Diversity of prokaryotic communities associated with sponges in Vietnam and their secondary metabolite biosynthetic potential

Ton That Huu Dat

Propositions

1. Efforts in cultivation of sponge symbionts need focus on the development of *in situ* cultivation methods.
   (this thesis)

2. Metagenomics reveals a striking number of secondary metabolite biosynthetic gene clusters, but translating these cluster genes into compounds is still at a standstill.
   (this thesis)

3. Marine microplastic pollution and its impacts on marine organisms are underestimated due to technology and resource limitations.

4. Open access helps scientists in low-income countries to more easily access publications, but also prevents them from publishing.

5. The uncontrolled development of hydropower makes floodings more severe and negatively impacts nature.

6. Technology helps football referees make more accurate decisions, but reduces the emotion of the game.

Propositions belonging to the thesis, entitled
Diversity of prokaryotic communities associated with sponges in Vietnam and their secondary metabolite biosynthetic potential

Ton That Huu Dat
Wageningen, 13 November 2023

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This research was conducted under the auspices of VLAG Graduate School (Biobased, Biomolecular, Chemical, Food and Nutrition Sciences)

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 13 November 2023
at 11 a.m. in the Omnia Auditorium.

Ton That Huu Dat
Diversity of prokaryotic communities associated with sponges in Vietnam and their secondary metabolite biosynthetic potential,
262 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)
With references, with summary in English

ISBN: 978-94-6447-908-9
DOI: https://doi.org/10.18174/639348

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Chapter 1

General Introduction

Marine sponges: origin and diversity

Sponges (phylum Porifera) are among the most ancient extant Metazoa and hold the basal position in the metazoan phylogeny (Borchiellini et al., 2001; Halanych, 2004; Klautau and Russo, 2016; Feuda et al., 2017). Sponges most likely originated from a benthic colony of choanoflagellate-like organisms that might have distinct cell types for feeding (protochoanocytes) and reproduction (protoarchaeocytes) (Valentine, 2004; Klautau and Russo, 2016). These organisms later developed multicellular bodies with differentiated cell types and an extracellular matrix to evolve into the metazoan ancestor, likely a marine sponge, more specifically, a larva of a homoscleromorph-like organism (Maldonado, 2004; Klautau and Russo, 2016). The earliest evidence of ancient sponges is a trace of 24-isopropylcholestane, a substance generated by the breakdown of lipids in sponges, in the fossil record from the Cryogenian Period of the Proterozoic Era (about 635 million years ago) (Love et al., 2009; Love and Summons, 2015; Gold et al., 2016). Furthermore, evidence of sponge skeletons is found in rocks from the Ediacaran Period (630 million to 542 million years ago) (Yin et al., 2015b; Gold et al., 2016). However, the most recent evidence suggests that sponges may have even appeared 890 million years ago in the early Neoproterozoic era (Zumberge et al., 2018; Turner, 2021).

Sponges are distributed globally (Van Soest et al., 2012; Bell et al., 2015). However, the majority of sponges live in the marine environment, and only a few species are found in the freshwater environment (Manconi and Pronzato, 2008; Van Soest et al., 2012; Prozanto et al., 2017). Particularly, in reef ecosystems, sponges are crucial components of the benthic community, contributing up to 80% of the biomass and play key functional roles in marine ecological processes (Bell, 2008; Wulff, 2016; Pawlik and McMurray, 2020). To date, sponges are still among the most diverse and abundant aquatic animals (Van Soest et al., 2012). According to the World Porifera Database, more than 9,500 valid sponge species have been identified so far (de Voogd et al., 2022).

There are five recognized classes of sponges mainly based on the composition of their skeletons, including Archaeocyatha (pleospongea), Calcarea (calcareous sponges), Hexactinellida (glass sponges), Demospongiae (demosponges), and the recently-recognized, encrusting sponges (Homoscleromorpha) (Van Soest et al., 2012; Klautau and Russo, 2016). Archaeocyatha have calcium carbonate skeletons and members of this class were crucial reef builders during the Cambrian period before becoming extinct (Wood et al., 1992; Antcliffe et al., 2019). Calcarea is the only living class that has skeletons made of calcite, which may form separate spicules or large masses. Demospongiae is the largest and most diverse class, representing up to 80% of the phylum (Van Soest et al., 2012) and is the only class that has colonized freshwater habitats and evolved carnivory. Demospongiae often have silicate spicules or spongin fibers or both within their soft tissues, but a few species also have massive

external skeletons made of aragonite, another form of calcium carbonate (Bergquist, 2001; Hooper et al., 2002). Hexactinellida is mostly represented by deep-sea sponges and has silicious spicules, with the largest spicules having six rays that may be individual or fused (Hooper et al., 2002). The main components of their bodies are syncytia in which large numbers of cells share a single external membrane (Bergquist, 2001). Homoscleromorpha was previously considered a subclass of Demospongiae, but it has recently been recognized as the smallest class of Porifera (Gazave et al., 2012; Morrow and Cárdenas, 2015). The class is notable by unique features such as flagellated pinacocytes and a basal membrane lining both choanoderm and pinacoderm, oval to spherical choanocyte chambers with large choanocytes, and viviparous cinctoblastula larvae (Van Soest et al., 2012). The skeleton of members of this class is composed of tetraxonic siliceous spicules with four equal rays (called calthrops) and derivatives with reduced (diods, triods) or proliferated rays (lophocalthrops).

Sponge identification and classification

Generally, animal identification and classification at the species level using morphological characters are still popular to date, applying morphology-based species concepts (De Queiroz, 2007). Nonetheless, strict morphological identification may result in misidentifications and incongruent evolutionary theories because distinct morphological features are often lacking or misinterpreted (Jenner, 2004). Likewise, traditional sponge identification mainly relies on morphological characteristics, such as spicule shapes and other skeletal features of sponges (Hooper and Van Soest, 2002; Uriz et al., 2003). Unfortunately, traditional sponge identification is seriously hampering due to the simplicity and plasticity of their morphological traits, as well as the absence of macroscopic hard structures in the majority of sponge species (Cárdenas and Rapp, 2012).
With the development of molecular techniques, DNA barcoding has been applied to identify sponges, such as sequencing fragments of the gene encoding the cytochrome oxidase subunit 1 (CO1), 28S ribosomal RNA (rRNA) and 18S rRNA. Nonetheless, even with molecular approaches, sponge identification is not always reliable. The different evolution rates of DNA barcoding genes and rampant polyphyly in sponges challenge species-level molecular resolution (Erpenbeck et al., 2007; Redmond et al., 2011). Therefore, for many sponge species, identification must combine histological and multi-locus phylogenetic approaches (Erpenbeck et al., 2006; Redmond et al., 2011; Morrow and Cárdenas, 2015; Yang et al., 2017). Chemosystematics has later been suggested as an alternative identification approach for such sponge species, albeit with limited success (Erpenbeck and van Soest, 2007).

Sponge body and aquiferous system

Sponges are among the structurally simplest metazoans and lack specialized tissues, organs, nerves, muscles, and even epithelia (Figure 1) (Ereskovsky and Dondua, 2006). Instead,

sponges have specialized cell types to perform specific functions. Typically, the outer layer (pinacoderm) is composed of flattened cells called pinacocytes (i.e., basopinacocytes, exopinacocytes, and endopinacocytes) (Ereskovsky and Dondua, 2006). This cell layer harbors many pores (ostia) that allow the surrounding seawater to enter inhalant canals in the sponge body (Van Soest et al., 2012). The seawater is then filtered through a cell layer of choanocytes, forming the choanoderm. Choanocytes are highly specialized collar cells that create water flow through the canals and capture food particles such as bacteria, archaea, viruses and unicellular algae (Hentschel et al., 2012). The captured particles are subsequently transported to the mesohyl and are digested by archaeocytes via phagocytosis or retained in the mesohyl as associated microbes (Hentschel et al., 2006; Wehrl et al., 2007). Finally, the filtered seawater is extruded from sponges by exhalant canals via exhalant pores (oscula) (Van Soest et al., 2012).

The network of canals and choanocyte chambers forms the aquiferous system of sponges. Based on its complexity, the aquiferous system of sponges is classified into five main types i.e., asconoid, syconoid, sylleibid, leuconoid, and solenoid (Figure 1) (Cavalcanti and Klautau, 2011). With highly efficient aquiferous systems, several certain sponge species are able to filter a large volume of seawater, up to 24,000 litres per kg per day (Vogel, 1977). Nonetheless, their water filtration ability varies widely among species and may be regulated in response to environmental changes through dilatation or restriction of ostia, canals, and oscula diameter (Asadzadeh et al., 2020; Dahihande and Thakur, 2021). Additionally, active pulsatile contractions may participate in enhancing the efficiency of pumping (Nickel et al., 2011). Surprisingly, one notable exception is that most carnivorous sponges lack an aquiferous system and its choanocytes. Therefore, rather than filter feeding, these carnivorous sponges have developed the capacity to passively capture tiny invertebrates such as crustaceans using filaments or other appendages, coupled with an adhesive surface and the ability of sponge cells to transport to and wrap prey objects (Hestetun et al., 2017; Godefroy et al., 2019).

The mesohyl is one of the most notable components of the sponge body. It is a fibril-rich, loosely organized layer between pinacoderm and choanoderm (Ereskovsky and Dondua, 2006). This layer contains a variety of cell types, including totipotent cells known as archaeocytes, cells involved in the synthesis of organic and inorganic skeleton synthesis (i.e., sclerocytes, spongocytes and lophocytes), cells supposed to be involved in chemical synthesis and exocytosis of granular and spherulous cells, as well as gametes, skeletal elements, and symbiotic microbes (Ereskovsky and Dondua, 2006; Renard et al., 2013).

The mesohyl serves as an endoskeleton and maintains the tubular shape of sponges (Renard et al., 2013). The skeletons of sponges are comprised of spicules and spongin fibres, which are organized in diverse forms to provide strength and flexibility (Hooper and Van Soest,

2002; Uriz et al., 2003). The tiny spicules (microscleres) are randomly distributed on the surfaces, whereas the larger spicules (megascleres) maintain the general shape of sponges (Uriz et al., 2003). Calcarea possess spicules composed of calcium carbonate in crystalline forms e.g., calcite and aragonite, whereas Hexactinellida, Homoscleromorpha, and Demospongiae possess siliceous spicules, which are mostly formed of silicic acid, minor amounts of sodium, potassium, iron, and chlorine-containing substances, and a small amount of organic matter, called spiculin, which forms an axial fibre (Uriz et al., 2003; Sperling et al., 2010). However, not all sponge species have spicules, e.g., families Oscarellidae, Aplysinidae, the subclasses Keratosa and Verongimorpha (except for the family Chondrillidae) lack spicules (Boury-Esnault et al., 1995; Erpenbeck et al., 2012; Gazave et al., 2012; Łukowiak et al., 2013).

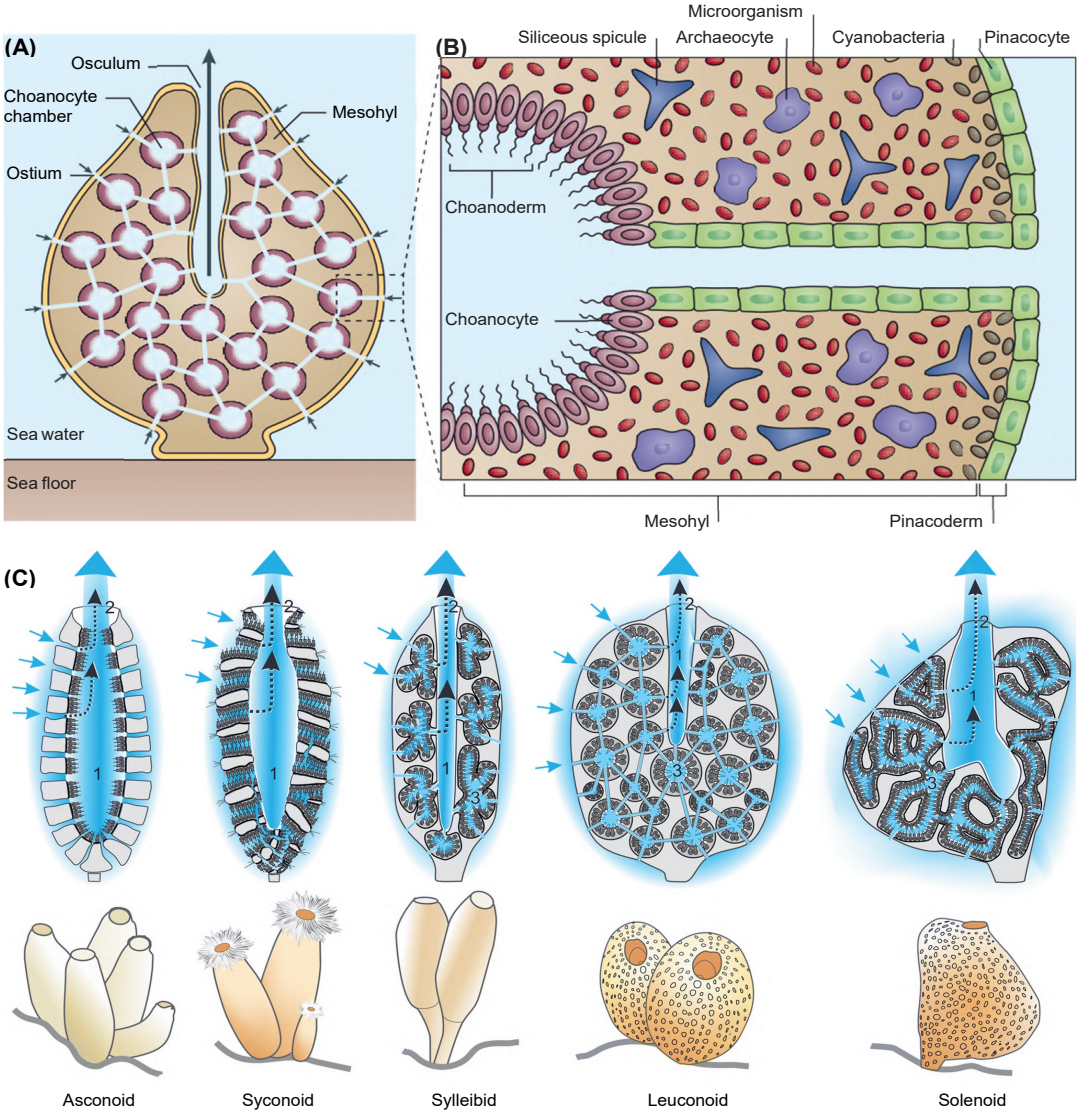


Figure 1. Body plan of marine sponges. A - Schematic overview of a typical demosponge.

B - Enlargement of the internal structure of a typical demosponge (Adapted from Hentschel et al. (2012) with permission from Nature Portfolio). C - Schematic representation of the five aquiferous systems. Arrows indicate the direction of the seawater flow into the spongocoel or atrium (1) and out through the oscule exhalant pore (2). In the more complex types, the water initially passes through a choanocyte chamber (3). (Adapted from Godefroy et al. (2019) with permission from Springer Nature.

Ecological roles of marine sponges

Sponges are globally distributed across marine and fresh-water ecosystems. They are key components of the benthic community in reef ecosystems, and playing numerous ecological functions (Bell, 2008; Wulff, 2016; Pawlik and McMurray, 2020). The key architectural roles of sponges on reefs include building and fortifying the reef framework with carbonate and siliceous skeletons, promoting reef-frame integrity, increasing coral survival by adhering living corals to the reef and protecting exposed skeletons against eroders, stabilizing rubble after physical damage and improving reef restoration by harnessing the ability of sponge to bind rubble (Bell, 2008; Wulff, 2016). Sponges also play vital biological roles on reefs such as providing shelter for symbiont species e.g., echinoderms, worms, mollusks, arthropods, fishes and multicellular algae, and food for other mobile organisms such as angelfishes, hawksbill turtles, and nudibranchs (Wulff, 2016).

