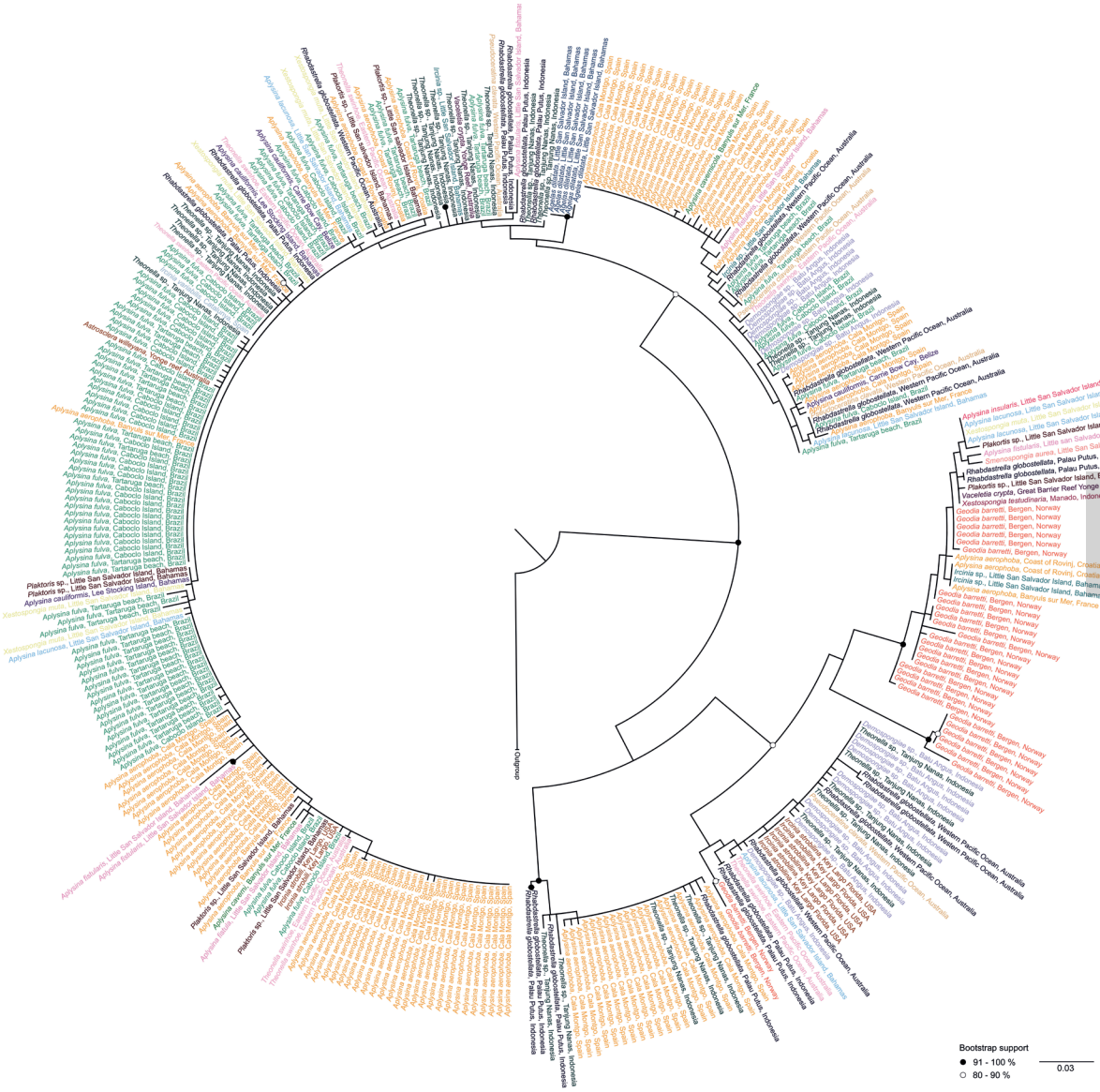

Figure S2 Detailed Maximum Likelihood phylogenetic tree containing downloaded sequences and novel sequenced clones indicating sponge hosts (coloured as in Figure 2) and country of sample origin. Bootstrapping was performed within RAxML v.8.2.7 (rapid bootstrap, 1000 replicates, GTR substitution model of evolution). Nine sequences (acc. AB297805, DQ302104, FJ481356, HE985107, JX280213, J280333, JQ844360, JQ844361, KF286181) were used as outgroup.

Figure S3 Phylogenetic network of the poribacterial OTUs. A) Coloured nodes are OTUs derived from the study of Lafi *et al*. (2009) and are coloured based on the initial assignment into four poribacterial groups. B) Coloured nodes are OTUs derived from the three Indonesian sponges sampled for this study.

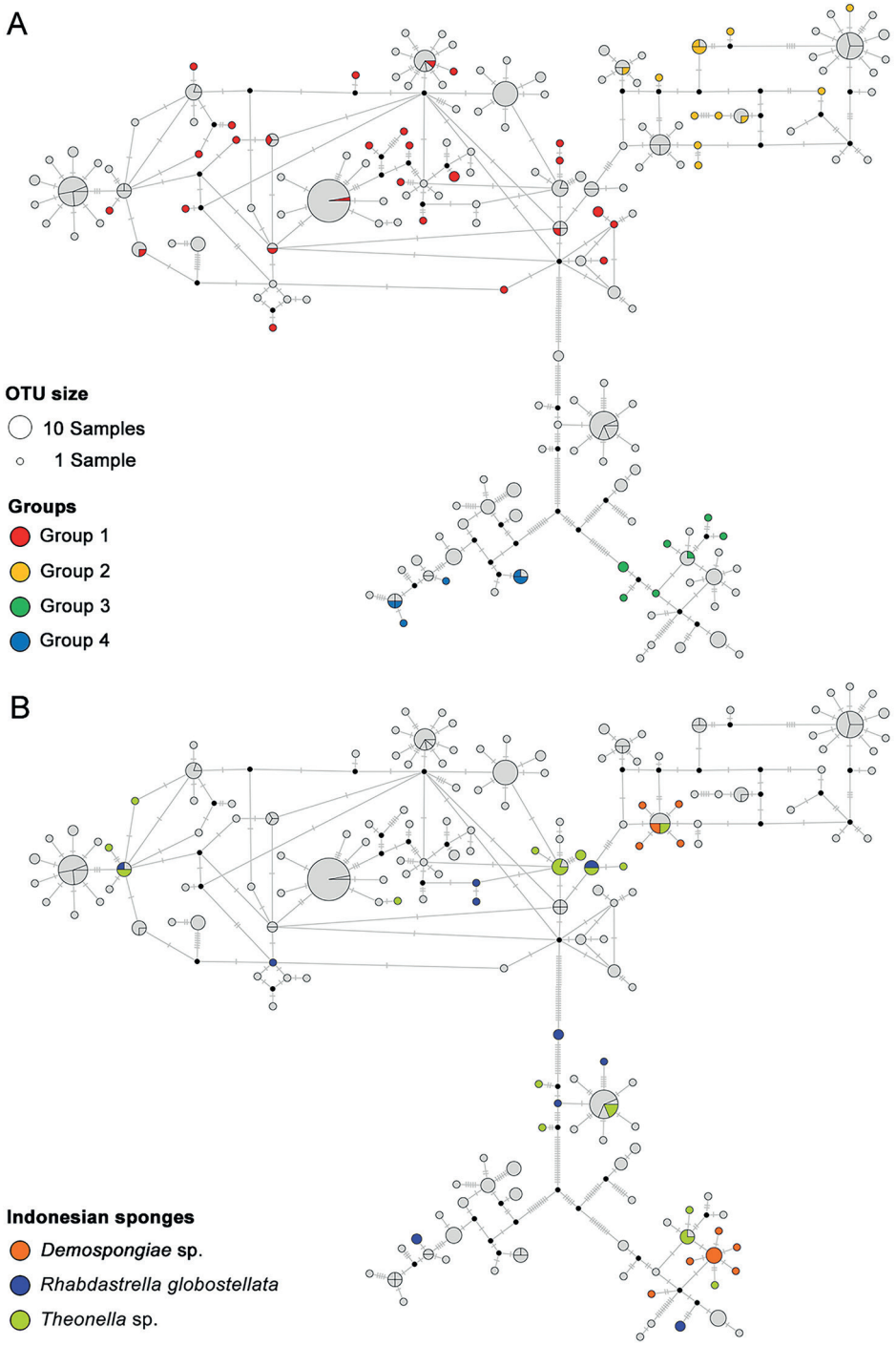

Table S1 Sponge identification results by COI and 28S gene molecular barcoding. Sequences were identified by BLAST searches against the nucleotide collection (nr / nt) and the 3 closest matches are depicted.

Accession	Sponge taxonomy	Sample ID	Locus	3 best BLAST hits per sequence	Query cover	Identity	BLAST hit accession numbers
KY625183	*Aplysina aerophoba*	Aa16	cytochrome oxidase subunit I gene	*Aplysina aerophoba* voucher BELUM<GBR>:Mc7167 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	100%	HQ379407.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	100%	EF043372.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	99%	EF043371.1
KY625184	*Aplysina aerophoba*	Aa17	cytochrome oxidase subunit I gene	*Aplysina aerophoba* voucher BELUM<GBR>:Mc7167 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	100%	HQ379407.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	100%	EF043372.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	99%	EF043371.1
KY625185	*Aplysina aerophoba*	Aa23	cytochrome oxidase subunit I gene	*Aplysina aerophoba* voucher BELUM<GBR>:Mc7167 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100%	100%	HQ379407.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	100%	EF043372.1
				Aplysina aerophoba cytochrome oxidase subunit I gene, partial cds; mitochondrial	99%	99%	EF043371.1
KY625186	*Rhabdastrella globostellata*	Put4	cytochrome oxidase subunit I gene	*Rhabdastrella globostellata* voucher USP<FJI>:9712SD114 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	85%	99%	HM592673.1
				Rhabdastrella sp. PC-2011 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	85%	99%	HM592676.1
				Rhabdastrella globostellata strain S1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	81%	99%	KT180228.1
KY625187	*Theonella* sp.	Tjn2	cytochrome oxidase subunit I gene	*Theonella cf. cylindrica* QMG301114 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	92%	99%	KJ494353.1
				Theonella deliqua isolate QMG329195 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	92%	99%	KJ494355.1
				Theonella swinhoei voucher ZMA:POR 16637 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	92%	99%	HM592745.1
KY625188	*Demospongiae* sp.	Bta10	28S large subunit ribosomal RNA gene	Demospongiae sp. GW3448 voucher SNSB-BSPG.GW3448 28S large subunit ribosomal RNA gene, partial sequence	85%	89%	KU060450.1
				Demospongiae sp. GW3262 voucher SNSB-BSPG.GW3262 28S large subunit ribosomal RNA gene, partial sequence	85%	88%	KU060391.1
				Demospongiae sp. GW3415 voucher SNSB-BSPG.GW3415 28S large subunit ribosomal RNA gene, partial sequence	85%	88%	KU060434.1

Table S2 Summary of publically available poribacterial sequences that were used in this study. Sequences were collected from the NCBI and SILVA nucleotide databases.

ARB DB ACC	Host	Location	Sequence Name	NCBI ACC	Authors
UncPor91	*Aplysina fistularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	AY485280	Fieseler et al. (2004)
UncPor97	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	AY485282	Fieseler et al. (2004)
UncPor45	*Aplysina insularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	AY485284	Fieseler et al. (2004)
UncPorib	*Smenospongia aurea*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	AY485285	Fieseler et al. (2004)
UncPor92	*Aplysina lacunosa*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	AY485286	Fieseler et al. (2004)
UncPor99	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	AY485287	Fieseler et al. (2004)
UncPor46	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	AY485288	Fieseler et al. (2004)
UncPori2	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	AY485289	Fieseler et al. (2004)
UncPor93	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	AY485290	Fieseler et al. (2004)
UncPo101	*Aplysina aerophoba*	France: Banyurs sur Mer	uncultured Poribacteria bacterium	AY485291	Fieseler et al. (2004)
UncPor47	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium 64K2	AY713479	Fieseler et al. (2006)
UncBacte	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori01	JQ844336	Jensen et al. (2016)
UncBact2	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori07	JQ844337	Jensen et al. (2016)
UncBact3	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori20	JQ844338	Jensen et al. (2016)
UncBact4	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori24	JQ844339	Jensen et al. (2016)
UncBact5	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori30	JQ844340	Jensen et al. (2016)
UncBact6	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori43	JQ844341	Jensen et al. (2016)
UncBact7	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori02	JQ844342	Jensen et al. (2016)
UncBact8	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori06	JQ844343	Jensen et al. (2016)
UncBact9	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori10	JQ844344	Jensen et al. (2016)
UncBac10	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori26	JQ844345	Jensen et al. (2016)
UncBac11	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori32	JQ844346	Jensen et al. (2016)
UncBac12	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori03	JQ844347	Jensen et al. (2016)
UncBac13	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori39	JQ844348	Jensen et al. (2016)
UncBac14	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori05	JQ844349	Jensen et al. (2016)
UncBac15	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori09	JQ844350	Jensen et al. (2016)

UncBac16	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori12	JQ844351	Jensen et al. (2016)
UncBac17	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori15	JQ844352	Jensen et al. (2016)
UncBac18	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori17	JQ844353	Jensen et al. (2016)
UncBac19	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori22	JQ844354	Jensen et al. (2016)
UncBac20	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori28	JQ844355	Jensen et al. (2016)
UncBac21	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori29	JQ844356	Jensen et al. (2016)
UncBac22	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori38	JQ844357	Jensen et al. (2016)
UncBac23	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori42	JQ844358	Jensen et al. (2016)
UncBac24	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori46	JQ844359	Jensen et al. (2016)
UncBac27	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori14	JQ844362	Jensen et al. (2016)
UncBac28	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori18	JQ844363	Jensen et al. (2016)
UncBac29	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori36	JQ844364	Jensen et al. (2016)
UncBac30	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori44	JQ844365	Jensen et al. (2016)
UncBac31	*Geodia barretti*	Norway: Korsfjord	Uncultured bacterium clone Pori48	JQ844366	Jensen et al. (2016)
PlaIrci7	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652464	Mohamed et al. (2010)
PlaIrci6	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652465	Mohamed et al. (2010)
PlaIrci5	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652469	Mohamed et al. (2010)
PlaIrci4	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652471	Mohamed et al. (2010)
PlaIrci3	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652472	Mohamed et al. (2010)
UncPlanc	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652473	Mohamed et al. (2010)
PlaIrci2	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652474	Mohamed et al. (2010)
PlaIrcin	*Ircinia strobilina*	USA: Conch Reef	uncultured Planctomycetales clone	FJ652475	Mohamed et al. (2010)
UncPor64	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356776	Hardoim et al. (2013)
UncPor19	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356777	Hardoim et al. (2013)
UncPo124	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356778	Hardoim et al. (2013)
UncPo137	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356779	Hardoim et al. (2013)
UncPor65	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356780	Hardoim et al. (2013)

UncPor20	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356781	Hardoim et al. (2013)
UncPo126	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356782	Hardoim et al. (2013)
UncPo139	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356783	Hardoim et al. (2013)
UncPor66	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356784	Hardoim et al. (2013)
UncPor21	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356785	Hardoim et al. (2013)
UncPo128	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356786	Hardoim et al. (2013)
UncPo141	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356787	Hardoim et al. (2013)
UncPor67	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356788	Hardoim et al. (2013)
UncPor22	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356789	Hardoim et al. (2013)
UncPo130	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356790	Hardoim et al. (2013)
UncPo143	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356791	Hardoim et al. (2013)
UncPor68	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356792	Hardoim et al. (2013)
UncPor23	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356793	Hardoim et al. (2013)
UncPo132	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356794	Hardoim et al. (2013)
UncPo145	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356795	Hardoim et al. (2013)
UncPor69	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356796	Hardoim et al. (2013)
UncPor24	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356797	Hardoim et al. (2013)
UncPo134	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356798	Hardoim et al. (2013)
UncPo147	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356799	Hardoim et al. (2013)
UncPor70	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356800	Hardoim et al. (2013)
UncPor25	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356801	Hardoim et al. (2013)
UncPo136	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356802	Hardoim et al. (2013)
UncPo149	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356803	Hardoim et al. (2013)
UncPor71	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356804	Hardoim et al. (2013)
UncPor26	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356805	Hardoim et al. (2013)
UncPo138	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356806	Hardoim et al. (2013)
UncPo151	*Aplysina fulva*	Brazil: Caboclo Island uncultured Poribacteria bacterium	FN356807	Hardoim et al. (2013)

UncPor7 2	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356808	Hardoim et al. (2013)
UncPor2 7	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356809	Hardoim et al. (2013)
UncPo14 0	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356810	Hardoim et al. (2013)
UncPo15 3	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356811	Hardoim et al. (2013)
UncPo14 2	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356812	Hardoim et al. (2013)
UncPo15 5	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356813	Hardoim et al. (2013)
UncPor7 3	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356814	Hardoim et al. (2013)
UncPor2 8	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356815	Hardoim et al. (2013)
UncPo14 4	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356816	Hardoim et al. (2013)
UncPo15 7	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356817	Hardoim et al. (2013)
UncPor7 4	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356818	Hardoim et al. (2013)
UncPor2 9	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356819	Hardoim et al. (2013)
UncPo14 6	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356820	Hardoim et al. (2013)
UncPo15 9	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356821	Hardoim et al. (2013)
UncPor7 5	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356822	Hardoim et al. (2013)
UncPor3 0	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356823	Hardoim et al. (2013)
UncPo14 8	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356824	Hardoim et al. (2013)
UncPo16 1	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356825	Hardoim et al. (2013)
UncPor7 6	*Aplysina fulva*	Brazil: Caboclo Island	uncultured Poribacteria bacterium	FN356826	Hardoim et al. (2013)
UncPor3 1	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356827	Hardoim et al. (2013)
UncPo15 0	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356828	Hardoim et al. (2013)
UncPo16 3	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356829	Hardoim et al. (2013)
UncPor7 7	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356830	Hardoim et al. (2013)
UncPor3 2	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356831	Hardoim et al. (2013)
UncPo15 2	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356832	Hardoim et al. (2013)
UncPo16 5	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356833	Hardoim et al. (2013)
UncPor7 8	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356834	Hardoim et al. (2013)

UncPo16 7	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356835	Hardoim et al. (2013)
UncPor7 9	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356836	Hardoim et al. (2013)
UncPor3 3	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356837	Hardoim et al. (2013)
UncPo15 4	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356838	Hardoim et al. (2013)
UncPo16 9	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356839	Hardoim et al. (2013)
UncPor8 0	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356840	Hardoim et al. (2013)
UncPor3 4	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356841	Hardoim et al. (2013)
UncPo15 6	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356842	Hardoim et al. (2013)
UncPo17 1	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356843	Hardoim et al. (2013)
UncPor8 1	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356844	Hardoim et al. (2013)
UncPor3 5	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356845	Hardoim et al. (2013)
UncPo15 8	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356846	Hardoim et al. (2013)
UncPo17 3	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356847	Hardoim et al. (2013)
UncPor8 2	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356848	Hardoim et al. (2013)
UncPor3 6	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356849	Hardoim et al. (2013)
UncPo16 0	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356850	Hardoim et al. (2013)
UncPo17 4	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356851	Hardoim et al. (2013)
UncPor8 3	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356852	Hardoim et al. (2013)
UncPor3 7	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356853	Hardoim et al. (2013)
UncPo16 2	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356854	Hardoim et al. (2013)
UncPo17 6	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356855	Hardoim et al. (2013)
UncPor8 4	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356856	Hardoim et al. (2013)
UncPor3 8	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356857	Hardoim et al. (2013)
UncPo16 4	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356858	Hardoim et al. (2013)
UncPor8 5	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356859	Hardoim et al. (2013)
UncPor3 9	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356860	Hardoim et al. (2013)
UncPo16 6	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356861	Hardoim et al. (2013)

UncPo177	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356862	Hardoim et al. (2013)
UncPor86	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356863	Hardoim et al. (2013)
UncPor40	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356864	Hardoim et al. (2013)
UncPo168	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356865	Hardoim et al. (2013)
UncPo178	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356866	Hardoim et al. (2013)
UncPor87	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356867	Hardoim et al. (2013)
UncPor41	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356868	Hardoim et al. (2013)
UncPo170	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356869	Hardoim et al. (2013)
UncPo179	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356870	Hardoim et al. (2013)
UncPor88	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356871	Hardoim et al. (2013)
UncPor42	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356872	Hardoim et al. (2013)
UncPo172	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356873	Hardoim et al. (2013)
UncPo180	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356874	Hardoim et al. (2013)
UncPor89	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356875	Hardoim et al. (2013)
UncPor43	*Aplysina fulva*	Brazil: Tartaruga beach	uncultured Poribacteria bacterium	FN356876	Hardoim et al. (2013)
UncMicro	*Aplysina aerophoba*	Croatia: Coast of Rovinj	uncultured microorganism clone	JN002374	Bayer et al. (2013)
UncBac32	*Vaceletia crypta*	Australia: Yonge Reef	uncultured bacterium	HE817855	Karlinska-Batres et al. (2013)
UncBac33	*Vaceletia crypta*	Australia: Yonge Reef	uncultured bacterium	HE817858	Karlinska-Batres et al. (2013)
UncPor90	*Aplysina cauliformis*	Bahamas: Lee Stocking Island	uncultured Poribacteria bacterium	KF286071	Olson et al. (2014)
UncPlan3	*Aplysina cauliformis*	Belize: Carrie Bow Cay	uncultured Planctomycetalis clone	KF286093	Olson et al. (2014)
UncPor44	*Aplysina cauliformis*	Bahamas: Lee Stocking Island	uncultured Poribacteria bacterium	KF286114	Olson et al. (2014)
UncPlan2	*Aplysina cauliformis*	Belize: Carrie Bow Cay	uncultured Planctomycetalis clone	KF286130	Olson et al. (2014)
UncPo175	*Aplysina cauliformis*	Belize: Carrie Bow Cay	uncultured Poribacteria bacterium	KF286195	Olson et al. (2014)
UncPo122	seawater	Pacific Ocean: Hawaii	uncultured Poribacteria bacterium	EU361158	Pham et al. (2008)
UncPo135	seawater	Pacific Ocean: Hawaii	uncultured Poribacteria bacterium	EU361159	Pham et al. (2008)
UncBac34	*Astrosclera willeyana*	Australia: Yonge Reef	uncultured bacterium	HE985121	Karlinska-Batres et al. (2013)
CanPorib	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-4C POR4C_contig00180standard	APGO01000272	Kamke et al. (2013)

CanPori3	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-4G POR4G_contig00091standard_C	AQPC01000005	Kamke et al. (2013)
CanPori4	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-4E, POR4E_contig00050standard_C	AQTV01000144	Kamke et al. (2013)
PorBacte	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Poribacteria bacterium WGA-4CII POR4CII_contig_6.7_C	ASZM01000007	Kamke et al. (2013)
CanPori5	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-3G	KC713965	Kamke et al. (2013)
CanPori6	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-4CII	KC713966	Kamke et al. (2013)
CanPori2	*Aplysina aerophoba*	Croatia: Coast of Rovinj	Candidatus Poribacteria sp. WGA-A3 POR_contig00110	ADFK02000001	Siegl et al. (2011)
UncClost	*Xestospongia testudinaria*	Indonesia: Manado Bay	uncultured Clostridium	JN596758	Montalvo et al. (2011)
UncPor48	marine sponge *Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076077	Taylor et al. (2007)
UncPori3	marine sponge *Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076078	Taylor et al. (2007)
UncPor94	marine sponge Plakortis sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076079	Taylor et al. (2007)
UncPo103	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076117	Taylor et al. (2007)
UncPor49	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076139	Taylor et al. (2007)
UncPori4	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076140	Taylor et al. (2007)
UncPor95	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076141	Taylor et al. (2007)
UncPo105	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076142	Taylor et al. (2007)
UncPor50	marine sponge *Agelas dilatata*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EF076143	Taylor et al. (2007)
UncCline	*Ircinia variabilis*	Spain: Tossa de Mar	uncultured clone	JX206704	Erwin et al. (2012)
UncPori5	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071627	Lafi et al. (2009)
UncPor96	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071628	Lafi et al. (2009)
UncPo107	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071629	Lafi et al. (2009)
UncPor51	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071630	Lafi et al. (2009)
UncPori6	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071631	Lafi et al. (2009)
UncPor98	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071632	Lafi et al. (2009)
UncPo109	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071633	Lafi et al. (2009)
UncPor52	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071634	Lafi et al. (2009)
UncPori7	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071635	Lafi et al. (2009)

UncPo100	*Rhabdastrella globostellata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071636	Lafi et al. (2009)
UncPo111	*Pseudoceratina clavata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071637	Lafi et al. (2009)
UncPor53	*Pseudoceratina clavata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071638	Lafi et al. (2009)
UncPori8	*Pseudoceratina clavata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071639	Lafi et al. (2009)
UncPo102	*Pseudoceratina clavata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071640	Lafi et al. (2009)
UncPo113	*Pseudoceratina clavata*	Palau: Ngetngod Reef	uncultured Poribacteria bacterium	EU071641	Lafi et al. (2009)
UncPor54	*Aplysina lacunosa*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071642	Lafi et al. (2009)
UncPori9	*Theonella swinhoei*	Pacific Ocean: Eastern Pacific Ocean	uncultured Poribacteria bacterium	EU071643	Lafi et al. (2009)
UncPo104	*Xestospongia muta*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071644	Lafi et al. (2009)
UncPo115	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	EU071645	Lafi et al. (2009)
UncPor55	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	EU071646	Lafi et al. (2009)
UncPor10	*Aplysina aerophoba*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	EU071647	Lafi et al. (2009)
UncPo106	*Aplysina aerophoba*	France: Banyurs sur Mer	uncultured Poribacteria bacterium	EU071648	Lafi et al. (2009)
UncPo117	*Aplysina cavernicola*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	EU071649	Lafi et al. (2009)
UncPor56	*Aplysina cavernicola*	France: Banyuls sur Mer	uncultured Poribacteria bacterium	EU071650	Lafi et al. (2009)
UncPor11	*Aplysina fistularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071651	Lafi et al. (2009)
UncPo108	*Aplysina fistularis*	Bahamas: Little san Salvador Island	uncultured Poribacteria bacterium	EU071652	Lafi et al. (2009)
UncPo119	*Aplysina fistularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071653	Lafi et al. (2009)
UncPor57	*Aplysina fistularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071654	Lafi et al. (2009)
UncPor12	*Aplysina fistularis*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071655	Lafi et al. (2009)
UncPo110	*Aplysina lacunosa*	Bahamas: Little san Salvador Island	uncultured Poribacteria bacterium	EU071656	Lafi et al. (2009)
UncPo121	*Aplysina lacunosa*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071657	Lafi et al. (2009)
UncPor58	*Aplysina lacunosa*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071658	Lafi et al. (2009)
UncPor13	*Aplysina lacunosa*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071659	Lafi et al. (2009)
UncPo112	*Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071660	Lafi et al. (2009)
UncPo123	*Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071661	Lafi et al. (2009)
UncPo125	*Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071662	Lafi et al. (2009)

UncPo114	*Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071663	Lafi et al. (2009)
UncPor14	*Plakortis* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071664	Lafi et al. (2009)
UncPor59	*Ircinia* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071665	Lafi et al. (2009)
UncPo127	*Ircinia* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071666	Lafi et al. (2009)
UncPo116	*Ircinia* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071667	Lafi et al. (2009)
UncPor15	*Ircinia* sp.	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071668	Lafi et al. (2009)
UncPor60	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071669	Lafi et al. (2009)
UncPo129	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071670	Lafi et al. (2009)
UncPo118	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071671	Lafi et al. (2009)
UncPor16	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071672	Lafi et al. (2009)
UncPor61	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071673	Lafi et al. (2009)
UncPo131	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071674	Lafi et al. (2009)
UncPor62	*Theonella swinhoei*	USA: San Diego	uncultured Poribacteria bacterium	EU071675	Lafi et al. (2009)
UncPor17	*Xestospongia muta*	Bahamas: Little san Salvador Island	uncultured Poribacteria bacterium	EU071676	Lafi et al. (2009)
UncPo120	*Xestospongia muta*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071677	Lafi et al. (2009)
UncPo133	*Xestospongia muta*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071678	Lafi et al. (2009)
UncPor63	*Xestospongia muta*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071679	Lafi et al. (2009)
UncPor18	*Xestospongia muta*	Bahamas: Little San Salvador Island	uncultured Poribacteria bacterium	EU071680	Lafi et al. (2009)

Table S3 Sequence similarity matrix indicating the average (± standard deviation) sequence similarity within and across the poribacterial groups.

	Group 1	Group 2	Group 3	Group 4	Group 4.1	Group 5
Group 1	98.73 (±0.72)					
Group 2	96.47 (±0.88)	98.37 (±0.96)				
Group 3	88.41 (±0.62)	89.30 (±0.53)	98.90 (±1.03)			
Group 4	88.52 (±0.72)	88.21 (±0.84)	93.42 (±0.65)	98.43 (±1.01)		
Group 4.1	87.92 (±0.70)	87.79 (±0.70)	92.54 (±0.83)	91.48 (±0.71)	99.15 (±1.22)	
Group 5	88.90 (±0.78)	88.69 (±0.79)	89.42 (±0.48)	89.63 (±0.66)	88.47 (±0.39)	99.19 (±0.95)

Data S1 Poribacteria ARB database containing 361 aligned poribacterial 16S rRNA gene sequences.
Available upon request

Data S2 Fasta file containing the Gblocks trimmed unique poribacterial sequences used for the generation of the phylogenetic network.
Available upon request

Data S3 Shared file contains the host identity and abundance of each unique OTU used for the generation of the phylogenetic network.
Available upon request

Chapter 4

Cultivation of Bacteria from *Aplysina aerophoba*: Effects of Oxygen & Nutrient Gradients

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Abstract

Sponge-associated bacteria possess biotechnologically interesting properties but as yet have majorly evaded cultivation. Thus, 'omics'-based information on the ecology and functional potential of sponge symbionts is waiting to be integrated into the design of innovative cultivation conditions. To cultivate bacteria derived from the marine sponge *Aplysina aerophoba*, nine novel media formulations were created based on the predicted genomic potential of the prevalent sponge symbiont lineage Poribacteria. In an attempt to maintain microbial metabolic interactions *in vitro*, a Liquid-Solid cultivation approach and a Winogradsky-column approach were applied. The vast majority of microorganisms appear viable after cryopreservation as determined by selective propidium monoazide DNA modification of membrane-compromised cells, however, only 2% of the initial microbial diversity could be recovered through cultivation. In total, 256 OTUs encompassing seven microbial phyla were cultivated. The diversity of the cultivated community was influenced by the addition of the antibiotic aeroplysinin-1 as well as by medium dilution, rather than carbon source. Furthermore, the Winogradsky-column approach reproducibly enriched distinct communities at different column depths, amongst which were numerous Clostridia and OTUs that could not be assigned to a known phylum. While some bacterial taxa such as *Pseudovibrio* and *Ruegeria* were recovered from nearly all applied cultivation conditions, others such as Bacteroidetes were specific to certain medium types. We conclude that the reason dominant sponge-associated microbial taxa remain uncultured is the inability to recreate cultivation conditions that resemble the sponge ecosystem. Nonetheless, alternative cultivation approaches could enrich for previously uncultivated microbes.

Introduction

Marine sponges represent the oldest, living lineage of the animal kingdom, with a longstanding association to microorganisms (Hooper & Van Soest, 2012; McFall-Ngai *et al.*, 2013). To date, 41 prokaryotic phyla have been found in association with sponges (Thomas *et al.* 2016) and accordingly, this vast genetic potential is hypothesized to be accountable for numerous interactions between sponge symbionts and their hosts (Webster & Thomas, 2016; Moitinho-Silva *et al.*, 2017; Chaib De Mares *et al.*, 2018). In recent years, omics-based methods (Horn *et al.*, 2016; Slaby *et al.*, 2017) as well as physiological *in situ* studies have shed some light on microbial processes in sponges. Microbes filtered from the seawater comprise the primary food source for most sponges, whilst specific microbes evade digestion by the sponge cells and get established in the mesohyl where they grow on metabolic waste products or host-derived carbohydrates (Vogel, 2006; M. W. Taylor *et al.*, 2007; Simpson, 2011; Kamke *et al.*, 2013; Bayer *et al.*, 2018b). Additionally, carbon fixation by photosynthesis (Burgsdorf *et al.*, 2015), nitrification (Bayer *et al.*, 2008; Hoffmann *et al.*, 2009), sulfur cycling (Keren *et al.*, 2015), phosphorus cycling (Fan Zhang *et al.*, 2015), vitamin synthesis by microorganisms, and microbial production of secondary metabolites for host defence (Kennedy *et al.*, 2007; Hochmuth & Piel, 2009; Freeman *et al.*, 2012; Indraningrat *et al.*, 2016) all occur in the sponge holobiont (Webster & Thomas, 2016). However, detailed disentanglement of microbial functionalities is majorly hindered by the uncultivability of its microbiome. Despite numerous approaches, none of the dominant sponge associated phylotypes belonging to the Acidobacteria, Chloroflexi, Cyanobacteria, Nitrospirae, Poribacteria or Thaumarchaeota could be

cultivated *in vitro* (Sipkema *et al.*, 2011; Lavy *et al.*, 2014; Steinert *et al.*, 2014; Keren *et al.*, 2015; Versluis *et al.*, 2017). One of the reasons might include the inability to recreate sponge-mesohyl conditions adequately, since sponges and their microbiome evolved complex networks of microbial cross-feeding and other interactions (Pande & Kost, 2017). However, by using conventional cultivation approaches, such interaction networks mostly get disrupted during the early stages of isolation.

Aplysina aerophoba

The Mediterranean sponge species *A. aerophoba* poses an interesting model for investigating the cultivability of sponge associated microorganisms due to its association with a highly diverse microbial consortium (Hentschel *et al.*, 2002; Schmitt, Hentschel, *et al.*, 2012; Slaby *et al.*, 2017). Furthermore, this sponge exhibits a rich biochemical arsenal comprising of high amounts of brominated alkaloids (Turon *et al.*, 2000), which correlate with the abundance of certain microbial taxa (Sacristán-Soriano *et al.*, 2011, 2016). Since FADH2-dependent halogenase gene fragments of microbial origin have been detected in *A. aerophoba*, microorganisms might be the actual producers of such brominated bioactives (Bayer *et al.*, 2013). One example is the antibiotic aeroplysinin-1 (AP), protecting damaged sponge tissue from bacterial infections (Ebel *et al.*, 1997; Thoms *et al.*, 2004; Niemann *et al.*, 2015). Despite various cultivation approaches utilizing antibiotics (Pimentel-Elardo *et al.*, 2003; Sipkema *et al.*, 2011; Versluis *et al.*, 2017), the inclusion of sponge-derived antimicrobials as a selection criteria have remained scarce.

Poribacteria

A dominant member of the *A. aerophoba* microbiome is the bacterial candidate phylum Poribacteria. This ubiquitous and widely distributed sponge-associated phylum represents a phylogenetically distant member of the Planctomycetes-Verrucomicrobia-Clamydiae superphylum (Fieseler *et al.*, 2006; Wagner & Horn, 2006). While Poribacteria has remained recalcitrant to cultivation despite multiple approaches (Pimentel-Elardo *et al.* 2003; Hardoim *et al.* 2014; Lavy *et al.* 2014), recent cultivation-independent studies illuminated on the lifestyle of this candidate phylum (Fieseler *et al.*, 2004, 2006, Kamke *et al.*, 2013, 2014; Jahn *et al.*, 2016) postulating a heterotrophic, aerobic metabolism with the genetic potential to degrade a wide range of carbohydrates and proteoglycans. Keeping the above in mind, this study addresses the discrepancy between the cultivable and total community of *A. aerophoba* and investigates several issues contributing to the current uncultivability of sponge-associated bacteria.

Firstly, we investigate whether sample processing and cryopreservation impacted the viability of sponge-associated bacteria.
Secondly, we explore the use of -omics data in defining nutrients and cultivation conditions for Poribacteria and the addition of a sponge derived antibiotic (aeroplysinin-1).
Lastly, we describe the first attempts to enrich for a complex microbial community maintaining at least some metabolic interactions of the sponge microbiome in a stratified cultivation system based on the Winogradsky-column approach (Madigan *et al.*, 2014; Rundell *et al.*, 2014) and within Liquid-Solid cultures.

Subsequently, 16S rRNA gene amplicon sequencing was used to investigate the diversity and composition of the total prokaryotic community of *A. aerophoba*, its viable fraction after cryopreservation, and its cultivable fraction.

Materials and Methods

Sample Collection and Sponge Tissue Processing

Three *A. aerophoba* individuals were collected in June 2014 by SCUBA diving in Cala Montgó, Spain (42.11N, 3.17E) at a depth of approximately 12 m. Tissue separation and cryopreservation was performed as previously described (Sipkema *et al.*, 2011), using a final concentration of 25% glycerol in sterile artificial sea water (ASW, 33 g/L, (Reef Crystals, Blacksburg, VA, USA) as cryoprotectant. Cryopreserved samples were transported to The Netherlands and stored at -80°C for several months. Four seawater samples (2 L each) were collected at the sampling site and filtered over 0.2 µm pore size polycarbonate filters. Filters were stored at -20°C until DNA extraction was performed.

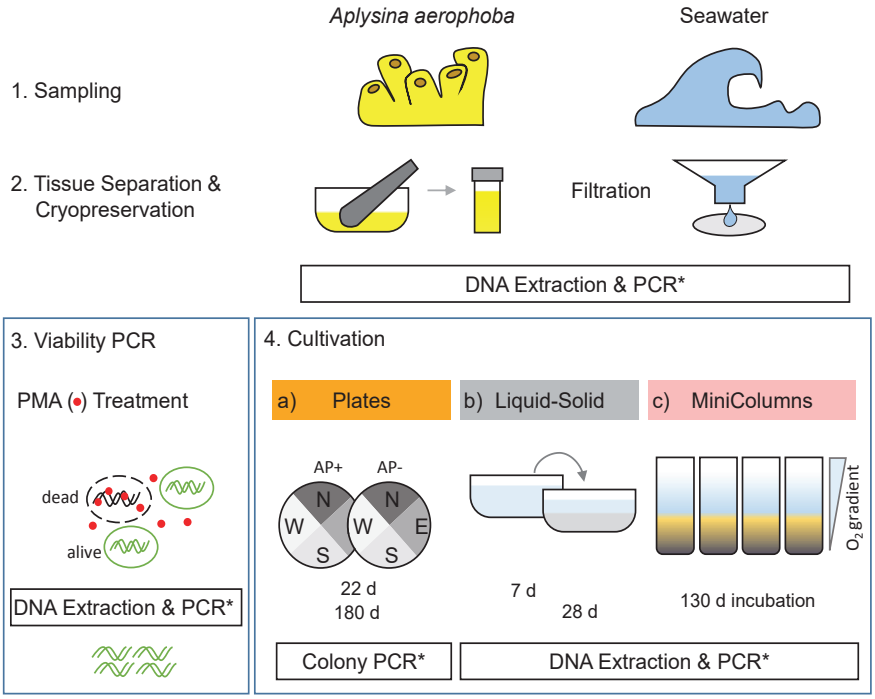

Figure 1 Experimental layout. Sponge samples (*A. aerophoba*) were cryopreserved and subsequently subjected to a viability test and three cultivation methods. For the a) Plates cultivation experiment, colonies were picked after 22 days and 180 days of incubation. In b) Liquid-Solid cultivation, cryopreserved material was incubated in liquid medium for 7 days, then transferred to Liquid-Solid medium for 28 days. In c) Winogradsky columns (MiniColumns), the established oxygen gradient was assessed and samples were taken after 130 days of incubation. Prokaryotic diversity of all samples was determined by sequencing 16S rRNA gene amplicons.

Viability of Microorganisms after Cryopreservation

A cryopreserved cell suspension of *A. aerophoba* (Aa18) was thawed and divided into four 150 µL aliquots. Two aliquots served as total prokaryotic community controls (Cryostock samples) and were stored at 4°C for a few hours until DNA extraction as described below. To assess the viable prokaryotic community after cryopreservation (Figure 1), 2 aliquots were treated with a propidium monoazide dye (PMAxx™, Biotium Hayward, CA, USA) following manufacturer's instructions (here referred to as PMA samples). PMA permanently modifies DNA of membrane-impaired, dead cells and thus only viable cells with intact membranes are amenable to PCR amplification and sequencing (Nocker *et al.*, 2007; Emerson *et al.*, 2017). After photo-activation of the dye (using the PMA-Lite™ LED Photolysis Device), cells were pelleted for subsequent DNA extraction.

Cultivation Setup

a) Plates

Applying single cell genomics, Kamke *et al.* (2013) postulated that Poribacteria can use a wide range of carbohydrates as energy source for its central metabolism. Poribacteria seem well adapted to degrade sponge mesohyl- and seawater-derived carbohydrates and proteoglycans including compounds such as uronic acids, glucose, N-acetylgalactosamine, xylose and galactoside polymers such as lactose and melibiose. The predicted carbohydrate degradation potential of Poribacteria (Kamke *et al.*, 2013) served as a basis for the design of 9 defined media formulations. The media differed only in carbon source, comprising of a variety of different polysaccharides and monomeric sugars. For each of the media, three carbon concentrations were applied: 1x (4 g/L), 10x diluted (0.4 g/L) and 50x diluted (0.08 g/L). Additionally, all media contained the same nitrogen, phosphorous and sulfur sources as well as micronutrients and trace elements (Table 1), which were defined in an attempt to meet metabolic needs of Poribacteria. All components were dissolved in artificial seawater (ASW). For medium dilutions (10x and 50x) all medium components were diluted except the micronutrients and trace metals, which were kept constant.

Media were solidified using 0.75% (w/v) gellan gum (GELRITE, Carl Roth, Karlsruhe, DE). Before autoclaving the pH was set to between 8 and 8.5, to achieve a final pH of approximately 8. After autoclaving, filter-sterilized cycloheximide (250 mg/L, anti-fungal), carbon source, micronutrient and trace metal solutions were added to the media and quickly poured in 90 mm diameter petri dishes. After cooling, collagen media plates were coated with 0.1% (1 mg/mL) solution of calf skin collagen (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Plates were divided in quadrants and kanamycin disks (30 µg Thermo Scientific™ Oxoid™ Kanamycin Antimicrobial Susceptibility Disks) were laid in the approximate center of each of the quadrants. For each medium and its three dilutions, three replicate Plates sets were used for incubation with (AP_positive) and without (AP_negative) aeroplysinin-1, and a negative control.

Table 1 Media compositions of the Plates. Concentrations are given for undiluted (1x) media. Nitrogen, sulfur and phosphorous sources were diluted for 10x and 50x diluted media, while other nutrients were kept constant in all media.

Carbon Source			Other Nutrients		
Medium	**Ingredients**	**[g/L]**	**Nutrient**	**Ingredients**	**[mg/L]**
Glc	N-acetylgalactosamine	2	Nitrogen	$NaNO_2$	250
	N-acetylglucosamine	2		Urea	250
Fru	Fucose	1	Phosphorous	Aminoethyl-phosphonate (2-AEPn)	250
	Fructose	1	Sulfur	Cysteine	40
	Glucose	1		Histidine	40
	Sucrose	1		Methionine	40
Gal	Lactose	1		$NaSO_4$	80
	Galactose	1	Micronutrients	BME Vitamin Solution	1 mL
	D-galactonate	1		B2	2
	Melibiose	1		B9	2
Aa	All amino acids	2		B12	5
	N-acetylglucosamine	2		FAD	1
Col	Collagen coated	3		NAD	2
	Galactose	1	Trace Metals	Customized Trace Metal Solution	1 mL
Muc	Glycoprotein: Mucin from porcine stomach	4	Customized Trace Metal Solution	for 1L	[mg/L]
Myo	Myo-inositol	4		$FeCl_2 * 4\ H_2O$	1500
Rha	L-rhamnose	1.33		7.7 M HCl	10 mL
	L-rhamonate	1.33		$CoCl_2 * 6\ H_2O$	190
	D-xylose	1.33		$MnCl_2 * 4\ H_2O$	100
All	N-acetylgalactosamine, N-acetylglucosamine, Fructose, Sucrose, Glucose, Lactose, Melibiose, Galactonate, Galacturonate, Glucuronate, Mucin, Myo-Inositol, Rhamnose, Xylose	0.30 each		$ZnCl_2$	70
				H_3BO_3	6
				Na_2MoO_4	36
				$NiCl_2 * 6\ H_2O$	24
				$CuCl_2 * 2\ H_2O$	2
				MoO_2	10
				$KMnO_4$	190
				NaI	5
				KBr	5

A cryopreserved *A. aerophoba* (Aa16) cell suspension was thawed, serially diluted in sterile ASW from 10^{-1} to 10^{-4}, and 50 µL of each dilution was inoculated on one of the quadrants of the Plates. For incubation with a sponge-derived antibiotic (AP_positive), the sponge cell inoculum (Aa17) was incubated with 5 mg/L of aeroplysinin-1 (Abcam, Bristol, UK, dissolved in DMSO), for 15 min at room temperature prior to inoculation. The negative control set was inoculated with sterile ASW. The remaining sponge cell suspension was stored at -20°C for total community DNA extraction. Plates were incubated at 20°C in the dark and colonies were picked after 20-25 and 180 days. None of the negative controls showed growth after 25 days. No colonies could be observed on the collagen-coated plates Col_10x and Col_50x, which were discarded from further analyses. A maximum of 48 individual colonies were picked per Plate, starting with the highest inoculum dilution quadrant, thus preferentially picking colonies derived from highly abundant community members. For each colony, media type and dilution, inoculum dilution, incubation time, morphology and distance from kanamycin patch was recorded. Individual colonies were picked using 200 µL pipet tips and transferred to both a sterile 1 mL 50% glycerol solution and to 100 µL nuclease free water in 96 well plates. The glycerol stocks were cryopreserved at -80°C, and nuclease free water was stored at -20°C as template for 16S rRNA gene amplification by colony PCR.

b) Liquid-Solid Media Cultivation

In order to investigate whether a liquid-solid interface within the cultivation medium would result in a different enriched community, 100 µL of cryopreserved *A. aerophoba* cell suspension (Aa18) was inoculated into liquid, 50x diluted "All amino acids medium" (Aa_50x medium) in duplicate. After 7 days of incubation, the biofilm was harvested, 1 mL of culture was cryopreserved by adding 1 mL of 50% glycerol in ASW and 1 mL was stored at -20°C for subsequent DNA extraction. Another 100 µL were transferred to petri dishes containing the solid Aa_50x medium overlaid by 5 mL of liquid Aa_50x medium. This Liquid-Solid culture was incubated for 4 weeks. Subsequently, the cultures were collected by carefully scraping the established biofilm off the submerged gelrite surface, and 1 mL of total Liquid-Solid culture was cryopreserved, while another 1 mL was stored at -20°C for DNA extraction.

c) Winogradsky Columns

Winogradsky columns, here referred to as MiniColumns, were prepared by filling glass culture tubes (25 mL) with 15 g of silicate sand (Sibelco, Antwerp, BE), and 75 mg of crystalline cellulose (5 mg/L) was mixed in as carbon source. For the water phase of the column 50x diluted marine broth (0.75 g/L), 0.5 g/L urea and 0.01 g/L $NaSO_4$ were dissolved in ASW. The pH was adjusted to 7.5, then 15 mL of this medium (referred to as MiniColumn Medium) was added on top of the silicate sand and the MiniColumns were autoclaved. After autoclaving, 1 mL/L of phosphate solution (5 mg/L NaH_2PO_4) and 1 mL/L BME vitamins (Sigma Aldrich, St. Louis, MO, USA) were added. Four replicate MiniColumns were inoculated with 300 µL of cryopreserved *A. aerophoba* sponge-cell suspension (Aa23). One MiniColumn was inoculated with sterile ASW as a negative control. The MiniColumns were closed with metal-caps and aluminum foil in order to allow for oxygen diffusion and incubated at a north side window. After 130 days, an oxygen microsensor (PreSens, Regensburg, DE) was inserted into the columns and oxygen concentrations were measured continuously from the water surface until the sediment fraction. Immediately afterwards, the MiniColumns were divided into 5 samples per column representing the surface (WS), the top (WT) and the bottom (WB) of the water phase as well as the top (ST) and the bottom (SB) of the sediment fraction. The

samples were cryopreserved and 1mL of water phase or 1g of sediment was stored at -20°C for subsequent DNA extraction.

DNA Extraction

Total community DNA was extracted from 1. cryopreserved sponge and bacteria cell suspensions, 2. filtered environmental seawater samples, 3. the four aliquots of the viability tests, 4. samples from 7 days and 4 weeks of Liquid-Solid cultivation, and 5. the MiniColumns samples (Figure 1). Seawater filters were cut into small fragments and suspended in 400 µL of STAR buffer (Roche Diagnostics Corporation, Indianapolis, IN, USA). Sediment samples of the MiniColumns were mixed with 1 mL of STAR buffer and vortexed thoroughly. After letting the sediment settle, the supernatant was transferred to bead-beating tubes. For all other sample types, cells were pelleted, resuspended in 400 µL of STAR buffer and transferred to bead-beating tubes, 2 mL sterile tubes filled with 0.1 g of 0.11 mm Zirconia beads (BioSpec Products, Bartlesville, OK, USA). Cell lysis was achieved by bead-beating using a FastPrep machine (Qbiogene) for two times 30 s at a speed setting of 5.5 m/s. Total community DNA was extracted using a Maxwell 16 Instrument in combination with the Maxwell® 16 Tissue LEV Total RNA Purification Kit (Promega, Madison, WI, USA) following manufacturer's instructions. The DNA was eluted in 30 µL DNAse free water, and quantified using Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis.

16S rRNA Gene Amplicon Sequencing

Amplicons of the V4 region of the 16S rRNA gene were generated from extracted DNA or directly (without DNA extraction) from colonies from the Plates experiment using a two-step PCR protocol. In the first step, PCR amplicons were generated using the primers 515f-806rB (Walters *et al.*, 2015) with an attached linker sequence (UniTag) (van Lingen *et al.*, 2017). PCR was performed in triplicate (single reactions for colony PCR) and the reaction mix contained 5 µL 5X Phusion HF buffer, 0.5 µL dNTPs (10 nM), 1 µL UniTag1-515f primer (5'- GAGCCGTAGCCAGTCTGC-GTGYCAGCMGCCGCGGTAA-3') (10 µM), 1 µL UniTag2-806rB primer (5'-GCCGTGACCGTGACATCG-GGACTACNVGGGTWTCTAAT-3') (10 µM), 0.25 µL Phusion Hot Start Polymerase (ThermoFisher Scientific), and 1-10 µL of extracted DNA (20 ng/µL). For colony PCR, 1 µL of template was used. Nuclease-free water (Promega) was added to yield a total reaction volume of 25 µL. The PCR program comprised of initial denaturation at 98°C for 30 sec (10 min for colony PCR), followed by 25 cycles of denaturation at 98°C for 45 sec, annealing at 50°C for 30 sec, elongation at 72°C for 10 sec, and a final extension step at 72°C for 7 min. All colony PCR reactions with a negative result were repeated. In the second step PCR a sample-specific barcode was added.

To reduce the number of barcoded samples for analysis of the Plates experiment, on average 40 colony PCR products were pooled at approximately equimolar amounts (based on gel band intensity) for the second step PCR. For the AP_negative set, one PCR product pool corresponded to the amplified colonies from one Plate. Due to the low yield of colonies from AP_positive Plates, not enough positive PCR products were available per pool, thus colonies from more than one Plate were pooled to keep the number of PCR products consistent within one sample for 16S rRNA gene profiling.

Second step PCR reactions were done in triplicate and contained: 10 µL 5X Phusion HF buffer, 1 µL dNTPs (10 nM), 5 µL of sample specific, mixed forward and reverse Unitag-Barcode primer, 0.5 µL Phusion Hot Start Polymerase (ThermoFisher ScientificTM), 28.5 µL nuclease-free water and 5 µL DNA template. The PCR program was initial denaturation at 98°C for 30 sec, followed by 5 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 20 sec, elongation at 72°C for 20 sec, and a final extension step at 72°C for 7 min. PCR products (~350 bp) were purified using the HighPrep™ PCR product purification kit (MAGBIO GENOMICS, Gaithersburg, MD, USA) and quantified using Qubit fluorometer BR assay kit (Molecular Probes by Life Technologies). Equimolar amounts of purified PCR amplicons were pooled into libraries and sent for sequencing using the Illumina MiSeq platform (GATC-Biotech, Konstanz, Germany).

Sequencing Data Analysis

Raw paired-end MiSeq sequencing reads were analysed using the NG-Tax pipeline (Ramiro-Garcia *et al.*, 2016) with default settings for filtering to reads with perfectly matching barcodes, which were used to demultiplex reads by sample. OTUs with 100% sequence identity occurring above a minimum 0.1% relative abundance threshold per sample were picked and subjected to non-reference based chimera checking. Taxonomy was assigned to OTUs using a customized version of the SILVA_128_SSU Ref database (Quast *et al.*, 2013). Demultiplexed, raw reads have been deposited at the European Nucleotide Archive (ENA) under accession number PRJEB31820 (http://www.ebi.ac.uk/ena/data/view/PRJEB31820).

Prokaryotic Community Analysis

The resulting biom tables and tree files were analyzed in R version 3.4.3 (https://www.r-project.org) using the phyloseq package version 1.20.0 (McMurdie & Holmes, 2013) and the microbiome package version 1.1.2 (Lahti et. al. 2017) for data import, storage, quality control, data transformations, subsetting, ordination methods and diversity analyses. OTUs classified as Chloroplasts were discarded from the analysis.

Diversity indices for total and viable community fractions were estimated as implemented in phyloseq and significance was tested using Kruskal-Wallis rank sum test. DESeq2 (Love *et al.*, 2014) as implemented in phyloseq was used to normalize the OTU table of viability-test samples and to detect differentially abundant taxa in duplicate samples. OTUs with $p_{adj} < 0.01$, corrected for multiple testing, were considered significantly differentially abundant.

The ape package version 5.0 (Paradis *et al.*, 2004) was used for phylogenetic tree handling and the picante package version 1.6-2 (Kembel *et al.*, 2010) was used to calculate Faith's phylogenetic diversity. Non-parametric tests on medians of phylogenetic diversity per sample group (Seawater, Sponges, Plates, MiniColumns and Liquid-Solid) were performed using Mann-Whitney test (Mann & Whitney, 1947). Sample counts were transformed to relative abundance and distances between samples were calculated using the weighted UniFrac distance metrics (Lozupone *et al.*, 2011) as implemented in phyloseq. Principal Coordinates analyses on weighted UniFrac distance metrics were performed to visualize beta diversity differences for sample groups. The degree of dispersion in beta diversity was calculated using the betadisper function, and adonis test with

999 permutations was used to test significance of associated variables affecting the clustering as implemented in the vegan package version 2.4-5 (Oksanen *et al.*, 2016). Venn-diagrams displaying shared and unique OTUs per sample group were created using Venny 2.1 (Oliveros, 2007) and redrawn using PowerPoint. Relative abundances of the top 100 cultivated taxa were visualized using the pheatmap package version 1.0.8 (Kolde, 2012a) and refined using Adobe Photoshop.

Canonical correspondence analysis (CCA) as implemented in phyloseq was conducted on relative abundance data to analyze the effect of the variable Medium dilution on the Plates sample set and of the variable Location within the MiniColumns sample set. Significance values were calculated using the anova.cca function as implemented in vegan. To assess the overall influence of aeroplysinin-1, relative abundances of taxa were calculated for the sum of reads of samples with or without the antibiotic. The linear model relating oxygen concentration and phylogenetic diversity was fitted as implemented in the R stats package. Ggplot2 version 2.2.1 (Wickham, 2016a) was used for data visualization. Full code and input files are available on GitHub (https://github.com/mibwurrepo/Gutleben_et.al_Cultivation_A.aerophoba_Bacteria).

Results

In total, 4 196 239 high-quality, denoised 16S rRNA gene sequences were obtained, with a minimum of 2 390 and a maximum of 344 380 sequences per sample. These sequences were clustered into a total of 587 OTUs.

Viability of Microorganisms after Cryopreservation

The viable fraction of cryopreserved *A. aerophoba* cell suspensions was analyzed by comparing the differences in community composition of the total (Cryopreserved samples) and viable fractions (PMA treatment of cryopreserved samples). The viable fractions contained fewer OTUs (n = 104) and exhibited lower phylogenetic diversity (PD_{avg} = 11.5) compared to the total communities (n = 109, PD_{avg} = 11.9), however, this difference was not significant (Kruskal-Wallis rank sum test). Of these, 100 OTUs (88.5%) were shared, while 9 OTUs and 4 OTUs were uniquely detected in cryopreserved samples and viable fractions, respectively. We used DESeq2 to identify 15 OTUs that were significantly ($p_{adj} < 0.01$) differentially abundant in viable fractions (Figure 2). In total, these OTUs comprised 12.4% of the total cryopreserved-, and 4.5% of the viable fraction's abundance. Of these, only Cyanobacteria OTU—451 and several Acidobacteria OTUs represented abundant members of the sponges' bacterial community that showed notable decrease in abundance in the viable fractions after PMA treatment. PAUC34f OTU—540 was the only taxon that showed a significant increase in abundance in the viable fractions, however, this OTU was absent or below the detection threshold in the total cryopreserved communities.

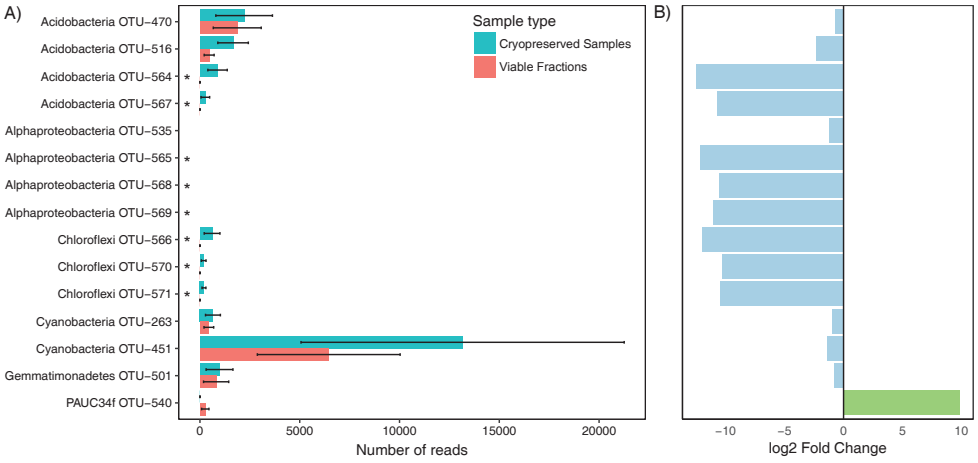

Figure 2 Bacterial taxa showing differences in abundances in viable fractions (PMA Treatment) as detected by DESeq2. A) Total read counts of significantly different (padj < 0.01) taxa from duplicate cryopreserved and viable fractions (PMA Treatment). Asterisks mark taxa falling below the detection threshold of 0.1% relative abundance in the viable fractions. B) Ratio of the log2 fold change of OTUs that increased (green) or decreased (blue) in viable fractions compared to cryopreserved samples.

Cultivation Overview

A. aerophoba-derived bacteria were grown in 3 different cultivation experiments: (a) a variety of "Plates" with media tailored towards the cultivation of Poribacteria, (b) a "Liquid-Solid" cultivation where bacteria were incubated in liquid medium and subsequently transferred to petri dishes that were covered with the same liquid growth medium and (c) in Winogradsky columns, hereafter referred to as "MiniColumns" (Figure 1). Table 2 provides an overview of the samples derived from cultivation experiments, referred to as "cultivation samples". For (a) the Plates, a cultivation sample corresponds to a pool of single colony PCR products, which, for the AP_negative set, corresponds to the colonies derived from one Plate with a specific carbon source and medium dilution. Due to the low yield of colonies from the AP_positive set, positive PCR products from more than one Plate were pooled (referred to as "mix" in Table 2). For (b) the Liquid-Solid cultivation, a cultivation sample corresponds to the prokaryotic community that was enriched after 7 d or 28 d of incubation. For (c) the MiniColumns, a cultivation sample corresponds to the community enriched in a specific fraction in one of the four replicate columns.