With their well-developed aquiferous systems, sponges efficiently filter a large amount of water and food particles, thereby having the potential to substantially influence nutrient cycling. Sponges do not only remove nutrients (e.g., carbon, nitrogen, phosphorus, sulfur, silicon) from the water column but also return processed nutrients e.g., particulate organic matter (POM), dissolved organic nitrogen (DON), dissolved organic carbon (DOC), and dissolved organic matter (DOM) back to the water column (Maldonado et al., 2012; Pawlik and McMurray, 2020). Recent findings indicated that sponges are key contributors in the central trophic pathway of DOM to higher trophic levels, the so-called 'sponge loop' (Figure 2) (de Goeij et al., 2013). In this ‘sponge loop’, sponges make DOM available to fauna by rapidly discharging filter cells as detritus, which is then consumed by reef fauna (de Goeij et al., 2013; Rix et al., 2018). Subsequent studies on the unprecedented losses of Caribbean coral reefs suggested a vicious-cycle hypothesis, where sponges and macroalgae exchange nutrients and dissolved organic carbon (DOC) in a positive feedback loop that causes a negative impact on reef-building corals through direct spatial competition (Pawlik et al., 2016). The detritus produced by either sponges or decomposing seaweeds may limit light penetration and increase stress on corals in Caribbean reefs (Mumby and Steneck, 2018). Significant uptake and depletion of silicon by sponges suggest that sponges may play crucial roles in global silicon cycling (Cerrano et al., 2004; Maldonado et al., 2011; Tréguer and De La Rocha, 2013; Pawlik and McMurray, 2020). Accordingly, abundant sponge populations

can have a considerable impact on the bentho-pelagic coupling by being involved in carbon, nitrogen, sulfur, phosphorus, and silicon cycles (Maldonado et al., 2005; Bell, 2008; Maldonado, 2016; Pawlik and McMurray, 2020).

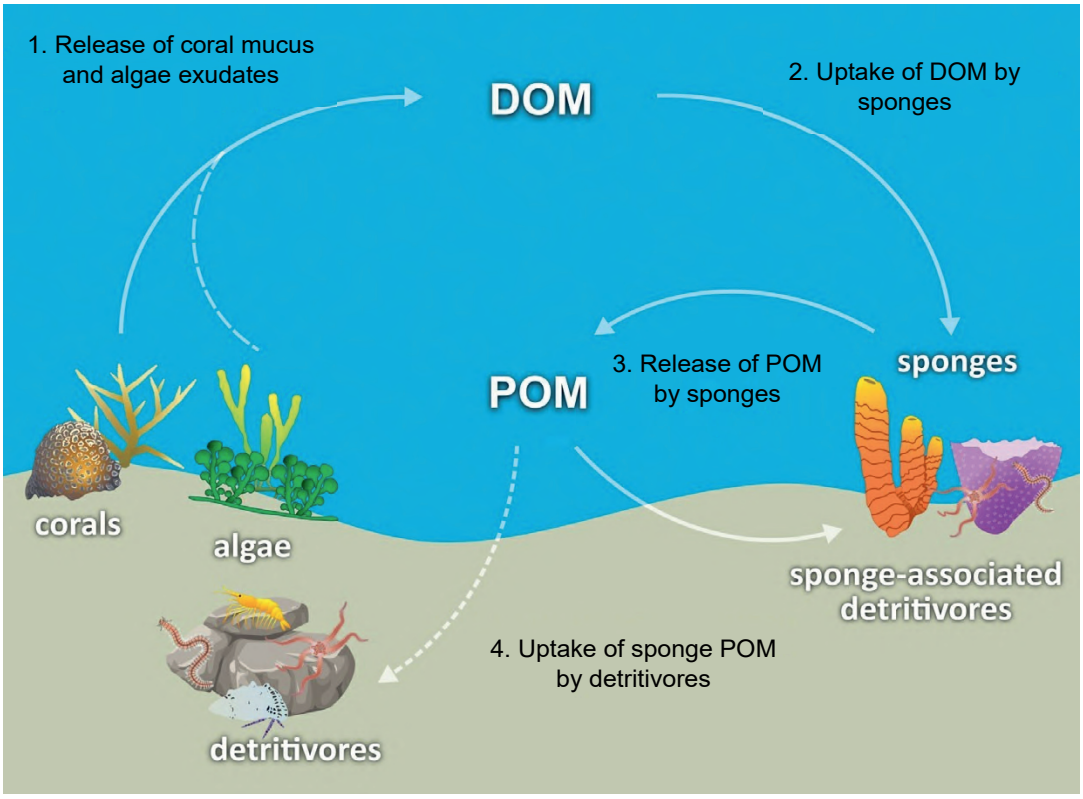


Figure 2. The steps of the sponge loop pathway: (1) corals and algae release exudates as dissolved organic matter (DOM), (2) sponges take up DOM, (3) sponges release detrital particulate organic matter (POM), (4) sponge detritus (POM) is taken up by sponge-associated and free-living detritivores. Adapted from Rix et al. (2018).

Chemical ecology of marine sponges

In marine habitats, sponges are often affected by predation by spongivorous animals, such as fishes, turtles, and nudibranchs (Ruetzler, 2003; Lesser, 2006; Wulff, 2006; Emmett et al., 2021). Several sponge species are particularly adapted to a higher risk of predation by using specialized structural and chemical defensive mechanisms (Burns et al., 2003; Burns and Ilan, 2003; Rohde and Schupp, 2011).

Structural components of the sponge, such as siliceous spicules, internal chitin fibers, and spongin elements may serve as physical protection against prospective predators (Chanas and Pawlik, 1995; Chanas and Pawlik, 1996; Hill and Hill, 2002; Burns and Ilan, 2003; Uriz et

al., 2003; Jones et al., 2005; Malcolm et al., 2005), which is similar to the functions of external spines of cactuses in terrestrial plants (Aliscioni et al., 2021). Therefore, herbivory defense mechanisms in plants are often used to illustrate the defensive strategies of sponges (Pawlik, 2011; Rohde and Schupp, 2011). Nonetheless, these physical defenses of sponges vary greatly and there is no consistent trend towards a generally defensive effect of these structural components (Chanas and Pawlik, 1995; Chanas and Pawlik, 1996; Burns and Ilan, 2003; Jones et al., 2005).

Chemical defense is a common mechanism of defense in numerous sponge species to prevent predation (Thacker et al., 1998; Wilson et al., 1999; Burns et al., 2003; Thoms and Schupp, 2007; Pawlik, 2011; Loh and Pawlik, 2014; Slattery et al., 2016). This is comparable to plants, which rely on defenses induced by inedible or toxic secondary products (Mithöfer and Maffei, 2017). In addition, the sponge-derived natural products protect sponges against overgrowth by other benthic sessile animals such as, e.g., corals, ascidians, bryozoans, algae, and other sponges (Kashman et al., 1980; Sullivan et al., 1983; Thompson et al., 1985; Thacker et al., 1998; Engel and Pawlik, 2000; Kubanek et al., 2002; Singh and Thakur, 2016; Thakur and Singh, 2016; El-Demerdash et al., 2018).

Experimental investigations with the addition of extracts and chemical compounds from sponges in artificial diets of omnivorous or spongivorous reef fish indicated that these compounds play an essential role in anti-predatory defenses (Waddell and Pawlik, 2000; Pawlik, 2011; Rohde and Schupp, 2011; Loh and Pawlik, 2014; Slattery et al., 2016). For example, an investigation with more than 70 Caribbean sponge species reveals that the majority of the investigated sponges (69%) are chemically defended (Pawlik et al., 1995). A later investigation of 109 Caribbean sponge species also revealed chemical defense in the majority of the studied sponges (57.5%) (Loh and Pawlik, 2014). Correspondingly, the analyses of fish guts revealed that sponges without known chemical defenses are the primary diet of sponge predatory fish (Pawlik, 2011). Concerning the proposed structural defense mechanisms of sponges, most results indicate that chemical defense is the main anti-predatory mechanism in sponges, which can be complemented by structural defenses, like spicules (Pawlik, 2011).

Natural products from marine sponges

The capacity of sponges to produce numerous secondary metabolites with potential biopharmaceutical applications is one of the primary drivers for the extensive studies on sponges. The oceans contain a largely untapped reservoir of secondary metabolites with over 39,000 marine natural compounds identified so far (Blunt and Munro, 2022). Among them, sponges contribute up to 30% of all known marine natural products. They have been the most prominent source of novel marine secondary metabolites during the past decades with more

than 200 new compounds reported each year (Hu et al., 2015; Carroll et al., 2021; Hong et al., 2022). These compounds include structurally diverse chemical classes, such as alkaloids, amino acid derivatives, lactones, macrolides, peptides, polyketides, sterols and terpenoids (Tasdemir et al., 2002; Sánchez et al., 2006; Molinski et al., 2009; Ebada et al., 2010; Han et al., 2019; Bian et al., 2020). Sponge-derived compounds have a high biotechnological potential (Proksch et al., 2003; Sipkema et al., 2005; Fenical, 2006; Anjum et al., 2016) with diverse pharmacological properties such as, e.g., antibacterial, antifungal, antitumor, antiviral, antifouling, and anti-inflammatory compounds (Sipkema et al., 2005; Mehbub et al., 2014; Dobson et al., 2015; Jaspars et al., 2016; Mehbub et al., 2016; Choudhary et al., 2017; Shady et al., 2017; Zhang et al., 2017a; Han et al., 2019; Khalifa et al., 2019; Zhu et al., 2019; Bian et al., 2020; Bai et al., 2021).

Numerous promising bioactive compounds have been isolated from marine sponges, for instance, the anticancer compound halichondrin B (Hirata and Uemura, 1986), the thrombin inhibitory molecule nazumamide A (Hayashi et al., 1992), the antibacterial compounds isoaaptamine and psammaplin A, the antifungal compounds lasonolide A, neopeltolide and aurantoside A (Sata et al., 1999; Laport et al., 2009), the antimalarial molecule (E)-oroidin, the antiprotozoal compounds plakortide B, parguesterol A and B with antituberculosus activity, the antiviral compounds manzamine A, mirabamides A, C, and D, and the anti-inflammatory and antipsoriatic compound avarol (Ferrándiz et al., 1994; Laport et al., 2009). However, very few clinical studies have been conducted on them (Ghareeb et al., 2020). This is because of the insufficient supply of pure compounds for clinical trials. Normally, marine organisms produce only minute concentrations of natural products, frequently less than 1 ppm of the wet weight (Mendola, 2003; Proksch et al., 2003). To date, only a few FDA-approved synthetic drugs are originated from sponge-derived compounds e.g., cytarabine (arabinosyl cytosine; Ara-C; cancer treatment) and vidarabine (arabinofuranosyladenine; Ara-A; viral disease treatment), eribulin mesylate, and palytoxin (Lindequist, 2016; Ghareeb et al., 2020). Hence, alternative strategies need to be developed to fulfil the demands for drugs, including, e.g., multi-omics approaches, cloning and heterologous expression, chemical synthesis and semi-chemical synthesis, and *in silico* approaches (Wright et al., 2014; Wohlleben et al., 2016; Loureiro et al., 2018; Wright, 2019; Kiriiri et al., 2020; Lin et al., 2020; Atanasov et al., 2021).

Sponge-associated microbes

The symbiotic relationship between sponges and their symbionts is evidenced early in the evolutionary history of marine animals. Evidence suggests that prokaryotes most probably established symbiosis in ancestral sponges during the late Precambrian (Sara et al, 1997). The immunological evidence obtained based on common immunological responses to bacteria from diverse sponges implies that bacterial symbionts may have originated from

about 600 million years ago (Wilkinson and Smith, 1984), making sponge-microbe partnerships one of the most ancient animal-microbe relationships known to date (Taylor et al., 2007). With recent advancements in molecular tools, particularly high throughput amplicon sequencing of marker genes and metagenomics, the insights into sponge microbiomes, such as the diversity, community structures and phylogenetic relationships of sponge-specific microbial community assemblages, as well as their potential establishment and maintenance mechanisms, are slowly being discovered (Hentschel et al., 2012; Thomas et al., 2016; Chaib De Mares et al., 2017).

As filter-feeding metazoans, marine sponges shelter diverse and abundant microbial communities. Nonetheless, the density of their associated microbial populations is dissimilar between various sponge species (Hentschel et al., 2006), leading to the division of sponges into "high-microbial-abundance" (HMA) and "low-microbial-abundance" (LMA) sponges (Reiswig, 1981; Hentschel et al., 2006). HMA sponges contain prokaryotic densities in the range of 10^8–10^{10} cells per gram of sponge wet weight (Hentschel et al., 2012), whereas LMA sponges contain only 10^5–10^6 prokaryotic cells per gram of sponge wet weight, being similar to prokaryotic densities in seawater (Taylor et al., 2007; Moitinho-Silva et al., 2017c). HMA sponges often host richer and more diverse microbial communities than LMA sponges, and several microbial taxa found differently abundant in either group have been used as indicator taxa in the HMA and LMA predictions by a machine learning study (Moitinho-Silva et al., 2017c). However, the LMA-HMA dichotomy in sponges should be considered carefully by a combination of electron microscopical and microbiome data, especially for sponge species that harbor intermediate prokaryotic loads (Bayer et al., 2014a; Gloeckner et al., 2014) or sponge species that have inconsistent microbiomes with HMA-LMA microbiome profiles (Noyer et al., 2010; Bayer et al., 2014a; Moitinho-Silva et al., 2014a). Furthermore, it should be noted that this division into HMA and LMA sponges generally does not follow discernable phylogenetic and geographic patterns (Bayer et al., 2014a; Gloeckner et al., 2014; Erwin et al., 2015).

Sponges host diverse microbial communities, encompassing all three domains of life including Bacteria, Archaea and Eukarya (Hentschel et al., 2012). To date, sponges have been reported to associate with more than 60 microbial phyla, including the most abundant taxa *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Crenarchaeota*, *Cyanobacteria*, *Firmicutes*, *Nitrospirae*, *Proteobacteria*, *Spirochaetes* and several candidate phyla such as Poribacteria, PAUC34f, and the so-called sponge-associated unclassified lineage (SAUL) (Hentschel et al., 2012; Reveillaud et al., 2014; Thomas et al., 2016; Moitinho-Silva et al., 2017b). Members of *Candidatus* Poribacteria were formerly considered to be exclusively found in sponges (Fieseler et al., 2004). However, the recent discovery of 13 putative *Candidatus* Poribacteria-related metagenome-assembled genomes from ocean water samples (Tully et al., 2018) resulted in the reclassification and

differentiation of sponge-associated *Entoporibacteria* and free-living *Pelagiporibacteria* within the phylum (Podell et al., 2019). In addition, *Candidatus* Tethybacterales, a newly identified sponge-specific order of ubiquitous sponge beta-proteobacterial symbionts, has been described with two families *Candidatus* Tethybacteraceae and *Candidatus* Persebacteraceae (Taylor et al., 2021). Interestingly, the *Candidatus* Tethybacterales are found in phylogenetically diverse sponges that represent both HMA and LMA sponges (Taylor et al., 2021). Overall, phylogenetic reconstructions based on more than 7500 near-full-length 16S rRNA gene sequences lead to the proposal of 173 globally distributed sponge-specific, monophyletic sequence clusters (Simister et al., 2012b). However, subsequent studies revealed that parts of the sponge-specific prokaryotes can also be found in very low abundances in various marine environments (Taylor et al., 2013; Thomas et al., 2016). Therefore, these clusters are not strictly "sponge-specific," but are better described as "sponge-enriched" (Taylor et al., 2013; Thomas et al., 2016). Nevertheless, these findings continue to support the general perception that the taxonomic composition of sponge-associated microbes can be distinguished from the microbial communities present in the nearby seawater and sediment e.g., (Vacelet and Donadey, 1977; Wilkinson, 1978b; a; c; Hentschel et al., 2002; Taylor et al., 2004b; Webster et al., 2010; Erwin et al., 2015; Moitinho-Silva et al., 2017c).

The diversity of the prokaryotic community appears to be influenced by sponge host phylogeny at different levels. Generally, the composition of sponge symbionts may be characterized by specialists (i.e. found only in one or a few sponge species) and generalists (i.e. found in numerous sponges) (Schmitt et al., 2012).