Table 2 Samples derived from three cultivation experiments (cultivation samples) and associated meta data. PD represents Faith's Phylogenetic Diversity.

a) PLATES

Sample ID	Medium	Medium Dilution	Aeroplysinin (AP)	Incubation Time [d]	PD
Glc_1x	Glc	1x	-	20	2.40
Fru_1x	Fru	1x	-	20	2.48
Gal_1x	Gal	1x	-	20	2.29
Aa_1x	Aa	1x	-	20	2.43
Col_1x	Col	1x	-	20	2.26
Muc_1x	Muc	1x	-	20	2.38
Myo_1x	Myo	1x	-	20	2.27
Rha_1x	Rha	1x	-	20	2.39
All_1x	All	1x	-	20	2.39
Glc_10x	Glc	10x	-	20	2.30
Fru_10x	Fru	10x	-	20	2.63
Gal_10x	Gal	10x	-	20	2.99
Aa_10x	Aa	10x	-	20	2.30
Muc_10x	Muc	10x	-	20	2.70
Myo_10x	Myo	10x	-	20	3.04
All_10x	All	10x	-	20	1.95
Glc_50x	Glc	50x	-	20	2.94
Fru_50x	Fru	50x	-	20	2.30
Gal_50x	Gal	50x	-	20	3.27
Aa_50x	Aa	50x	-	20	3.21
Muc_50x	Muc	50x	-	20	3.26
Myo_50x	Myo	50x	-	20	2.94
Rha_50x	Rha	50x	-	20	3.03
P13II	mix	mix	mix	20	1.73
P14I	mix	mix	+	24	1.54
P14II	mix	mix	+	24	2.61
P15I	mix	mix	+	24	2.37
P15II	mix	mix	+	24	2.34
P16I	mix	mix	+	24	2.29
P16II	mix	mix	+	24	2.35
P17I	mix	mix	+	24	2.55
P17II	mix	mix	+	24	2.68
P18I	mix	mix	+	24	2.24
P19I	mix	mix	+	24	2.74
P19II	mix	mix	+	24	2.69
2ndP1I	mix	mix	-	180	4.11
2ndP1II	mix	mix	-	180	3.94
2ndP2I	mix	mix	mix	180	4.30
2ndP2II	mix	mix	-	180	3.84

b) LIQUID-SOLID

Sample ID	Medium	Medium Dilution	Type	Incubation Time [d]	PD
A7i	Aa	50x	Liquid-Culture	7	1.73
A7ii	Aa	50x	Liquid-Culture	7	2.51
Aw4i	Aa	50x	Liquid-Solid	28	1.79
Aw4ii	Aa	50x	Liquid-Solid	28	2.32

c) MINICOLUMNS

Sample ID	Location	O2 conc. [%]	Inc. Time [d]	PD
1WS	Water_Surface	65.9	130	3.08
2WS	Water_Surface	41.2	130	3.08
3WS	Water_Surface	36.8	130	2.85
4WS	Water_Surface	6.8	130	1.99
1WT	Water_Top	25.4	130	2.84
2WT	Water_Top	26	130	3.00
3WT	Water_Top	30.6	130	2.94
4WT	Water_Top	1.8	130	2.76
1WB	Water_Bottom	0.1	130	3.57
2WB	Water_Bottom	15.3	130	3.00
3WB	Water_Bottom	30.6	130	2.96
4WB	Water_Bottom	1.8	130	3.06
1ST	Sediment_Top	0.1	130	2.55
2ST	Sediment_Top	6.4	130	2.48
3ST	Sediment_Top	19.8	130	2.22
4ST	Sediment_Top	0.4	130	2.20
1SB	Sediment_Bottom	0.1	130	3.09
2SB	Sediment_Bottom	3.2	130	2.62
3SB	Sediment_Bottom	0.3	130	2.46
4SB	Sediment_Bottom	0.2	130	2.13

In order to compare the microbial diversity of *A. aerophoba*, the surrounding seawater and the microbial communities that enriched within the different cultivation approaches, Faith's phylogenetic diversity was calculated (Figure 3 A). The total number of unique OTUs recovered by cultivation, irrespective of the method applied (Mann-Whitney Test $p < 0.05$), was higher compared to the sponge samples (Figure 4 A). However, the phylogenetic diversity was substantially lower, indicating a strong bias towards cultivating only certain phylogenetic groups.

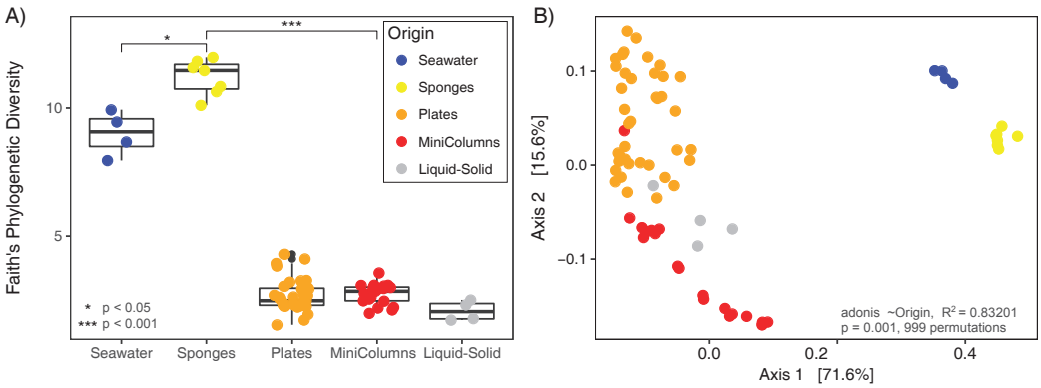

Figure 3 Diversity analyses of the bacterial communities derived from environmental samples and the different cultivation approaches. A) Faith's Phylogenetic Diversity and B) Principal Coordinates Analysis on weighted UniFrac distances.

Sponges, seawater and cultivation samples showed little overlap in microbiome structure (Figure 3B), and sample origin significantly (adonis $R^2 = 0.83201$, $p = 0.001$) explained the separation. We observed large heterogeneity in regard to microbial community compositions for cultivation samples, which spread widely in the ordination space (Figure 3 B). Microbial communities derived from Plates are well separated from the MiniColumn samples, while the Liquid-Solid samples are positioned in between. Even though large heterogeneity of recovered bacteria was also observed within each of the cultivation method sample groups (Plates, Liquid-Solid, MiniColumns), the degree of dispersion in beta diversity was not significantly different (permutation test of beta dispersion F.Model = 2.1915, Pr(>F) = 0.084). This means that all groups exhibit similar levels of variation within the group, confirming that the differences between cultivation method sample groups are caused by the cultivation method and not by chance.

In total, the three cultivation experiments yielded 256 cultured OTUs from seven bacterial phyla, namely Actinobacteria (1.2% of cultivated OTUs), Bacteroidetes (5.1%), Firmicutes (29.7%), Planctomycetes (3.1%), Proteobacteria (56.6%), Tenericutes (0.4%) Verrucomicrobia (0.4%) and an unidentified phylum related to the Planctomycetes (3.5%). Amongst the Proteobacteria, Alphaproteobacteria (44.5%) and Gammaproteobacteria (10.9%) dominated the cultivated fraction. No Poribacteria OTUs were detected in any of the cultivation experiments. On average, 17 OTUs were detected per cultivation sample, with a minimum of seven and a maximum of 36 OTUs. This confirmed that the number of OTUs recovered per colony PCR pool from the Plates was in the range of the expected numbers based on the amount of colonies pooled into one sample.

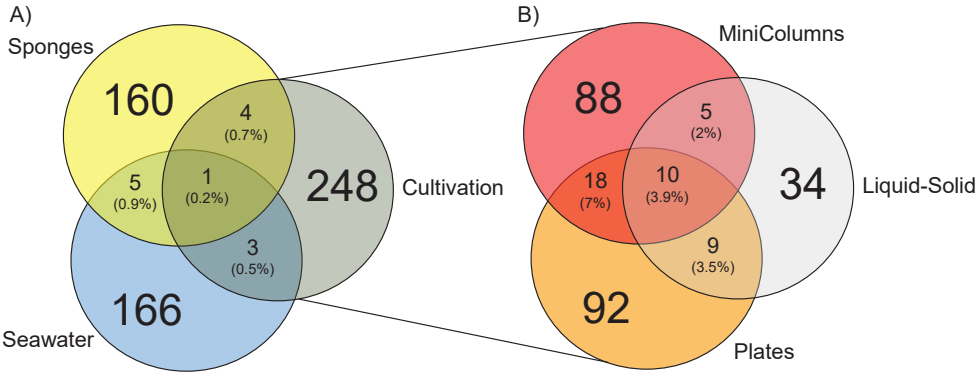

Figure 4 Venn diagrams displaying A) OTUs unique and shared (% of number of OTUs) between environmental samples and cultivation experiments and B) OTUs unique and shared between the three different cultivation methods.

Of the 256 OTUs recovered by cultivation, 4 OTUs, classified as *Pseudovibrio* sp., *Halomonas* sp., Flavobacteriaceae family and *Lutimonas* sp. were shared between the sponge tissue and the cultivated fraction (Figure 4 A). Another three OTUs, *Halomonas* sp., *Idiomarina* sp. and *Hyphomonas* sp. were shared between the seawater and the cultivated fraction. One OTU, *Ruegeria* sp. (OTU-3) was shared between seawater, sponge tissue and the cultivated fraction. The different cultivation approaches shared merely 10 OTUs, classified as *Ruegeria* sp., *Pseudovibrio* sp., *Microbulbifer* sp. and 5 other Rhodobacteraceae family OTUs, indicating that most bacteria were only obtained by one cultivation approach (Figure 4 B).

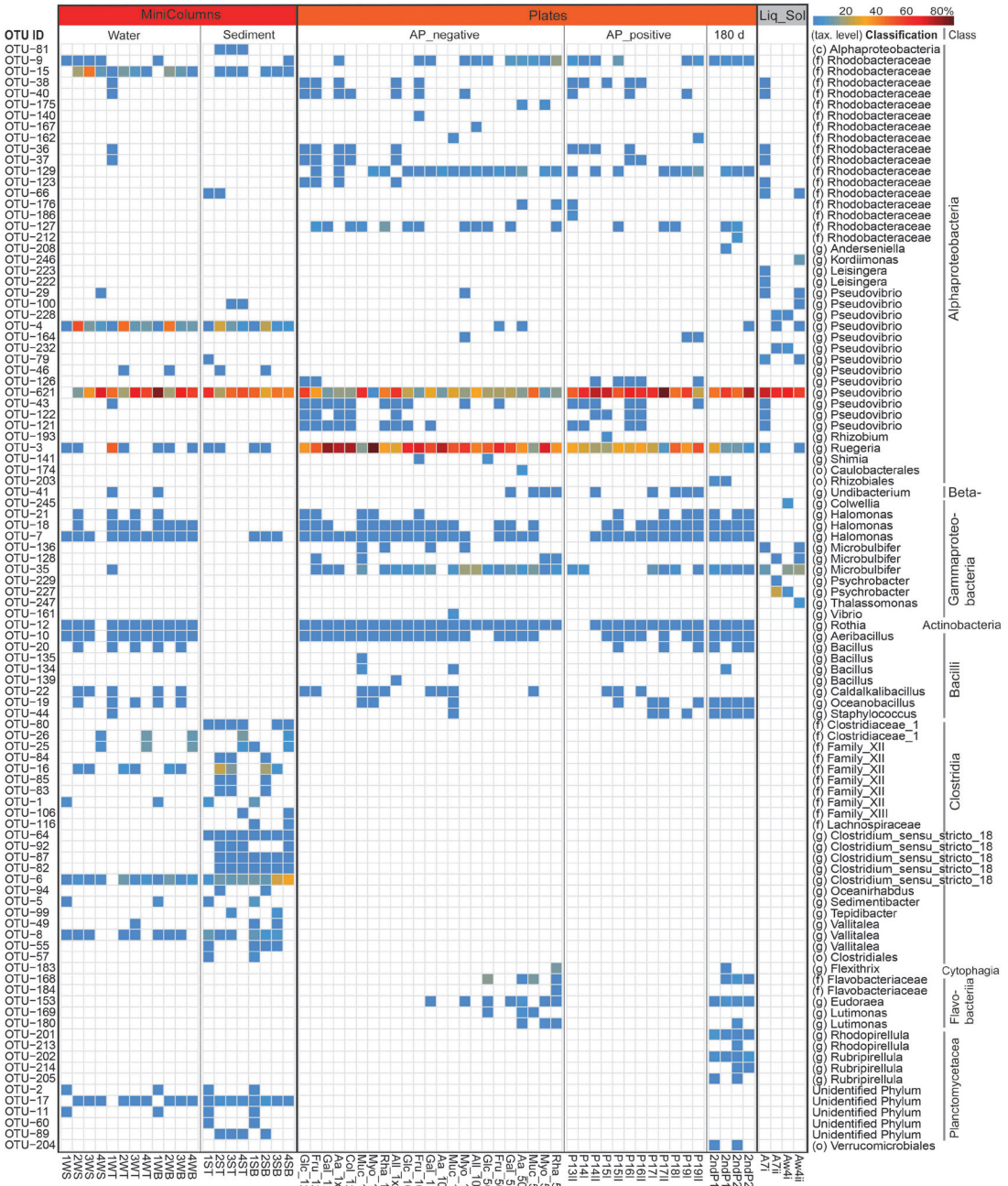

Figure 5 Heatmap showing the relative abundance of the top 100 cultivated OTUs, representing 94–100% of the total number of reads in individual samples. Color scale shows relative abundance (%) within each sample. Rows of the heatmap are ordered by taxonomic classification, and OTUs were classified up to class (c), family (f) or genus (g) level. SampleIDs reflect cultivation media and can be obtained from Table 1.

Pseudovibrio sp. OTU-621 was recovered from all but one cultivation condition and dominated the cultivable fractions from 3.0 to 89.7% relative abundance per sample (Figure 5). In addition to the high relative abundance of OTU-621, a large number of other OTUs from the genus *Pseudovibrio* were recovered from a variety of growth media and conditions. Further, *Ruegeria*, *Bacilli* and various *Halomonas* OTUs were also found on many conditions. Overall, many recovered taxa had a preference for one of the cultivation methods. This difference was especially pronounced by the exclusive presence of Clostridia OTUs in the anaerobic sediment fractions of the MiniColumns. These sediment fractions were also enriched for OTUs that could not be assigned to a phylum (OTU-2, 17, 11, 60, 89). Online BLAST results showed that these OTUs exhibited maximally 89% nucleotide sequence similarities to unidentified Planctomycetes (e.g., accession no. AY162124, AY344414 and AY344412). Furthermore, an OTU belonging to the rarely cultivated phylum Tenericutes was detected with approximately 2% relative abundance in three samples derived from MiniColumn3. On the other hand, Flavobacteriia OTUs and *Microbulbifer* OTUs were nearly absent in the MiniColumns, but were recovered at high relative abundances from Plates. A prolonged incubation time of 180 days led to the recovery of Verrucomicrobia and Planctomycetes on Plates. Subsequent near full length 16S rRNA gene sequencing of these colonies revealed mixed sequences, indicating that these did not initially grow as pure strains. The Liquid-Solid approach resulted in high recovery of *Microbulbifer* and other Gammaproteobacteria from the genus *Psychrobacter*, of which the latter was detected solely in the Liquid-Solid cultivation.

Impact of Aeroplysinin-1 (AP), Medium Dilution and Carbon Sources in the Plates Experiment

From Plates 1758 colonies were picked, 1129 colonies from the AP_negative set, and 629 colonies from the AP_positive set. The 16S rRNA gene of 1463 colonies could be successfully PCR-amplified and sequenced.

Aeroplysinin-1 (AP) presence led to a lower number of colonies and impacted their size and morphology. Most colonies were small and translucent on AP_positive plates, where on AP_negative plates larger and partly pigmented colonies could be observed (Supplementary Figure S1). No DMSO-control was included, however previous studies indicated no toxic effect below a concentration of 1% DMSO (Wadhwani *et al.*, 2009). Since the final concentration of DMSO was only 0.3% in the inoculum suspension, we conclude the low yield of colonies on AP_positive plates was due to the toxicity of the antibiotic.

While Bacteroidetes were completely inhibited by AP, most other taxa grew also in the presence of the antibiotic (Figure 5). The *Rhizobium* OTU-193 on P15I was the only OTU exclusively detected on a AP_positive plate. Overall, the addition of AP resulted in a consistently higher (65% vs 32%) relative abundance of *Pseudovibrio* sp. OTU-621, while reducing the relative abundance of *Ruegeria* sp. OTU-3 from 49% to 29% (Supplementary Figure S2). PCoA analysis on weighted UniFrac distances revealed that AP significantly (adonis $R^2 = 0.25784$, $p = 0.001$) affected the composition of OTUs recovered (Figure 6).

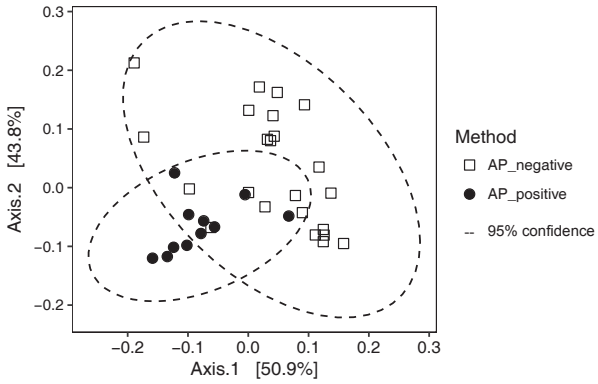

Figure 6 PCoA analysis on weighted UniFrac distances illustrating the effect of incubation with (AP_positive) and without (AP_negative) aeroplysinin-1 on the cultivated communities derived from the Plates.

To assess the influence of medium dilution and carbon source, samples from the Plates experiment were subset to only AP_negative since for this part of the dataset the variables medium dilution and carbon source could be consistently assigned. Overall, phylogenetic diversity increased significantly ($p < 0.01$) in 50x diluted media compared to the 1x diluted media, whereas carbon source did not significantly impact the recovered microbial diversity (Figure 7 A). Consistently, medium dilution (adonis $R^2 = 0.25771$, $p = 0.015$) and not carbon source (adonis $R^2 = 0.47646$, $p = 0.165$) was the significant variable explaining community composition patterns in constrained ordination analysis (Figure 7 B).

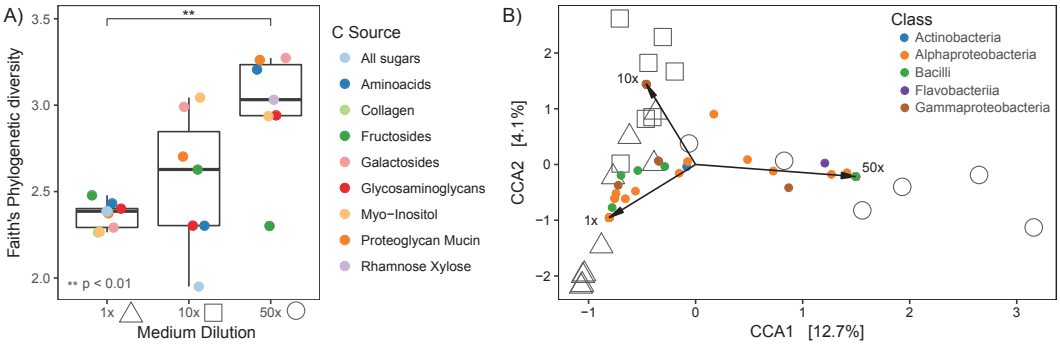

Figure 7 A) Faith's phylogenetic diversity of different medium dilutions and carbon sources. B) Canonical correspondence analysis (CCA) biplot of cultivated microbial communities on Plates and indicative bacterial taxa.

Certain bacterial classes were associated to specific medium dilutions such as Flavobacteriia or Bacilli, which were mainly recovered on 50x or 1x - 10x diluted media, respectively. Proteobacteria were cultivated on all medium dilutions. Phylogenetic diversity was highest in the 50x diluted media containing galactosides, followed by mucin and amino acids, which also showed the highest relative abundance of Flavobacteriia.

The Liquid-Solid Cultivation Approach

Since on Plates, the 50x diluted aminoacids medium (Aa_50x) resulted in high cultivated diversity, this medium was used to create liquid cultures. We observed biofilm formation on the bottom of the liquid culture wells after 7 days, upon which we transferred the cultures to solid media, overlaid with the same liquid media for a total of 4 weeks of incubation. The cultures were dominated by *Pseudovibrio* OTUs after 7 days as well as after 4 weeks. Nonetheless, the Liquid-Solid cultivation resulted in the recovery of genera that were not detected in other cultivation methods, such as the gammaproteobacterial genera *Colwellia*, *Thalassomonas* and *Psychrobacter*, as well as the alphaproteobacterial genera *Leisingera* and *Kordiimonas*. Faith's phylogenetic diversity was not significantly different than in the other cultivation approaches (Figure 3 A), indicating that a variety of microorganisms were present, although in low abundances.

Impact of Oxygen Concentrations in the Winogradsky Columns

After 130 days of incubation, the oxygen gradient along the depth of the MiniColumns was measured. The oxygen concentration in the headspace of all columns was 88.4% (± 6.4%) air saturation, and the four replicate columns had different oxygen profiles along the column: MiniColumn4 was completely anaerobic at the top of the water column, while other columns had oxygen concentrations around 30 - 40% at the water surface with a gradual decrease to complete anoxia in the sediment fractions (Figure 8 A). The negative control maintained a 100% oxygen saturation along the whole depth of the column (data not shown). Despite visual differences amongst the columns (Supplementary Figure S3), identical locations in different columns promoted the growth of very similar microbial communities (Figure 5).

Location within the column was the main driver of community profile differences, with a range of Clostridia OTUs and unidentified OTUs related to the Planctomycetes representing indicative taxa for the anaerobic sediment fractions of the columns (Figure 8 C). Linear model regression analysis revealed a positive, though not significant correlation of oxygen concentration on phylogenetic diversity (PD) estimates. Both high (PD = 3.56) and low (PD = 1.99) estimates were found for anaerobic conditions, while microaerophilic parts of the columns enriched for intermediately diverse communities (Figure 8 B).

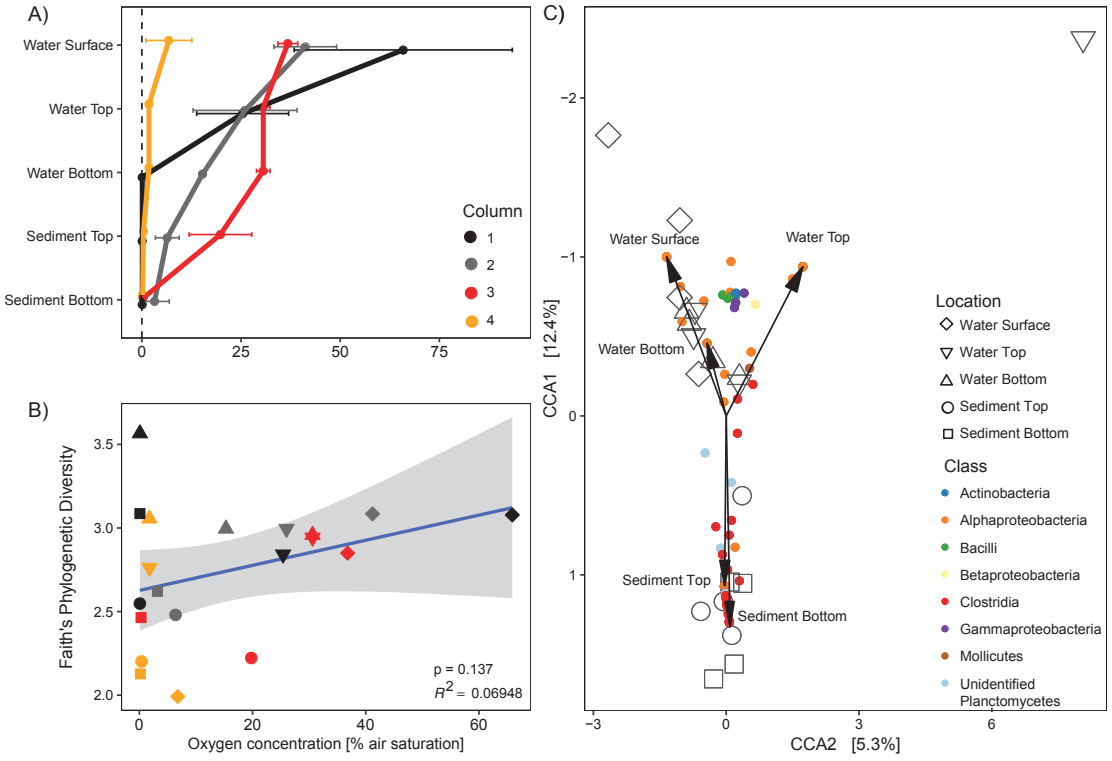

Figure 8 A) Dissolved oxygen concentration along the depth of four replicate MiniColumns. B) Regression analysis of phylogenetic diversity and oxygen concentration. Grey area marks 95% confidence region. C) CCA triplot of enriched microbial communities (large symbols) in relation to indicative bacterial taxa (labelled at Class level) and location in the MiniColumns (arrows).

Discussion

In recent years, culture-independent methods have by far outnumbered cultivation-based efforts to investigate the microbial diversity in sponges. Even though multi-omics based methods can unravel numerous functional, taxonomic and ecological traits of sponge associated microbial communities (M. W. Taylor *et al.*, 2007; Thomas *et al.*, 2016; Slaby *et al.*, 2017), these findings remain hypothetical unless validated by experimental evidence. However, valuable insights from using molecular tools can be used to create novel cultivation conditions (Gutleben *et al.*, 2018) in the quest to improve cultivability of sponge associated microorganisms.

Sponge-associated Microorganisms Remain Viable after Cryopreservation

Since sponge-associated microorganisms are yet mostly recalcitrant to cultivation efforts, we aimed to investigate if the often inevitable cryopreservation impacted the viability of sponge associated microorganisms. We did not detect significant differences in phylogenetic diversity and observed

species richness (OTU counts) between the total and viable communities after cryopreservation. We can thus conclude that the majority of sponge-associated microorganisms survive sample processing and storage at -80°C for several months. Based on our results, the current uncultivability of these microorganisms is most likely not due to cryopreservation of samples before the start of cultivation experiments. To the best of our knowledge, only Esteves *et al.* (2016) analyzed the viable fraction of two Australian sponge species, after tissue separation and cell fractionation to obtain microbial pellets. Overall, they observed a loss of OTUs of up to 51% in the viable fraction, while we observed a 5% loss. This difference might be due to the many steps of filtration and centrifugation involved in the cell fractionation as done by Esteves *et al.* (2016), which might negatively affect the viability of microorganisms, as well as lead to the loss of rare taxa.

Most Cultivated Taxa Differ from Sponge-associated Bacteria

Here we described three different attempts to cultivate sponge tissue-derived bacteria, which all resulted in the recovery of communities with a very different structure and composition compared to the original sponge samples and surrounding seawater samples (Figure 3). The majority of recovered OTUs could only be detected by cultivation and not in the sponge tissue. As negative controls in the cultivation experiments consistently showed no microbial growth, we conclude that these cultivated OTUs must be derived from rare members of the sponge community that were below the applied detection threshold of 0.1% relative abundance. This is also supported by the observation that in the Plates experiment, the 1000-fold diluted quadrants often contained few or no colonies, while 10-fold diluted quadrants teemed with colonies from rare community members (Supplementary Figure S1).

Defining cultivability as the number of taxa that could be cultured divided by the total number of taxa detected in the sponge tissue, we report here a recovery of 2% of the sponge-associated taxa by cultivation. This falls into the range of other sponge-microbe cultivation experiments (Sipkema *et al.*, 2011; Lavy *et al.*, 2014; Keren *et al.*, 2015; Esteves *et al.*, 2016; Versluis *et al.*, 2017), which report a recovery rate of 0.1 to 14%. Even though different sponge species harbor an individual and highly divergent microbiota, it has been shown that their cultivable fractions are comparable (Li *et al.*, 2007; Hardoim *et al.*, 2014) since rare generalists are proliferating under laboratory conditions.

Despite employing newly designed media containing various unusual carbon sources, the novelty of recovered isolates was moderate. Except for the unidentified OTUs (OTU-2, 17, 11, 60, 89) related to Planctomycetes which were not obtained as pure isolates, the 100 most dominant cultivated OTUs revealed a sequence identity of >98% to the closest cultivated relative in the NCBI database. This may be partially explained by the high degree of sequence conservation of the V4 region of the 16S rRNA gene (Sun *et al.*, 2013). Nevertheless, 5 OTUs that were present in the sponge tissue were also recovered by cultivation. Amongst these shared taxa, the Alphaproteobacteria *Pseudovibrio* and *Ruegeria* OTUs were the most frequently cultivated and grew on almost every cultivation method applied in this study. *Pseudovibrio* species represent low abundant sponge symbionts which are known as versatile, opportunistic bacteria capable to adapt to a wide range of cultivation conditions (Muscholl-Silberhorn *et al.*, 2008; Bondarev *et al.*, 2013; Versluis *et al.*, 2017; Fróes *et al.*, 2018). With this study we could furthermore add aeroplysinin-1 to the list of antibiotic resistances exhibited by *Pseudovibrio* species (Versluis *et al.*, 2017). The genus *Ruegeria* is part of

the abundant seawater-dwelling Roseobacter lineage (Buchan *et al.*, 2005; Wagner-Döbler & Biebl, 2006) and has frequently been isolated from sponges and other marine environments (Mitova *et al.*, 2004; Muscholl-Silberhorn *et al.*, 2008; Esteves *et al.*, 2013; Rua *et al.*, 2014). Two other shared OTUs (OTU-168, 169) belong to the class Flavobacteriia, which are regularly detected in and isolated from marine sponges, however, their roles as sponge symbionts remain to be investigated (Lavy *et al.*, 2014; Montalvo *et al.*, 2014; Horn *et al.*, 2016; Yoon *et al.*, 2016; Versluis *et al.*, 2017).

Increased incubation time of 180 days resulted in a broader detected cultivated microbial diversity, as evidenced by the detection of Planctomycetes and Verrucomicrobia OTUs on Plates. To our knowledge, this is the first report of an *A. aerophoba* derived member of the Verrucomicrobia phylum detected during cultivation, and the OTU-204 exhibits only 91% sequence similarity to other cultured sponge-derived Verrucomicrobia: *Rubritalea marina* (Scheuermayer *et al.*, 2006) and *Rubritalea spongiae* (Yoon *et al.*, 2007). Planctomycetes have been obtained from *A. aerophoba* before (Pimentel-Elardo *et al.*, 2003), however, the unidentified OTU-2, which was enriched up to 5% relative abundance in sediment fractions of the MiniColumns, exhibited only 89% sequence similarity to the closest cultivated neighbor, Planctomycetes obtained from Sargasso Sea bacterioplankton (AY162124). The nearest uncultured phylogenetic relatives of OTU-2 have been found associated to diseased tissue of a Caribbean coral (AF544881, 96% sequence identity, Pantos *et al.* 2003), as well as in seawater from 3000m depth close to the Mariana Trench (AB703899, 96% sequence identity, Nunoura *et al.* 2015).

In this study, we extended the attempt of Lavy *et al.* (2014) to design cultivation media for the candidate phylum Poribacteria. The predicted genetic potential of urea and organic phosphorous source utilization, as well as the potential to degrade a variety of carbohydrates was taken into account (Siegl *et al.*, 2011b; Kamke *et al.*, 2013) to design nine defined media. Additionally, trace metal solutions and micronutrient solutions were tailored towards meeting potential co-factor requirements of annotated Poribacteria enzymes (Siegl *et al.*, 2011b; Kamke *et al.*, 2013). Furthermore, all media were diluted to account for Poribacteria potentially being oligotrophs, and incubation time was prolonged to 180 days to account for potential slow growth (Vartoukian *et al.*, 2010; Prakash *et al.*, 2013). Gellan gum was used as solidifying agent to avoid inhibitory effects of agar (Janssen *et al.*, 2002; Overmann, 2010). However, none of the incubation conditions applied enabled the cultivation of Poribacteria. Further adaptations of the cultivation conditions might resolve this in the future, such as selective enrichment based on the predicted Wood-Ljungdahl pathway (Siegl *et al.*, 2011b). Also, the inclusion of siderophores (Vartoukian *et al.*, 2016) or the provision of helper strains might aid in the cultivation of this sought-after microbial candidate phylum (Morris *et al.*, 2008; Davis *et al.*, 2014; Pande & Kost, 2017).