Ecological functions of sponge-associated microbes

Marine sponges host dense and diverse microbial communities, leading to the assumption that the sponge microbiota carries out functions that support sponge physiology and ecology (Figure 3). Nevertheless, the knowledge on symbiont functions remains limited to date, which is partly because all of predominant sponge symbionts remained recalcitrant to cultivation. More recently, advances in multi-omics techniques have revealed genetic features of sponge-associated microbes that facilitate their symbiotic lifestyle, contributing 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). Sponge symbionts may contribute to sponge chemical defenses against predators and biofouling via production of numerous bioactive compounds (Thoms and Schupp, 2007; Lopanik, 2014; Rohde et al., 2015; Helber et al., 2018). In addition, sponge symbionts can play a role in the host sponge life cycle by promoting larval settlement (Song et al., 2021).

Sponge metabolism and metagenomic studies indicated that nutrient cycles in marine

sponges are mediated by their symbionts with both heterotrophic and autotrophic lifestyles (Hoffmann et al., 2009; Webster and Thomas, 2016; Moitinho-Silva et al., 2017a; Chaib De Mares et al., 2018; Zhang et al., 2019), including participation in the carbon cycle (Burgsdorf et al., 2015; Rix et al., 2020), nitrogen cycle (Hoffmann et al., 2009; Schläppy et al., 2010; Moeller et al., 2019), phosphorus cycle (Colman, 2015; Zhang et al., 2015), and sulfur cycle (Hoffmann et al., 2005; Jensen et al., 2017; Tian et al., 2017; Engelberts et al., 2020), as well as supplying vital vitamins and amino acids to the host sponges (Fan et al., 2012; Hentschel et al., 2012; Fiore et al., 2015; Webster and Thomas, 2016; Karimi et al., 2019). Microbial symbionts also contribute to the removal of metabolic waste from host sponges (Hallam et al., 2006; Hoffmann et al., 2009). A number of meta-transcriptomic and metaproteomic studies on sponges have also revealed gene and protein expression patterns of complex carbon, nitrogen, and silicon cycles in their associated microbes (Liu et al., 2012; Bayer et al., 2014c; Moitinho-Silva et al., 2014a; Moitinho-Silva et al., 2014b; Burgsdorf et al., 2022). Particularly, nitrification and denitrification may occur in both HMA and LMA sponges, confirming that the various nitrogen processes may be performed in separate niches within sponges (Schläppy et al., 2010).

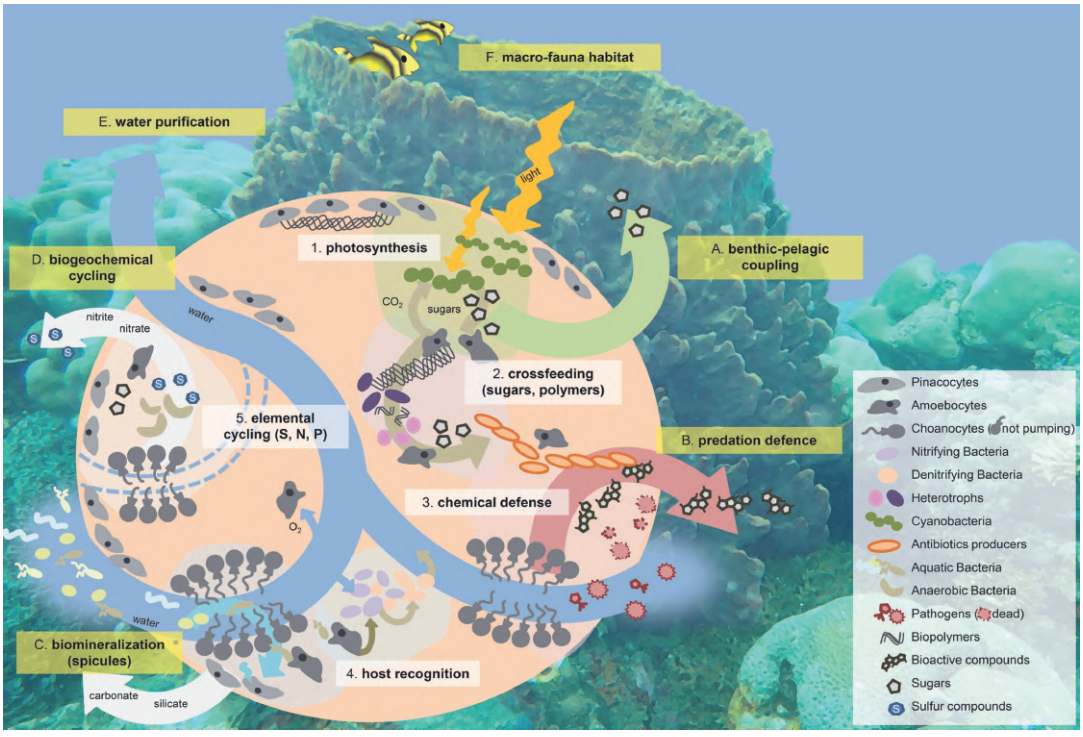


Figure 3. Diagram of the components and their possible functions in a marine sponge-associated microbial community. Adapted from Gutleben (2019).

Ecological roles of several predominant sponge-symbiont taxa have also been elucidated in recent experimental and multi-omics studies. For instance, the cyanobacterium *Candidatus*

Synechococcus spongiarum might provide photosynthate, fix nitrogen, and shield the sponge from excessive light (Usher, 2008; Webster and Taylor, 2012; Freeman et al., 2013), aid in chemical defense (Unson et al., 1994), and produce and store polyphosphate granules (Zhang et al., 2015; Astudillo-García et al., 2018). *Chloroflexi* might synthesize amino acids from ammonia, aid in co-factor biosynthesis and secondary metabolite production (Sacristán-Soriano et al., 2011; Bayer et al., 2018), and degrade sponge-derived carbohydrates (Chaib De Mares et al., 2018). *Candidatus* Poribacteria might degrade sponge-derived polysaccharides, perform autotrophic carbon fixation, contribute to nitrogen-cycling, produce numerous vitamins (Kamke et al., 2013), metabolize sulfates, uronic acid, and propanediol inside their microcompartments (Jahn et al., 2016). SAUL symbionts may play a crucial role in the degradation of sponge carbohydrates and the storage of phosphate for the sponge during periods of phosphate limitation (Astudillo-García et al., 2018). *Tethybacterales*, a newly identified order, might oxidate carbon monoxide, assimilate sulfur, and produce essential amino acids (Gauthier et al., 2016; Taylor et al., 2021; Waterworth et al., 2021).

Sponge-associated bacteria have to evade phagocytosis by host sponges and adapt to the symbiotic life. Although the ways in which sponges recruit and maintain their specific microbiome remain mostly unknown to date, several mechanisms involved in the sponge-microbe association have been suggested in recent studies. Sponge-associated microbes often contain genes encoding a variety of symbiotic factors, including proteins that respond to micro-environmental stressors such as heat, osmotic shocks, and oxidative stress (Astudillo-García et al., 2018). Several sponge symbionts possess proportionally high numbers of genes responsible for the stable insertion of mobile DNA as a strategy to rapidly adapt to the host sponges (Burgsdorf et al., 2015). Moreover, sponge symbiont genomes are enriched in clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CAS) and restriction-modification (RM) systems, which may provide defense against foreign DNA such as phages and plasmids (Bolotin et al., 2005; Haft et al., 2005; Horn et al., 2016; Robbins et al., 2021). Furthermore, sponge microbial metagenomes are enriched in tetratricopeptide repeat, ankyrin repeat, and leucine-rich repeat domains, which are important in protein interactions between prokaryotes and eukaryotes (Thomas et al., 2010a; Fan et al., 2012; Burgsdorf et al., 2015; Degnan, 2015; Karimi et al., 2017; Moeller et al., 2019; Waterworth et al., 2021). The expression of such proteins can modulate phagocytosis by amoebal cells and might represent a mechanism for symbionts to evade digestion by their sponge host (Nguyen et al., 2014; Reynolds and Thomas, 2016). Metatranscriptomic evidence also supported a high expression of genes encoding membrane transport-associated proteins, transposases, and several proteins mediating prokaryote-eukaryote interactions (Jahn et al., 2016). Transposases may facilitate the evolutionary adaptation of symbionts to the host environment through mediating horizontal gene transfer or removing non-essential genes (Fan et al., 2012), whereas transport systems may participate in the uptake of specific

host-derived nutrients (Díez-Vives et al., 2018; Karimi et al., 2018; Moeller et al., 2019; Taylor et al., 2021). In certain cases, sponge-associated symbionts might reduce their genomes to remove genes that are not essential for their symbiotic lifestyle i.e., those coding for chemotaxis and motility (Karimi et al., 2018; Konstantinou et al., 2021).

To date, studies into the co-evolution and interaction of sponges and their symbionts follow a holobiont model to elucidate the importance of symbionts in marine organisms. Pita et al. (2018) introduced the concept of the nested ecosystem in a review on the sponge holobiont, which was expanded upon by Vanwonterghem and Webster (2020) in the context of coral reef communities. This concept suggests that microbiome functioning at the organismal level may modulate holobiont performance, which in turn affects its interactions with the surrounding environment. In this way, functional microbiome components influence ecosystem health and functioning via cascading effects involving primary production, nutrient cycling, disease control, and community structure (Pita et al., 2018; Vanwonterghem and Webster, 2020).

Transmissions of sponge-associated microbes

The high stability and specificity of sponge-microbe associations raise the question about how those associations are established and maintained. Horizontal transmission is the mode in which the sponges acquire free-living symbionts from the surrounding environments. Horizontal transmission in sponges is evidenced by the discovery of phylogenetically highly related sponge-associated microbes in geographically distant sponges (Hentschel et al., 2006; Erwin and Thacker, 2008; Taylor et al., 2021) with sponge-specific sequences being also found in the surrounding seawater (Webster et al., 2010; Taylor et al., 2013). For example, for *Candidatus* Tethybacterales it has been suggested that it is acquired from the environment through horizontal transmission (Taylor et al., 2021) due to their distribution in globally and phylogenetically diverse sponges, including both HMA and LMA sponges (Webster et al., 2001a; Thiel et al., 2007; Cleary et al., 2013; Croué et al., 2013; Gauthier et al., 2016; Waterworth et al., 2017; Cleary et al., 2019; Taylor et al., 2021), and its presence at very low abundances in marine water and sediment samples (Taylor et al., 2021). In general many sponge symbiont sequences are also found in the surrounding seawater at very low relative abundance (Webster et al., 2010; Taylor et al., 2013; Thomas et al., 2016; Turon et al., 2018; Oliveira et al., 2020; Taylor et al., 2021), implying that the marine environment may serve as a ‘seed bank’ for uptake by sponges and subsequent colonization of the sponge.

Conversely, vertical transmission represents the transfer of symbionts via the gametes or larvae of the sponges. This mode of transmission is evidenced by numerous studies that found microbes in various sponge reproductive stages (Uriz et al., 2001; Usher et al., 2005; Schmitt et al., 2007; Sharp et al., 2007; Schmitt et al., 2008; Lee et al., 2009; Uriz et al., 2012;

Engelberts et al., 2022). Recent studies have suggested that several members of *Candidatus* Tethybacterales might be vertically transmitted in sponges such as *Tethya rubra* (Waterworth et al., 2017), *Tedania* sp. (Wu et al., 2018), and *Amphimedon queenslandica* (Fieth et al., 2016). However, it has been noted that vertical transmission of bacterial symbionts to sponge larvae is inconsistent and unfaithful, and these symbionts are not as specific to a particular host species (Björk et al., 2019). An investigation of global patterns in vertical transmission in sponges indicated that vertical transmission is widespread but not universal (Carrier et al., 2022). For instance, several sponges including, e.g., *Halisarca dujardini*, *Mycale laxissima*, and *Petrosia ficiformis* have been reported not to vertically transmit microbial symbionts (Korotkova and Aizenshtadt, 1976; Lepore et al., 1995; Maldonado and Riesgo, 2009). A study on vertical transmission by looking at microbial communities in sponge parent–offspring pairs suggested two vertical transmission ways, i.e., direct and indirect vertical transmissions. In direct vertical transmission, symbionts are transferred through multiple sponge generations, whereas in indirect vertical transmission, symbionts from the adult parent may be acquired from the environment and subsequently incorporated into the oocytes (Björk et al., 2019).

A mixture of both vertical and horizontal transmission has been suggested by recent studies, referred to as the so-called leaky vertical transmission (Schmitt et al., 2012; Thacker and Freeman, 2012). It has been reported that microbes that are horizontally transmitted in some sponges are phylogenetically related to microbes that are vertically transmitted in other sponges (Sipkema et al., 2015). The mixture of vertical and horizontal transmission has also been reported in the sponge *Ircinia campana*, in which the non-core microbiome might be acquired by vertical transmission, whereas the core microbiome might be acquired by horizontal transmission (Griffiths et al., 2019). These findings support the hypothesis that horizontal transmission is a ubiquitous mechanism of symbiont acquisition in sponges and plays a crucial role in forming the core microbiomes, whereas vertical transmission appears as a rather complementary acquisition mechanism (Turon et al., 2018; Griffiths et al., 2019; de Oliveira et al., 2020). A recent review of transmission modes of sponge microbiomes suggests that the mixture of vertical and horizontal transmissions is a common transmission mechanism for most sponges (de Oliveira et al., 2020).

Cultivation of sponge-associated microbes

Although technological advances in DNA sequencing now easily allow the illustration of microbial biodiversity and predict function in environments, microbial cultivation is critical for annotating novel or unknown genes, and is a reliable strategy for validating hypotheses resulting from multi-omics studies (Pham and Kim, 2012; Muller et al., 2013). Furthermore, microbial cultures allow microbial metabolism and interactions to be studied in depth, and open the path for biotechnological exploitation of microbes (McLain et al., 2016; Wohlleben

et al., 2016). Cultivation of sponge-associated microbes that produce bioactive metabolites is of particular interest because it has the potential to alleviate the compound supply problem for the pharmaceutical development pipeline (Brinkmann et al., 2017; Steinert et al., 2018; Atanasov et al., 2021). Furthermore, the cultivation of sponge-associated microbes would allow to experimentally explore the sponge holobiont.

Unfortunately, the number of living microbes in most ecosystems often exceeds the number that can be cultivated by orders of magnitude, and sponges are no exception. This fact has been dubbed as the "great plate-count anomaly" (Staley and Konopka, 1985; Nichols, 2007) or "mysterious dark matter" of the microbial world (Rinke et al., 2013; Lok, 2015). Even with the considerable advances in cultivation techniques (Bruns et al., 2002; Zengler et al., 2005; Aoi et al., 2009; Ferrari and Gillings, 2009; Nichols et al., 2010; Park et al., 2011; Jung et al., 2014; Rettedal et al., 2014; Tillich et al., 2014; Lewis et al., 2021), this bottleneck remains unsolved. The fact that the majority of microbes is resistant to culture can be attributed to a variety of reasons, but the most crucial reason is our failure to precisely reproduce the bio-chemical and physico-chemical conditions that characterize the microbial native environment *in vitro* due to our insufficient knowledge about the native conditions. The chemistry of micro-habitats, interactions with the host and other microbes, as well as interactions between biotic and abiotic factors in the native environment are parameters that are particularly challenging to mimic (Alain and Querellou, 2009).