Effects of Micro- and Macroenvironmental Cultivation Conditions on Cultivated Taxa

We observed that the sponge-derived antibiotic aeroplysinin-1 (AP) strongly inhibited microbial growth and led to decreased cultivated diversity on the Plates. The number, size and pigmentation of colonies was negatively impacted, hinting at an overall cellular toxicity of this antibiotic (Supplementary Figure S1). AP seemed to affect taxa differently, as observed by the consistently higher relative abundance of the sponge-associated *Pseudovibrio* sp. OTU-621 in the presence of AP as compared to the seawater-derived *Ruegeria* sp. OTU-3. *Ruegeria* species seem to be

negatively affected by AP, which supports the notion that this antibiotic contributes to protecting the sponge from seawater-derived bacterial infections (Lipowicz *et al.*, 2013). Furthermore, all cultivable members of the phylum Bacteroidetes were completely inhibited by the antibiotic.

Within the AP_negative set, the highest media dilutions (50x) supported the growth of the most diverse microbial community, and almost exclusively supported the growth of Flavobacteriia. This observation supports the notion that many marine organisms require oligotrophic conditions for successful cultivation and might be inhibited by increased substrate concentrations (Stevenson *et al.*, 2004; Hanson *et al.*, 2007; Pham & Kim, 2012), which was also demonstrated for sponge-derived bacteria (Hentschel *et al.* 2001; Muscholl-Silberhorn *et al.* 2008). Medium dilution was the only significant factor driving the development of specific communities, while the impact of the specific carbon source was not significant within the Plates experiment. This could indicate that many marine heterotrophic microorganisms are equipped with the genetic potential to degrade a wide range of carbohydrates, which was reported for e.g. Poribacteria, *Pseudovibrio* or marine Flavobacteriia (Kamke *et al.*, 2013; Barbeyron *et al.*, 2016; Alex & Antunes, 2018), and are more influenced by the concentration of the nutrient.

Overall, the recovered microbial communities clustered based on the cultivation method applied, indicating that macro-environmental conditions such as liquid (water samples in MiniColumns, Liquid-Solid cultivation) or solid medium interface had a more pronounced effect than micro-environmental conditions such as carbon source. For example, the Aa_50x medium applied as Plates enriched for Flavobacteriia, while in the Liquid-Solid cultivation the same medium supported the growth of the gammaproteobacterial genera *Psychrobacter*, *Thalassomonas* and *Colwellia*, which were not detected in other cultivation experiments within this study. Liquid cultivation approaches have only rarely been applied to cultivate sponge-associated bacteria (Sipkema *et al.*, 2011), and have been shown to result in lower species diversity as when compared to solid agar plates (Schoenborn *et al.*, 2004). However, liquid cultivation can result in the recovery OTUs not detected in other cultivation methods (Sipkema *et al.*, 2011), which we could also observe in this study, e.g. the genera *Psychrobacter* or *Leisingera*.

Impact of Oxygen Concentrations in the Winogradsky Column Approach

To our knowledge, this is the first report applying a Winogradsky column approach to sponge-derived samples. Winogradsky columns are enclosed, self-sustaining microbial ecosystems, where chemical gradients create niches for different microorganisms and metabolic interactions are maintained (Madigan *et al.*, 2014; Rundell *et al.*, 2014).

During incubation, oxygen gradients from moderately aerobic in the water columns to complete anoxia in the sediment fractions developed. Community composition varied between the aerated parts of the columns, which exhibited higher abundances of majorly aerobic bacteria such as Actinobacteria and Bacilli, and the anaerobic parts of the columns, which were dominated by Clostridia. The unidentified OTU-2 related to Planctomycetes was enriched with up to 5% relative abundance in the anaerobic sediment fractions of the MiniColumns, hinting towards an anaerobic lifestyle of this microbe. Most fractions of the MiniColumns also exhibited high relative abundances of *Pseudovibrio*, confirming the facultative anaerobic lifestyle of this microbial genus (Shieh *et*

al., 2004). The overall phylogenetic diversity of enriched microorganisms was positively, however not significantly, correlated with oxygen concentration, even though some exceptional samples exhibited high phylogenetic diversity albeit anoxic conditions (Figure 8). These results indicate that anaerobic cultivation approaches can yield a comparable diversity, with distinct and potentially novel recovered taxa, as indicated by Lavy *et al.* (2014). In their study, six out of eight novel taxa with <94% sequence identity to the closest cultivated strains were recovered from strictly anaerobic conditions. Also for *A. aerophoba* and other sponges, periodically occurring tissue anoxia has been observed, indicating a potential niche for anaerobic microbes (Hoffmann *et al.*, 2005, 2008) and the need to further explore anaerobic cultivation techniques for culturing sponge-associated microorganisms.

Conclusions

Based on the results presented we conclude that sponge-associated microbes remain viable during sampling, tissue separation and cryopreservation. In this study we showed that medium dilution rather than media diversification leads to increased diversity of recovered bacterial isolates and that the sponge-derived antibiotic aeroplysinin-1 has a strong impact on the number and morphology of microbial colonies. Only five OTUs overlapped between cultivated bacteria and the sponge tissue, accounting for 2% of the bacterial richness from *A. aerophoba*.

The previously unreported use of a Winogradsky column approach for cultivating sponge microbes could enrich for novel OTUs. This indicates the potential of such stratified cultivation systems for exploring the dynamics of sponge associated microbial communities independently from the host under controlled *in vitro* conditions. Winogradsky columns supplemented with different substrates could become a promising tool to investigate whether sponge-derived microbes can form self-sustainable microbial ecosystems and study their metabolic interactions across aerobic as well as anaerobic niches. To date, the majority of sponge-associated microbes remain uncultivated, calling for further novel media formulations and incubation strategies in the quest to recreate conditions that resemble the sponge ecosystem and thus increase the cultivability of sponge-associated bacteria.

Acknowledgements

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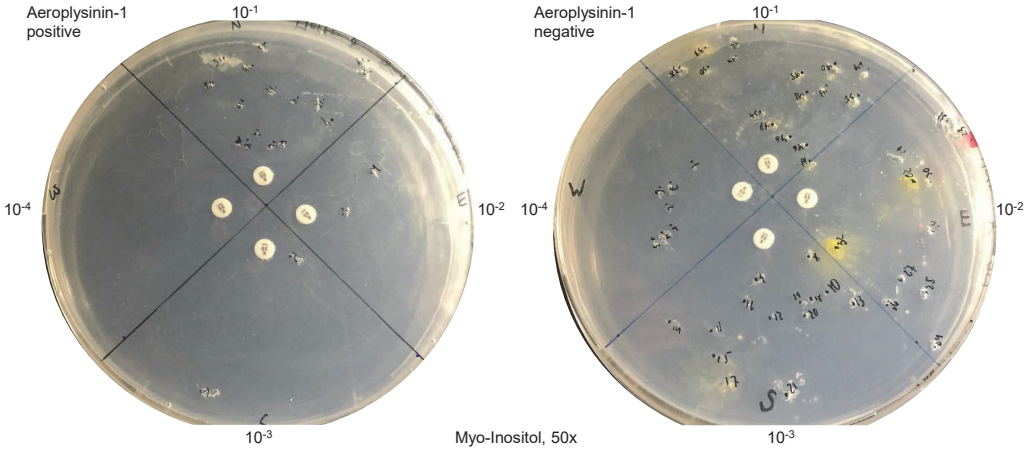
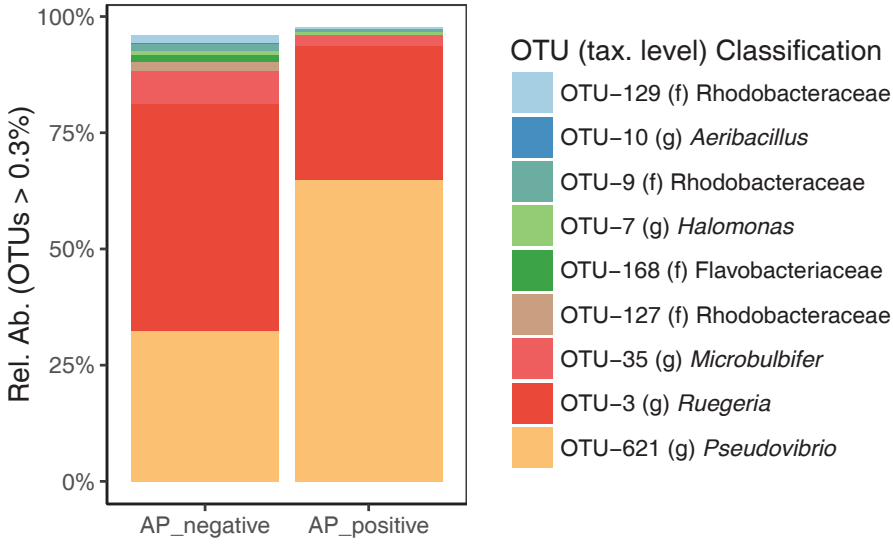
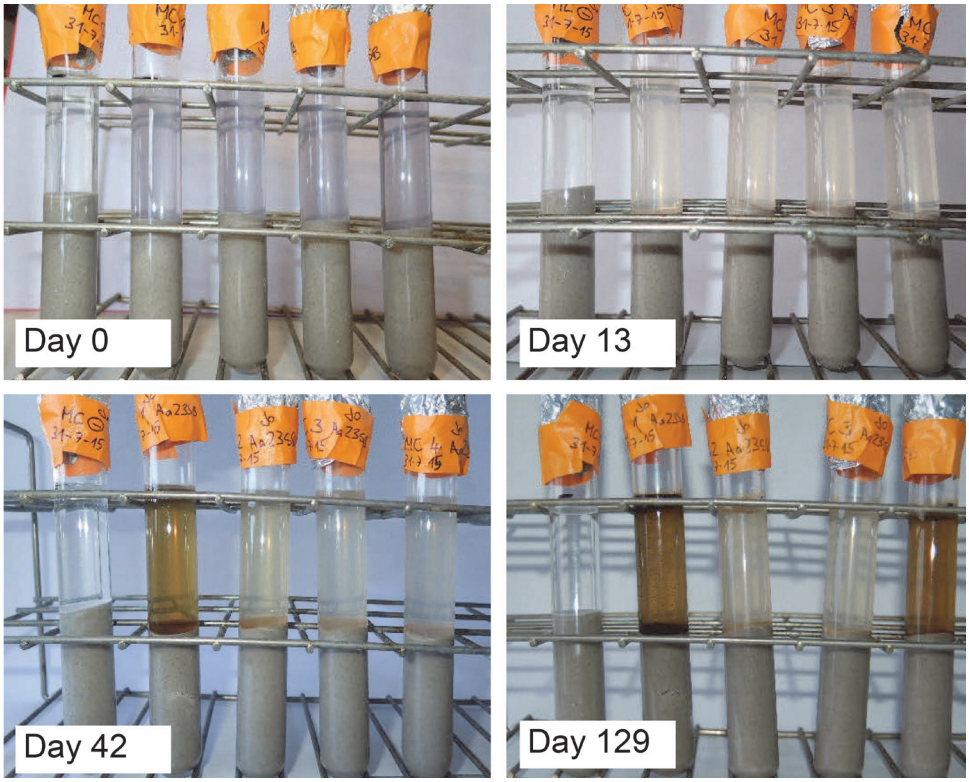
Supplementary Information



Supplementary Figure S1 Example of aeroplysinin-1 (AP) effect on colony number, size and morphology on two Plates containing the same medium.



Supplementary Figure S2 Relative abundances of the most abundant taxa from the Plates, summarized from incubation with (AP_positive) and without (AP_negative) aeroplysinin-1.



Supplementary Figure S3 Photographs of the MiniColumn cultivation experiment over time.

Chapter 5

The Multi-Omics Promise in Context: From Sequence to Microbial Isolate

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Abstract

The numbers and diversity of microbes in ecosystems within and around us is unmatched, yet most of these microorganisms remain recalcitrant to *in vitro* cultivation. Various high-throughput molecular techniques, collectively termed multi-omics, provide insights into the genomic structure and metabolic potential as well as activity of complex microbial communities. Nonetheless, pure or defined cultures are needed to 1) decipher microbial physiology and thus test multi-omics-based ecological hypotheses, 2) curate and improve database annotations, and 3) realize novel applications in biotechnology. Cultivation thus provides context. In turn, we here argue that multi-omics information awaits integration into the development of novel cultivation strategies. This can build the foundation for a new era of omics information-guided microbial cultivation technology and reduce the inherent trial-and-error search space. This review discusses how information that can be extracted from multi-omics data can be applied for the cultivation of hitherto uncultured microorganisms. Furthermore, we summarize ground-breaking studies that successfully translated information derived from multi-omics into specific media formulations, screening techniques and selective enrichments in order to obtain novel targeted microbial isolates. By integrating these examples, we conclude with a proposed workflow to facilitate future omics-aided cultivation strategies that are inspired by the microbial complexity of the environment.

Introduction: The Importance of Cultivation in the Age of Multi-Omics

The diversity and ubiquity of the microbial world that is observed in environmental samples through recent advances in high-throughput sequencing technologies is astounding. The multi-omics revolution dramatically reshaped the field of microbial ecology and led to the realization that in most ecosystems, the existing microorganisms outnumber those that are accessible through cultivation by orders of magnitude. Only a minor part of the microorganisms observed in an environmental sample can be grown and maintained axenically or in defined communities under laboratory conditions. This phenomenon, termed "the great plate count anomaly" (Staley, 2002; Nichols, 2007) has resulted in the development of numerous innovative cultivation techniques using advanced technologies like microfluidics (Ma *et al.*, 2014; Boitard *et al.*, 2015), cultivation chips (Ingham *et al.*, 2007; Hesselman *et al.*, 2012; Gao *et al.*, 2013), manipulation of single cells (Ben-Dov *et al.*, 2009; Park *et al.*, 2011) and high throughput cultivation termed "culturomics" (Lagier *et al.*, 2012). These techniques immensely expanded the number of novel species brought into culture, as reviewed in several recent publications (Alain *et al.*, 2009; Zengler, 2009; Overmann, 2010; Dewi Puspita *et al.*, 2012; Dini-Andreote *et al.*, 2012; Pham & Kim, 2012; Stewart, 2012; Allen-Vercoe, 2013; Harwani, 2013; Narihiro & Kamagata, 2013). To date, we count approximately 11,000 isolated species distributed over 30 bacterial and 5 archaeal phyla that have been validly classified (List of Prokaryotic Names with Standing in Nomenclature (LPSN; http://www.bacterio.net, accessed August 3, 2016)). In spite of the tremendous progress in cultivation technology, the "great plate count anomaly" remains in place, as the rate of discovery of microbes that are as yet uncultivable outpaces the rate of isolating novel species. During the past decades, microbiologists have extensively characterized microbial community composition based on the sequencing of universal marker genes, in most cases PCR-amplified regions of the small subunit ribosomal RNA

(rRNA) gene (Lane *et al.*, 1985). This led to an estimated rate of approximately 40 000 novel prokaryotic species being discovered per year, and a total of 400 000 species of bacteria and archaea are predicted to be discovered by 2017 (Yarza *et al.*, 2014). To put this in perspective, the number of named bacterial phyla increased from 12 to 92 in the last four decades. Additionally, archaea were discovered as a separate domain in 1977 and since then expanded to 26 recognized phyla to date (Woese & Fox, 1977; Dick & Baker, 2014; Youssef *et al.*, 2015; Hug *et al.*, 2016). In stark contrast, less than 6% of the total number of bacterial and archaeal species included in the SILVA REF 114 database has been validly classified by physiological tests of isolates, listed in LPSN (Parte, 2014).

Not surprisingly, sequencing instead of culturing became the trend in the field of microbial ecology after the revolution in sequencing technology. In the present review, we subsume the terms metagenomics, metatranscriptomics and metaproteomics under the term "multi-omics" (Zhang *et al.*, 2010). Metabolomics is not discussed in this review because examples where this technology has successfully been embedded in novel cultivation strategies remain very scarce to date. Metagenomics is defined as the comprehensive sequence analysis of total DNA isolated from environmental samples (Handelsman *et al.*, 1998; Marchesi & Ravel, 2015). Metatranscriptomics is the analysis of total environmental RNA. It provides insights into the local and taxon-specific expression levels of genes (Frias-Lopez *et al.*, 2008) at the community or even the single cell level (Shi *et al.*, 2015). Lastly, metaproteomics enables linking genotypes to phenotypes by detecting functional catalytic components of microbial communities (Wilmes *et al.*, 2015). Multi-omics studies have been readily applied to characterize the diversity and metabolic potential of microbial communities in a wide range of different environments. These include soils (Dini-Andreote *et al.*, 2012; Fierer *et al.*, 2012), wastewater treatment bioreactors (Speth *et al.*, 2016), marine sediments (Plewniak *et al.*, 2013; Urich *et al.*, 2014) and eukaryotic host-associated microbiomes (Erickson *et al.*, 2012; Radax *et al.*, 2012; Sessitsch *et al.*, 2012; Segata *et al.*, 2013; Fuerst, 2014), thereby rapidly increasing our knowledge and understanding of microorganisms and their key roles in biogeochemical cycling processes and eukaryotic host functioning and health. In addition, with metagenomics whole genomes of newly discovered uncultured species can be resolved, allowing to predict the metabolic capabilities of these microorganisms in natural or man-made ecosystems (Tyson *et al.*, 2004; Siegl *et al.*, 2011; Hug *et al.*, 2012; Albertsen *et al.*, 2013; Wilson & Piel, 2013; McLean *et al.*, 2013; Walker *et al.*, 2014; Narihiro *et al.*, 2014; Nielsen *et al.*, 2014; Urich *et al.*, 2014; Afshinnekoo *et al.*, 2015; Brown *et al.*, 2015).

Facing these datasets with reconstructed genomes of hundreds of microbial species and the hypotheses they give rise to, cultivation of microorganisms is more valuable than ever. Cultivation of microorganisms currently is the most reliable way to validate ecological hypotheses raised from multi-omics data. In addition, cultivation is important for the annotation and functional characterization of novel genes (Muller *et al.*, 2013). With available cultures, bacterial metabolism can be studied at the biochemical level, revealing as-yet-unknown physiological traits under varying incubation conditions. Furthermore, multi-species interactions, evolutionary principles, population dynamics and pathogenicity can only be experimentally validated when isolates are available (Fig. 1). Lastly, stable cultures pave the way towards applications in biotechnology, for instance regarding the quest for novel bioactive compound discovery and production, bioremediation and ecosystem engineering. In fact, multi-omics and microbial cultivation studies should be

acknowledged as two sides of the same coin (Leadbetter, 2003; Overmann, 2010). It has been suggested that – in many instances - multi-omics information can provide valuable insights for culturing additional environmental microbes (Allen-Vercoe, 2013; Narihiro & Kamagata, 2013), however, actual examples of attempts to link multi-omics information with cultivation technology have remained scarce.

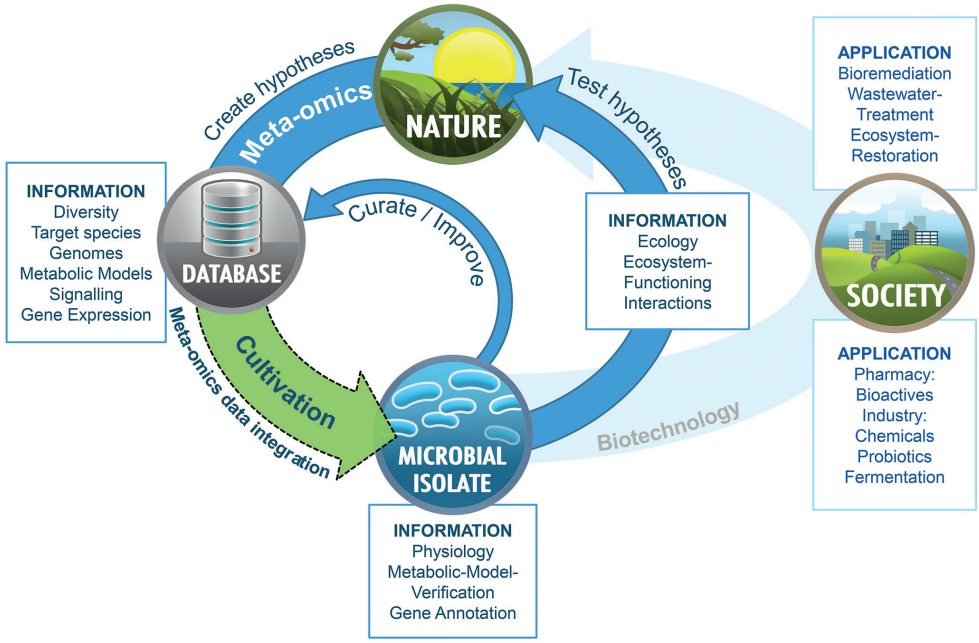

Figure 1 A model depicting the positive feedback loop between multi-omics data generation and isolation of as yet uncultured microorganisms. The rise of multi-omics tools has led to a better understanding of microbial life in nature, the resource for novel biotechnological applications needed by our society. The tedious cultivation of microorganisms often represents the first milestone in novel biotechnological process development and facilitates testing of ecological hypotheses. Multi-omics information, curated by physiological characterization of already available microbial isolates, represents a huge pool of knowledge about the yet-uncultured microbial world. Hence, integrating multi-omics data directed at culturing novel environmental bacteria (arrow in green) brings multi-omics data into context and has the potential to boost biotechnological innovation for the benefit of society and nature conservation.

In this review we discuss the available strategies that allow bridging the gap between current microbial cultivation and multi-omics approaches. In particular, we focus on the progress and pitfalls in the quest to integrate specific multi-omics-derived information with respect to enhancing microbial cultivation success. Secondly, we provide a literature survey, summarizing pioneering multi-omics-based cultivation experiments. Finally, we examine the current developments, extracting the experimental milestones that were achieved and propose a generalized workflow for future multi-omics inspired cultivation approaches.

Information Associated With and Extracted From Multi-Omics Data

Genomes from Metagenomes

Metagenomics provides information about the collective set of genes in a given community. For many purposes, it is required that these gene pools are separated and assigned to particular taxa. Depending on the complexity of the community and the depth of the analyses, sometimes even whole genomes can be assembled (Tyson *et al.*, 2004; Brown *et al.*, 2015). Taxonomy assignment to reads or (assembled) contigs from metagenomes can be done either based on phylogenetic affiliation (Darling *et al.*, 2014) or through a process called binning. Binning can be performed using sequence composition–dependent or –independent methods, or a combination of both (Albertsen *et al.*, 2013). Composition-dependent methods use information such as GC content and tetranucleotide frequency patterns to sort reads within a metagenome into 'bins' containing sequences with similar characteristics (Parks *et al.*, 2011), but these can be limited by local sequence deviations within genomes. On the other hand, composition-independent methods use the assumption that reads/contigs with similar coverage profiles originate from the same microbial population and represent a proxy for its abundance. Generally, combining information on differential coverage profiles with composition-based approaches has been shown to improve binning fidelity (Imelfort *et al.*, 2014).
On the basis of assembled genomes or genome fragments, predictions regarding the ecology, physiology and genetic potential of individual community members are feasible. For example, inferences concerning the metabolic pathways for nitrogen and carbon cycling, respiration mechanisms and the degradation of particular toxic compounds can be based on the relative abundance or even the presence and absence of the relevant genes (Barone *et al.*, 2014; Narihiro *et al.*, 2014). With the constantly improved methods for sequence generation and bioinformatic analysis, near complete genome assembly is slowly becoming a standard method (Hug *et al.*, 2016). This allows insights into the metabolic potential of environmental communities with the potential to unravel the factors preventing cultivation to date, especially in combination with metabolic reconstruction.

Genome-Scale Reconstruction of Metabolic Capacities and Pathways

Predicting metabolic capabilities and other phenotypical features of microorganisms based on genomic data is achieved by means of genome-scale metabolic models. These models are tools that are commonly used for linking genomic data to biochemical reaction networks controlling cellular processes (Bordbar *et al.*, 2014). They can be utilized to understand the relationships between genotype and phenotype and can provide a framework for the integration of transcriptomic, proteomic and metabolomic data (Joyce & Palsson, 2006). Such data integration thus offers an overview of *in silico* predicted cellular physiological and genetic responses to environmental changes in the microbial habitat. The process of genome-scale metabolic model construction has been reviewed extensively (Oberhardt *et al.*, 2009; Santos *et al.*, 2011; Bordbar *et al.*, 2014). Briefly, it requires an initial draft genome-derived metabolic reconstruction based on gene annotation data that is coupled to information on pathways such as found in the KEGG database (Kanehisa *et al.*, 2006) where genes are linked to functional categories. Following this, a model can be proposed that generates predictions about the phenotypes conferred by the analyzed genomes. The models can be conceptual, but their analysis can be taken further using a mathematical representation

of the bio-transformations and metabolic processes encoded within the organism's genome. The latter is typically achieved using constraint-based methods, which impose constraints that consider network stoichiometry, thermodynamics, flux capacity and sometimes transcriptional regulation (Reed, 2012). Multiple tools are available that either aid in the metabolic reconstruction from an assembled genome directly (The SEED) (Henry *et al.*, 2010), or combine the information of multiple existing, manually curated models (Notebaart *et al.*, 2006; Santos *et al.*, 2011). Potentially relevant in the context of improving *in vitro* culturability of microorganisms, Carr & Borenstein, (2012) implemented NetSeed, a modelling tool, which predicts the compounds an organism needs to obtain from its environment. Based on NetSeed data, the Minimal ENvironment TOol (MENTO), predicts minimal nutritional requirements for the microorganisms at stake (Zarecki *et al.*, 2014), making this a potentially useful tool in designing culture media. Currently, genome-scale metabolic models exist almost exclusively for targeted, single species and their respective genomes. However, methods for metabolic reconstruction of complex communities start to appear for well-studied ecosystems like the human gut (Magnúsdóttir *et al.*, 2016), a development that is worth persuing since the input data for such analyses is accumulating rapidly in databases. Metabolic models based on genome - scale reconstructions can be seen as collections of hypotheses, which can be systematically identified, tested and resolved to provide feedback for model refinements (Oberhardt *et al.*, 2009). Therefore, for example, models of interacting species can be used to predict cross-feeding phenotypes, which would require simultaneous cultivation for growth. An interactive and iterative approach, including experiments and further model development, is expected to improve the accuracy of the predictions, in turn allowing to refine media for cultivation of yet uncultured target microorganisms (Fig. 1).

Active Metabolic Functions at Community Level: Metatranscriptomics and Metaproteomics

Metatranscriptomics provides a snapshot of gene expression levels in a community. Application of metatranscriptomics-derived information provides another piece of the puzzle in the quest to establish robust cultivation conditions, as it allows to distinguish - under given environmental conditions - the active and passive community members and their expressed metabolic pathways (Frias-Lopez *et al.*, 2008). It can even point at gene categories that are apparently required for growth and have not (yet) been highlighted in concurrent metagenomics-based studies (Radax *et al.*, 2012). Furthermore, comparison of metatranscriptomes derived from samples subjected to different cultivation or environmental conditions can provide correlations between gene expression and environmental variables (Bomar *et al.*, 2011). Thus metatranscriptomics, and also metaproteomics, can assist us in understanding the abundance and function of the expressed genes and corresponding proteins in microbial habitats of interest (Keller & Hettich, 2009). Responses to certain stress levels, alternating metabolic strategies (e.g. aerobic or anaerobic respiration *vs* fermentation), defense mechanisms like antimicrobial compound production patterns, or metabolite export and uptake can be differentiated. For example, information about active growth versus sole biomass maintenance can be obtained from a given community (Belnap *et al.*, 2010; Erickson *et al.*, 2012). This can provide detailed insights in the metabolic status of microbial communities and their adaptations towards differential conditions, representing valuable information for improving the cultivability of specific community members.

Cooperative and Antagonistic Interactions within Microbial Communities

Within microbial communities, distinct microorganisms often compete for limited resources. However, many processes occur in a cooperative manner since individual species often lack the ability to produce all essential components needed for survival. These needs are met by other microorganisms, enabling such interdependent microbes to live efficiently by clipping down the required number of encoded and expressed genes within individual community members in nutrient-limited environments (Morris *et al.*, 2012). Consequently, microorganisms communicate by trading metabolites and/or signaling molecules. Processes driven by signaling molecules include, e.g., biofilm formation, virulence factor secretion, bioluminescence, antibiotic production and exoenzyme production (West *et al.*, 2007). Bacteria perform these actions aided by a process of cell-to-cell communication, like quorum sensing, where bacteria synchronously control gene expression in response to changes in cell density (Ng & Bassler, 2009).

Typically, intra-species interactions among bacteria include, but are not limited to, signaling molecules such as N-acyl-homoserine lactones (N-AHLs), autoinducer-2 (AI-2) and antimicrobial compounds including peptides (Yim *et al.*, 2006; Bassler, 2009). Through genomic information, it has become apparent that quorum sensing-related mechanisms are widespread in the bacterial world. For instance, the gene responsible for the production of AI-2 (LuxS) is present ubiquitously across the bacterial domain and found in over half of all sequenced bacterial genomes (Federle, 2009; Pereira *et al.*, 2013).

Hence, in cultivation attempts, the absence of metabolites or signaling molecules that are usually provided by other community members can have pronounced effects on the growth of target organisms and thus cultivation success (Bassler, 2009). This will particularly affect host-associated bacteria, which have highly specialized genomes (McCutcheon & Moran, 2012). Such complex relationships are difficult to reproduce in traditional microbial cultivation approaches where cells are physically separated from each other and inter- and intra-species exchange of metabolites and/or signaling compounds is disrupted during the first stage of isolation.

To this end, the aforementioned genome-based model predictions of auxotrophies, i.e. dependencies on external supply of specific compounds, may indicate which metabolic pathways have to be complemented in order to allow for *in vitro* growth of the targeted organism. Moreover, the discovery of phylogenetically and structurally novel signaling molecules, that provide the cues for metabolic activity, is common in microbial multi-omics data (Kimura 2014), potentially leading to success when integrated in cultivation methods (Bruns *et al.*, 2002a; Nichols *et al.*, 2008; Vartoukian *et al.*, 2010; Sipkema *et al.*, 2011).