In addition to traditional agar-plating techniques, numerous additional isolation approaches have been used to culture sponge symbionts, including the use of liquid cultures, floating filters (Sipkema et al., 2011), microcapsule-based cultivation (Gerardo Toledo et al., 2012), diffusion growth chambers (Steinert et al., 2014), *in situ* cultivation devices (Jung et al., 2014; Knobloch et al., 2019; Jung et al., 2021; Jung et al., 2022), the addition of antibiotics (Versluis et al., 2017), sodium pyruvate, catalase (Olson et al., 2000), alpha-butyrolactone (Selvin et al., 2009), sponge extracts (Webster et al., 2001a; Sipkema et al., 2011; Steinert et al., 2014; Esteves et al., 2016), spongin-based sponge skeleton (Kaboré et al., 2019), and arsenic (Keren et al., 2015; Ray et al., 2016) in culture media, as well as the incorporation of sponge-derived physiological and genomic information (Lavy et al., 2014; Gutleben et al., 2020). Such tremendous culture efforts often yield large numbers of isolates, many of which are new species and even produce novel secondary metabolites. Depending on the sponge species, the cultivability of sponge symbionts ranges from 0.1 to 14% of the total microbial diversity found in sponges by molecular methods (Webster and Hill, 2001; Li et al., 2007a; Muscholl-Silberhorn et al., 2008; Abdelmohsen et al., 2010; Santos et al., 2010; Sipkema et al., 2011; Öztürk et al., 2013; Hardoim et al., 2014; Lavy et al., 2014; Rua et al., 2014; Steinert et al., 2014; Keren et al., 2015; Esteves et al., 2016; Versluis et al., 2017).

To date, multi-omics tools and physiological studies have provided more information on the

in vivo conditions of the sponge mesohyl, which is extremely beneficial for enhancing sponge-symbiont cultivability. For instance, the detection of momentarily anoxic regions occurring during non-pumping periods of sponges (Hoffmann et al., 2005; Hoffmann et al., 2008) and various nitrogen cycling-related genes and taxa implies nitrogen cycling to be a significant process in the sponges (Bayer et al., 2008b; Moitinho-Silva et al., 2014a; Slaby et al., 2017). Complex carbohydrates that form the extracellular matrix of sponge mesohyl may be an energy-source degradable by sponge-symbionts (Kamke et al., 2013; Fiore et al., 2015; Bayer et al., 2018; Chaib De Mares et al., 2018). Additionally, it should be noted that the doubling-time of environmental microbes adapted to oligotrophic conditions is typically longer than the duration of many cultivation experiments (Zengler et al., 2002; Alain and Querellou, 2009).

A large number of studies on sponge-associated microbes has revealed the discrepancy of microbial communities recovered by cultivation-dependent and -independent approaches (Sipkema et al., 2011; Versluis et al., 2017; Knobloch et al., 2019; Gutleben et al., 2020; Anteneh et al., 2022; Jung et al., 2022), and only a few true sponge symbionts have been successfully cultured so far. For instance, members of the alphaproteobacterial genus *Pseudovibrio* are isolated frequently from various sponges (Muscholl-Silberhorn et al., 2008; Santos et al., 2010; Bondarev et al., 2013; Lavy et al., 2014; Versluis et al., 2017). *Pseudovibrio* can produce sponge-derived brominated alkaloids (Nicacio et al., 2017) and has been evidenced to be vertically transmitted via larvae in certain cases (Enticknap et al., 2006). Moreover, cyanobacterial strains of the genus *Leptolyngbya* have been isolated from various sponge species, which can absorb and store phosphorus from seawater (Lafi et al., 2005; Zhang et al., 2015; Konstantinou et al., 2018; Konstantinou et al., 2021). Unfortunately, these cultured sponge symbionts are present at low relative abundances in sponges, hence, their roles in the sponge-microbe symbiosis are under debate, whereas the predominant sponge-associated microbial phyla e.g., *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Nitrospirae*, *Candidatus* Poribacteria, and *Thaumarchaeota* have not yet been cultured *in vitro* (Sipkema et al., 2011; Lavy et al., 2014; Steinert et al., 2014; Keren et al., 2015; Versluis et al., 2017). This fact points to a pressing need for continued efforts towards the development of innovative approaches for culturing sponge symbionts.

Biosynthesis of secondary metabolites from sponge-associated microbes

Microbial secondary metabolites, also referred to as natural products, are an important source of bioactive compounds for healthcare and agriculture applications (Butler et al., 2014; Katz and Baltz, 2016; Newman and Cragg, 2016; Singh et al., 2017). These valuable secondary metabolites represent a group of low-molecular weight and structurally diverse compounds including polyketides (PKs), non-ribosomal peptides (NRPs), ribosomally synthesized and post-translationally modified peptides (RiPPs), terpenoids, and a plethora of hybrids. To this

end, polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene clusters are the two main clusters responsible for the biosynthesis of numerous secondary metabolites (Weissman, 2015). So far, thousands of natural products have been shown to be biosynthesized by PKS and NRPS (Brakhage, 2013), including several important currently available antibacterial, antifungal, antiviral, immunosuppressant, and anticancer drugs (Felnagle et al., 2008).

Polyketides are typically produced by PKS assembly lines that are composed of different functional modules, in which each module is responsible for the incorporation and tailoring of a two-carbon unit into the final pathway product (Sánchez et al., 2006). NRPS also forms multienzyme assembly lines that are similar to those of PKS and produces peptide-containing compounds (Miller and Gulick, 2016b). Notably, many bacteria have been found to possess hybrid NRPS-PKS enzymes (Masschelein et al., 2013; Mizuno et al., 2013; Komaki et al., 2014; Komaki et al., 2018; Rischer et al., 2018). These studies indicated that both PKS and NRPS function simultaneously in the same assembly line, however, the products synthesized by most of the hybrid PKS/NRPS enzymes are still unknown.

Recent (meta)genomic studies revealed that sponge symbionts contained highly diverse biosynthetic gene clusters of secondary metabolites (Wakimoto et al., 2014; Wilson et al., 2014; Ueoka et al., 2015; Freeman et al., 2016; Nakashima et al., 2016; Lackner et al., 2017b; Mori et al., 2018; Tianero et al., 2019; Storey et al., 2020; Dharamshi et al., 2022; Loureiro et al., 2022), including PKSs (Piel et al., 2004; Helfrich and Piel, 2016; Gavriilidou et al., 2020; Helfrich et al., 2021), NRPSs (Gavriilidou et al., 2020; Podell et al., 2020; Storey et al., 2020; Gavriilidou et al., 2021; Loureiro et al., 2022), terpenes (Thomas et al., 2010b; Matobole et al., 2017; Jackson et al., 2018; Karimi et al., 2019; Gavriilidou et al., 2020; Gavriilidou et al., 2021), bacteriocins (Phelan et al., 2013; Jackson et al., 2018; Karimi et al., 2019; Zhou et al., 2019; Gavriilidou et al., 2021), lantipeptides (Harjes et al., 2014; Ian et al., 2014; Jackson et al., 2018; Gavriilidou et al., 2020; Guerrero-Garzón et al., 2020; Matroodi et al., 2020), and other secondary metabolites e.g., aryl polyenes, ladderanes and phosphonates (Naughton et al., 2017; Gavriilidou et al., 2020). Many valuable bioactive compounds from sponges and their symbionts are known to be biosynthesized by PKSs (e.g., diffusomycin, psymberin, onnamide A, calyculin A, swinhoeiamide A, swinhodile A, clavosine A, geoetricin A, misakinolide A, oocydin A, mycalamide A) (Piel et al., 2004; Helfrich and Piel, 2016; Helfrich et al., 2021). Recently, Phelan et al. (2013) isolated a new bacteriocin i.e., subtilomycin from *Bacillus subtilis* MMA7 associated with the sponge *Haliclona simulans* and found that the subtilomycin biosynthetic gene cluster is widespread among *B. subtilis* strains isolated from different shallow and deep sea sponges.

Some biosynthetic gene clusters appeared to be sponge-specific, i.e. only detected in sponges. For example, sponge-derived ketosynthase (KS) sequence analysis revealed the presence of

'sponge-specific' PKS groups. Fieseler et al. (2007) detected a cluster of sponge-specific PKS sequences, termed "symbiont ubiquitous type I PKS" (*Sup*). In another study, Sala et al. (2013) detected another sponge-specific KS group, named "sponge (symbiont) widespread fatty acid synthase" (*Swf*), in HMA sponges. Furthermore, Borchert et al. (2016) investigated the diversity of PKS genes in the microbiome of three deep-sea sponges *Inflatella pellicula*, *Poecillastra compressa*, and *Stelletta normani* and also found sponge-specific KS groups in the investigated sponges. A recent comparative metagenomics study by Loureiro et al. (2022) found a novel group of NRPS-like ether lipid-associated gene cluster families widespread across three sponge microbiomes. These findings imply PKS and NRPS produced by sponge symbionts may play an important role in sponge holobiont defense or communication.

Demand for novel antibiotics and sponge-associated microbes as an untapped reservoir of antimicrobials

Before the 'antibiotic era', infectious diseases were the leading causes of mortality among the human population (Adedeji, 2016; Holmes et al., 2017). The discovery of Penicillin by Alexander Fleming in 1928 and subsequent developments of numerous antibiotics resulted in significant advances in life expectancy and quality of life (Fleming, 1929; Hutchings et al., 2019). These discoveries together with improvements in healthcare in the following decades, in the so-called "Golden era of antibiotic discovery", resulted in a decline in the prevalence of infectious diseases as a main cause of morbidity in the general population (Smith et al., 2012; Adedeji, 2016). Unfortunately, shortly after the discovery of Penicillin, attention was raised to the potential emergence of bacterial resistance to antibiotics. The concern was not just regarding Penicillin, but to all antibiotics being used (Levy, 2002; Ventola, 2015; Brown and Wright, 2016; Abadi et al., 2019; Dhingra et al., 2020). In reality, the evolution of antibiotic resistance has been sped up by over and misuse of antimicrobials, particularly in the medical, agricultural and aquacultural sectors (Davies and Davies, 2010; White, 2012; Ventola, 2015). The ability of pathogenic bacteria to gain resistance to numerous antibiotics is facilitated by mutation, conjugation, transformation and transduction (Aminov, 2010).

It is believed that the world is currently in the 'post-antibiotic era', in which the prevalence of antibiotic-resistant pathogenic microbes is sharply rising, and infectious diseases are once again becoming a leading cause of human death (Alanis, 2005; Ashkenazi, 2013). The fast emergence of antibiotic resistance among opportunistic human pathogens is a serious threat to the healthcare system and a global socioeconomic burden (Barriere, 2015; Ahmad and Khan, 2019; Murray et al., 2022). Unfortunately, only a limited number of new antimicrobial agents have been released on the market in recent decades, failing to keep up with the rising incidence of morbidity and mortality due to infections caused by resistant pathogenic microbes (Taylor and Wright, 2008; Ventola, 2015; Dheman et al., 2021). Therefore, further

attempts to discover novel antimicrobials are urgently required.

Currently, most of the used antimicrobials are derived from microbes in temperate and tropical terrestrial habitats (Montaser and Luesch, 2011; Mullis et al., 2019). Thus, unexplored habitats and ecological niches may be untapped reservoirs of uncovered novel antibiotics, such as marine sponges and their symbionts, deep seas, hydrothermal vents, saline brines sediments, and hot springs (Thornburg et al., 2010; Montaser and Luesch, 2011; Anjum et al., 2016; Brinkmann et al., 2017; Mahajan and Balachandran, 2017; Sayed et al., 2020). For instance, the oceans are unexhaustive sources of secondary metabolites with over 39,000 marine natural compounds identified so far (Blunt and Munro, 2022), of which approximately 30% originated from sponges. However, drug development from sponges is hampered by sustainable supply (Thoms and Schupp, 2005; Sagar et al., 2010; Indraningrat et al., 2016). As a result, the search for bioactive molecules has been shifted to sponge-associated microbes since many sponge-derived natural products are of microbial origin (Schmidt et al., 1998; Piel et al., 2004; Newman and Hill, 2006; Waters et al., 2014; Tianero et al., 2019). Extensive efforts to search for antimicrobial compounds from sponge-associated microbes based on cultivation approaches have led to the discovery of numerous antimicrobial compounds (Juliana et al., 2014; Indraningrat et al., 2016; Brinkmann et al., 2017; Fehmida et al., 2017), with many potent antimicrobial compounds such as the antiviral sorbicillactone A, chartarutine B, and truncateol M (Bringmann et al., 2003), the antibacterial subtilomycin, mayamycin, kocucin, sydonic acid, and valinomycin (Phelan et al., 2013), the antifungal saadamycin (El-Gendy and El-Bondkly, 2010) and the antiprotozoal manzamine A (Waters et al., 2014). However, none of these antimicrobial compounds have entered preclinical and clinical trials or are approved as drugs on the market (Indraningrat et al., 2016). Furthermore, novel antimicrobials from sponge-associated microbes have been mostly unexplored due to the majority of sponge-associated microbes remaining recalcitrant to cultivation. As outlined above, cultivation-independent approaches, e.g., metagenomics, have revealed highly diverse gene clusters encoding novel natural products from the uncultivable sponge-associated symbionts (Schirmer et al., 2005; Siegl and Hentschel, 2010; Yung et al., 2011; Wilson et al., 2014; Freeman et al., 2016; Loureiro et al., 2022). Such gene clusters may become interesting targets for discovery of novel antimicrobials from yet uncultivable sponge-associated microbes through heterologous expression in suitable hosts (Alt and Wilkinson, 2015; Loureiro et al., 2018).

Studies in sponges and sponge-associated microorganisms in Vietnam

Vietnam is one of the sixteen greatest biologically diverse countries in the world with over 50,000 identified species (Thuaire et al., 2021). For marine ecosystems, Vietnam's coastal region is within the Coral Triangle, which includes four out of 35 global biodiversity hotspots with unique biota and is a high-priority area for marine conservation (Selig et al., 2014). With

a long coastline of 3,260 km and a sea area of over one million square kilometers, Vietnam's coastal region is composed of 20 typical marine ecosystems and more than 3,000 inshore and offshore islands and islets (Lutaenko et al., 2011; de Queiroz et al., 2013; Mau et al., 2015). This coastal region holds high biodiversity conservation values with over 11,000 marine species, including 6,000 benthic animals, 2,038 fish, 94 mangroves 662 seaweeds, 15 seagrasses, 537 phytoplankton species, 659 zooplankton species, 346 hard corals, 43 seabirds, and 32 marine mammals, reptiles, etc. (Thuaire et al., 2021). Furthermore, the coastal areas with their abundant biodiversity resources are important sources of livelihood for approximately 20 million people in 125 coastal districts (MONRE, 2014). Nonetheless, just like other countries, human activities have significant negative impacts on biodiversity, particularly the loss of seagrass areas and coral reefs (Thuaire et al., 2021).

Compared to other marine animals, studies on sponges in Vietnam's sea have only been carried out mainly in the last two decades. Recent investigations on several sea areas of Vietnam have identified 100 sponge species in Nha Trang bay, 112 sponge species in Con Co island, 54 sponge species in the limestone islands, and 66 sponge species in coastal regions of North Central Vietnam (Calcinai et al., 2020; Huong et al., 2020; Do Cong, 2022). Currently, more than 223 sponge species have been identified in Vietnam with 124 genera, 65 families, 18 orders, and 4 classes (Thai, 2013; Do Cong, 2022); however, their diversity is still greatly underestimated due to many sea areas of Vietnam that have still not been investigated, and these studies focus only on shallow sponges.

Along with studies on sponge taxonomy, investigations of bioactive compounds from marine sponges in Vietnam have received considerable attention since 2006. So far, more than 250 natural products have been isolated and their chemical structures elucidated from about 30 sponge species, mainly collected in the sea of Northern Vietnam, including more than 50 new compounds (Kiem et al., 2019). Vietnamese sponge-derived compounds have diverse structures and include various classes such as steroids, sesquiterpenes, triterpenes, alkaloids, and other compounds. These compounds have been targeted for evaluation of their anticancer and anti-inflammatory activities, many of which exhibited outstanding anticancer and anti-inflammatory effects (Kiem et al., 2019), whereas their pharmacological properties e.g., antibacterial, antifungal, antivirus, etc. have not been studied yet. The potential of bioactive natural products from Vietnamese sponges is still largely underestimated because only about 10% identified sponge species in Vietnamese sea were investigated for their natural products, and the investigated sponge species are mostly from the sea of Northern Vietnam, whereas the majority of sponge species, especially from the sea of Southern Vietnam have yet to be investigated for their natural products.

Contrary to studies on sponge taxonomy and sponge-derived natural products, Vietnamese sponge-associated microbiota and their natural products remain largely unexplored and have

only been studied in the last few years. These studies focused on the isolation of sponge-associated microbes that produce bioactive compounds (Trinh et al., 2016; Cao et al., 2019; Cao et al., 2020; Quang et al., 2020; Trinh et al., 2020; Chau et al., 2021; Quang et al., 2021a; Kim et al., 2022; Phan et al., 2022; Trinh et al., 2022). Interestingly, several new antimicrobial and cytotoxic compounds have been isolated successfully from Vietnamese sponge-associated microbes (Cao et al., 2019; Cao et al., 2020; Trinh et al., 2020; Quang et al., 2021a; Kim et al., 2022; Trinh et al., 2022), revealing that microbes associated with Vietnamese sponges may be a promising source of new bioactive compounds. A few investigations of Vietnamese sponge microbiota by cultivation-independent methods have elucidated several interesting insights into Vietnamese sponge microbiomes such as host-specificity, the influence of environmental quality on their microbiomes, as well as the role of seawater in bacteria recruitment and microbiome stability in sponges (Turon et al., 2018; Turon et al., 2019a; Turon et al., 2019b; Turon and Uriz, 2020).

Despite these first data on Vietnamese sponges, many sea areas, as well as many sponge species in Vietnam, remain unexplored and offer large opportunities for further investigation of Vietnamese sponge-associated microbial communities and their antimicrobials to assess their potential for developing novel antimicrobial compounds.

Aims and Thesis Outline

Marine sponges host dense microbial communities in their tissue and these symbionts are potential producers of bioactive compounds. The Vietnamese sea is known to have a high biodiversity of marine organisms, including marine sponges. However, studies on Vietnamese sponges have only received attention in the last two decades and many aspects of Vietnamese sponges remain largely unexplored, especially sponge microbiomes as well as their natural products. The study presented here aims to provide a better understanding of prokaryotes associated with Vietnamese sponges and their secondary metabolite gene clusters and antimicrobial activity by applying cultivation-dependent and -independent approaches (Figure 4).

Chapter 1 introduces marine sponges and their associated microbial communities within their evolutionary and ecological contexts. This general information serves as a starting point for our current understanding of the sponge-microbe association. These sponge-associated microbial symbionts can be studied using both cultivation-dependent and cultivation-independent techniques that have been applied in the research described in this thesis.

Chapter 2 describes the prokaryotic community composition of 18 Vietnamese sponge species using 16S rRNA gene Illumina MiSeq sequencing. Insights of prokaryotic communities associated with sponges e.g., the most abundant Operational Taxonomic Units

(OTUs), the influence of host identity, sponge-enriched OTUs, and aerobic nitrifying taxa, are discussed.

Chapter 3 reports on the diversity of cultivable bacteria associated with 18 Vietnamese sponge species and compares it with the diversity of bacteria associated with the same Vietnamese sponge species studied by 16S rRNA gene Illumina MiSeq sequencing as described in **Chapter 2**. The bacterial strains associated with the 18 Vietnamese sponge species are also evaluated for their antimicrobial properties against microbial indicator strains including Gram-positive bacteria, Gram-negative bacteria and a yeast. In addition, a bacterial strain exhibiting good antimicrobial activity is subjected to cultivation and isolation of its secondary metabolites. The compounds are then evaluated for antimicrobial effects against a variety of microbial indicator strains.

Marine sponges are known to contain diverse and dense associated microbiota; however, the majority of the bacteria remain recalcitrant to cultivation. Therefore, **Chapter 4** provides a comprehensive review of the cultivability of sponge-associated bacteria. This chapter (i) discusses cultivation techniques that have been used to cultivate sponge-associated bacteria, (ii) evaluates and analyzes the cultivable bacterial diversity from sponges and their associated culture conditions, and (iii) highlights promising cultivation strategies for cultivating currently uncultivable bacteria from sponges. Additionally, isolated sponge-associated bacterial taxa that were previously marked as "sponge-specific" and "sponge-enriched" are discussed in this chapter.

Recent reviews have reinforced that sponge-associated bacteria are a valuable source of structurally diverse secondary metabolites with potent biological properties, which make these bacterial communities promising sources of new drug candidates (Cheng et al., 2020). However, the overall diversity of secondary metabolite biosynthetic potential present in bacteria is difficult to access due to the fact that the majority of bacteria are not readily cultured in the laboratory, as shown also in **Chapter 3** and **Chapter 4**. Thus, **Chapter 5** uses a cultivation-independent approach through metagenomics to investigate the diversity of secondary metabolite biosynthetic gene clusters (BGCs) from the metagenomes of bacterial communities associated with three of the 18 Vietnamese sponge species: *Clathria reinwardti*, *Rhabdastrella globostellata*, and *Spheciospongia* sp. KS sequences related to 'sponge-specific' clusters i.e., *SupA* and *SwfA* from the three sponge-associated bacterial communities are also discussed in this chapter.

Chapter 6 provides a general discussion, integrating the results obtained in **Chapters 2-5**, and portraying them in the light of recent studies related to aspects of sponge prokaryotic symbionts and their antimicrobial secondary metabolites as well as biosynthesis of bacterial secondary metabolites from sponges.

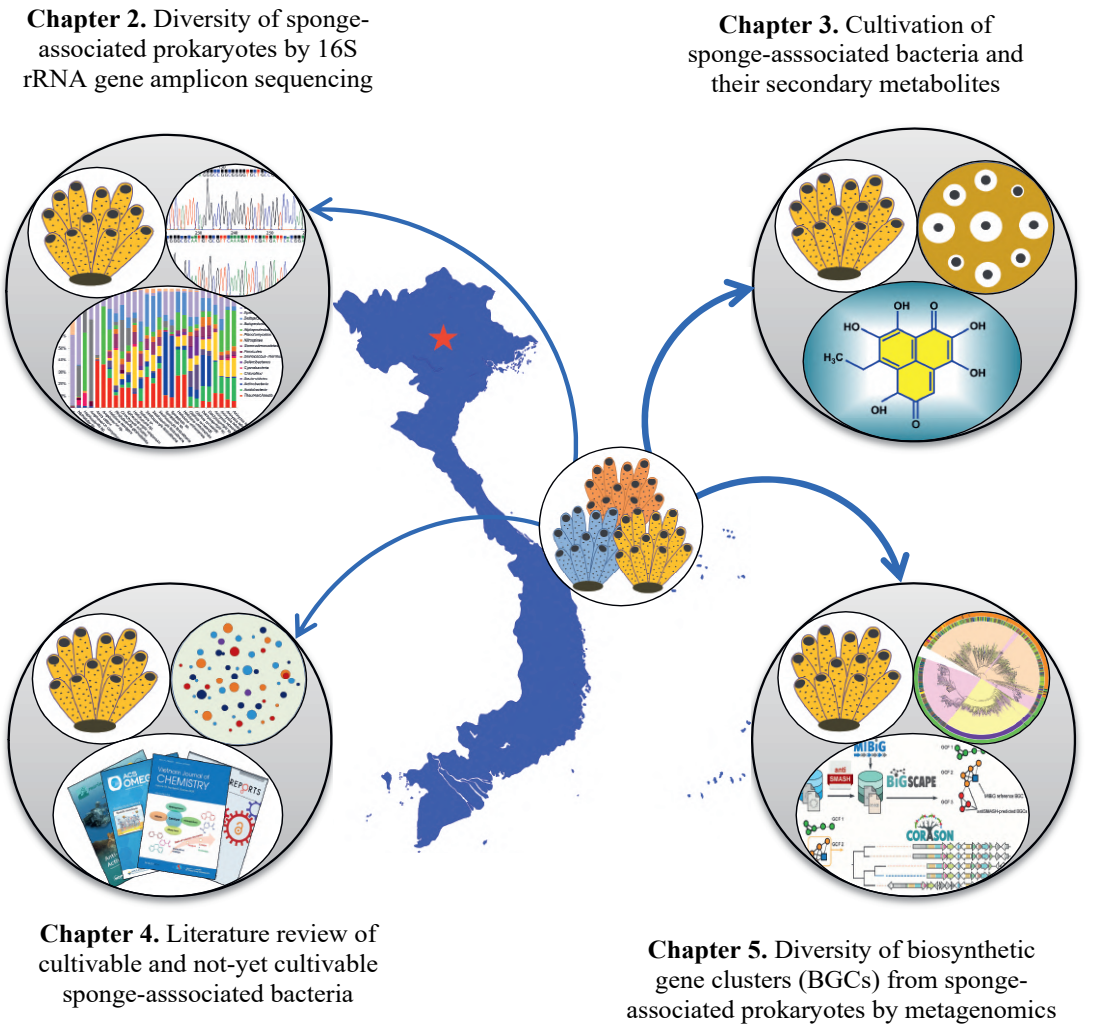
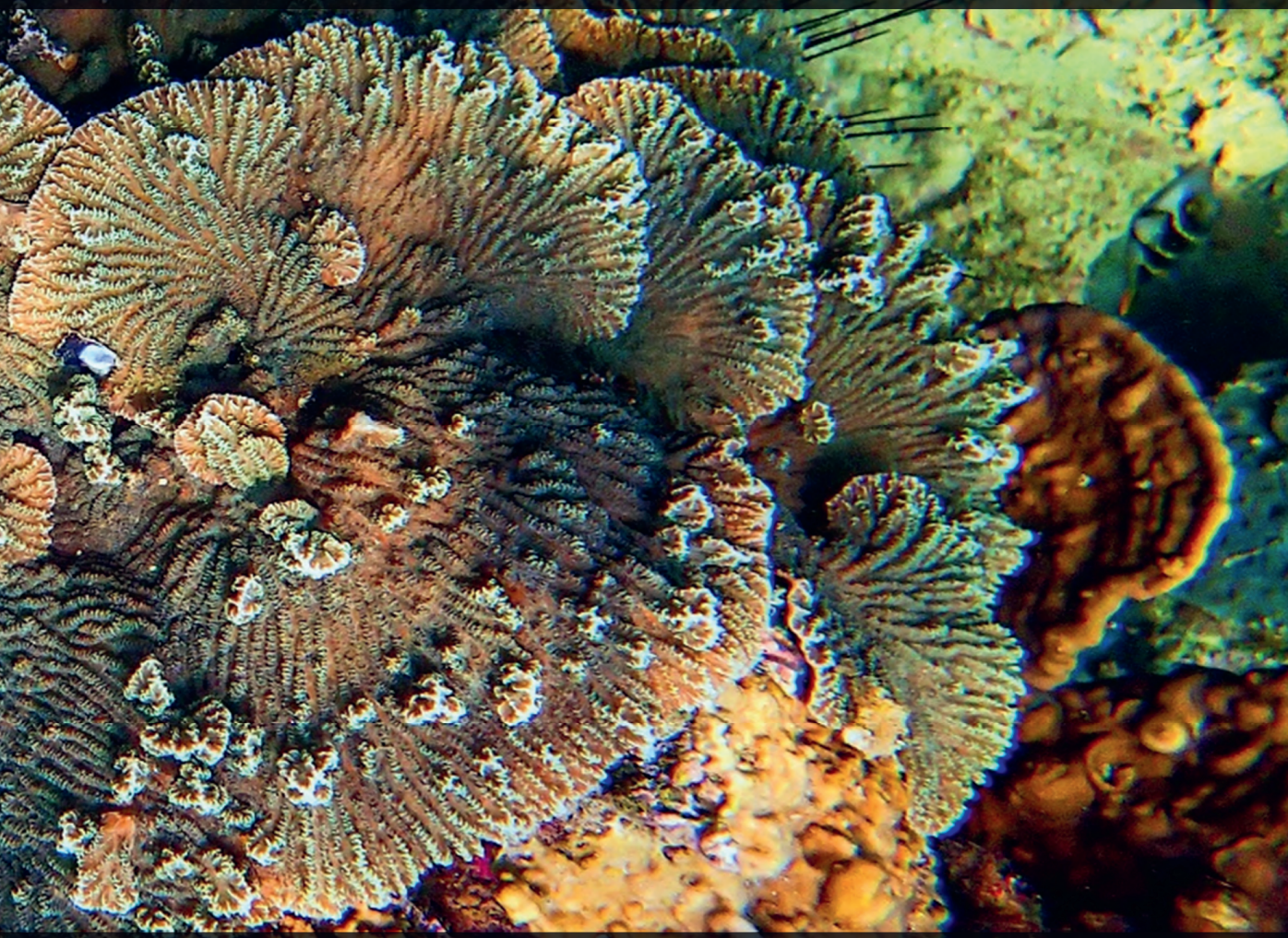


Figure 4. Overview of the main chapters in this thesis

Chapter 2

Archaeal and bacterial diversity and community composition from 18 phylogenetically divergent sponge species in Vietnam



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This chapter was published as Dat et al. (2018)
PeerJ 6: e4970. https://doi.org/10.7717/peerj.4970

Abstract

Sponge-associated prokaryotic diversity has been studied from a wide range of marine environments across the globe. However, for certain regions, e.g. Vietnam, Thailand, Cambodia and Singapore, an overview of the sponge-associated prokaryotic communities is still pending. In this study we characterized the prokaryotic communities from 27 specimens, comprising 18 marine sponge species, sampled from the central coastal region of Vietnam. Illumina MiSeq sequencing of 16S rRNA gene fragments was used to investigate sponge-associated bacterial and archaeal diversity. Overall, 14 bacterial phyla and one archaeal phylum were identified among all 27 samples. The phylum *Proteobacteria* was present in all sponges and the most prevalent phylum in 15 out of 18 sponge species, albeit with pronounced differences at the class level. In contrast, *Chloroflexi* was the most abundant phylum in *Halichondria* sp., whereas *Spirastrella* sp. and *Dactylospongia* sp. were dominated by *Actinobacteria*. Several bacterial phyla such as *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Deferribacteres, Gemmatimonadetes*, and *Nitrospirae* were found in two-thirds of the sponge species. Moreover, the phylum *Thaumarchaeota* (Archaea), which is known to comprise nitrifying archaea, was highly abundant among the majority of the 18 investigated sponge species. Altogether, this study demonstrates that the diversity of prokaryotic communities associated with Vietnamese sponges is comparable to sponge-prokaryotic assemblages from well-documented regions. Furthermore, the phylogenetically divergent sponges hosted species-specific prokaryotic communities, thus demonstrating the influence of host identity on the composition and diversity of the associated communities. Therefore, this high-throughput 16S rRNA gene amplicon analysis of Vietnamese sponge-prokaryotic communities provides a foundation for future studies on sponge symbiont function and sponge-derived bioactive compounds from this region.

Introduction

Sponges (Porifera) are the sister group to all other metazoans (Simion et al., 2017), and are dated back at least 600 million years ago (Maloof et al., 2010; Yin et al., 2015a). Marine sponges are distributed across a wide range of habitats, from polar regions, and temperate benthic communities to subtropical and tropical coral reefs (Van Soest et al., 2012). They play an important ecological role in the carbon, nitrogen and sulfur cycles in benthic ecosystems, and both aerobic and anaerobic processes have been observed (Bell, 2008; Maldonado et al., 2012). Moreover, the sponge microhabitat provides diverse niches for a wide range of different potential symbionts, including archaea, bacteria, microalgae, fungi, unicellular eukaryotes, as well as macrofaunal communities that can constitute a large fraction of the total sponge biomass (Sarà, 1971; Vacelet and Donadey, 1977; Wilkinson, 1978c; d; Santavy, 1985; Santavy and Colwell, 1990; Taylor et al., 2007; Bell, 2008; Duris et al., 2011; Lattig and Martín, 2011; Thomas et al., 2016).