Antimicrobial compounds play a major role in the environment, not only as defense mechanisms against competing organisms, but also as intra-species signaling molecules (Goh *et al.*, 2002; Yim *et al.*, 2006, 2007; Voolaid *et al.*, 2012). Therefore, antibiotic resistance genes are also widespread; they can be extracted from multi-omics data (Medema & Fischbach, 2015), and have recently been shown to be expressed in a broad range of different natural environments (Versluis *et al.*, 2015). Often, antibiotics show poor activity against oligotrophic and slow-growing organisms (Lewis, 2007), many of which are potential targets for cultivation studies. Thus antibiotics and their production and resistance loci detectable in multi-omics data can be used as selection criteria for

the isolation of target organisms. This may include the prevention of overgrowth by fast-growing microorganisms (Hameş-Kocabaş *et al.*, 2012; Sizova *et al.*, 2012; Rettedal *et al.*, 2014; Keren *et al.*, 2015) or selection for specific phenotypic traits such as those characteristic for Gram-positive bacteria or production of antibiotic-resistant spores.

Habitat Complexity and Current Multi-Omics Based Cultivation Studies

Different habitats require different strategies to obtain meaningful multi-omics data. Here, we categorize microbial habitats based on their complexity in order to interpret available literature data on the use of multi-omics data leading to cultivation successes. The complexity of a microbial habitat may include aspects of species diversity, fluctuations in environmental conditions and interactions among the community members. Temporal and spatial instability of environmental parameters favor the evolution of large genomes in a community (Guieysse & Wuertz, 2012; Bentkowski *et al.*, 2015). The presence of large genomes in a sample makes it more difficult to resolve individual genomes using a given multi-omics strategy and reduces the strength of resulting hypotheses that lay the foundation for cultivation experiments. However, both environmental instability and the intricacy of interactions amongst microbes in an environment have been scarcely addressed in the multi-omics literature. Therefore, we use the parameter species diversity as a proxy for microbial habitat complexity (Fig. 2).

Low-diversity environments are characterized by the predominance of just one or a few microbial species. These microbes may be host-associated or free-living in environments with conditions tolerated by only a handful of species, such as in acid mine drainage biofilms or hot springs. Currently, the majority of the successful –omics information based cultivation examples are from such low-diversity environments, since according multi-omics data can be analyzed more thoroughly with currently available bioinformatic tools. On the contrary, most environments support the growth of a myriad of species resulting in a high microbial diversity, such as in soils, seawater or animal intestines (Torsvik, 2002). In the following section, we elaborate how multi-omics information can be used as a basis for cultivation of targeted microorganisms from an environment with a given complexity, supported with key examples from literature.

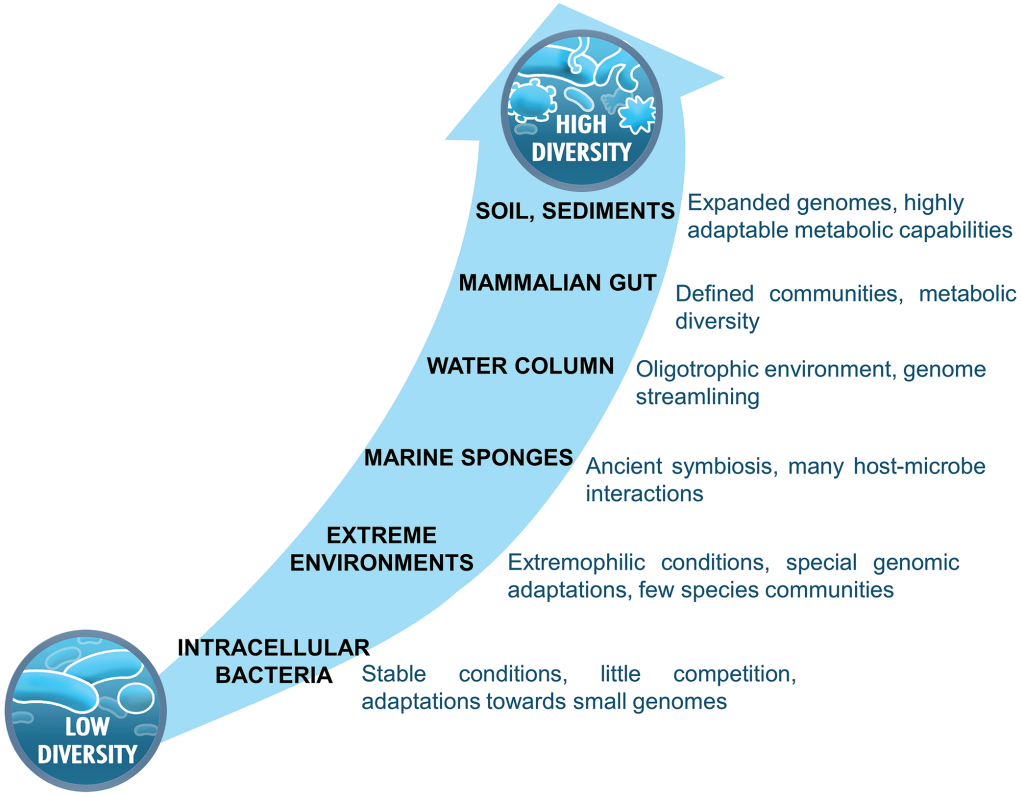

Figure 2 Schematic depiction of selected environments discussed in this review according to the diversity of the residing microbial community.

Low Diversity Microbial Habitats

Host-associated

This category contains examples of both intra- and extracellular host-dependent or host-favored microbes that share their habitat with only few or no other species. From intracellularly occurring microbes, genomic information can be readily obtained after purifying the microbes from host cell material by e.g. cell disruption and differential centrifugation as was done for the Q (query) fever-causing obligate intracellular pathogen *Coxiella burnetti* (Cockrell *et al.*, 2008). In general, evolution favors the reduction of genome sizes, and hence these microbes often have only a limited set of specialized metabolic pathways that support the host associations (Dutta & Paul, 2012). Media and cultivation conditions have to be carefully adapted to the microbes' demands, and multi-omics data can be instrumental in identifying nutritional requirements.

An early example of genome-inspired medium design was the development of a defined medium for *Xylella fastidiosa*, a slow-growing plant pathogen inhabiting the xylem of citrus plants (Lemos *et al.*, 2003; Almeida *et al.*, 2004; Janse & Obradovic, 2010). Even though the organism had

been isolated with empirical methods before (Wells *et al.*, 1987), and its genome was sequenced in 2000 (Simpson *et al.*, 2000), Lemos and colleagues (2003) revealed that a relatively simple medium composition supported growth. They prepared five minimal media that differed in amino acid composition and concentration based on the presence or absence of genes for amino acid biosynthetic pathways in the *X. fastidiosa* genome. Some enzymes required for the biosynthesis of serine, cysteine and methionine were missing, and the addition of these amino acids resulted in faster growth of *X. fastidiosa*. Furthermore, myo-inositol, a specific precursor of plant cell wall polysaccharides, was added since it was hypothesized that *X. fastidiosa* metabolizes this compound based on the presence of the enzyme inositol monophosphatase. However, not all metabolic reactions the organism was performing were predicted from the genome. For example, despite a predicted serine auxotrophy, growth occurred in serine-free media. Potential reasons for this incongruence include incorrect gene annotation, multifunctional enzymes, unknown metabolic pathways or enzymes encoded by analogous genes (Lemos *et al.*, 2003). This example shows how a variety of genome-tailored minimal medium designs can result in improved growth rates, but advocates at the same time that currently not all metabolic capabilities of the target organisms can be predicted correctly from genomic data.

Another example is provided by the first axenic culture of *Tropheryma whipplei*, the causative agent of Whipple's disease. This obligate intracellular pathogen had resisted axenic cultivation for almost a century and was growing only in association with eukaryotic cells until its 0.9 Mb genome, obtained by differential centrifugation, was published in 2003 (Bentley *et al.*, 2003). Analyzing metabolic models based on the genome revealed the (partial or complete) absence of biosynthetic pathways for 16 amino acids, which suggested that *T. whipplei* obtains these from its host. As a follow-up, Renesto and colleagues (2003) designed a medium providing the 16 amino acids, inoculated it with supernatant of *T. whipplei*-infected fibroblasts and established an axenic culture of *T. whipplei*.

The Q (query) fever-causing obligate intracellular pathogen *Coxiella burnetti* was recently liberated from its host into axenic culture (Omsland *et al.*, 2009). In this case, information derived from multiple sources was used to optimize the medium that supported growth: Replicating niche characteristics (low pH, salt concentrations) and incorporating the genome-predicted metabolic capacities (amino acid uptake and metabolism) resulted in the formulation of the initial growth medium. A subsequent comparison of the transcriptomes from *C. burnetti* incubated in suboptimal medium and *C. burnetti* growing in Vero cells suggested a deficiency in amino acids for the bacteria growing in the designed medium. The addition of casamino acids and L-cysteine to the initial medium yielded an approximately 13-fold increase in protein synthesis, but not substantial growth. Further genome inspections revealed that terminal oxidases associated with both aerobic and microaerobic respiration was encoded, suggesting that *C. burnetti* could respire in low-oxygen environments. Incubations under different oxygen tensions showed an increased ability of *C. burnetti* to oxidize essential substrates under microaerophilic conditions. Finally, *C. burnetti* was incubated in amino acid-supplemented growth medium under 2.5% oxygen tension conditions, where 3 logs of growth were observed after 6 days of incubation. Thus, fine tuning growth conditions concurrently with designing media based on predicted metabolic capabilities led to the successful axenic cultivation of *C. burnetti*.

Bomar and colleagues (2011) analyzed the metatranscriptomes of two extracellular bacterial symbionts that make up the entire gut microbiota of the leech *Hirudo verbana*. The high expression levels of genes encoding mucin (and glycan) degrading enzymes suggested that mucins constitute the main carbon and energy source for the one as-yet-uncultured *Rikenella*-like leech symbiont. Replacement of glucose by mucin in the culture medium resulted in an isolate that was identical to the target bacterium from the leech gut. Hence, by identifying genes that are highly expressed in their original environment, key physiological properties of the target organisms can be predicted and used in targeted isolation approaches.

Extreme Environments

Slightly more complex, but still considered low-diversity environments are habitats that are characterized by extreme conditions. Hot springs, acid mine drainage biofilms or hypersaline lakes allow only a few highly adapted species to thrive. Such extremophilic organisms harbor a high potential for industrial processes and compounds. Furthermore, fundamental research interests in evolution, abiotic to biotic element cycling and possibilities for extraterrestrial life have made such extreme habitats popular study sites for decades. The relatively simple communities have led to many ground-breaking results, also in the field of molecular microbial ecology.

Tyson and colleagues (2005) established one of the first breakthroughs in a metagenome-derived cultivation approach by reconstructing the genome of an as-yet-uncultured member of the Nitrospirae from a metagenome of an acid mine drainage biofilm. The reconstructed genome revealed a single nitrogen fixation operon. Based on this information, the authors developed a nitrogen-free medium. The cultivation conditions were further set up to match prevailing environmental conditions, i.e. high metal concentrations, pH 0.8 and 37°C. They successfully obtained axenic cultures of a *Leptospirillum* group III member by means of repeated sequential batch dilution series. This iron-oxidizing Nitrospirae isolate was described as *Leptospirillum ferrodiazotrophum* sp. nov. (Tyson *et al.* 2005). In a follow-up study, the entire acid mine drainage biofilm was targeted, including its complete metabolic functions (Belnap *et al.*, 2010). Hence, a cultivation system was designed that maintained and propagated the biofilm *in vitro*. Metabolic labeling-based proteomic analysis after 12 days of growth confirmed also the presence and activity of low-abundance community members. Additionally, autotrophic primary production and stress responses were monitored. Modifying the cultivation conditions led to enhanced growth and decreased the abundance of stress response proteins as monitored by metaproteomics (Belnap *et al.*, 2010). Thus metaproteomics data as monitoring tool enabled the customization of cultivation conditions towards the metabolic demands of the targeted communities. To our knowledge, this is the only example of the use of metaproteomics for improving culturability, which might be due to the inherent complexity of community metaproteomes and the current analytical limitations of this technology (Wilmes *et al.*, 2015).

Nanoarchaeota have first been described as obligate extracellular symbionts of Crenarchaeota from submarine hydrothermal vents (Huber *et al.*, 2002). Using specific primers, Nanoarchaeota have since then been detected in many environments including terrestrial hot springs and hypersaline lakes with culture-independent methods (Casanueva *et al.*, 2008). Single-cell sorting and whole genome amplification of antibody-labeled archaea from the Obsidian Pool in Yellow Stone National park revealed 16S rRNA gene sequences of a novel nanoarchaeal organism as well as sequences

from its putative crenarchaeal host, an uncultured member of the Sulfolobales (Podar *et al.*, 2013). Both nanoarchaeal and crenarchaeal whole genome amplification products were assembled into near-complete genomes and their metabolic capacities were reconstructed. The nanoarchaeon's fragmented, extremely small genome lacked many essential biosynthetic pathways which indicated that the nanoarchaeon cannot live autonomously and hence depends on the presence of a crenarchaeal associate. Potential glycolysis and gluconeogenesis pathways, however, are retained in the nanoarchaeon, suggesting the use of peptides or complex sugars as energy source (Podar *et al.*, 2013; Wurch *et al.*, 2016). On this basis, Wurch *et al.* (2016) established enrichment cultures containing yeast extract, casamino acids and sucrose or glycogen in anoxic Brock medium with low pH and 80-85°C and obtained stable communities with increasing relative abundance of nanoarchaeota, as monitored by qPCR. After two rounds of dilution to extinction, they transferred a single crenarchaeal cell carrying a nanoarchaeon into liquid medium using optical tweezers, thereby obtaining a pure co-culture of the crenarchaeon host (*Acidilobus* sp. 7A) and its associated nanoarchaeon (strain N7A). This first isolated geothermal nanoarchaeon, proposed as "*Candidatus* Nanopusillus acidilobi" represents the smallest cultured organism to date (100-300 nm cell size), and available cultures now allow experimentation to reveal its particular metabolism and adaptation features. Many more ultra-small bacteria and archaea with similar ectosymbiotic or ectoparasitic lifestyles might await discovery and identification of their hosts, something that - apart from single cell genomics - is only feasible by direct cultivation (Delafont *et al.*, 2015; He *et al.*, 2015; Wurch *et al.*, 2016).

We conclude that genomic information of microbes derived from low diversity environments can often be obtained relatively easily and that individual genome reconstructions are often feasible (Fig. 3). Analyzing metabolic networks for the presence or absence of essential biosynthetic pathways, presence/absence of specific catabolic pathways and uptake systems can reveal which pathways have to be complemented by the culture medium, as well as help to choose specific carbon sources, electron donors and electron acceptors. Genome-inspired medium design in combination with fine tuning cultivation conditions can assist in isolating as-yet-uncultivated, even host-associated microorganisms with reduced genomes. In cases where genomic information is inconclusive, metatranscriptomics or metaproteomics can be applied, indicating which bacteria are active in selected conditions. This will reveal the active metabolic functions and hint towards substrates that are preferably catabolized by the organisms under study.

High Diversity Microbial Habitats

This category contains examples of microorganisms sharing their habitat with a large variety of co-occurring species. Hence, interpreting multi-omics data represents a true challenge. Monitoring the efficiency of cultivation media through molecular and multi-omics methods for the growth of targeted species or specific microbial consortia is a common denominator for the examples in this category.

Host-associated High Diversity Habitats

Tian and colleagues (2010) used DGGE (denaturing gradient gel electrophoresis, a molecular community diversity estimation method) of PCR-amplified 16S rRNA gene fragments to compare cultivated communities to microbial profiles of the human oral cavity. They developed a cultivation

medium that propagated a diverse community, which resembled the composition of the oral community the most. This *in vitro* grown community also contained phylotypes of the -until then- uncultivated candidate phylum TM7 (He *et al.*, 2015). Some of the phylotypes contained a mutation in their 16S rRNA gene sequence that had been previously linked to streptomycin resistance (Hugenholtz *et al.*, 2001). Indeed, addition of streptomycin to the culture medium led to an enrichment of a specific TM7 phylotype (named TM7x), which was physically associated with a previously uncultured Actinomyces species. This stable co-culture enabled the complete sequencing of TM7's highly reduced genome, providing insights into the growth conditions and lifestyle of this human-associated epibiotic organism.

In addition to oral microbial communities, gut ecosystems provide a broad range of metabolic niches that are inhabited by diverse microbial communities (Vieira-Silva & Rocha, 2010; Fodor *et al.*, 2012). Especially the human intestinal tract has been studied intensively, and many species have been cultured (Lagier *et al.*, 2012; Rajilić-Stojanović & de Vos, 2014). The challenges of assembling genomes from such high-diversity environments are currently being overcome (Nielsen *et al.*, 2014; White *et al.*, 2016), allowing metabolic networks to be established for target species as well as for whole communities (Abubucker *et al.*, 2012; Magnúsdóttir *et al.*, 2016). Based on metabolic network information of target species, enrichment strategies can be designed that exclusively support the metabolism of the selected bacteria.

For example, nucleotide composition-based sequence binning enabled Pope and colleagues (2011) to assemble approximately 2 Mb (~90%) of the genome of an as-yet-uncultured member of the Succinivibrionaceae from a wallaby foregut metagenome that comprised sequences of more than 500 different species. The assembled genome was used to partially reconstruct the metabolic pathways of the bacterium as well as to search for putative antibiotic resistances. This predicted the utilization of starch as a sole carbon source and urea as a non-protein nitrogen source. A defined medium containing starch, urea and bacitracin was then developed, which led to highly-enriched and (later) axenic cultures of the targeted phylotype. Further physiological characterization was consistent with the genome-based predictions, confirming that this bacterium is dependent on CO_2 to support its succinate biosynthesis and produces succinate as major fermentation end product, further explaining the basis of the low methane emissions from herbivorous marsupials.

The inclusion of selection criteria such as antibiotics or other bactericidal compounds in the isolation strategy can increase the success of isolation manifold and select for specific phenotypic traits such as sporulation. Following up on the metagenomics-derived observation that many members of the human intestinal microbiota unexpectedly possess extensive genomic sporulation capacity, a pre-treatment using ethanol enriched for spores from the fecal samples, and enabled the subsequent isolation of 45 novel candidate species (Browne *et al.*, 2016). Overall, their isolates represented approximately 90% of the overall relative abundance at the species level in the individuals from which they were obtained and revealed novel insights into the transmission mode of human-associated strict anaerobes (Browne *et al.* 2016).

Free-Living, Highly Complex Communities

Highly diverse environments, such as water columns or soils and sediments pose particular challenges for current multi-omics approaches for multiple reasons. Firstly, such environments support the growth of microbial species with expanded genomic repertoires allowing them to adjust to oligotrophy and varying abiotic conditions (Konstantinidis & Tiedje, 2005). Secondly, these environments are exposed to fluctuations in temperature, light, water content or salinity in daily or seasonal cycles, creating niches for a myriad of closely related species, making it difficult to separate genomes at strain or species level. As a consequence, the amount of sequencing needed to cover genomes substantially is astronomical and existing computational power needed to resolve such complex datasets exceeds current capacities (White *et al.*, 2016).

Based on rRNA gene cloning, the SAR11 clade was found to be the most ubiquitous bacterium in ocean waters, yet recalcitrant to isolation. Hence, Rappé and colleagues (2002) employed a high-throughput dilution-to-extinction method to natural marine communities and inoculated a series of low-nutrient media with around 20 cells per well in microtiter plates. After 23 days of incubation, they obtained axenic cultures of eleven SAR11 strains, including the proposed "*Candidatus* Pelagibacter ubique", allowing for *in vitro* studies with an organism of global biogeochemical significance. Further applications of this cultivation method led to the successful propagation of up to 14% of the cells of coastal waters (Connon & Givannoni, 2002). This empirical approach constituted a milestone in our quest to isolate the abundant bacteria in a given habitat. The analysis of two "*Candidatus* Pelagibacter ubique" genomes revealed an incomplete set of genes for assimilatory sulfate reduction, suggesting that the organism requires reduced sulfur compounds (e.g. methionine) for growth (Tripp *et al.*, 2008). Furthermore, a fragmented glycolysis pathway and the absence of glycine and serine biosynthesis pathways suggested a metabolic dependency on low-molecular-weight organic acids as carbon sources. This information was used for the design of a defined medium, and it was shown that the addition of glycine and pyruvate as well as inorganic micro- and macronutrients and vitamins were required for robust growth of SAR11 isolates (Carini *et al.*, 2012).

To our knowledge, multi-omics information-assisted cultivation approaches from highly diverse habitats such as soils and sediments remain scarce to date. The current pitfalls of multi-omics data generation and analysis are especially noticeable when it comes to multi-omics guided microbial isolation from such highly complex environments where extremely intricate community member interactions are expected (Traxler & Kolter, 2015). However, recent breakthroughs are promising, and with the constantly improving methods for sequence generation and bioinformatics analysis, reasonably complete genome reconstruction is slowly becoming a standard method (Hug *et al.*, 2016).

Pitfalls to Current Multi-Omics Methods and Ways around the Limitations

First, one classical problem of metagenomics and metatranscriptomics based gene targeting is the difficulty of assigning observed functions to specific taxa (Dutilh *et al.*, 2007). However, recent increases in obtainable sequence read length and assembled fragments have resulted in major improvements. Besides, computational developments are paving the way to make better use of currently available short reads. One recently developed pre-assembly method, coined latent strain analysis (LSA) (Brian Cleary *et al.*, 2015), separates the reads into 'biologically informed' partitions, enabling the assembly of individual genomes from metagenomes. This is promising, since a large number of samples from high-complexity environments could enable a resolution high enough to assemble genomes of bacterial taxa present even at low abundances.

Second, a direct translation of genomics-based data to cultivation conditions and cultivation media is still difficult. For example, Lavy and colleagues (2014) designed a medium based on genomic data of "*Candidatus* Poribacteria sp. WGA-4E" obtained through single-cell genomics of cells retrieved from the Red Sea sponge *Theonella swinhoei* (Siegl *et al.*, 2011) However, the bacterium could not be brought into culture. Challenging in such endeavors is to decide on the concentrations of medium components and the combination thereof, since the (bio-) chemical composition of natural environments is often unknown despite carefully collected metadata. High concentrations of substrates can be toxic or inhibiting for environmental bacteria derived from nutrient-limited environments (Connon & Givannoni, 2002), or favour less-abundant, fast-growing organisms. Two different issues can be identified here: one is the isolation of community members, and another one deciphering optimal growth conditions. For the former, the concentration ranges of medium components are likely quite large, and inspiration from media that work for related microbes can be used as a proxy, although also potential competition by other, faster growing community members has to be taken into account. For the latter, further refinement of the media by factorial trial and error may be required. In fact, supplying cultivation media with ingredients that are not predicted as required according to an organism's biosynthetic pathways can still be growth promoting (Lemos *et al.*, 2003).

Third, information about the active contribution of community members to the overall nutrient cycling in an ecosystem is less frequently available as compared to their genomic potential. Bacteria can exist in a metabolically inactive dormant state, especially in nutrient-scarce environments that are subjected to regular disturbances, such as influx of toxic compounds (Epstein, 2009; Buerger *et al.*, 2012). This is gradually overcome by comparing gene expression or protein data with (meta) genomic data, which may reveal discrepancies between the most abundant and the most active organisms in a community.

Lastly, functional traits and metabolic pathways are inferred from the annotated portion of the metagenome. This fraction is a proportion of the total due to a variety of factors. First, metagenomic library construction relies on the accuracy of DNA extraction methods, which are prone to problems, such as incomplete cell extraction, cell lysis or DNA degradation (Wesolowska-Andersen *et al.*, 2014). In addition, depending on the microbial diversity of the environment, approximately 30 to 50% of the genes found are of undetermined function altogether (Ellrott *et al.*, 2010). Finally, many annotations present in databases are not accurate (Schnoes *et al.*, 2009). Microbial cultivation

itself is one way that can positively impact experimental validation of gene annotations, through an 'ecologically-validated' positive feedback loop. As a consequence, predictions of metabolic capabilities from multi-omics data can be expected to further improve. This in turn has the potential to bring a larger number of uncultured species into cultivation (Fig. 1).

In order to avoid some of the pitfalls mentioned above, we want to stress the importance of collecting bio- and physicochemical metadata at an appropriate temporal and spatial resolution in order to link multi-omics data to environmental cues. Microorganisms inhabit microenvironments strongly influenced by the structure of the environment, and they respond to conditions and resources at scales ranging from micrometers to a few meters (Franklin & Mills, 2003; Cardon & Gage, 2006). In the case of metagenomics and metatranscriptomics data, the standard of minimum information about any sequence is called MIMS (Minimum Information about a Metagenome Sequence) (Yilmaz *et al.*, 2011), developed by the Genomics Standards Consortium (GSC, http://gensc.org). The metadata that describes the sampled environment usually includes collection date, specification of the environment (biome) and the location where samples were collected. We here advocate that additional parameters such as temperature, pH, oxygen concentration and biochemical data on nutrient or salt concentrations should be included as much as possible as they provide important environmental descriptors that can assist in interpreting multi-omics results and setting the appropriate cultivation conditions.

Envisioned Strategies for Omics-Aided Cultivation Approaches

Even though each microbial habitat requires tailored study designs and challenges the creativity and inventiveness of individual researchers, we propose a more generic workflow based on the examples summarized in this review (Table 1) as a guidance for future multi-omics based cultivation experiments.

This workflow starts with sampling the environment of interest and collecting metadata at appropriate temporal and spatial resolution in order to link multi-omics data to environmental cues (Fig. 3). Estimating the species diversity of a given sample based on 16S rRNA gene analysis, even though not a multi-omics approach *sensu strictu*, is helpful to determine, which of the complementary techniques of single-cell genomics and metagenomics to follow. For low diversity samples, metagenomics might enable the recovery of complete genomes of dominant species due to high copy numbers of those genomes in the samples. Applying metagenomics to high-diversity habitats may require a pre-treatment step such as cell size or cell density sorting, resulting in an enrichment of the microorganisms of interest. At the same time, the pre-treated samples can be used as pre-enriched inocula for cultivation. In addition, sequencing a large number of samples might also improve the odds for full genome recovery, given that availability of metagenomes from samples with different relative abundances aid in binning and genome reconstruction. Single cell genomics is an alternative option to reconstruct draft genomes of the (target) microorganisms from highly complex microbial habitats.

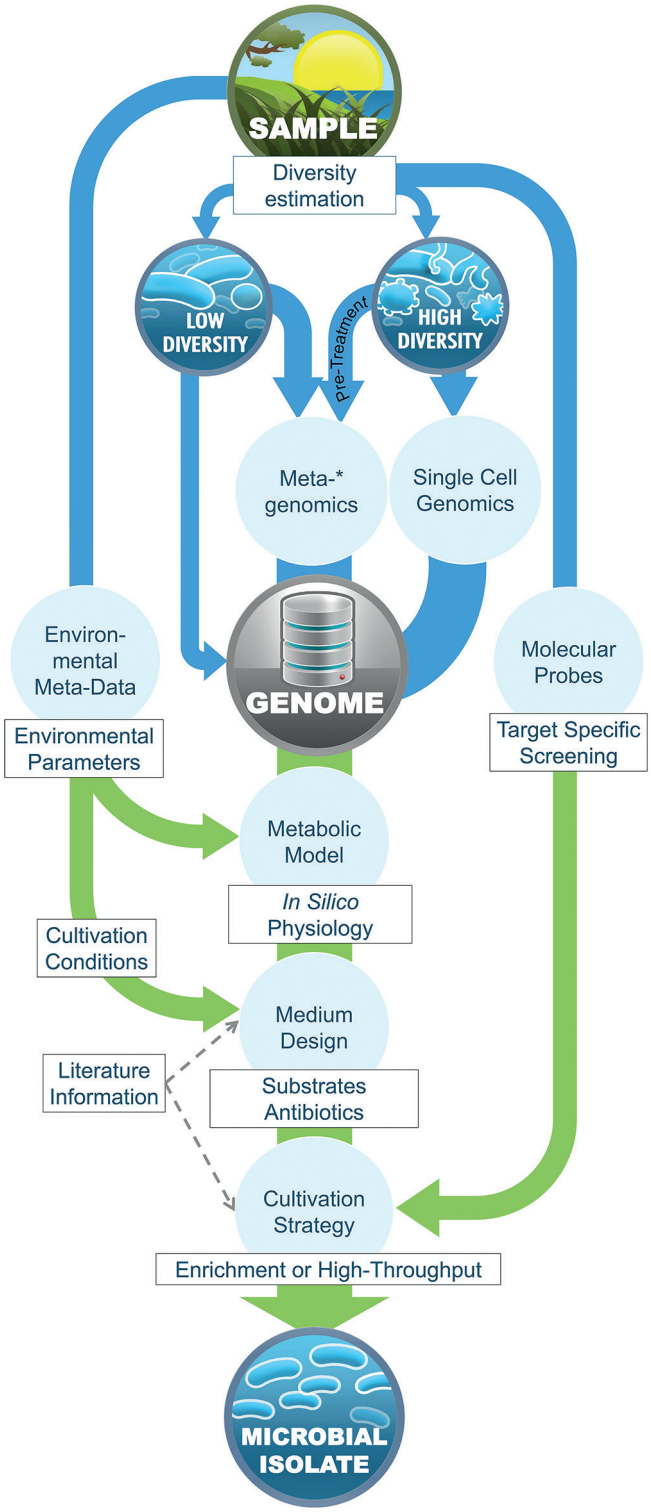

Figure 3
Proposed workflow for integrating multi-omics data into microbial cultivation. Arrows indicate the flow of information, blue for multi-omics strategies and green for microbial cultivation (in correspondence to Figure 1). *The sampling strategy prior to metagenomic or single cell genomics differs for low and high diversity environments, the latter requiring a larger number of samples for enhanced genome recovery.

The recovered genomes constitute a firm basis for the construction of metabolic models, however, the natural environment of the target organism and the quality of the draft genomes should be kept in consideration when examining resulting metabolic models, i.e. mind the gaps! Metabolic models illustrate the targets' genomic signatures of aerobic or anaerobic respiration, fermentation pathways, possible electron donor or acceptor molecules, carbohydrate metabolism and biosynthetic pathway deficiencies, which constitute useful information for medium design. Media formulations that were used to isolate phylogenetically related organisms represent a valuable source of inspiration for the compositional details of trace metal, salts and vitamin concentrations. We propose to aim for selective media compositions for initial isolation of the target. Identifying genetic traits that distinguish the target from other organisms such as antibiotic resistance markers or auxotrophies can represent valuable selection criteria, inhibiting undesired, fast growing organisms from the sampled community. Designing optimal growth media should only be considered after initial diversity reduction or isolation of the target since optimal growing conditions can be similar for many untargeted microbes from the environment, which may overgrow the organism of interest.

To summarize, the predicted metabolic substrates and the resulting products, in conjunction with the environmental metadata, can be translated into medium designs that can be subjected to the currently available multitude of novel cultivation strategies such as microfluidics, cultivation chips, manipulation of single cells and high throughput cultivation, mentioned in the introduction. Lastly, 16S rRNA gene-based techniques such as fluorescent *in situ* hybridization (FISH) or qPCR screening enable tracking and quantification of the target organisms throughout the process of sampling and subsequent enrichment, cultivation and recovery. We surmise that, on the basis of such highly rational cultivation approaches (Fig. 3), a plethora of novel target species will be brought into culture.