Due to the abundance and composition of the associated prokaryotes, most sponges can be grouped in either high microbial abundance (HMA) or low microbial abundance (LMA) species (Gloeckner et al., 2014; Moitinho-Silva et al., 2017c). HMA sponges contain a high concentration of microorganisms (10^8-10^{10} microorganisms per gram sponge tissue), whereas the number of microorganisms in LMA sponges is much lower with only 10^5-10^6 microorganisms per gram sponge tissue (Hentschel et al., 2006). In addition, LMA sponges harbor less diverse microbial communities at the phylum-level than HMA sponges (Schmitt et al., 2011; Bayer et al., 2014b; Moitinho-Silva et al., 2014a). However, exceptions that deviate from this pattern have been reported (e.g., Easson and Thacker, 2014; Thomas et al., 2016). Several sponge microbiota studies revealed that sponge-associated prokaryotic communities are distinct from benthic and planktonic communities (e.g., Naim et al., 2014; Alex and Antunes, 2015a; Rodríguez-Marconi et al., 2015; Weigel and Erwin, 2015), and that, besides evolutionary history of the sponge species, host identity is the most important factor for structuring the sponge-prokaryotic communities (Easson and Thacker, 2014; Steinert et al., 2016; Thomas et al., 2016). Previous studies report that sponge-associated prokaryotic communities are stable despite geographic differences and can exhibit monophyletic "sponge-specific" 16S rRNA gene sequences that are not present in the surrounding environments (Hentschel et al., 2002; Taylor et al., 2007; Simister et al., 2012b). However, recent studies using deep sequencing revealed that some sequences in these monophyletic 16S RNA gene clusters are also found in seawater and sediment samples, albeit at very low abundances. Therefore, these clusters are not strictly "sponge-specific", but better described as "sponge-enriched" (Taylor et al., 2013; Thomas et al., 2016). Although our knowledge about the prokaryotic communities associated with sponges has substantially improved in recent decades, our understanding about the ecological function of sponge symbionts is still limited. Animals typically excrete ammonium as inorganic metabolic waste rather than nitrate, thus the release of nitrate from sponges has been considered as sign for microbial nitrification in sponges (Corredor et al., 1988; Diaz and Ward, 1997; Jiménez and Ribes, 2007). Recent studies provided evidence of nitrification related to sponges symbionts based on incubation experiments as well as molecular markers (i.e., genes encoding for nitrification, for instance the *amoA* gene or *nxrB* gene) (Diaz and Ward, 1997; Bayer et al., 2008a; Steger et al., 2008; Hoffmann et al., 2009; Feng et al., 2016). Studies investigating microbial nitrifiers in sponges reported many potential sponge symbionts that are related to nitrification such as *Nitrosopumilus* (AOA: ammonia-oxidizing archaea), *Nitrosococcus*, *Nitrosospira* (AOB: ammonia-oxidizing bacteria), and *Nitrospira* (NOB: nitrite-oxidizing bacteria) (Kowalchuk and Stephen, 2001; Steger et al., 2008; Hoffmann et al., 2009; Turque et al., 2010; Cuvelier et al., 2014; Kennedy et al., 2014; Pfister et al., 2014; Medema et al., 2015; Feng et al., 2016).

Bacterial and archaeal diversity in sponges has been characterized from a wide range of

marine regions across the globe including the Atlantic, Pacific and Indian Oceans as well as the Mediterranean, Red Sea, the Caribbean, the Yellow Sea and the South China Sea (Li et al., 2007b; Li et al., 2011a; Giles et al., 2013; Thomas et al., 2016). Vietnam exhibits a high diversity of marine sponges with at least 229 species, belonging to 124 genera, 65 families, 18 orders, and 4 classes (Quang, 2013), however, sponge-associated prokaryotic communities from Vietnam still remain unexplored. Many sponges in Vietnam have been identified as potential sources for new bioactive compounds: new muurolane-type sesquiterpenes were isolated from the sponge *Dysidea cinerea* (Kiem et al., 2014), new anticancer sterols from an *Ianthella* sp. (Nguyen et al., 2009), antifouling 26,27-Cyclosterols from *Xestospongia testudinaria* (Nguyen et al., 2013), and new sesquiterpenes and bis-sesquiterpene from *Dysidea fragilis* (Cuc et al., 2015; Nguyen et al., 2015; Kiem et al., 2016). However, the true producers of many bioactive compounds from sponges are sponge-associated microorganisms rather than the sponges themselves (Unson and Faulkner, 1993; Fisch et al., 2009; Indraningrat et al., 2016).

Therefore, we aimed to characterize the yet uninvestigated prokaryotic diversity and composition of Vietnamese sponges, and to provide a foundation for future studies focusing on sponge-associated bioactive compounds. We collected 27 sponge specimens, comprising 18 sponge species, from the central coastal region of Vietnam. Bacterial and archaeal diversity and sponge-specific community composition was characterized using Illumina MiSeq sequencing of PCR-amplified 16S ribosomal RNA (rRNA) gene fragments. To the best of our knowledge, among these 18 sponge species, six species (i.e., *Clathria reinwardti, Haliclona amboinensis, Cinachyrella schulzei, Haliclona fascigera, Tespios aploos,* and *Axos cliftoni*) are investigated for the first time regarding their prokaryotic communities.

Materials and Methods

Sampling

Sponge specimens (n = 27) were collected by scuba diving from May to September 2015 at three locations from the central coastal region of Vietnam at 5-10 m depth (Supplementary Table S1). Lang Co Bay is located in the Phu Loc district within the Thua Thien Hue province. Con Co is a small island in the Quang Tri province with an area of 2.3 km^2. Hon Mun Island is a marine conservation area in the Nha Trang Bay, Khanh Hoa province. Lang Co Bay was sampled in May 2015, Con Co Island in August 2015 and Hon Mun Island in September 2015. The distance between sampling sites ranges from 150 km to 540 km. The specimens were transferred directly to zip-lock bags containing seawater to prevent contact of sponge tissue with air. All samples were immediately transported to the laboratory, rinsed three times with sterile artificial seawater (Instant Ocean Aquarium Sea Salt Mixture: Instant Ocean), and stored at -80°C until DNA extraction.

DNA extraction and PCR amplification of 16S rRNA genes

Sponge samples prior to extraction were processed using a modified protocol from (Abe et al., 2012). In brief, the specimens were rinsed three times with sterile artificial seawater to remove any debris attached to the sponge. Then the specimens were further cleaned with a sterile scalpel in order to remove any sediment and other organisms strictly attached to the sponge. Finally, a piece of sponge tissue was ground in TEN buffer (3.5% sodium chloride, 10 mM tris-hydroxymethyl-aminomethane, 50 mM ethylenediaminetetraacetic acid, pH 8.5) with a sterilized mortar and pestle. Cell suspensions were filtered through a large nylon mesh (20 µm) to remove potential contaminants. The filtrates were then centrifuged at 8,000 g for 15 min at 4°C. The pellets were used to extract total genomic DNA using the ZymoBead™ Genomic DNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The concentration of the extracted DNA was determined with a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and its integrity was examined by gel electrophoresis on a 1% (w/v) agarose gel. The extracted DNA was dissolved in TE buffer and stored at -20°C until further analysis.

The prokaryotic communities were characterized by Illumina MiSeq sequencing of 16S rRNA gene fragments using a two-step amplification procedure. The V4 region of the 16S rRNA genes was amplified by PCR using the 2nd version of the bacterial primers 515F/860R (Apprill et al., 2015), which were added to the 3' end of Unitag1 and Unitag2, respectively (Supplementary Tables S2, S3). The PCR amplification was performed in a volume of 40 µL containing 8 µL of 5X Phusion HF green buffer (Thermo Scientific, Waltham, USA), 1 µL of a 10 mM dNTP mixture (Promega Benelux B.V., Leiden, the Netherlands), 0.4 µL of Phusion Hot Start II High-Fidelity DNA polymerase (2 U/µL; Thermo Scientific, Waltham, USA), 1 µL of a 10 µM solution of each primer, 1 µL template (20 ng/µL), and 27.6 µL nuclease free water. The PCR was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 98°C for 5 s, annealing at 56°C for 20 s, elongation at 72°C for 20 s, and a final elongation at 72°C for 5 min. Subsequently, the first PCR product was used as template in a second PCR in order to add sample specific barcodes (8 nucleotides). The second PCR consisted of 10 µL Phusion HF green buffer (Thermo Scientific, Waltham, USA), 1 µL dNTP mixture (Promega, Benelux B.V., Leiden, The Netherlands), 0.5 µL Phusion Hot Start II DNA polymerase (Thermo Scientific), 31 µL nuclease free water, 2.5 µL 10 µM forward primer (barcode-linker-Unitag1), 2.5 µL 10 µM reverse primer (barcode-linker-Unitag2) (Supplementary Table S3) and 2.5 µL of the first PCR product. The PCR conditions were 98°C for 30 s, followed by 5 cycles at 98°C for 10 s, 52°C for 20 s and 72°C for 20 s, and final elongation at 72°C for 10 min. All PCR products were analyzed on a 1.8% (w/v) agarose gel to verify the products. The PCR products were purified using the HighPrep^{TM} PCR clean-up protocol-MagBio kit (Magbio, London, UK), and quantified using the Quant-iTdsDNA high-sensitivity assay kit and the Qubit fluorometer 2.0 (Invitrogen, Grand Island, NY, USA). Finally, samples were

pooled in equimolar concentrations to ensure equal representation of each sample (including two mock communities as an internal standard to compare expected with observed 16S rRNA gene composition). The pooled library was purified, concentrated and quantified again with the HighPrep™ PCR clean-up kit (Magbio, London, UK) and the Quant-iTdsDNA high-sensitivity assay kit (Invitrogen, Grand Island, NY, USA). The samples were sequenced on the Genome Sequencer Illumina MiSeq at GATC Biotech, Germany. Sequencing data was deposited in the NCBI Sequence Read Archive under BioProject ID: PRJNA354731 with accession numbers: SRS1815731- SRS1815757 (Supplementary Table S3).

Sequence data analyses

Illumina sequencing data was processed and analyzed using the NG-Tax pipeline (Ramiro-Garcia et al., 2016). Briefly, paired-end libraries were combined, and only read pairs with perfectly matching primers and barcodes were retained. To this end, both primers were barcoded to facilitate identification of chimeras produced during library generation after pooling of individual PCR products. Reads were trimmed to 100 bp to avoid overlap in forward and reverse reads, which would affect the quality filtering. However, both quality reads (forward/reverse) were subjected to further analyses. Demultiplexing, OTU picking, chimera removal and taxonomic assignment were performed within one single step using the *OTU_picking_pair_end_read* script in NG-Tax. Reads were ranked per sample by abundance and OTUs (at a 100% identity level) were added to an initial OTU table for that sample starting from the most abundant sequence until the abundance was lower than 0.1%. The final OTU table was created by clustering the reads that were initially discarded as they represented OTUs < 0.1% of the relative abundance with the OTUs from the initial OTU table with a threshold of (98.5% similarity) (Ramiro-Garcia et al., 2016). Taxonomic assignment using the most abundant sequence of each OTU was done utilizing the UCLUST algorithm (Edgar, 2010) and the Silva 111 SSU Ref database (Yilmaz et al., 2014).

Prokaryotic community analysis

Community composition summaries at phylum and class levels were created using the *summarize_taxa_through_plots.py* script from QIIME version 1.9.1 (Caporaso et al., 2010). Good's coverage index, rarefaction curves, and alpha diversity metrics (e.g., Shannon, inverse Simpson, and evenness) were calculated using the QIIME script *alpha_rarefaction.py*. Alpha diversity indices calculated from prokaryotic communities of sponge-species with replicates were tested by the Kruskal-Wallis test using function *kruskal.test* within the FSA package (Ogle, 2017) of R v.3.3.1 (R Core Team, 2016). Heatmaps were generated for a) prokaryotic composition at phylum and class level, b) the most abundant OTUs containing at least 2.5% of the reads in at least one of the samples, c) archaeal OTUs, and d) OTUs related to known nitrifying taxa using the *heatmap.2* function of the gplots package in R (Warnes et al., 2016). For distance-based multivariate analyses, the 16S rRNA gene OTU table was standardized using the *decostand* function (method =

"hellinger") of the vegan package in R (Oksanen et al., 2016). Hierarchical cluster analysis was performed using the functions *vegdist* (method = "bray") and *hclust* (method = "average"). The non-metric multidimensional scaling (NMDS) plot was created via the function *metaMDS* (Bray-Curtis distances) of the vegan package (Oksanen et al., 2016). Multivariate analysis based on Bray-Curtis dissimilarities of sponge-associated prokaryotic communities for sponge-species with replicates were performed using the functions *betadisper*, *permutest*, and the permutational multivariate analysis of variance (*adonis*) functions of the vegan package.

Sponge-enriched OTUs

The 91 most abundant OTUs of our study (i.e., at least 2.5% of all reads in at least one of the samples) were used to identify OTUs which are significantly enriched in the 3569 sponge specimens (comprising 269 sponge species) from the sponge microbiome project (Moitinho-Silva et al., 2017b). In brief, the representative sequences (n = 91) of the most abundant OTUs from our study were subjected to a BLAST search (Altschul et al., 1990) against a curated sponge microbiome database, containing 64424 high quality deblurred subOTU sequences that were extracted from the sponge Earth Microbiome Project (EMP) database (https://github.com/amnona/SpongeEMP). The curated spongeEMP BLAST database and additional information describing the database creation can be accessed here: https://github.com/marinemoleco/spongeEMP_BLASTdb. The sponge microbiome project subOTU sequences with 100% similarity to the present 91 query sequences were uploaded to the spongeEMP online server (www.spongeemp.com) in order to identify OTUs that are significantly enriched in sponge EMP specimens ($p < 0.05$ for the category "host-associated" in the field env_package).

Molecular sponge identification

For the taxonomic identification of the sponge specimens, near full length 18S rRNA gene and cytochrome *c* oxidase subunit I (COI) gene fragments from all samples were PCR amplified using the primer sets EukF/EukR (Medlin et al., 1988) for the 18S rRNA gene, and jgLCO1490/jgHCO2198 (Geller et al., 2013) for COI (Supplementary Table S2). PCR products were cloned into pGEM-T Easy vector systems (Promega, Madison, WI, USA) according to the manufacturer's protocol. Positive clones were selected and sequenced using the T7/SP6 primer pair. Sequences were trimmed (error probability limit 0.1) using Geneious 4.8.3 (Kearse et al., 2012). The cloning vector was identified using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and removed. For the 18S rRNA gene, forward and reverse sequences were assembled to obtain near full length fragments. The 18S rRNA sequences from this study and the three most similar sequences obtained through blasting against the NCBI nr/nt database were added to a set of existing sponge 18S rRNA sequences (Sipkema et al., 2009), and subsequently aligned using the MAFFT (v.7.222) program with the FFT-NS-i strategy (Katoh and Standley, 2013). The same strategy was

followed for COI sequences obtained in this study. The additional three COI gene sequences were added based on the highest BLAST similarity to our sequences as obtained from the NCBI database (nr/nt database), and then aligned as described above. Phylogenetic trees for the 18S rRNA and COI genes were created using RAxML version 8.0.0 (Stamatakis, 2014) with the GTRGAMMA model and 1000 bootstrap replicates. The final sponge taxonomy was determined based on the best position in both phylogenetic trees and their BLAST identity to sequences of sponge species deposited in the NCBI database. Specimens were identified to species level if their identities were at least 99% with sequences in the NCBI database. However, three species showed high identity (≥99%) with sequences from sponge species in the NCBI database, which known distribution typically does not include the Pacific Ocean according to the World Porifera Database (http://www.marinespecies.org/porifera/). For those samples, it was decided not to classify down to species level and maintain the generic status instead. All generated sequences were deposited in GenBank under the accession numbers: KX894454-KX894480 (18S rRNA genes) and KX894481-KX894504 (COI genes).

Results

Molecular sponge taxonomy

A total of 27 sponge specimens were collected from the central coastal region of Vietnam and identified by sequence analysis of 18S rRNA and COI taxonomic marker genes (Supplementary Figures S1 and S2). Eighteen phylogenetically divergent sponge taxa were identified: *Axinyssa* sp., *X. testudinaria*, *C. reinwardti*, *Spirastrella* sp., *Dactylospongia* sp., *H. amboinensis*, *C. schulzei*, *Niphatidae* sp., *H. fascigera*, *Amphimedon* sp. 1, *Amphimedon* sp. 2, *Haplosclerida* sp., *R. globostellata*, *Spheciospongia* sp., *Halichondria* sp., *Tedania* sp., *T. aploos*, and *A. cliftoni* (Supplementary Table S1).