Conclusion: The Era of Multi-Omics-Based Microbial Cultivation

In this decade, the huge increase in sequence data from genomes, metagenomes, metatranscriptomes and metaproteomes continues to unveil the enormous variety of as-yet-unexplored metabolic potential in nature. The massive amount of publicly available multi-omics data sets transits many environmental bacteria from the unknown-unknown to the known-unknown search space. Now, the decadal challenge is to further scrutinize such data sets and use them to serve our ecological and exploratory questions about members of microbiomes and their roles in the natural habitats we are studying. But, multi-omics methods have much more potential than just serving as explanatory tools. They provide hypotheses that await testing using advanced cultivation technologies to bring a range of previously recalcitrant extant microbes into cultivation. From targeted isolation (Huber *et al.*, 1995; Davis *et al.*, 2014) via multi-omics inspired medium development (Bomar *et al.*, 2011; Pope *et al.*, 2011) to high- throughput screening of a myriad of colonies or enriched liquid cultures (Lagier *et al.*, 2012; Ma *et al.*, 2014), the possibilities for data integration are plentiful. Given the fact that bacterial cultivation is time-consuming and tedious, the additional sources of information derived from high-end molecular tools provide highly practical advantages that may lead to important breakthroughs and should not be ignored. The integration of multi-omics knowledge in cultivation studies increases the chances of success and decreases the search space in the quest for new microbial isolates.

Table 1. Summary of existing literature on the use of multi-omics for isolating as-yet uncultured microorganisms.

Organism (description)	Multi-omics strategy	Multi-omics derived information	Cultivation medium development
			Low-Diversity Environmental Habitats
Xylella fastidiosa (Extracellular pathogen)	Empirical isolation, genome sequence	(Partial) Absence of amino acid biosynthesis pathways	5 different minimal media, growth measurements
Tropheryma whipplei (Intracellular pathogen)	Genome sequencing, Metabolic models	(Partial) Absence of 9 amino acid biosynthesis pathways, absence of tricarboxylic acid cycle	Cell-culture medium supplemented with FCS, glutamine and human non-essential amino acids
Coxiella burnetii (Intracellular pathogen)	Genome sequencing, Metabolic models	Expression microarrays, genomic reconstruction, and metabolite typing	Replicating niche characteristics (low pH, salts)
Rikenella-like bacterium (extracellular symbiont)	Metatranscriptomics on leech gut content when organism was proliferating rapidly	Mucin and glycans as main carbon and energy source	Replacing glucose by mucin
Leptospirillum ferrodiazotrophum (acid mine drainage biofilm)	Metagenome assembly and genome reconstruction	Single nitrogen fixation (nif) operon belonging to one uncultured community member	Nitrogen free medium replicating environmental conditions
Entire acid mine drainage biofilm	Metabolic labeling-based quantitative proteomic analysis: autotrophic primary production and stress response monitoring	Stress response and oxidative damage repair protein expression, heat shock and osmotic shock protein expression (Hsp20), growth rate and primary production measurements	Extreme environment imitation, modifications of cultivation medium to even more natural conditions
Nanopusillus acidilobi (Nanoarchaeon)	Single-cell sorting of antibody-labeled cells and whole genome amplification	Physiological host-dependency, peptide and complex sugar degradation pathways	Replicating niche conditions and addition of casamino acids, sucrose and glycogen
			High-Diversity Environmental Habitats
Candidate Phylum TM7 Phylotype (human oral microbiome)	SNP in 16S rRNA sequence	Streptomycin resistance	Addition of Streptomycin to enrichment culture
Succinivibrionaceae clade (Wallaby foregut microbiome)	Genome assembled from metagenome	Utilization of starch as sole C source, Urea as non-protein N source, antibiotic resistance	Defined minimal medium, semi-continuous batch culture enrichments
45 novel candidate species	Identifying genomic signatures from publicly available metagenomes	highly conserved sporulation and germination pathways	Ethanol pretreatment of samples
SAR11 strains	Dilution to Extinction	Natural Sea water medium	High abundance in sea water
Pelagibacter ubique	Genome sequencing, Metabolic models	Fragmented glycine and serine biosynthesis pathways	Multiple growth media, optimization for high OD

Key Medium Component(s)	Problems and bottlenecks	Reference
Low-Diversity Environmental Habitats		
Amino acids, myoinositol, vitamins	Not all biosynthetic pathways visible in genome	(Lemos et al. 2003)
Amino acids (histidine, tryptophan, leucine, arginine, proline, lysine, methionine, cysteine, asparagine, glutamine)	Distinct morphology and restriction profiles in axenic culture	(Renesto et al. 2003)
Microaerobic conditions 2.5% oxygen), casamino acids and L-cysteine, high chloride, citrate buffer (pH ~ 4.75), 3 complex nutrient sources (neopeptone, FBS, and RPMI cell culture medium)	None mentioned	(Omsland et al. 2009)
Mucin	Initial goal of study was not microbial isolation.	(Bomar et al. 2z011)
Nitrogen free, low pH, metal rich, 37°C, nitrogen fixing chemolithoautotrophs respiring ferrous iron	Plating impossible due to low pH. Multiple replicates needed to ensure isolate purity	(Tyson et al. 2005)
40°C, pH 1, higher medium recharge rate, lower N and P salt concentrations	Interpretation of protein abundance changes caused by media modification difficult for rare organisms	(Belnap et al. 2010)
Optical tweezer isolation and co-cultivation with its host archaeon	Obligate host-dependency	(Wurch et al. 2016)
High-Diversity Environmental Habitats		
Streptomycin	Epibiotic parasitic organism, physically associated to *Actinomyces* host species	(He et al. 2015)
Starch, urea and the antibiotic bacitracin	CO_2 concentration dependent growth	(Pope et al. 2011)
Pre-selection of ethanol resistant spores	None mentioned	(Browne et al. 2016)
Oligotrophic media, extended cultivation time	Natural niche conditions	(Rappé et al. 2002)
Micro- and macronutrients, glycine and pyruvate	Fine tuning of micronutrient media composition	(Carini et al. 2012)

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We thank Toni Clariana and A. A. Kampfraath for help and critical comments on designing and drawing.

Chapter 6

General Discussion

The Importance of Sponges for Marine Biotechnology

The ongoing antibiotic resistance crisis lead to the global re-emerging threat of infectious disease caused by multi-drug-resistant pathogens (Aslam *et al.*, 2018). Furthermore, numerous other diseases such as Alzheimer's, cancer or numerous viral infections still await the discovery of successful treatments. This challenges policy makers, pharmaceutical industries and academic researchers alike to find novel therapies and medicines. In recent years, the focus has shifted to marine environments in the quest to discover novel drug candidates. Marine environments, despite their large contribution to the global biosphere, are currently still vastly understudied since the underwater technology to explore the sea has only been developed recently. Thus increasing our understanding of marine environments has become a focus for policies of the European Union, out of biotechnological as well as fundamental research interest (European Commission, 2017). The EU supports the topic "Blue Growth" by funding several FP7 and H2020 projects including MaCuMBA, www.macumbaproject.eu; PharmaSea, www.pharma-sea.eu; MicroB3, www.microb3.eu; Bluegenics, http://www.bluegenics.eu, MarPipe, http://www.marpipe.eu; and BluePharmTrain https://www.bluepharmtrain.eu (this thesis). These projects are aimed at overcoming bottlenecks in the marine biodiscovery pipeline and advance environmentally friendly drug discovery and development approaches.

The results are astonishing: For example, since the establishment of marine natural product discovery in the 1960s, over 15 000 bioactive compounds have been isolated from marine invertebrates, of which more than 30% have been derived from marine sponges (Brinkmann *et al.*, 2017).
Apart from the large biotechnological potential exhibited by highly potent bioactive compounds detected in sponges, these animals and their associated microbes are interesting from several fundamental aspects: First of all, sponges are the oldest, still existing metazoans on Earth, and represent the last common ancestor of all animals, including humans. Despite their simplistic body plan and lack of differentiated tissue, sponges select and maintain a highly species-specific microbial community that is relevant to the integrity of the sponge as an animal (Vacelet, 1975; Wilkinson, 1978a, 1978b, 2006; Hentschel *et al.*, 2012; Thomas *et al.*, 2016). Thus, the characterization of the sponge holobiont can provide a baseline for an increased understanding of animal-microbe interactions and provide clues about the origin and maintenance of symbiosis between metazoans and prokaryotes.

Thus, the aims of this thesis were to study sponge-associated microorganisms with focus on both biotechnological interests, such as the cultivation of potentially bioactives-producing bacteria or the discovery of novel biotechnologically interesting enzymes (**Chapter 2** and **4**), as well as from a fundamental, ecological perspective such as investigating global diversity patterns of specific sponge-associated bacteria (**Chapter 3**). Finally, defining methods to translate the findings of multi-omics research into strategies for isolating and growing novel microorganisms under defined conditions was the focus of **Chapter 5**, in an attempt to link fundamental research to biotechnological applications.

In **this chapter** I would like to address several points, for which the research described in this thesis worked towards resolving. Without claiming to provide all the answers, I anticipate to summarize the current state of knowledge and provide directions for future research endeavors.

Assessing Sponge-Associated Microbiomes

The bacterial communities of sponges have been under extensive scrutiny over the recent years, and a consensus about the dominant microbial taxa inhabiting numerous species has emerged. A total of 41 microbial phyla (including candidate phyla) have been reported across 81 globally distributed sponge species, and their community structure varies greatly from the surrounding seawater (Thomas *et al.* 2016 and Figure 1). Within this thesis, a particular focus was paid to *Aplysina aerophoba*, due to its fairly easy accessibility in the Mediterranean and its emergence as a model species in the field of sponge microbiology. We found its microbiome composed of 18 classified bacterial phyla when amplifying the V1-V3 region of microbial 16S rRNA genes (27f-338r primers) and subsequent 454 pyrosequencing (**Chapter 2**). By amplifying the V4 region of the 16S rRNA genes (EMP primers) and subsequent Illumina MiSeq sequencing, we detected Thaumarchaeota and 21 classified bacterial phyla (**Chapter 4**). Of these, the most abundant phyla and their contribution to the overall community composition is shown in Figure 1.

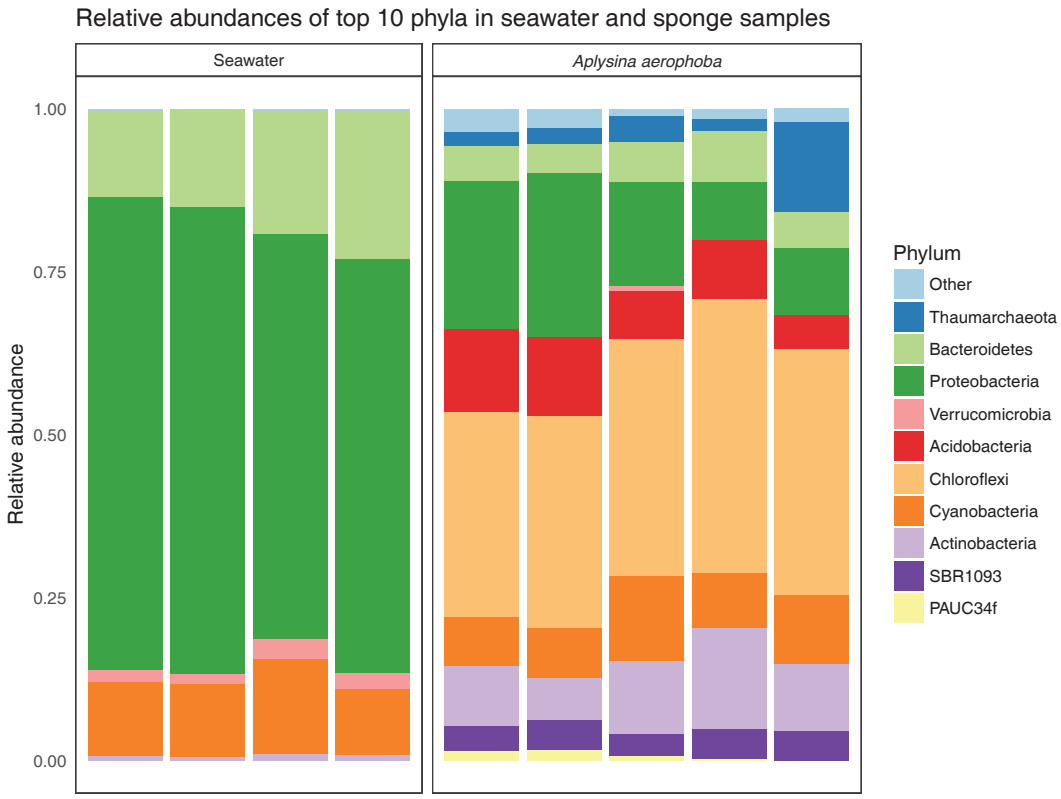


Figure 1 Relative abundance of the top 10 most abundant microbial phyla from seawater and *Aplysina aerophoba* individuals analyzed in **Chapter 4**.

Despite methodological differences, the results from different approaches characterizing 16S rRNA gene profiles are highly reproducible, yielding detection differences only in low-abundance phyla such as Fusobacteria, Parcubacteria, PAUC34f, Hydrogenedentes and Saccharibacteria (Figure 2), some of which are part of the recently described candidate phyla radiation (Brown *et al.*, 2015). These results are in accordance with other 16S rRNA gene based investigations, indicating that these methods are well established for molecular profiling of sponge microbiomes (Thomas *et al.*, 2016; Chaib De Mares *et al.*, 2017; Steinert *et al.*, 2017; Dat *et al.*, 2018).

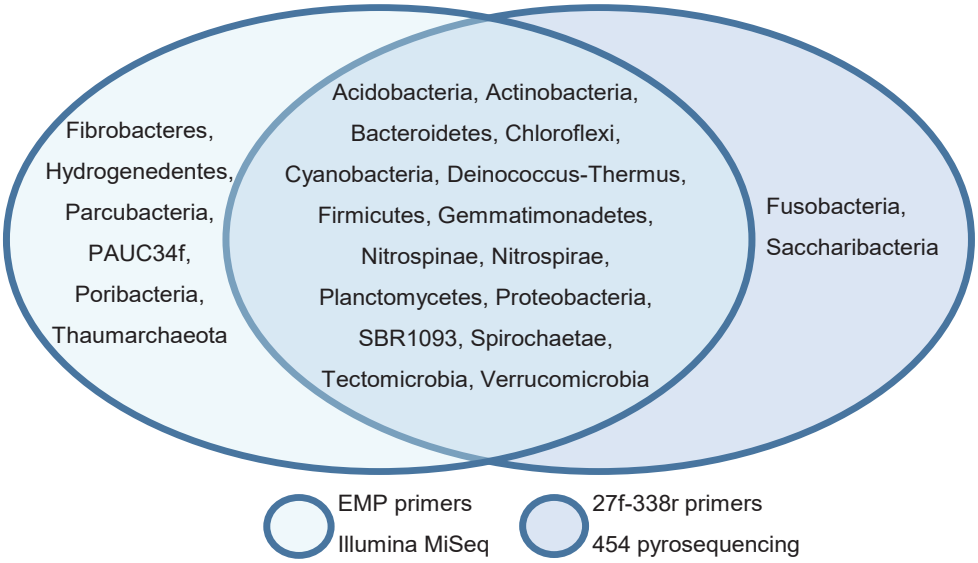


Figure 2 Venn diagram displaying the detection of microorganisms associated to *Aplysina aerophoba* with the different universal 16S rRNA gene primers and sequencing methods used in **Chapter 2** and **4.** The vast majority of the abundant microbial taxa are detectable with both methods (overlap).

However, whether 16S rRNA gene based microbial profiling studies reflect the true microbial diversity in marine sponges is under debate, since amplicon sequencing data is inherently sensitive to several methodological biases. Sample processing and DNA extraction methods might influence the quality and quantity of microbial DNA obtained from samples (Simister *et al.*, 2011; Wesolowska-Andersen *et al.*, 2014). Furthermore, template concentration was shown to significantly impact microbial profile variability in many samples, where especially low template concentrations are particularly susceptible to stochastic processes during PCR (Kennedy *et al.*, 2014). Universal primers are designed based on known bacterial taxa and thus can fail to amplify abundant bacteria from understudied microbial systems containing numerous novel taxa, such as sponge microbiomes. Metagenome-based studies reported that more than 15% of the bacterial domain might possess divergent 16S rRNA gene sequences which include self-splicing introns or

even protein sequences and thus would evade detection in typical amplicon surveys (Brown *et al.*, 2015). How many of these so-called candidate phyla radiation organisms inhabit marine sponges remains currently unknown.

More importantly, primer bias has been shown to severely affect the detection of Poribacteria, which are estimated to comprise up to 20% of the sponge-associated community by primer- and probe-independent assessments of microbial abundance (Bayer, Kamke, *et al.*, 2014). Poribacterial 16S rRNA genes show more than 25% sequence divergence compared to any other bacterial phylum (Lafi *et al.*, 2009), and contain several mismatches to the widely used Earth Microbiome Project (EMP) primer set (Walters *et al.*, 2015). Thus, microbial profiling studies using the EMP primers might underestimate the abundance of Poribacteria in sponges (Bayer, Kamke, *et al.*, 2014; Podell *et al.*, 2019). Nonetheless, even though relative abundance measures might not be accurate, the EMP primers can successfully amplify poribacterial sequences in DNA of most high-microbial abundance sponges (Thomas *et al.* 2016 and Figure 2). In preparation for the cultivation experiments described in **Chapter 4**, this was confirmed with an *in silico* primer specificity analysis on the poribacterial 16S rRNA sequences within the SILVA database using TestPrime 1.0 (http://www.arb-silva.de/search/testprime/). Out of a total of 49 sequence accessions classified as Poribacteria in the non-redundant SILVA SSU r132 database, a maximum of 45 sequences could be matched with 2 or 3 mismatches in the primer binding regions, resulting in a coverage of more than 90% (Table 1).

Table 1 *In silico* PCR results of EMP primer coverage on Poribacteria 16S rRNA gene sequences listed in the SILVA SSU r132 database (re-analyzed 25-3-2019).

Number of primer mismatches	Taxonomy	Coverage (%)	Primer Specificity	Poribacteria Accessions	Match	Mismatch
0	Poribacteria	12.2	21.9	49	6	43
1	Poribacteria	89.8	15.2	49	44	5
2	Poribacteria	91.8	12.1	49	45	4
3	Poribacteria	91.8	7.9	49	45	4

Additionally, we confirmed *in vitro* that the EMP primers could successfully amplify poribacterial DNA. Poribacterial 16S rRNA genes were amplified from extracted DNA of two *A. aerophoba* individuals used for inoculating the cultivation experiments (**Chapter 4**). Amplification of 16S rRNA genes using Poribacteria-specific primers (POR389f, POR1130r) (Fieseler *et al.*, 2004) was conducted as described in Chapter 3. The resulting products were then used as template for PCR-amplification with EMP primers (EMP 515f, EMP 806r), following the PCR protocol described in **Chapter 4** (Figure 3 A). The resulting PCR products were visualized on agarose gel as described in **Chapter 4**, and matched the expected size (Figure 3 B).

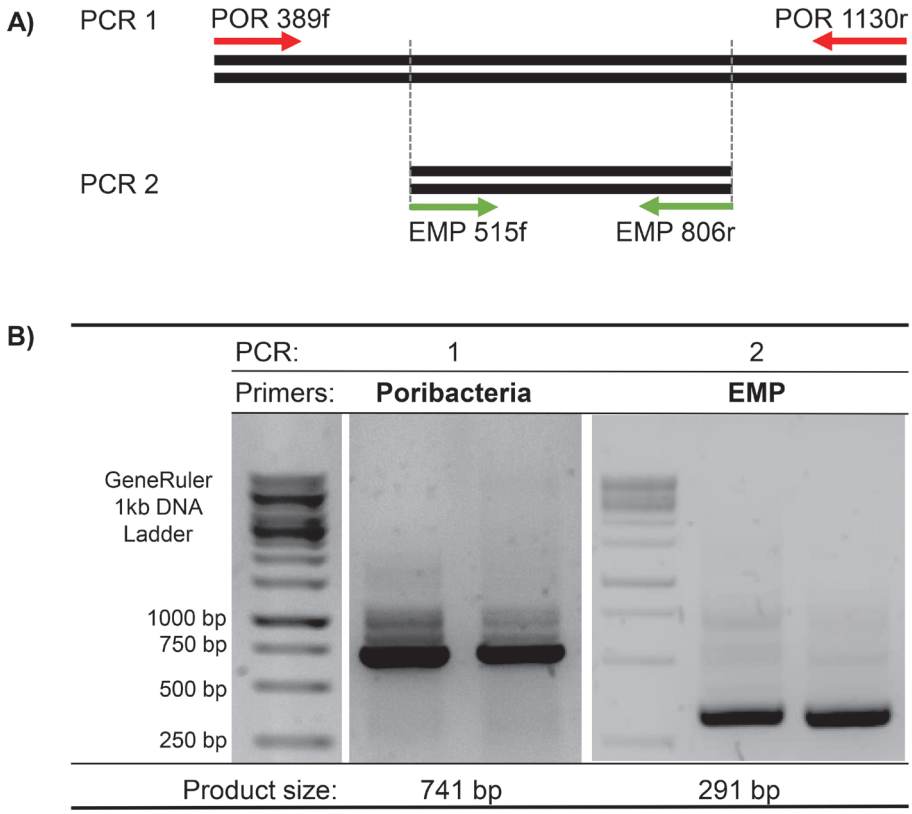


Figure 3 A) Schematic representation of the nested PCR approach used to validate poribacterial 16S rRNA gene amplification with EMP primers (green). B) Agarose gel visualization of PCR-amplified fragments confirmed the expected amplicon size.

Since the EMP primers enabled us to simultaneously identify all other potentially novel bacteria that grew successfully, we hypothesized that if Poribacteria grew under any of the cultivation conditions applied, we would be able to detect them using the EMP primers.

Recently, the discovery of putative Poribacteria-related genomes in samples derived from the open ocean sparked Podell *et al.* (2019) to develop novel universal primers that correct for the mismatches in poribacterial 16S rRNA genes. They report an approximately fivefold higher relative abundance of Poribacteria in various sponge species as compared to EMP primer results (Podell *et al.*, 2019). Thus, these primers should be considered in future diversity analyses since they might be better suitable for reflecting the composition of sponge microbiomes.

Origin of the Sponge Holobiont

Both ecological (temperature, geography) and evolutionary (phylogenetic) processes seem to shape the sponge microbiome (Lurgi *et al.*, 2019). Abiotic factors appear to influence the variable sponge-associated community, while the core microbiome, consisting of only a small number of microbial species in HMA sponges, seems to be phylogenetically related and co-evolving with the host (Lurgi *et al.*, 2019). Since the discovery of Poribacteria as an important member of sponge-associated communities (Fieseler *et al.*, 2004), ample research was accumulated on this lineage and e.g. shed light on its metabolism (Kamke *et al.*, 2013, 2014) and cell compartmentalization features (Jahn *et al.*, 2016), despite the lack of cultivated representatives (**Chapter 4**). Putative symbiotic genomic factors such as repeats and eukaryote-like domains have been detected that might facilitate interactions with their host and prevent phagocytosis (Kamke *et al.*, 2014). It is generally believed that Poribacteria are commensalistic symbionts that aid in and benefit from digesting food particles (Siegl *et al.*, 2011). Thus, these bacteria presumably contribute to the host's metabolism, and, due to vertical transmission (Webster & Blackall, 2009; Schmitt, Tsai, *et al.*, 2012), possibly also play a role in the evolutionary success of their hosts. Despite representing a model organism for investigating sponge-microbial associations, the precise functions of Poribacteria within sponges remain to be elucidated. Due to skewed abundance values of Poribacteria when amplified with universal primers (see above), Poribacteria have been found to either not be part of the sponge core community (Schmitt, Tsai, *et al.*, 2012) or constitute a minor part (Turon *et al.*, 2018; Lurgi *et al.*, 2019). However, poribacterial sequences were detected in 23 phylogenetically diverse HMA sponges when screened with Poribacteria-specific primers (Hardoim *et al.*, 2013; Lafi *et al.*, 2009; **Chapter 3**), and Poribacteria are generally believed to be an abundant key symbiont in HMA sponges. Poribacteria have been shown to be vertically transmitted through sponge larvae, however, the ocean water represents an additional seed bank for sponge-associated microorganisms, where 'sponge-specific' sequence clusters are regularly detected at low abundances (Webster *et al.* 2010). Due to their large water throughput, sponges are exposed to a large influx of seawater bacteria stimulating horizontal transmission of symbionts. Overlap has been detected between the sponge core microbiome and the seawater core microbiome (Turon *et al.*, 2018; Lurgi *et al.*, 2019), and sponge-specific mechanisms might act to enrich specific bacterial communities within the tissue, which might aid in maintaining similar microbial communities over large geographical distances. Thus, the horizontal acquisition of poribacterial phylotypes from the ambient seawater might constitute a reason for the lack of host-specificity amongst poribacterial taxa (**Chapter 3** and Figure 4).

These findings intrigue to ask questions about the evolutionary origin of sponge-microbe symbiosis. Sponges are considered the oldest metazoan lineage (Wörheide *et al.*, 2012, 2014; Simion *et al.*, 2017), and the genetic infrastructure for recognizing microorganisms and establishing symbiosis, such as cell receptors, an innate immune system and an extracellular matrix, were inferred to be present already in early sponges (Taylor, Radax, *et al.*, 2007). However, there is little evidence dating the origin of sponge-microbial symbiosis. Despite the large record of sponge fossils (Antcliffe *et al.*, 2014; Yin *et al.*, 2015), thus far there is a lack of paleontological reports investigating the presence of microorganisms within ancient sponges. As a result of vertical transmission, sponge-associated microbial 16S rRNA genes seem to have diverged between sponge species, but remained more similar to other sponge-derived sequences as compared to those of free-living microorganisms

(Schmitt, Tsai, *et al.*, 2012). Due to large (>10%) sequence divergences and assuming that an individual sponge-specific microbial lineage is derived from only one or a few colonization events, these associations must be extremely ancient (Webster *et al.*, 2010; Schmitt, Tsai, *et al.*, 2012). As a consequence, current hypotheses state that the intrinsic microbial associations to sponges must have originated in the Precambrian era (Taylor *et al.* 2007; Hentschel *et al.* 2012, Figure 4).

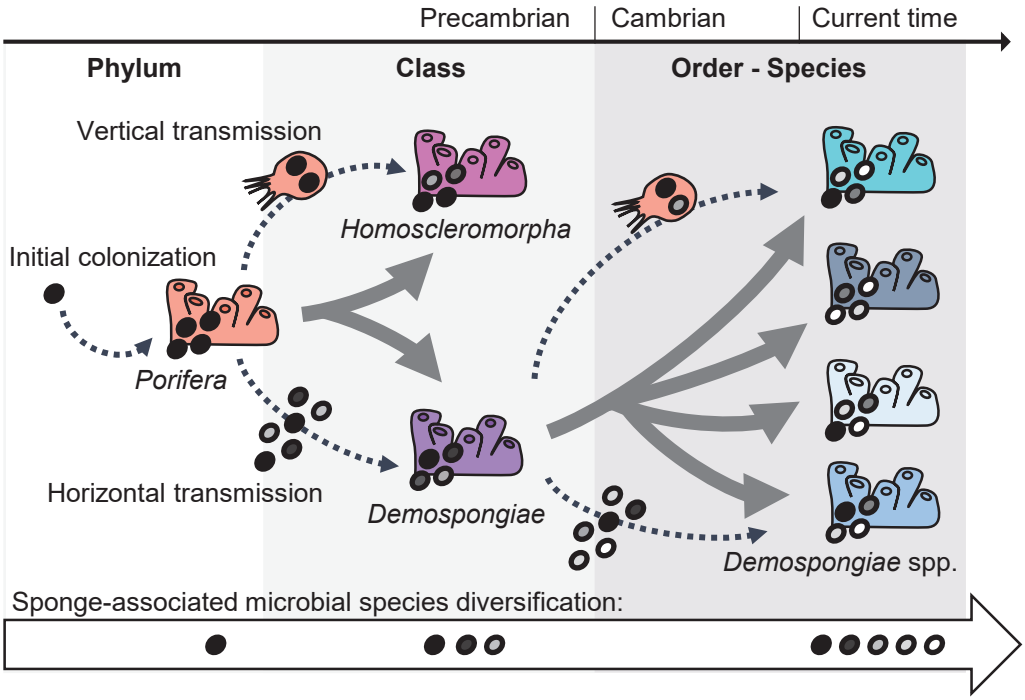


Figure 4 Schematic depiction of the hypothesized diversification of the Porifera holobiont. Vertical transmission via sponge larvae guaranteed symbiont transfer to the offspring. Horizontal transmission via the seawater allowed the colonization of different sponge species by similar microbes. Vertical and horizontal transmission can occur simultaneously in all sponge species. Microbial symbionts diversified within their sponge host over time into distinct taxa but remained more similar to each other than to free-living microorganisms. This graph summarizes hypotheses from Wilkinson 1983; Zilber-Rosenberg & Rosenberg 2008; Schmitt et al. 2012; Schuster et al. 2018 and **Chapter 3**.

The only published study trying to find evidence for this early association between sponges and microbes reported the detection of the same bacterial species in nine sponge species from the Great Barrier Reef, Australia, and the Mediterranean Sea, France (Wilkinson, 2006). The consequent hypothesis stated that symbionts associated to sponges before the separation of the phylum Porifera into classes and orders during the Precambrian (600 million years ago). In this thesis, **Chapter 3** discusses the global diversity and phylogenetic relationships of Poribacteria, and neither co-speciation with the sponge host nor a biogeographical correlation could be detected. Amongst the class Demospongiae, 22 taxa from eight marine regions were sampled, all of which inhabited

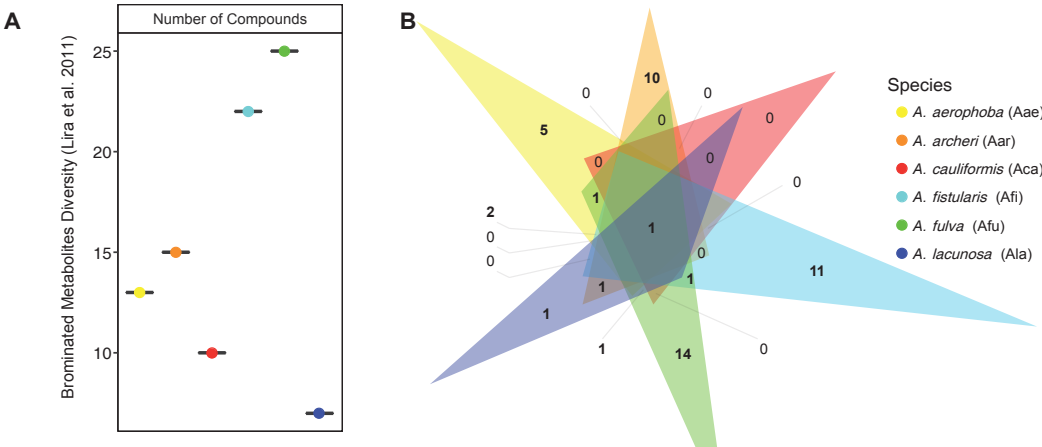
symbionts from several phylogenetic groups of Poribacteria. A recent time-calibrated phylogeny dates the emergence of Demospongiae to the Precambrian, and estimates the divergence into several sponge orders during the Paleozoic era, between 252 and 540 million years ago (Schuster *et al.*, 2018). Since all sampled HMA Demospongiae host Poribacteria, combining these results supports the hypothesis that sponge-poribacterial symbiosis was established before the evolutionary split of the Porifera phylum into different orders, or even classes (Demospongiae and Homoscleromorpha, Figure 4). Thus it might be possible to conclude that animal-bacterial associations are very ancient and have been a consistent factor during the evolutionary history of sponges and all other animals.

Bioactives and their Potential Microbial Producers

Their rich arsenal of molecules with potent bioactive properties have been a major reason for the scientific interest in sponges and their microbial associates. Originally it was hypothesized that the sponge cells produce these secondary metabolites as a means to deter predators and pathogens since brominated secondary metabolites were found to be located in spherulous cells of *Aplysina fistularis* (Thompson *et al.*, 1983) and *Aplysina aerophoba* (Turon *et al.*, 2000). *Aplysina* species are especially renown for the production of a variety of brominated alkaloids (Lira *et al.* 2011, Figure 4). However, many of these compounds bear remarkable similarities to bacterial compounds (Piel, 2009), and thus microbial origin was suggested and confirmed for several sponge-derived metabolites (Unson *et al.*, 1994; Piel, 2009).

Only in a few cases these secondary-metabolite-producing microbes have been identified. The filamentous cyanobacterium *Oscillatoria spongeliae* has been shown to contain the major brominated bioactive compound found in the sponge *Dysidea herbacea* (Unson *et al.*, 1994). More recently, the yet uncultured filamentous bacterium *Candidatus* Entotheonella factor has been demonstrated to be the producer of almost all polyketide and modified peptide families reported from the sponge *Theonella swinhoei* and contains numerous gene clusters for yet unknown, additional metabolites (Piel, 2009; Wilson *et al.*, 2014; Freeman *et al.*, 2017).

Halogenase genes are often indicative of secondary metabolite gene clusters, and are often part of the genetic toolbox required for the production of brominated metabolites (Van Pée & Patallo, 2006; Smith *et al.*, 2017). Apart from sharing several halogenase genes as well as prokaryotic OTUs, the Caribbean *Aplysina* species investigated in **Chapter 2** were also reported to share several brominated bioactive compounds (Figure 5). Thus, these observations could hint towards a connection between the two measured genes and the bioactive compounds detected, thus providing leads towards identification of the actual microbial secondary metabolite producers within *Aplysina* species.



C

Substance Code	Compound Name	Aae	Aar	Aca	Afi	Afu	Ala
21	2-(3,5-Dibromo-4,4-dimethoxy-1-hydroxy-2,5-cyclohexadien-1-yl) acetamide or 2-(3,5-Dibromo-1-hydroxy-4,4-dimethoxycyclohexa-2,5-dienyl)acetamide						
29	(7S*,11R*)-5-[3,5-Dibromo-4-[(2-oxo-5-oxazolidinyl)]methoxyphenyl]-2-oxazolidinone						
41	Aerothionin						
46	Fistularin-3						
52	11-Oxoaerothionin						
100	(+) or (-) Aeroplysinin 1						

Figure 5 Summary of brominated secondary metabolites detected in *Aplysina* species investigated in this thesis. Data was obtained from Lira *et al.* (2011). A) Total number of compounds detected per *Aplysina* spp. B) Venn diagram displaying unique and shared compounds in the investigated species. C) Table listing compounds detected in 3 or more of the *Aplysina* spp.

In *A. aerophoba*, sponge-associated Chloroflexi of the Caldilineae and SAR202 lineages were shown to encode several polyketide synthase (PKS) as well as non-ribosomal peptide synthase (NRPS) gene clusters potentially involved in secondary metabolite production (Siegl & Hentschel, 2010; Bayer *et al.*, 2018). Furthermore, the abundance of a *Chloroflexus* phylotype and the concentration of brominated alkaloids were found to be strongly correlated (Sacristán-Soriano *et al.*, 2011). In **Chapter 2** we found the abundance of several Chloroflexi OTUs strongly positively correlated with the abundance of several novel halogenase gene variants in Caribbean *Aplysina* species. This is a further indication that these dominant symbionts might be actively involved in bioactives production. On the other hand, genes that could be involved in the degradation of aromatic compounds were also discovered in sponge-associated Chloroflexi (Bayer *et al.*, 2018), indicating that these bacteria might also be able to break down bioactive substances produced by the sponge host or other microbes, thus enabling their survival within the tissue. Without cultivated representatives, however, the role of Chloroflexi in bioactive compound production remains speculative.

We detected also other taxa to be correlated with the abundance of certain halogenase genes. Most of these groups have been reported as secondary metabolite producers previously and include Acidobacteria (Parsley *et al.*, 2011), Actinobacteria (Valliappan *et al.*, 2014), Alphaproteobacteria (Muscholl-Silberhorn *et al.*, 2008), and Tectomicrobia (Wilson *et al.*, 2014), just to name a few (**Chapter 2**, Figure 3). However, caution should be taken in interpreting these results, since rare members of microbiomes might have an over-proportional role as essential hidden drivers of microbiome functions such as host health and defense or biogeochemical cycling (Jousset *et al.*, 2017). Such microbes that are rare in abundance, but highly prevalent, are *Pseudovibrio* species, which are detected in extremely low abundance when sponge microbiomes are investigated with molecular methods (≤ 0.1%, **Chapter 2** and ≤ 0.42% relative abundance, **Chapter 4**). However, *Pseudovibrio* strains get isolated in basically every cultivation-based study on sponges and other marine invertebrates (Webster & Hill 2001; Muscholl-Silberhorn *et al.* 2008; Sipkema *et al.* 2011; Versluis *et al.* 2017; Alex & Antunes 2018, **Chapter 4**). These bacteria possess numerous genetic features for symbiotic interactions (Bondarev *et al.*, 2013) and seem to be an integral part of the healthy sponge microbiome since their abundance decreases in diseased sponges (Sweet *et al.*, 2015). Recently it was shown that a sponge-associated *Pseudovibrio* strain produces several bromotyrosine-derived alkaloids in culture, which were previously only isolated from marine sponges directly (Nicacio *et al.*, 2017). Our finding that *Pseudovibrio* appears to be resistant to the sponge-derived antibiotic aeroplysinin-1 is in line with these results (**Chapter 4**). Thus, we might need to re-evaluate the importance of these microbes within the sponge holobiont and include them in the list of potential sponge-derived bioactives producers.

Cultivation

Bioprospecting of marine environments and especially sponges has been highly prolific in the quest to discover novel potent bioactive compounds displaying cytotoxic, antimicrobial and antiviral properties (Mehbub *et al.*, 2014). However, the majority of these compounds are structurally too complex for chemical synthesis and are not undergoing clinical trials due to supply issues (Singh & Thakur, 2016). The advancements of sequencing and bioinformatic technologies have enabled us to identify the enormous gene clusters responsible for the synthesis of these metabolites (Loureiro *et al.*, 2018). Heterologous expression of secondary metabolite gene clusters is one option to solve the supply issue, however, thus far success has been limited. Multi-modular enzyme complexes such as polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) proteins are often subjected to complex regulatory mechanisms and require post-translational modifications as well as specific substrate pools in order to actively produce secondary metabolites (Loureiro *et al.*, 2018). Furthermore, the heterologous expression host must also be resistant towards the resulting bioactives, which reduces the choice of bacterial host strains to only few (Steinert *et al.*, 2018).
Sponge aquaculture has also been proposed as a means to produce sufficient quantities of bioactive compounds (Sipkema, 2004). Trials at several marine locations have reported high unpredictability of the behavior and growth of cultivated sponges, and these in situ systems are very vulnerable to environmental impacts such as extreme weather conditions, changing seawater temperatures and pollution (Mendola, 2003; Page *et al.*, 2005; Osinga *et al.*, 2010). In vitro cultivation of sponge cells has resulted in little progress other than the establishment of temporary cultures (Grasela *et al.*, 2012), and to date, no sponge cell line is available (Munroe *et al.*, 2019).

Thus, cultivating the actual bacterial producers of the bioactive compounds of interest provides an attractive solution for the sustainable production of these compounds (Kennedy *et al.*, 2007; Hentschel *et al.*, 2012). Cultivation of sponge-associated microbes as 'biological fermenters' has thus received intense attention from the scientific community. While applications of multi-omics methods to investigate marine sponges have provided numerous hypotheses on the genetic and functional potential of sponge-associated microbes, the importance of bacterial cultivation must not be underestimated (Chaib De Mares, 2018). Cultivation allows for the acquisition of phenotypic information, important for testing and validating multi-omics generated hypotheses. Microbial cultures are essential for determining gene functions as well as characterizing the metabolic and physiological properties of microbes (**Chapter 5**) since currently, a huge knowledge gap remains between the assessment of genomic potential and the functional assignment of genes and proteins (Prakash *et al.*, 2013). In regard to the sponge holobiont, continuous efforts to culture the hitherto uncultivable symbiotic microorganisms are necessary if we want to understand the metabolism of sponges and their microbiome in its entirety (Karimi *et al.*, 2019).

Cultivating bacteria has a long history, with Robert Koch being the first to develop a method of isolating and cultivating bacteria in pure culture in the 1880s (Madigan *et al.*, 2014). Since then, microbial cultivation remained an indispensable aspect of microbiological studies, enabling the description of more than 22 500 microbial species (https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html, February 2019). Ongoing efforts increase this number continuously. However, already Robert Koch realized that some media were unsuitable to support the growth of certain microorganisms, which later was described as the "great plate-count anomaly" (Staley, 2002; Nichols, 2007).

In order to address this uncultivability problem of the majority of environmental microorganisms, researchers progressed cultivation technology by applying innovative methods such as high-throughput cultivation using microdroplets with single encapsulated cells in a flow-through reactor (Zengler *et al.*, 2005), polycarbonate membrane systems (Ferrari & Gillings, 2009), hollow fiber membrane chambers (Aoi *et al.*, 2009), or large and miniature diffusion chambers (Belanger *et al.*, 2010; Steinert *et al.*, 2014). All these cultivation devices were placed back into the originating environment for incubation, providing the microorganisms with their natural substrates, while physically separating them from it. These studies yielded numerous novel bacterial species within these *in situ* cultivation devices, but reported difficulties to maintain their growth later on. Such approaches manifest out of our poor understanding of the chemical composition as well as substrate availability and concentration in the majority of environments. Further fundamental environmental parameters that need to be considered for cultivation experiments include temperature, pH, osmotic conditions, co-existing species and many more (Stewart, 2012). Attempting to incorporate all these variables results in a multidimensional matrix of possibilities that cannot be exhaustively addressed within a reasonable timeframe.

While there are several studies attempting the cultivation of sponge-associated organisms, they have recently become overshadowed by studies applying molecular methods to investigate sponge-microbes. However, multi-omics studies provide valuable information which can be implemented into the design of media formulations, cultivation setups and screening strategies, thus paving the way towards information-based cultivation strategies (**Chapter 5**). To date, the information

collected by molecular methods has only rarely been implemented in cultivation-based studies (**Chapter 5**) and even less so in studies targeting sponge-associated microorganisms (Lavy *et al.*, 2014).

With the cultivation experiments reported in this thesis, we aimed to address several points that were put forward as possible reasons for the uncultivability of microbes, and to translate multi-omics-derived information on sponge-associated microbes into cultivation strategies targeting the isolation of Poribacteria:

Oligotrophy and Difficult Substrates

Many bacteria, especially within the marine environment, survive despite extremely low nutrient concentrations in their surroundings. These microbes genetically adapted to such scarce conditions through decreased cell size, slow growth rates, highly efficient nutrient uptake systems and the lack of motility (Overmann *et al.*, 2017). By applying highly oligotrophic conditions in the cultivation setup, abundant marine organisms such as "*Candidatus* Pelagibacter ubique" (SAR11 clade) and oligotrophic marine Gammaproteobacteria (Cho & Giovannoni, 2004) could be successfully isolated. In **Chapter 4** we could show that low nutrient concentrations (0.08 g/L carbon source) would support the highest diversity of cultivated microbes, and that especially marine members of the Flavobacteriia would not grow at high nutrient concentrations. These findings are in line with results from other cultivation experiments on marine sponges, where diluted complex media enriched for a higher number of colonies than undiluted media (Lavy *et al.*, 2014; Versluis *et al.*, 2017), or low concentrations of peptone and starch were most successful for isolating diverse microbes (Sipkema *et al.*, 2011).

Another method to supply low amounts of substrate is using polymeric carbohydrates as carbon source. Such complex compounds such as chitin, cellulose or peptidoglycans are difficult to degrade by microorganisms, and especially at the beginning of an incubation only low amounts of hydrolytic exoenzymes are present (Overmann *et al.*, 2017). Furthermore, complex carbohydrates represent carbon sources that could be encountered by organisms in their natural environments. Proteoglycans are important components of the animal extracellular matrix, and the intercellular milieu in sponges is rich in collagen and spongin fibres containing glucosamine, uronic acids, fucose, arabinose and mannose (Simpson, 2011). Poribacteria have been predicted to possess the genomic potential necessary to utilize these sugars for their energy metabolism (Siegl *et al.*, 2011; Kamke *et al.*, 2013). However, spongin fibres are extremely resistant to chemical as well as enzymatic degradation (Simpson, 2011), and only few enzymes can effectively degrade collagen (Yu-Zhong Zhang *et al.*, 2015). Concordantly, we did not detect any microbial colonies growing on 10x or 50x diluted media Plates that were coated with a thin layer of collagen (**Chapter 4**). This indicates that the inoculated bacteria could not utilize the provided collagen and relied on diffusion of the underlying galactose as substrate, which was only effective in the undiluted (1x) Plates. Another set of plates was supplemented with the proteoglycan mucin as sole carbon source, resulting in the growth of an above-average microbial diversity in 10x and 50x media dilutions (**Chapter 4**). Mucin medium was also reported as amongst the most successful for cultivating diverse microbial OTUs from *Haliclona* sp. (Sipkema *et al.*, 2011), and resulted in the isolation of novel Flavobacteriia from *A. aerophoba* (Versluis *et al.*, 2017). Thus, using such unusual carbohydrates has a high potential

for isolating novel marine microorganisms. Furthermore, beyond focusing on deciphering novel bioactive molecules (Yousaf *et al.*, 2002; Lira *et al.*, 2011; De Caralt *et al.*, 2013; Niemann *et al.*, 2015; Puyana *et al.*, 2015), future efforts should evaluate the chemical composition of sponges and identify potential substrates for the inhabiting microorganisms.

Maintaining Microbial Interactions

Environmental bacteria do not occur alone, but are embedded in intricate ecological networks with numerous interactions. *In situ* cultivation devices as described above successfully maintain such microbial interactions, while traditional cultivation methods rely on isolation, which results in the accumulation of potentially toxic metabolic waste products on media plates without the presence of a microbial neighbor that utilizes them. Furthermore, numerous examples of fastidious bacteria have lost the ability to perform essential metabolic functions and depend on microbial neighbors to supplement them with certain growth factors or nutrients (Stewart, 2012). Thus, maintaining microbial interactions in cultivation systems might prove valuable in the quest to cultivate yet uncultivable microorganisms (Overmann *et al.*, 2017).

An approach to enrich for a complex microbial community in a stratified cultivation system while maintaining the metabolic interactions of the sponge microbiome is described in **Chapter 4**. Winogradsky columns (in **Chapter 4** referred to as MiniColumns) are enclosed, self-sustaining microbial ecosystems, consisting of a sediment and a water phase in a clear glass column, and are inoculated with a mixed microbial community (Madigan & Martinko, 2006; Rundell *et al.*, 2014). With time, chemical gradients of oxygen or H_2S concentrations get established, which creates unique, vertically distributed niches favoring the growth of different microorganisms (Madigan & Martinko, 2006). With the input of light, essential nutrients such as carbon, nitrogen and sulfur are recycled and metabolic interactions are maintained within the column (Rundell *et al.*, 2014). Since *Aplysina aerophoba* harbors a diverse microbial community including phototrophs, ammonium oxidizers, carbohydrate degraders and sulfate reducers (Bayer *et al.*, 2008; Hardoim *et al.*, 2013; Chaib De Mares *et al.*, 2017) that might interact and exchange nutrients, we hypothesized that the Winogradsky column approach could facilitate the enrichment of sponge-specific microorganisms by supporting the metabolic interactions found within the sponge. Even though the dominant microorganisms found in sponges did not successfully grow in this system, we could enrich for diverse microbial communities within different fractions of the MiniColumns (**Chapter 4**). In accordance to other microbial diversity investigations of Winogradsky columns, we found the MiniColumns dominated by the phyla Proteobacteria and Firmicutes, with Clostridia representing biomarkers for the deepest fractions of the columns (Rundell *et al.*, 2014). Furthermore, the results indicated that a few taxa were highly abundant, with 11 OTUs from Alphaproteobacteria, Clostridia and an Unidentified organism related to the Planctomycetes (**Chapter 4**) constituting over 94% of the overall abundance, while a large number of OTUs were rare (110 OTUs ≤1% relative abundance).

Despite the fact that the overall community structure in the replicate columns was rather similar, they established individual dissolved oxygen concentration profiles (**Chapter 4**, Figure 8A) and several OTUs occurred in only one of the columns (Figure 6). This indicates that complex dynamics caused by interspecies interactions and community-environment feedback, as well as stochastic processes might create individual selective environments. These might result in the microbial

community developing in different directions, as has been observed before (Becks *et al.*, 2005; Benincá *et al.*, 2008; Pagaling *et al.*, 2014). Some species interactions might also be mediated by the physical environment. For example, the depletion of oxygen resulting from sulfate reduction and cellulose degradation can favor the selection of anaerobes in the sediment fractions of the MiniColumns.

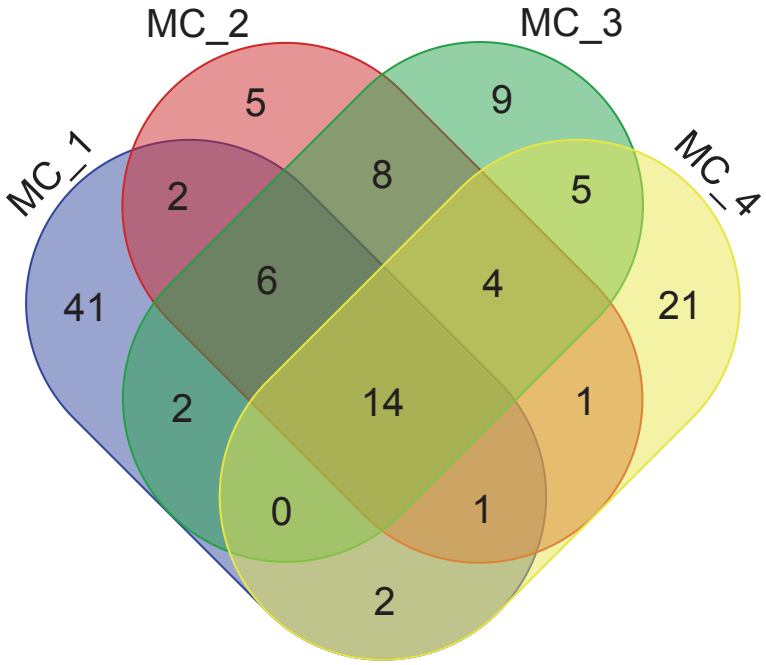


Figure 6 Venn diagram depicting shared and unique microbial OTUs detected in four replicate MiniColumns (MC, **Chapter 4**). Diagram was created using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Two MiniColumns exhibited green pigmentation at the end of the incubation period (**Chapter 4**, Supplementary Figure S3). This indicates that aerobic (MiniColumn 1) and anaerobic (MiniColumn 4) phototrophic microorganisms enriched within the columns, even though their identity could not be elucidated. Neither Cyanobacteria nor chloroplast sequences were detected in these columns. However, several lineages within the alphaproteobacterial order Rhodobacterales contain phototrophic organisms (Imhoff *et al.*, 2018), of which numerous OTUs were enriched within the MiniColumns.

Network analysis of significant co-occurrence patterns obtained from sequencing data can be used to investigate potential interactions between microbial taxa (Röttjers & Faust, 2018). Strong patterns of co-occurance were revealed in a network analysis on the relative abundances of all genera detected in the MiniColumns, hinting at putative ecological interactions among microbial lineages, which seem to be driven by the presence or absence of oxygen (Figure 7). The abundances of mostly aerobic classes such as Bacilli were positively correlated, while anaerobic Clostridia co-

occurred with each other. This indicates lifestyle preferences, shared niches and possible inter-dependencies amongst the taxa enriched in the columns. A significant positive co-occurance correlation of an unidentified organism (orange) to Clostridia provides an additional hint towards a presumable anaerobic lifestyle of this organism (Figure 7).

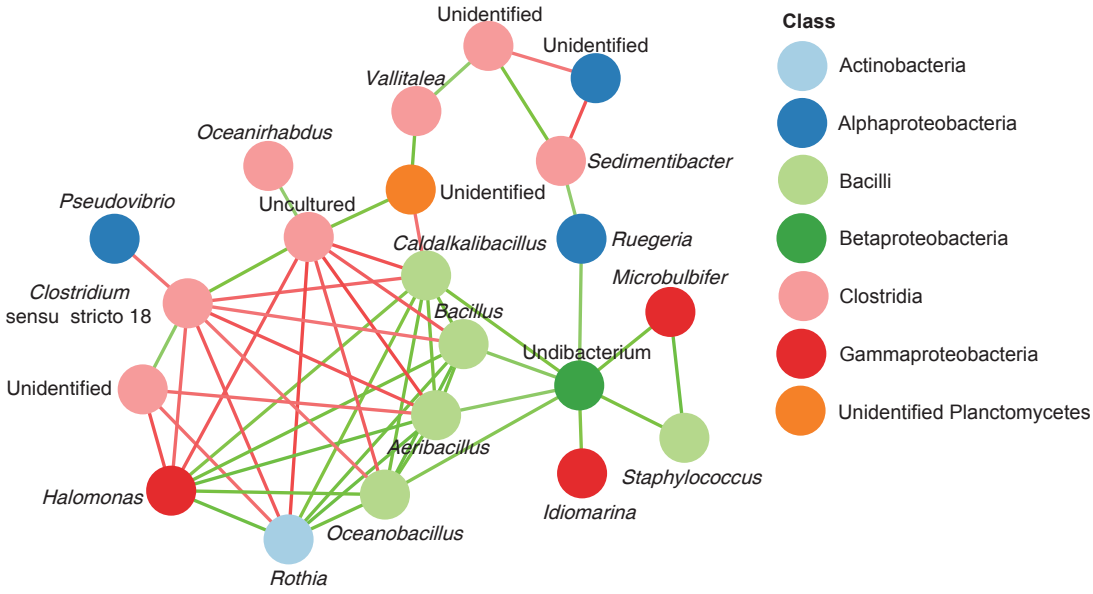


Figure 7 Network analysis of co-occuring taxa in the four Winogradsky columns (MiniColumns, **Chapter 4**). OTUs were clustered on Genus level and read counts were transformed to relative abundance. Spearman correlations were calculated and visualized using Cytoscape. Nodes are colored by Class and edges are colored by positive (green) or negative (red) significant (P-value <0.05) Spearman correlation (>0.5) of co-occurance.

Since many novel and naturally abundant types of microorganisms cannot be isolated and can only be cultivated in cocultures with other bacteria (Kaeberlein *et al.*, 2002; Lewis *et al.*, 2006; Hahn, 2009; Dedysh, 2011), future cultivation experiments on fastidious microorganisms should continue to implement methods allowing microbial interactions within artificial cultivation environments. The application of Winogradsky columns represent cultivation systems that are easily manipulated and possess the potential to enrich for novel microorganisms.

Prolonged Incubation Time

Natural microbial environments are often characterized by substrate limitations, fluctuating environmental conditions and the presence of numerous competing species. In order to guarantee their long-term survival, environmental organisms adjusted with slow growth rates, temporal metabolic inactivity, dormancy and spore formation (Epstein, 2009). When inoculated on artificial

growth media, microbes might only stochastically awake from their dormant states and require time to adjust their metabolism to the novel environmental conditions they encounter. Many members of rarely isolated microbial groups such as Acidobacteria, Chloroflexi and Planctomycetes require several months of incubation on appropriate cultivation media to form visible colonies, and continue to grow slowly also when isolated (Davis *et al.*, 2005). Thus, elevated incubation time and patience of researchers might prove crucial when aiming to cultivate fastidious organisms. In the Plates experiment in **Chapter 4** we harvested microbial colonies after 22 days of incubation, which resulted majorly in the detection of fast-growing microbes such as *Pseudovibrio* and *Ruegeria*. However, we kept incubating the Plates and picked further colonies after 180 days of incubation, which resulted in an increased phylogenetic diversity (**Chapter 4**, Table 2) and the detection of Planctomycetes and Verrucomicrobia. These rarely cultivated organisms are renowned for their fastidiousness and slow growth rates (Pimentel-Elardo *et al.*, 2003; Davis *et al.*, 2005; Bergmann *et al.*, 2011; Lage & Bondoso, 2012). It might thus be plausible to hypothesize that in order to cultivate dominant sponge-associated lineages such as Chloroflexi, Acidobacteria and Poribacteria, even longer incubation times are necessary. However, it is challenging to avoid the overgrowth and dominance of more readily cultivable bacteria such as *Pseudovibrio*, while simultaneously, research projects are usually under time constraints that render long-term cultivation studies difficult to justify.

The More the Better

Interestingly, in **Chapter 4** we observed little overlap in isolates from the different culturing methods, suggesting that multiple approaches may yield greater diversity, i.e. the more cultivation conditions applied, the higher the cultivated microbial diversity. This has been observed in cultivation studies addressing oral (Sizova *et al.*, 2012) and gut microbiota (Lagier *et al.*, 2012) of humans, as well as sponge microbiota (Sipkema *et al.*, 2011) and many more. Within the Plates experiments in **Chapter 4**, 9 different media x 3 medium dilutions x 2 presence/absence of aeroplysinin x 2 incubation time points already resulted in 108 different cultivation conditions. The MiniColumns were divided into 5 samples per column, where different oxygen concentrations and other chemical gradients also provided numerous distinct niches, and the water-gelrite interface within the Liquid-Solid experiments constituted further unprecedented incubation conditions. Taken together, several hundred cultivation setups were probed for the enrichment of sponge associated microorganisms, which resulted in a total of 256 cultured OTUs from 7 bacterial phyla. In accordance to other sponge-microbiota cultivation experiments, conditions such as nutrient concentration, liquid or solid interface and antibiotics seemed to be the main driver for isolating diverse microbes, while metabolically highly versatile bacteria such as *Pseudovibrio* or *Ruegeria* species could successfully grow on almost any condition applied (Sipkema *et al.*, 2011; Lavy *et al.*, 2014; Keren *et al.*, 2015, 2016; Esteves *et al.*, 2016; Versluis *et al.*, 2017; Karimi *et al.*, 2019). Cultivation approaches targeting specific sponge-associated microbial lineages or functions generally report successful isolation of such target groups, e.g. Actinobacteria (Kuo *et al.*, 2019), Planctomycetes (Pimentel-Elardo *et al.*, 2003), Cyanobacteria (Konstantinou *et al.*, 2018), fungi (Naim *et al.*, 2017) or anaerobic (Brück *et al.*, 2010) and dehalogenating organisms (Ahn *et al.*, 2003). However, also with these approaches, the cultivated microorganisms were mostly not identical to the OTUs detected with molecular methods in the sponge inoculum.

Simultaneously, the importance of high sampling effort should be highlighted. In natural environments, bacterial communities are dominated by a few species accounting for the majority of biomass, with a large number of rare species that are only present in low abundance (Jousset *et al.*, 2017; Shetty, 2019). Microbial cultivation environments are no exception; the vast majority of diversity of microbial OTUs are represented by only a small number of colonies (Sipkema *et al.* 2011; Lavy *et al.* 2014; Versluis *et al.* 2017, **Chapter 4**). Thus, thousands of colonies have to be picked and identified in order to find a handful of novel and potentially interesting microbial isolates (Sipkema *et al.* 2011; Lavy *et al.* 2014, **Chapter 4**), while still a chance remains that novel taxa are overlooked due to undersampling. Next generation sequencing methods enable deep sampling of cultivation-derived communities, but extended waiting periods for obtaining results represent additional challenges that complicate a timely screening of enrichment cultures or microbial isolates.

Attempts to Translate Multi-Omics Information into Cultivation Conditions for Poribacteria

At the time we designed the experimental setup for the Plates experiment (**Chapter 4**), six single-cell genomics-derived genomes were available for Poribacteria, with sizes up to 5.4 Mbp and genome coverage of up to 98.5% (Kamke *et al.*, 2013). The predicted metabolic capabilities therein were used as starting hypotheses for the design of growth media tailored towards the metabolic requirements of Poribacteria, expanding on previous attempts (Lavy *et al.*, 2014). Genomic features indicate the importance of heterotrophy and the presence of numerous glycoside hydrolases hint at a specialization towards degrading diverse complex carbohydrates and glycoproteins. We thus supplied our media with different aminosugars, monomeric sugars, and complex carbohydrates that could potentially be hydrolysed by the predicted enzymes (**Chapter 4**, Table 1). Additionally, the presence of uronic acid degradation pathways and specific sulfatases point towards the ability of Poribacteria to degrade glycoproteins from the host matrix, leading us to also utilize mucin and collagen. An enzymatic pathway hinting towards the utilization of 2-aminoethylphosphonate (2-AEPn) suggests that Poribacteria might rely on organic phosphorus (Kamke *et al.*, 2014) which constitutes an important phosphorus source in marine environments (Mukhamedova & Glushenkova, 2000; Dyhrman *et al.*, 2009). Similarly, we included both organic sulfur sources in form of the amino acids cysteine, histidine and methionine, as well as inorganic sulfur ($NaSO_4$) in our media. Three urease subunits (Siegl *et al.*, 2011) also point towards utilization of this metabolic waste product from sponge metabolism, which we included as nitrogen source. Even though Poribacteria have the genomic potential to produce numerous vitamins (B12, B2, B7, B1, B6), the addition of vitamins can be crucial for isolating environmental bacteria (Rappé *et al.*, 2002; Giovannoni, 2012; Kéki *et al.*, 2013). Trace metals are often essential for enzymatic functions and in the poribacterial gene annotations we observed numerous enzymes requiring iron as co-factor. We based the composition of the trace metal solution on solution SL-10 developed by DSMZ (https://www.dsmz.de/) with the addition of iodide and bromide which constitute important elements in marine environments. Since agar had been shown to inhibit the growth of several fastidious organisms (Davis *et al.*, 2005; Tanaka *et al.*, 2014), gelrite was used as a solidifying agent (Rygaard *et al.*, 2017). Nonetheless, we could not detect Poribacteria after 22 and 180 days of incubation. Future studies attempting the cultivation of Poribacteria should aim to design autotrophic cultivation setups that require microbes to perform CO_2 fixation via the Wood-Ljungdahl pathway, which was detected in Poribacteria (Siegl *et al.*, 2011).