Sponge-associated prokaryotic communities

In total 5,326,187 high-quality reads were retained after quality filtering and were clustered into 926 OTUs. For all samples, rarefaction curves indicated near saturation, and coverage >99% (Table 1, Supplementary Figure S3). OTUs were classified into 14 bacterial phyla and one archaeal phylum (Figure 1). Of all classified reads 88.2% belonged to the domain Bacteria, and 11.8% reads to the domain Archaea. Alpha diversity measures (i.e., Shannon, inverse Simpson, evenness) of the prokaryotic community associated with each specimen showed a wide range of diverse metric values. Evenness values ranged from 0.79 to 0.97, Shannon from 2.08 to 5.95, and inverse Simpson from 2.16 to 40 (Table 1). In our study, *T. aploos*, *Amphimedon* sp. 1, *Amphimedon* sp. 2, and *Niphatidae* sp. exhibited the lowest alpha diversity indices of the associated prokaryotic communities of all sponge species, whereas *Tedania* sp., *Dactylospongia* sp., and *C. reinwardti* exhibited the highest alpha diversity indices. The Kruskal-Wallis test for sponge species with duplicates showed significant

differences among all prokaryotic alpha diversity indices (i.e., Shannon, inverse Simpson, OTU richness, evenness) (Supplementary Table S4).

Table 1. Sequence statistics and alpha diversity of Vietnamese sponge-associated prokaryotic communities, including the total number of OTUs, coverage, and alpha diversity metrics

Sample	Taxon	No. of OTUs	Coverage	Evenness	Shannon	Inverse Simpson
AMC	*Amphimedon* sp. 1	47	99.8	0.87	2.25	2.16
AMQ	*Amphimedon* sp. 2	31	99.8	0.84	2.14	2.90
AXT.1	*Axinyssa* sp.	94	99.6	0.94	4.95	18.52
AXT.2	*Axinyssa* sp.	107	99.5	0.94	4.98	17.86
AXT.3	*Axinyssa* sp.	85	99.6	0.94	4.70	15.15
AXT.4	*Axinyssa* sp.	99	99.6	0.94	4.84	15.87
AXC	*Axos cliftoni*	92	99.7	0.95	4.56	10.64
CIS	*Cinachyrella schulzei*	115	99.4	0.95	4.87	10.75
CLR.1	*Clathria reinwardti*	145	99.3	0.96	5.84	27.03
CLR.2	*Clathria reinwardti*	176	99.5	0.97	5.95	38.46
DAS.1	*Dactylospongia* sp.	157	99.2	0.96	5.81	30.30
DAS.2	*Dactylospongia* sp.	141	99.4	0.96	5.70	29.41
HAS	*Halichondria* sp.	108	99.5	0.94	4.63	11.24
HAA.1	*Haliclona amboinensis*	118	99.5	0.93	4.62	10.99
HAA.2	*Haliclona amboinensis*	92	99.5	0.93	4.33	9.26
HAF	*Haliclona fascigera*	89	99.6	0.93	4.77	15.39
CRV	*Haplosclerida* sp.	58	99.8	0.90	3.58	6.80
NIS	*Niphatidae* sp.	35	99.8	0.86	2.08	2.36
RHG	*Rhabdastrella globostellata*	146	99.3	0.96	5.36	15.63
SPV	*Spheciospongia* sp.	122	99.2	0.96	5.66	26.31
SPS.1	*Spirastrella* sp.	98	99.6	0.93	4.37	9.71
SPS.2	*Spirastrella* sp.	76	99.7	0.92	3.93	7.94
TES	*Tedania* sp.	165	99.9	0.97	6.04	40.00
TEA	*Terpios aploos*	19	99.9	0.79	2.12	3.47
XES.1	*Xestospongia testudinaria*	92	99.6	0.95	4.86	13.16
XES.2	*Xestospongia testudinaria*	121	99.6	0.95	4.96	12.35
XES.3	*Xestospongia testudinaria*	117	99.5	0.95	5.02	16.39

Proteobacteria was the most abundant taxon in 15 of the 18 sponge species, making up 23.6% (*Dactylospongia* sp.) to 90.5% (*Amphimedon* sp. 1) of the total community in a sample. Although the phylum *Proteobacteria* was predominant in most sponge species,

individual sponge species hosted different abundant proteobacterial classes: *Alpha-*, *Beta-*, and *Gammaproteobacteria* (Figure 1). In contrast, *Chloroflexi* was the most abundant phylum in *Halichondria* sp., whereas *Spirastrella* sp. and *Dactylospongia* sp. were dominated by *Actinobacteria*. Other major phyla such as *Acidobacteria, Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes* were present at high relative abundances in different sponges (on average 4.5% - 8.7% for all samples). Remaining phyla (e.g., *Nitrospirae*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Spirochaetes*) were detected at a lower relative abundance (<2.5%).

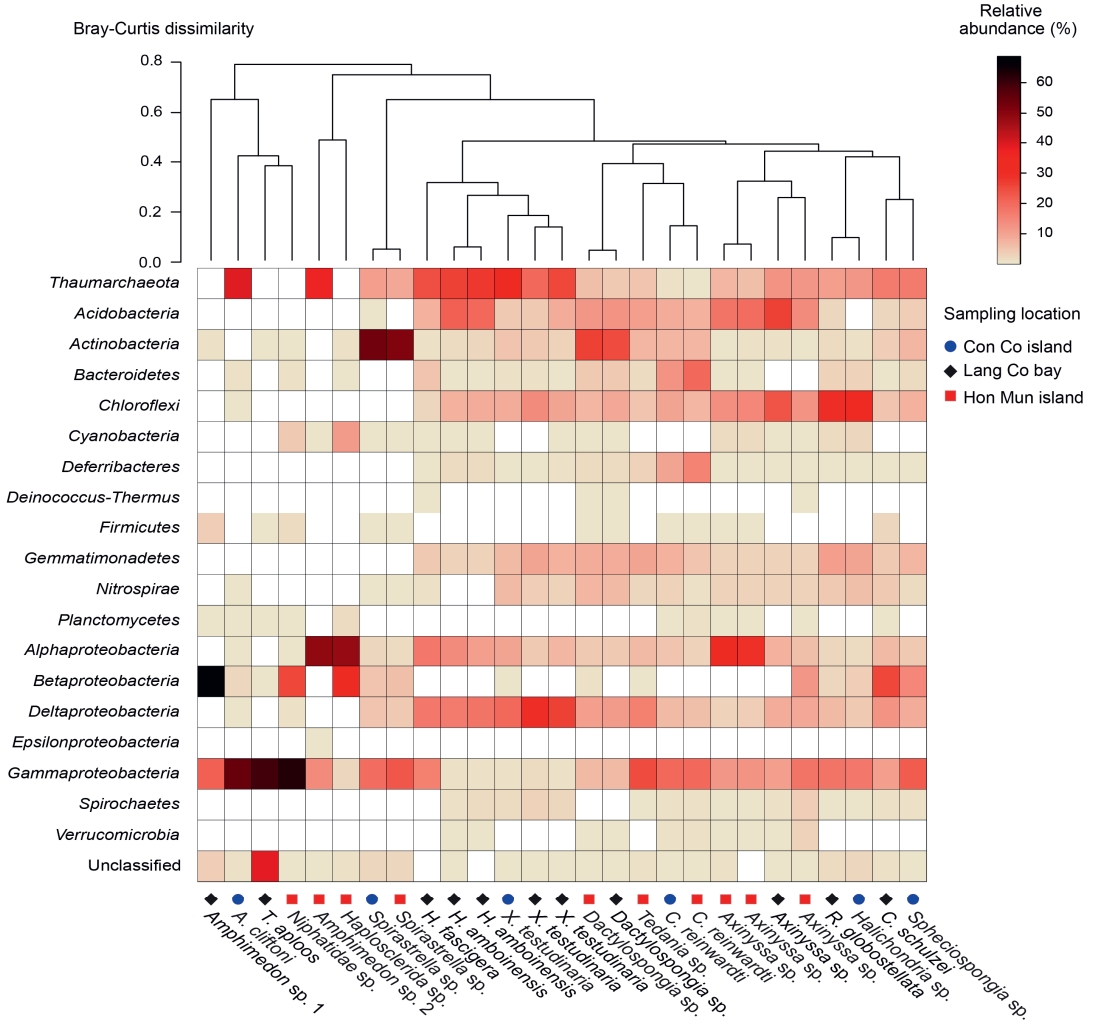


Figure 1. Heatmap of the prokaryotic composition and relative abundance of sponge-associated prokaryotes at phylum level (at class level for the phylum Proteobacteria). Samples were grouped using hierarchical clustering based on the Bray–Curtis distance matrix calculated from relative OTU abundances.

In this study, all archaeal OTUs (n=73) belonged to the phylum *Thaumarchaeota* (Supplementary Figure S4). The *Thaumarchaeota* were found in a wide range of species (14 out of 18 species as well as all specimens of the same species if replicates were available) with relative abundances ranging from 0.4% (*C. reinwardti*) to almost 40% (*A. cliftoni*), with the majority of samples exhibiting relative abundances of archaea greater than 10%. Most archaeal OTUs were assigned to Marine Group I (n = 68), and only five OTUs were assigned to the Soil Crenarchaeotic Group (SCG). The OTUs belonging to Marine Group I were found in all the 14 species that contained archaea, whereas the OTUs belonging to Soil Crenarchaeotic Group (SCG) were found in only two species (Supplementary Figure S4).

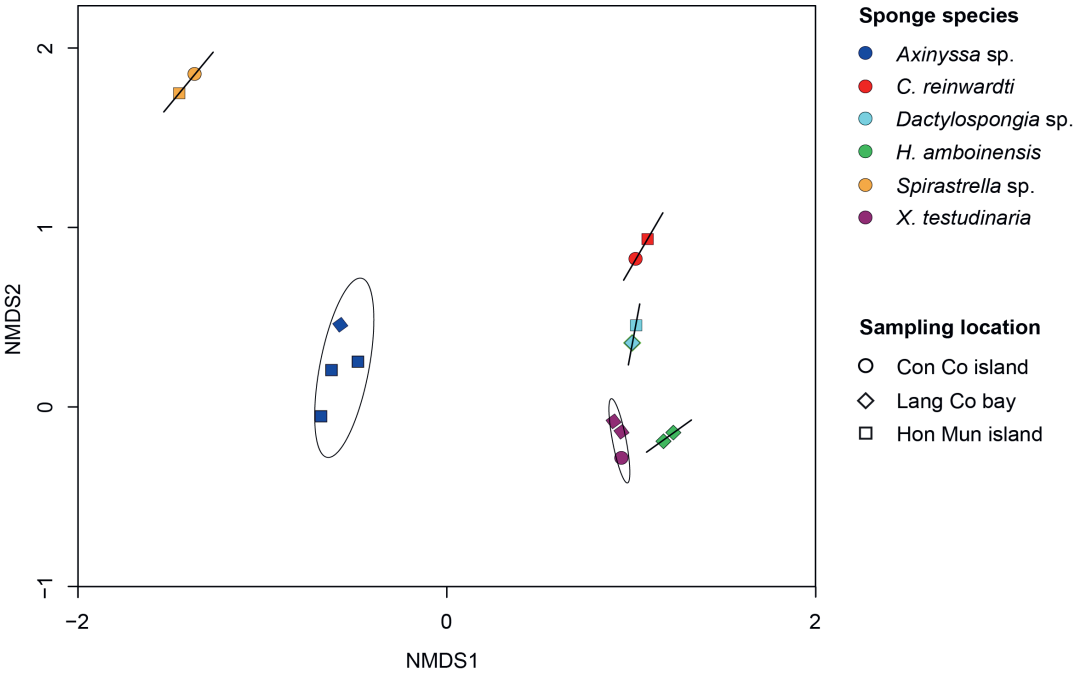


Figure 2. Non-metric multidimensional scaling (NMDS) plot derived from Bray–Curtis distances of sponge prokaryotic communities at OTUs level, NMDS stress value = 0.116. The samples of the same species were grouped with ordination ellipse using function ordiellipse of vegan package

Non-metric multidimensional analysis of the sponge-associated prokaryotic community based on Bray-Curtis dissimilarity showed that replicate specimens of a species (i.e., *Axinyssa* sp., *C. reinwardti*, *Dactylospongia* sp., *H. amboinensis*, *Spirastrella* sp., and *X. testudinaria*) clustered together (Figure 2). The analysis using adonis based on Bray-Curtis dissimilarity showed strong support for the effect of host-identity on their prokaryotic communities for these species (R^2 = 0.94, $p < 0.001$) (Supplementary Table S5). This was further supported by hierarchical clustering with sponge samples belonging to the same species clustering together in spite of different sampling locations (Supplementary Figure S5).

In addition, the analysis of mock communities showed the good correlation between observed and expected profile. The Pearson correlation coefficients between observed and expected profiles at genus level of two mock communities in our study were 0.7094 and 0.8308.

Analysis of the most abundant OTUs

The 91 OTUs with a relative abundance of at least 2.5% in one of the samples belong to 13 different phyla (Figure 3). Among these 13 phyla, the following families and genera were most prominent: *Rhodobacteraceae, Nitrosomonadaceae, Sva0996 marine group, Nitrosococcus,* and *Caldilinea*. Approximately two-thirds of these 91 OTUs were shared at least among two sponge species (Figure 3). These OTUs belonged to prokaryotic taxa such as *TK85*, *Sva0996 marine group*, *Defluviicoccus*, *Desulfurellaceae*, *Nitrospinaceae*, *Sh765B-TzT-29*, *BD2-11*, *Nitrospira*, *Marine Group I*. Furthermore, 75 out of the 91 OTUs are 100% similar to OTUs present in the sponge microbiome database, of which 45 OTUs were significantly enriched in the sponge microbiome project specimens (Figure. 3). The significantly enriched OTUs were mainly found in *Actinobacteria* (*Sva0996 marine group*), *Bacteroidetes* (*Rhodothermaceae*), *Alphaproteobacteria* (Defluviicoccus, Rhodobacteraceae), *Detaproteobacteria* (*Desulfurellales*, *Sh765B-TzT-29*), *Gammaproteobacteria* (Pseudomonas), *Deferribacteres* (PAUC34f), *Gemmatimonadetes* (BD2-11 terrestrial group), *Nitrospirae* (*Nitrospira*), *Thaumarchaeota* (*Marine group I*).

Discussion

Microbial communities in Vietnamese marine sponges

To the best of our knowledge, of the 18 sponge species in the present study, the associated prokaryotic communities were examined for the first time for the following six species: *C. reinwardti*, *C. schulzei*, *H. amboinensis*, *H. fascigera*, *A. cliftoni* and *T. aploos*. Moreover, this is the first time that the associated prokaryotic community of a sponge from the genus *Axos* has been described. All 15 prokaryotic phyla detected belong to the 41 phyla that have so far been detected in marine sponges (Thomas et al., 2016). The prokaryotic taxa with a high relative abundance in the present study (e.g., *Nitrospinaceae, PAUC34f, Caldilineaceae*, *Nitrosomonadales*, *Rhodobacteraceae*, *Endozoicomonas*, *Rhodospirillaceae*) are also abundant in other marine sponges, which can be found in vastly different marine regions (Rodríguez-Marconi et al., 2015; Steinert et al., 2016; Thomas et al., 2016). Regarding the six newly studied sponges, in four of them (i.e., *C. reinwardti, H. amboinensis, H. fascigera, C. schulzei*) all prokaryotic phyla were also found in other sponge species belonging to the same sponge genera (Sipkema et al., 2009; Erwin et al., 2011; Cleary et al., 2013; Khan et al., 2013; Naim et al., 2014; Alex and Antunes, 2015a; Jasmin et al., 2015; Thomas et al., 2016). In contrast, three additional bacterial phyla (i.e., *Actinobacteria, Firmicutes, Planctomycetes*) have not been found to be associated with the genus *Terpios* in a previous

study (Tang et al., 2011).