The cultivation attempts described in **Chapter 4** highlight the need to keep on innovating, since none of the dominant microbial symbionts of sponges has been cultured to date. Nonetheless, despite the challenges inherent to studies addressing sponge-microbiota cultivation, combined efforts resulted in a large number of microbial cultures and isolates derived from marine sponges (Dat *et al.* 2019, **Chapter 4**).

What Did We Culture

The results of cultivation experiments conducted in this thesis are in line with most previous sponge-associated microbial cultivation studies. At phylum level, the dominant cultivated bacterial taxa belonged to the Proteobacteria (Gamma and Alpha), Actinobacteria, Firmicutes and Bacteroidetes (Sipkema *et al.*, 2011; Jung *et al.*, 2014; Steinert *et al.*, 2014; Esteves *et al.*, 2016; Silva *et al.*, 2016; Versluis *et al.*, 2017). Additionally, we could detect growth of the phyla Tenericutes, Planctomycetes, Verrucomicrobia and an unidentified phylum related to the Planctomycetes. Figure 8 depicts the microbial phylogenetic diversity obtained by cultivation from **Chapter 4**. Even though many of these OTUs might represent novel taxa, no efforts were made to further characterize them, since many of them could not be detected in the sponge samples and were thus considered to constitute extremely rare, if not completely coincidental, members of the *Aplysina aerophoba* microbiome. The ecological roles of many of these cultivated organisms derived from the sponge microbiome, some of which are discussed below, remain to be elucidated in future studies.

Pseudovibrio species dominated the cultivation experiments, and this genus has been shown to be metabolically highly versatile, being adapted to symbiosis with marine invertebrates. This alphaproteobacterial lineage has been repeatedly isolated from sponges and also detectable with molecular methods (**Chapter 4**). It appears to be a commensalistic or even beneficial symbiont with the genomic potential to attach and interact with eukaryotic cells, supply the host with vitamins and cofactors and produce sponge-specific secondary metabolites (Bondarev *et al.*, 2013; Nicacio *et al.*, 2017; Alex & Antunes, 2018; Fróes *et al.*, 2018; Versluis *et al.*, 2018). Its facultative anaerobic lifestyle, fast growth rate and wide substrate utilization capabilities furthermore render *Pseudovibrio* species valuable candidates for industrial upscaling in order to produce sponge-derived secondary metabolites.

Ruegeria species are globally distributed marine bacteria found primarily attached to particles in the upper open oceans (Sonnenschein *et al.*, 2017). Several strains produce the antibacterial compound tropodithietic acid, which renders them potential candidates for probiotics strategies against fish pathogens in aquaculture (Porsby *et al.*, 2008). This might allow *Ruegeria* species to dominate cultivation experiments alongside *Pseudovibrio*, since their bioactivity might inhibit the growth of competing microorganisms in their vicinity. *Ruegeria* species have been regularly isolated from marine sponges (Mitova *et al.*, 2004; Zan *et al.*, 2012; Rua *et al.*, 2014; Naim, 2015; Versluis *et al.*, 2017), and these strains seem to show genetic adaptions to symbiosis, such as a lack of motility and chemotaxis traits, enriched sulfur metabolism uptake and utilization genes, and distinct natural product biosynthesis gene clusters (Karimi *et al.*, 2018, 2019).

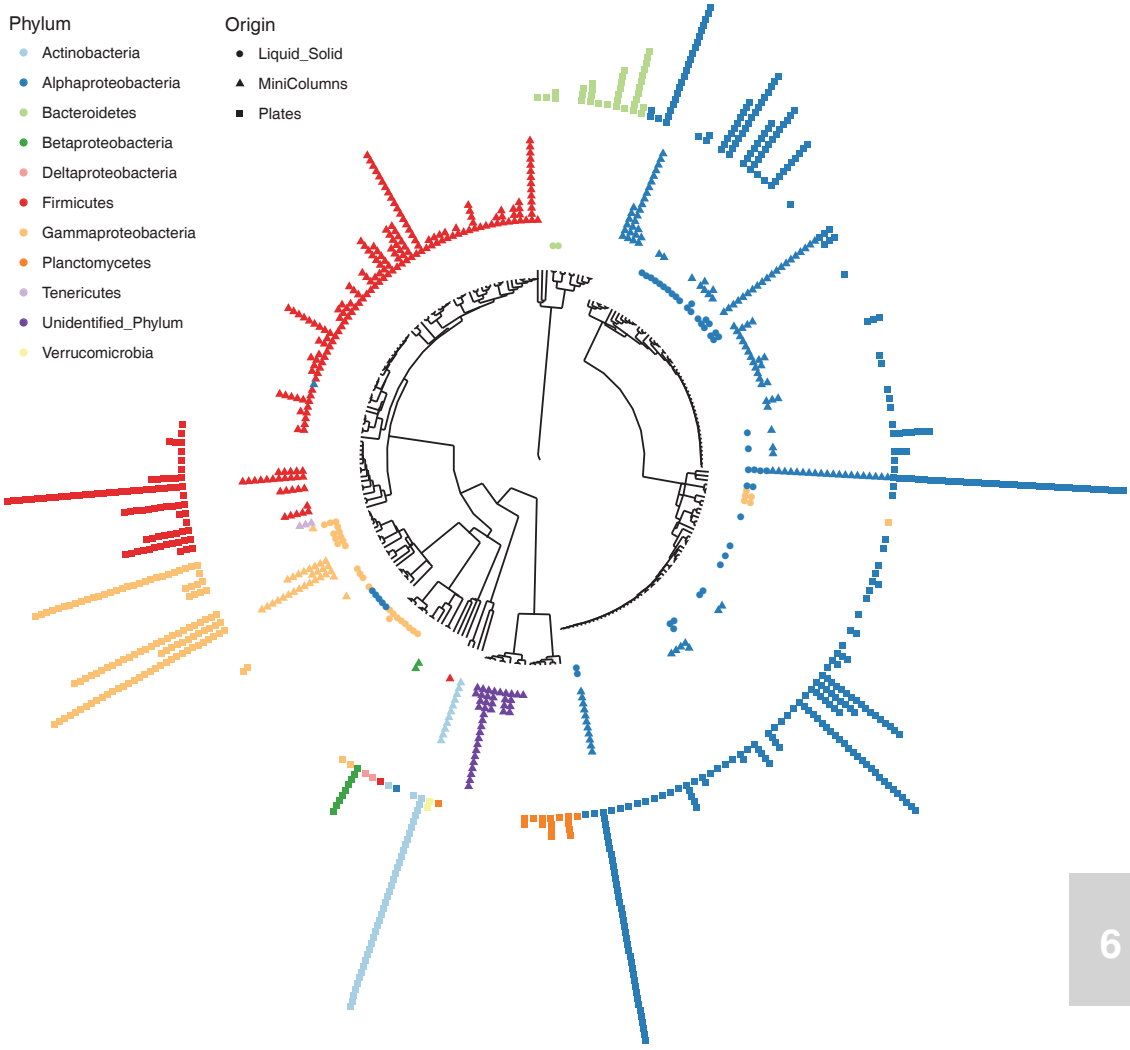


Figure 8 Subset of phylogenetic tree depicting phylogenetic diversity of cultivated microbial phyla (Class for Proteobacteria) from **Chapter 4**. Shapes indicate the different cultivation experiments. Number of shapes for each taxon depicts number of samples where taxon was detected. Tree was constructed using the Neighbour-joining method without distance corrections as implemented in Clustal Omega (McWilliam *et al.*, 2013).

Several phylogenetically novel members of the Flavobacteriia were isolated on oligotrophic media and are currently investigated further. Flavobacteriia, the biggest family within the Bacteroidetes phylum, display several unique characteristics such as the genetic ability to degrade algae-, plant-, bacteria- and vertebrate carbohydrates. Flavobacteriia are furthermore characterized by a novel type 9 secretion system that secrets proteins enabling their gliding motility (Gavriliidou *et al.*, 2019). Bacteroidetes seem to be part of the healthy sponge microbiome (Thomas *et al.*, 2016), and several members have been cultured (Versluis *et al.*, 2017; Yokoyama & Miki, 2017), however,

Flavobacteriia have also been isolated from sponges maintained in aquaculture for several months (Mohamed *et al.*, 2008), and found associated to diseased sponges (Webster, Xavier, *et al.*, 2008). Cytophagia, further cultivated members of the Bacteroidetes, have also been detected primarily in diseased sponges (Webster, Cobb, *et al.*, 2008; Webster, Xavier, *et al.*, 2008). Thus, Flavobacteriia and Cytophagia might actually be sponge pathogens and their role in sponge symbiosis remains currently unresolved.

Numerous Clostridia, some of which could only be taxonomically assigned until family level, were enriched in the anaerobic sediment fractions of the MiniColumns. Firmicutes have been associated to diseased specimen of *A. aerophoba* (Webster, Xavier, *et al.*, 2008) and significantly changed in their relative abundance after transplantation of sponge specimens (Meyer *et al.*, 2016). More commonly however, Clostridia represent dominant members of the microbial communities inhabiting marine sediments (Choi *et al.*, 2016) and might thus be only coincidentally present in sponges at the time of sampling. The spore-forming capabilities of this generally obligate anaerobic lineage (Yutin & Galperin, 2013) probably rendered Clostridia resistant to oxygen during sponge processing and cryopreservation and enabled their growth in the MiniColumns once the oxygen was depleted.

Actinobacteria are renown to be prolific producers of secondary metabolites, and are the source of about 70% of natural bioproducts currently in clinical use (Manivasagan *et al.*, 2014). Also sponge-associated Actinobacteria were reported to exhibit potent antimicrobial properties (Gandhimathi *et al.*, 2008; Abdelmohsen *et al.*, 2010; Grkovic *et al.*, 2014). We detected one actinobacterial OTU from the *Rothia* genus, which could successfully grow in numerous cultivation conditions within the Plates and MiniColumns experiments (Figure 8). *Rothia* strains are regularly isolated from marine sponges (Khan *et al.*, 2011; Abdelmohsen *et al.*, 2014), but also from plants (Xiong *et al.*, 2013) and some species are human pathogens (Tomczak *et al.*, 2013).

Extended incubation times allowed the growth of the rarely cultivated bacterial phyla Planctomycetes, Tenericutes and Verrucomicrobia. Planctomycetes, characterized by peptidoglycan-free cell walls, cell compartmentalization and budding mode of reproduction, have been isolated from *A. aerophoba* and other sponges before (Pimentel-Elardo *et al.*, 2003; Sipkema *et al.*, 2011; Izumi *et al.*, 2013). These highly oligotrophic bacteria represent close relatives of Poribacteria. However, the Planctomycetes OTUs detected in low abundance in *A. aerophoba* were different from the cultivated representatives, which were most closely related to *Rubripirellula* species commonoly associated to macroalgae (Bondoso *et al.*, 2014; Youssef & Elshahed, 2014).

Tenericutes are a unique class of bacteria that are typically parasites or commensals of eukaryotic hosts and lack a cell wall (Skennerton *et al.*, 2016). They have been found associated to mussles from Indonesian marine lakes (Cleary *et al.*, 2015), and to marine sponges (Garate *et al.*, 2016), but to date, relatively little is known about their role in environmental ecosystems or within sponges.

Verrucomicrobial 16S rRNA genes have been detected in marine animals, but only few cultivated representatives exist (Spring *et al.*, 2016). While this fastidious phylum is present in numerous sponge species, Verrucomicrobia have thus far only been cultivated from *Axinella polypoides* (Scheuermayer *et al.*, 2006), *Haliclona* (gellius) sp. (Sipkema *et al.*, 2011) and *Aplysina aerophoba*

(**Chapter 4**). None of these cultured representatives however match verrucomicrobial OTUs detectable in the sponge.
Taken together, these results indicate that the field of sponge microbiology in respect to microbial cultivation is still in its infancy and future efforts are necessary to advance cultivation technology in order to achieve a holistic understanding of the sponge holobiont and its metabolism.

Future Avenues for Cultivating Sponge-associated Microorganisms

The advances in sequencing technologies have provided large amounts of information on the genetic potential, as well as expressed functions of sponge-associated microbes (Chaib De Mares *et al.*, 2018; Kiran *et al.*, 2018). Through the advance of analysis methods and the integration of metagenomics with metatranscriptomics and metaproteomics (Chaib De Mares, 2018), a more and more complete and refined picture of microbial metabolism and possible interactions with other microbes or the sponge host can be drawn. Future projects targeting the isolation of sponge-associated microorganisms should consult multi-omics-derived information, which can narrow down the search space in the quest to define appropriate cultivation conditions (**Chapter 5**). Despite the great potential, to date, this field of information-based cultivation technology is still in its infancy.

Anaerobic and Autotrophic Conditions

The sponge as an animal needs oxygen for its central metabolism, however, sponges are known to tolerate very low oxygen conditions and presumably evolved long before atmospheric oxygen levels reached current concentrations (Mills *et al.*, 2014). In consequence, an anaerobic world has been discovered in high-microbial abundance sponges, which are often characterized by dense body tissue (Hoffmann *et al.*, 2005, 2008) and numerous anaerobic microbial metabolic pathways have been described using multi-omics approaches, such as denitrification and sulfate reduction (Taylor, Radax, *et al.*, 2007; Thomas *et al.*, 2010; Bayer, Moitinho-Silva, *et al.*, 2014). Additionally, the phylogeny and genomic features of numerous sponge-associated microorganisms point towards an anaerobic, or at least facultative anaerobic lifestyle (Hentschel *et al.*, 2006; Lavy *et al.*, 2014; Alex & Antunes, 2018; Kiran *et al.*, 2018). Strictly anaerobic cultivation led to the first and only report on the isolation of a sponge-associated member of the Chloroflexi (Brück *et al.*, 2010). Furthermore, the genes involved in autotrophic carbon fixation via the Wood-Ljungdahl pathway have been described for the otherwise presumably heterotrophic Poribacteria (Siegl *et al.*, 2011). Thus, anaerobic microbial cultivation could provide an interesting avenue for obtaining sponge-associated microorganisms while simultaneously selecting against dominant aerobic seawater-dwelling bacteria. A large challenge for anaerobic cultivation of sponge-associated microorganisms however is appropriate sample collection and processing, since infrastructure for anaerobic sample handling needs to be installed in laboratories in proximity to the sampling site.

Concurrently, instead of aiming to cultivate diversity, cultivation experiments could aim at the isolation of microorganisms that are capable of specific metabolic processes, such as denitrification, anammox or comammox. Multi-omics studies have discovered numerous sponge-associated microbes involved in aerobic nitrogen cycling, such as chemolithotrophic Nitrospira and

Thaumarchaea (Feng *et al.*, 2016; Moitinho-Silva *et al.*, 2017). Bacteria performing such metabolic activities have been cultivated from other environments previously and according isolation methods have been established (Strous *et al.*, 1999; Heylen *et al.*, 2006; Börner, 2016).

Mimick the Sponge Environment

Further improvements on mimicking conditions within a marine sponge might also prove beneficial. For example, the cultivation of sponge-associated bacteria in biomass-retaining bioreactors might provide better control over nutrient supply, oxygen concentrations and metabolic waste product removal, while simulating a flow comparable to what microorganisms might experience within the sponge. Furthermore, several sponge symbionts seem to possess the genetic potential for both heterotrophic and autotrophic lifestyles, which they might alternate during pumping and non-pumping periods of the sponge (Siegl *et al.*, 2011; Tian *et al.*, 2014). Bioreactor cultivation would enable regular fluctuations of oxygen supply, thus selecting for microbes capable of dealing with such changing environmental conditions. Bioreactor cultivation of entire sponges has been attempted previously (for a review, see Schippers *et al.* 2012), but to the best of my knowledge, enrichments of sponge microbes in batch- or steady-state bioreactors have not been attempted yet.

Focus on the Sponge Host

In addition, the importance of the sponge host should not be underestimated. The sponge holobiont evolved in intricate association with microorganisms and numerous modes of interaction have been identified that mediate between microorganisms and their sponge host. For example, sponge extract seems to contain chemical compounds that facilitate bacterial starvation recovery (Jung *et al.*, 2019). Proteins involved in eukaryote-prokaryote interactions such as eukaryotic-like proteins (ELPs), ankyrin repeats (ANKs) and tetratricopeptide repeats (TPRs) are regularly detected in sponge-associated bacteria and have been shown to modulate phagocytosis by amoebal cells (Nguyen *et al.*, 2014; Reynolds & Thomas, 2016). Potentially sponge-derived auto inducers such as cAMP and acyl homoserine lactones, as well as sponge extracts have also been found to enhance the cultivation potential of sponge-associated bacteria (Esteves *et al.*, 2016). Additionally, sponge-associated microorganisms seem to chemotactically navigate towards sponge-extracts in the sea (Tout *et al.*, 2017). However, cultivating sponge cells has been difficult thus far (Grasela *et al.*, 2012; Munroe *et al.*, 2019), and to date, only one genome of a low microbial abundance sponge has been published (Srivastava *et al.*, 2010). As a result, the contribution of the sponge animal to the sponge-microbe symbiosis can currently only be speculated about. Future efforts should also aim to collect more genetic and functional information on the sponge animal in order to gain insights into the role of the host within the holobiont.

Concluding, countless possibilities have remained unexplored in the context of sponge-associated microbial cultivation. But, on the bright side, it should be noted that the steadily increasing accumulation of multi-omic data and the resulting conclusions are continuously filling knowledge gaps, enabling the formulation of more concrete hypotheses on the metabolism of sponge-associated microorganisms. Incorporating these multi-omics-leads into cultivation efforts promises us to grasp the metabolic and functional complexity of sponges and their associated microbiomes in the future.

Concluding Remarks

Sponges harbor dense and diverse microbial communities with unique functional capabilities that render these extremely ancient aquatic animals exciting study objects. Especially during the last decade, the expansion of available multi-omics and bioinformatic tools has enabled unprecedented insights into the genetic foundation of associations between sponges and their microorganisms. Apart from identifying dominant microbial taxa, this enabled us to start understanding the metabolism and ecological importance of marine sponges and their microbiomes. However, the picture is far from complete. The discovery of a new phylogenetic group of Poribacteria in **Chapter 3** indicates that there is still large potential for identifying novel microbial diversity even within model sponges such as *Aplysina aerophoba*. Going beyond microbial diversity, we have begun to characterize sponge-associated microbiomes at a functional level. The large number of novel halogenases discovered in several *Aplysina* species in **Chapter 2** illustrate sponge microbiomes as a great, yet mostly untapped source of novel enzymes with interesting biotechnological properties. Taking this even further, the field of sponge microbiology is currently deciphering metabolic and functional interactions in the sponge microbiome. However, these multi-omics based findings remain mostly hypothetical. In **Chapter 4**, we describe several approaches to cultivate sponge-associated microorganisms, which would provide a basis for experimental validation of multi-omics based predictions. Microbial cultivation, however, is tedious and requires further attention and effort. Thus, the future challenge lies in creating a synergy between cultivation-dependent and cultivation-independent methods (**Chapter 5**), in order to form a holistic framework for studying sponge associated microbiomes in their entirety.

Appendices

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Summary

The research described in this thesis had the goal to increase our understanding of marine sponge microbial ecology by integrating both cultivation-dependent and –independent approaches. Simultaneously, steps towards accessing the biotechnological potential of sponge-associated microbes are presented in this work. Thus, the insights presented here will deepen our understanding of sponge microbial ecology as well as provide directions for further bioprospecting efforts targeting marine sponges, especially *Aplysina* species.

Sponges harbor dense and diverse microbial communities, and are key members of marine ecosystems. **Chapter 1** introduced the sponge, provided an overview of the importance of these animals in their environment and summarized the current knowledge on functional aspects of their associated microbiomes. This chapter furthermore outlined the biotechnological potential inherent to sponge-associated microorganisms, such as the production of secondary metabolites with antibiotic, antiviral and anticancer properties. Furthermore, a brief introduction of microbial cultivation was given, and previous efforts on obtaining sponge-associated microbes in culture were highlighted.

In many cases, the microbes inhabiting sponges have been demonstrated to be the actual producers of often halogenated bioactive secondary metabolites. Microorganisms attach halogen atoms such as chlorine or bromine to organic scaffolds using specialized enzymes, including halogenases. Such enzymes are of major biotechnological interest for the production of pharmaceutical or agrochemical compounds, since they halogenate regioselectively and under mild reaction conditions. In **Chapter 2**, six sponge species from the genus *Aplysina* were screened for flavin-dependent tryptophan halogenase sequence variants as well as the composition and structure of their bacterial communities using a PCR-based approach. In these sponge species from the Mediterranean and Caribbean seas we detected four phylogenetically diverse clades of putative tryptophan halogenase protein sequences, of which most were only distantly related to previously reported halogenases. The Mediterranean *A. aerophoba* harbored unique halogenase sequences, whereas the Caribbean species shared numerous sequence variants. By correlating the relative abundances of halogenases with those of bacterial taxa, we could identify prominent sponge-associated taxa belonging to Chloroflexi and Acidobacteria as putative owners of corresponding halogenase-encoding genes and therefore likely to be involved in the production of halogenated secondary metabolites in *Aplysina* spp.

Certain microorganisms have been found to be highly specific in their association to marine sponges and are rarely detected in other habitats. As such, members of the candidate phylum 'Poribacteria' are considered promising model microorganisms for studying the origin of host-microbe interactions in sponges. In **Chapter 3**, we investigated the global diversity and phylogenetic distribution of Poribacteria among different sponge hosts. By generating a phylogenetic network, we could decipher the genetic distances between poribacterial phylotypes and visualize their distribution amongst numerous sponge species. In total, 361 poribacterial 16S rRNA gene sequences were examined, and neither co-speciation with the host, nor biogeographical correlations could be detected. However, analyses resulted in the discovery of a novel phylogenetic clade of Poribacteria, which might represent a link between the previously established clades. We expanded the number

of Sanger-sequenced poribacterial 16S rRNA genes by approximately one third and could thus contribute to mapping the global diversity and distribution of this sponge-associated bacterial candidate phylum.

Chapter 4 describes several approaches to increase the cultivability of bacteria associated to the sponge *Aplysina aerophoba*. Alternative cultivation setups such as a Winogradsky-column approach, a liquid-solid media approach as well as media based on multi-omic-derived information on the metabolism of Poribacteria were applied. We found that most bacteria remained viable after cryo-preservation, however, only 2% of the initial diversity detected in *A. aerophoba* could be recovered through cultivation. We observed that medium dilution, rather than carbon source, most strongly affected the composition of cultivated microbial communities. Furthermore, the sponge-derived antibiotic aeroplysinin-1 negatively affected microbial growth, and selectively inhibited several taxa such as Flavobacteriaceae. The Winogradsky-column approach led to enrichment of distinct microbial communities at different locations in the columns, which included members of the class Clostridia and OTUs that were distantly related to the Planctomycetes. *Pseudovibrio* and *Ruegeria* spp. were obtained under almost all cultivation conditions applied, while other taxa such as Bacteroidetes were more specific to certain media types. Even though the predominant sponge-associated microorganisms remained uncultured, we could enrich 256 OTUs encompassing seven microbial phyla.

Microbial cultivation is often a tedious game of trial-and-error, however, pure or defined cultures are needed to decipher microbial physiology, curate and improve gene- and protein database annotations and realize novel biotechnological applications. **Chapter 5** discusses a novel approach to microbial cultivation: Multi-omics-derived information has accumulated exponentially over the last decade and provides a plethora of information awaiting integration into the development of novel cultivation strategies. This review summarizes ground-breaking studies that translated information derived from multi-omics into successful isolation strategies for previously unculturable microorganisms. Such strategies include specific media formulations, screening techniques and selective enrichments. Inspired by the microbial complexity of the environment, we integrated these inventive methods and concluded with proposing a workflow for future omics-aided cultivation experiments: Initial diversity estimation results in deciding the method for obtaining the genome of the targeted organism. Based on the resulting metabolic model, media can be formulated, while environmental parameters are included into defining cultivation conditions. Molecular probes can assist targeted screening strategies of novel high-throughput cultivation methods. This multi-omics integration should increase the chances of isolating novel microbial species.

Finally, **Chapter 6** integrated the findings from the previous chapters and portrayed them in the light of recent work from within the field of sponge microbiology and ecology. Using Poribacteria as an example, hypotheses on the origin of sponge-microbe symbiosis were combined and discussed. In short, mixed vertical and horizontal symbiont transmission resulted in a lack in host-species specificity and might indicate that sponge-poribacterial symbiosis originated in the Precambrian, before the phylum Porifera diverged into different classes. Additionally, aspects such as methods for assessing sponge-associated communities, or factors influencing microbial cultivability were elaborated on in more detail. Furthermore, this chapter summarized the successes, as well as failures, of isolating sponge-associated microorganisms and highlighted future avenues for

bringing these fastidious organisms into culture. In this thesis, we aimed to create a synergy between cultivation-dependent and cultivation-independent methods, by incorporating genomic predictions on carbohydrate metabolism as well as micro- and macro-environmental parameters into defining novel cultivation strategies for sponge-associated microorganisms. However, the inherent search space remains far from exhausted: Future efforts should integrate additional multi-omics-predicted metabolic capabilities such as anaerobic metabolic pathways or autotrophy into cultivation strategies, since cultivated representatives of sponge-associated microbes will reveal novel ways to tap into their biotechnological potential.

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"*Unconcerned but not indifferent is a good attitude to not get too overwhelmed with PhD life*".
(Detmer)

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List of Publications

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Gutleben, J., Koehorst, J. J.[4], McPherson, K.[1], Pomponi, S.[6,8], Wijffels, R. H., Smidt, H., Sipkema, D. (2019) Diversity of Tryptophan Halogenases in Sponges of the Genus *Aplysina*. FEMS Microbiology Ecology 95 (8).

Gutleben, J., Loureiro, C.[1], Ramírez Romero, L. A.[1], Shetty, S. A. [1], Wijffels, R. H., Smidt, H., Sipkema, D. Cultivation of Bacteria from *Aplysina aerophoba*: Effects of Oxygen and Nutrient Gradients (manuscript submitted to Frontiers in Microbiology)

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About the Author

Johanna Gutleben was born on the 2nd of March 1990 in Innsbruck, Austria. She started studying Environmental, Process and Biotechnology at MCI Management Center Innsbruck, where she obtained her BSc degree in in 2011. From 2011 until 2013 she participated in an Erasmus Mundus European Master program in Animal Breeding and Genetics, for which she studied at Wageningen University and AgroParisTech, Paris, France.

During her first year in Wageningen, she conducted thesis research on the diversity of micro-eukaryotic communities in microbial mats. In the course of this research she worked at NIOZ (The Royal Netherlands Institute of Sea Research) in Yerseke under the supervision of Henk Bolhuis, as well as at the Animal Genetics group at WUR under the supervision of Ole Madsen and Hilde van Pelt (IMARES). During the second year of the master studies she conducted thesis research on the influence of antibiotic treatments on coral mucus associated microbial communities. This research was carried out at the Oceanographic Institute of Nha Trang, Vietnam, under the supervision of Yvan Bettarel (IRD) and Hanh Nguyen, as well as at Montpellier University 2, France, under the supervision of Thierry Bouvier (CNRS).

In February 2014, Johanna started in the Molecular Ecology group of the Laboratory of Microbiology at Wageningen University as a PhD fellow in the Research and Training Network BluePharmTrain, which was coordinated by Detmer Sipkema. BluePharmTrain aimed at using marine sponges for the development of new pharmaceuticals. Johanna's project focussed on developing methods for cultivating novel microorganisms from sponges, as well as on further characterizing the microbial diversity and biotechnological potential associated to marine sponges. She put special attention on the Mediterranean species *Aplysina aerophoba*, which was one of the target species of the BluePharmTrain project. Her research was supervised by Detmer Sipkema, as well as Prof. Hauke Smidt and Prof. René Wijffels, and most of this work is described in this thesis.

At the moment, Johanna works and lives on her family's farm in the Austrian Alps and is currently exploring options for future challenges.

SENSE

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

for specialised PhD training

The Netherlands research school for the
Socio-Economic and Natural Sciences of the Environment
(SENSE) declares that

Johanna Gutleben

born on 2 March 1990 in Innsbruck, Austria

has successfully fulfilled all requirements of the
educational PhD programme of SENSE.

Wageningen, 21 November 2019

The Chairman of the SENSE board

Prof. dr. Martin Wassen

the SENSE Director of Education

Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Johanna Gutleben** has successfully fulfilled all requirements of the educational PhD programme of SENSE with a work load of 51.9 EC, including the following activities:

SENSE PhD Courses

- Environmental research in context (2014)
- Spring School Host-Microbe Interactomics (2014)
- Principles of Ecological Genomics (2015)
- SENSE writing week (2017)
- Research in context activity: 'Organization Committee of the "Laboratory of Microbiology PhD Trip" and "Blue Symposium, Sponges, Corals and the World" (2017)'

Other PhD and Advanced MSc Courses

- BluePharmTrain Summer School: Research and Time Management & Science Ethics, MATIS (2014)
- BluePharmTrain workshop Biochemistry and Metagenomics, University of Cork (2014)
- Information Literacy, Wageningen University (2014)
- Introduction to R for statistical analysis, Wageningen University (2014)
- Introduction to Marine Biology – From Elements to Ecosystems, HYDRA Institute (2014)
- BluePharmTrain summer school Entrepreneurship, Cardiff University (2015)
- BluePharmTrain workshop Host-microbe interactions, Technical University Tallinn (2015)
- BluePharmTrain workshop Scientific Illustrations, ETH Zurich (2016)
- BluePharmTrain Summer School: Statistical analysis using R, Wageningen University
- Adobe InDesign Essential Training, Wageningen University (2016)
- (Meta-)Genomic Data analysis, University of Groningen, (2016)
- SIAM Writing Week, MIB/SIAM (2018)

Management and Didactic Skills Training

- Supervising 6 MSc student with thesis (2014-2018
- Teaching in the BSc/ MSc course 'Microbial Ecology' (2014-2017)
- Teaching in the BSc/ MSc course 'Research Methods in Microbiology' (2016)

Oral Presentations

- *Cultivation of sponge microorganisms,* LIPI Bogor, 6 November 2015, Cibinong, Indonesia
- *Co-Cultivation of Sponge Cells and Microorganisms.* 2nd International Symposium of Sponge Microbiology, 26-29 October 2014, Baltimore, United States of America
- *Integrating (Meta)genomics data into sponge symbiont cultivation.* 100 years of Microbiology Wageningen University and Research, 21 October 2017, Wageningen, The Netherlands

SENSE coordinator PhD education

Dr. ir. Peter Vermeulen

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