Furthermore, we exhibited that members of the *Thaumarchaeota* were associated with a wide range of present Vietnamese sponges and that *Thaumarchaeota* accounted for a high relative abundance (> 10%) in over half of the prokaryotic communities (15 out of 27 samples). This phylum has also been detected in many other sponge taxa from the Pacific, Atlantic, Antarctic ocean, Mediterranean Sea, Caribbean and Floridian reefs as well as in cold-water sponges (Webster et al., 2001b; Margot et al., 2002; Lee et al., 2011; Radax et al., 2012; Dupont et al., 2013; Cuvelier et al., 2014; Polónia et al., 2014; Alex and Antunes, 2015a; Rodríguez-Marconi et al., 2015), and with particular high abundances in deep-sea sponges that generally exceed relative abundances in the seawater (Jackson et al., 2013; Kennedy et al., 2014). Members of the Thaumarchaeota - formerly Marine Group I Crenarchaeota - are capable of oxidizing ammonia and play an important role in the nitrogen cycle in marine environments (Capone et al., 2008; Prosser and Nicol, 2008; Ward, 2011). These ammonia-oxidizing archaea are the primary ammonia-oxidizing components in marine systems, displaying *amoA* abundances of up to 10^8 copies L^{-1} (Wuchter et al., 2006; Tolar et al., 2013). It has been reported that nitrogen input such as ammonia is one of the factors influencing the abundance of *Thaumarchaeota* (Herfort et al., 2007; Kirchman et al., 2007; Hatzenpichler, 2012; Hong and Cho, 2015; Oton et al., 2015). The environmental monitoring data at the sampling locations from our study revealed varying concentrations of inorganic nitrogen. For example, the ammonia concentration varied from 0.05 to 0.3 mg/L in Quang Tri, from 0.03 - 0.1 mg/L in Lang Co bay and was lowest (0.003 to 0.008 mg/L) in Hon Mun island as this island is in the protected area (Linh et al., 2015; QCEEM, 2015; MONRE, 2016). However, besides nitrogen input, other environmental factors may influence the abundance of *Thaumarchaeota* lineages, such as pH, depth, oxygen levels, as well as other organic substrates (Di et al., 2010; Verhamme et al., 2011; Isobe et al., 2012; Tolar et al., 2013; Yao et al., 2013; Hong and Cho, 2015).

Finally, Bray-Curtis dissimilarity analysis of the prokaryotic communities in those sponge species with replicates (i.e., *Axinyssa* sp., *C. reinwardti*, *Dactylospongia* sp., *H. amboinensis*, *Spirastrella* sp., and *X. testudinaria*) showed similar pattern of host-specificity, which was already found in other studies (e.g., Pita et al., 2013b; Easson and Thacker, 2014; Naim et al., 2014; Steinert et al., 2016; Thomas et al., 2016). In addition, specimens sampled from different locations at different time points still clustered together, supporting earlier observations of stable host-specific prokaryotic communities despite geographic and temporal differences (Taylor et al., 2007; Pita et al., 2013b; Hardoim and Costa, 2014; Erwin et al., 2015).

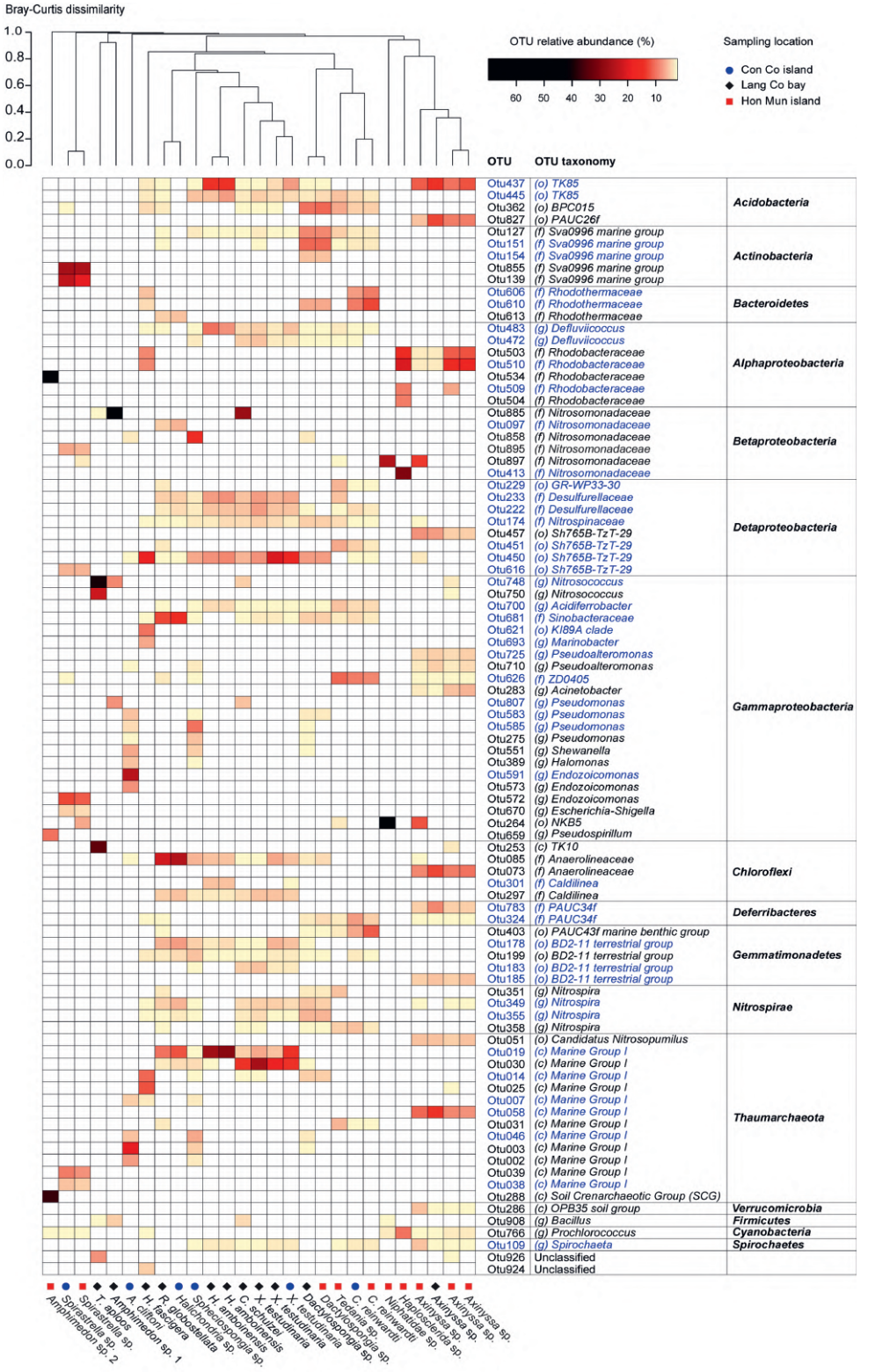

Bray-Curtis dissimilarity
1.0
0.8
0.6
0.4
0.2
0.0
OTU relative abundance (%)
60 50 40 30 20 10
Sampling location
Con Co island
Lang Co bay
Hon Mun island
OTU
OTU taxonomy
Otu437 (o) TK85
Otu445 (o) TK85
Otu362 (o) BPC015
Otu827 (o) PAUC26f
Acidobacteria
Otu127 (f) Sva0996 marine group
Otu151 (f) Sva0996 marine group
Otu154 (f) Sva0996 marine group
Otu855 (f) Sva0996 marine group
Otu139 (f) Sva0996 marine group
Actinobacteria
Otu606 (f) Rhodothermaceae
Otu610 (f) Rhodothermaceae
Otu613 (f) Rhodothermaceae
Bacteroidetes
Otu483 (g) Defluviicoccus
Otu472 (g) Defluviicoccus
Otu503 (f) Rhodobacteraceae
Otu510 (f) Rhodobacteraceae
Otu534 (f) Rhodobacteraceae
Otu509 (f) Rhodobacteraceae
Otu504 (f) Rhodobacteraceae
Alphaproteobacteria
Otu885 (f) Nitrosomonadaceae
Otu097 (f) Nitrosomonadaceae
Otu858 (f) Nitrosomonadaceae
Otu895 (f) Nitrosomonadaceae
Otu897 (f) Nitrosomonadaceae
Otu413 (f) Nitrosomonadaceae
Betaproteobacteria
Otu229 (o) GR-WP33-30
Otu233 (f) Desulfurellaceae
Otu222 (f) Desulfurellaceae
Otu174 (f) Nitrospinaceae
Otu457 (o) Sh765B-TzT-29
Otu451 (o) Sh765B-TzT-29
Otu450 (o) Sh765B-TzT-29
Otu616 (o) Sh765B-TzT-29
Deltaproteobacteria
Otu748 (g) Nitrosococcus
Otu750 (g) Nitrosococcus
Otu700 (g) Acidiferrobacter
Otu681 (f) Sinobacteraceae
Otu621 (o) Kl89A clade
Otu693 (g) Marinobacter
Otu725 (g) Pseudoalteromonas
Otu710 (g) Pseudoalteromonas
Otu626 (f) ZD0405
Otu283 (g) Acinetobacter
Otu807 (g) Pseudomonas
Otu583 (g) Pseudomonas
Otu585 (g) Pseudomonas
Otu275 (g) Pseudomonas
Otu551 (g) Shewanella
Otu389 (g) Halomonas
Otu591 (g) Endozoicomonas
Otu573 (g) Endozoicomonas
Otu572 (g) Endozoicomonas
Otu670 (g) Escherichia-Shigella
Otu264 (o) NKB5
Otu659 (g) Pseudospirillum
Gammaproteobacteria
Otu253 (c) TK10
Otu085 (f) Anaerolineaceae
Otu073 (f) Anaerolineaceae
Otu301 (f) Caldilinea
Otu297 (f) Caldilinea
Chloroflexi
Otu783 (f) PAUC34f
Otu324 (f) PAUC34f
Deferribacteres
Otu403 (o) PAUC43f marine benthic group
Otu178 (o) BD2-11 terrestrial group
Otu199 (o) BD2-11 terrestrial group
Otu183 (o) BD2-11 terrestrial group
Otu185 (o) BD2-11 terrestrial group
Gemmatimonadetes
Otu351 (g) Nitrospira
Otu349 (g) Nitrospira
Otu355 (g) Nitrospira
Otu358 (g) Nitrospira
Nitrospirae
Otu051 (o) Candidatus Nitrosopumilus
Otu019 (c) Marine Group I
Otu030 (c) Marine Group I
Otu014 (c) Marine Group I
Otu025 (c) Marine Group I
Otu007 (c) Marine Group I
Otu058 (c) Marine Group I
Otu031 (c) Marine Group I
Otu046 (c) Marine Group I
Otu003 (c) Marine Group I
Otu002 (c) Marine Group I
Otu039 (c) Marine Group I
Otu038 (c) Marine Group I
Otu288 (c) Soil Crenarchaeotic Group (SCG)
Thaumarchaeota
Otu286 (c) OPB35 soil group
Verrucomicrobia
Otu908 (g) Bacillus
Firmicutes
Otu766 (g) Prochlorococcus
Cyanobacteria
Otu109 (g) Spirochaeta
Spirochaetes
Otu926 Unclassified
Otu924 Unclassified
Amphimedon sp. 2
Spirastrella sp.
T. aplos
Amphimedon sp. 1
A. clinoni
H. fascigera
R. gladiospongia
Haliclona cinerea
Sphecidospongia sp.
H. amboinensis
C. schulzei
X. testudinaria
X. testudinaria
X. testudinaria
Dactylospongia sp.
Dactylospongia sp.
Tedania sp.
C. reinwardti
C. reinwardti
Niphatidae sp.
Axinyssa sp.
Axinyssa sp.
Axinyssa sp.
Axinyssa sp.

Figure 3. Heatmap of the most abundant OTUs (≥2.5% of the total reads in at least one of the samples). Samples were grouped using hierarchical clustering based on the Bray–Curtis distance matrix calculated from the relative abundances of these OTUs. If applicable, OTU taxonomy was assigned to the phylum (p), class (c), order (o), family (f), or genus (g) by NG-tax. The blue colors of OTU names indicate that these OTUs significantly enriched in the sponge microbiome project database (http://www.spongeemp.com).

Sponge-enriched OTUs

Previous studies have identified "sponge-specific" and/or "sponge coral-specific" clusters based on monophyletic 16S rRNA gene clusters derived from sponge-associated microorganisms (Hentschel et al., 2002; Taylor et al., 2007; Simister et al., 2012b; Steinert et al., 2014). However, many prokaryotic members of sponge-specific clusters were also found in seawater and sediment samples, albeit at low abundances, therefore these clusters should be considered as "sponge-enriched" instead of "sponge-specific" (Taylor et al., 2013; Thomas et al., 2016). Approximately half of the most abundant OTUs in the present study are significantly enriched in sponges belonging to the large collection of sponge microbiome project specimens (Moitinho-Silva et al., 2017b). These sponge-enriched OTUs belong to certain phyla and classes (e.g., *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Deferribacteres*, *Gemmatimonadetes*, *Nitrospirae*, *Thaumarchaeota*), which comprised many "sponge-specific" sequences in past studies (Taylor et al., 2007; Simister et al., 2012b). At lower taxonomic levels, many sponge-enriched OTUs represent still uncultured prokaryotes; hence their ecological functions are still unknown (e.g., *Sva0996 marine group*, *Sh765B-TzT-29*, *PAUC34f*, *Marine Group I*). While uncultured, these taxa are often associated with sponge hosts (Moitinho-Silva et al., 2014a; Thomas et al., 2016; Steinert et al., 2017; Verhoeven et al., 2017). In contrast, other sponge-enriched OTUs in our study belong to known nitrification and denitrification related prokaryotic taxa (e.g., *Nitrosomonadaceae*, *Desulfurellaceae*, *Nitrospinaceae*, *Nitrosococcus*, *Nitrospira*), suggesting that these symbionts may play a role in nitrogen cycling processes.

Aerobic nitrification in Vietnamese sponges

Ammonia is known as metabolic waste products excreted by sponge cells, and the microbial nitrifiers may be key components in ammonia waste removal (e.g., Jiménez and Ribes, 2007; Bayer et al., 2008a; Hoffmann et al., 2009). Taxonomic analysis of OTUs at lower levels showed that a large number of OTUs (n = 78) belonged to prokaryotic taxa whose representatives are known aerobic nitrifiers: *Candidatus* Nitrosopumilus (AOA), *Nitrosococcus*, *Nitrosomonadaceae* (AOB), and *Nitrospira* (NOB: nitrite-oxidizing bacteria) (Supplementary Figure S6). Recent studies have demonstrated that some *Nitrospira* are also capable of performing complete ammonium oxidation (Comammox) (e.g., Daims et al., 2015; Van Kessel et al., 2015; Pinto et al., 2016). These OTUs were found in most of the Vietnamese sponges studied here (17 out of 18 species and 26 out of 27 samples), of which

some species harbored a large proportion (> 20% in prokaryotic communities) of these taxa (i.e., *Amphimedon* sp. 1, *T. aploos*, *C. schulzei*, *Haplosclerida* sp., *H. amboinensis*, *Niphatidae* sp.). However, the sponges investigated harbored different prokaryotic partners putatively able to perform aerobic nitrification: *Amphimedon* sp. 1, *C. schulzei*, *Haplosclerida* sp., and *Niphatidae* sp. predominantly hosted *Nitrosomonadaceae*, *T. aploos Nitrosococcus*, *H. amboinensis Candidatus* Nitrosopumilus, and *Dactylospongia* sp. *Nitrospira*. To date, all AOA found by these studies belonged to the phylum *Thaumarchaeota*, including the genera *Nitrosopumilus* and *Cenarchaeum*, whereas the NOB detected in sponges mostly belonged to *Nitrospira* (Kowalchuk and Stephen, 2001; Steger et al., 2008; Hoffmann et al., 2009; Turque et al., 2010; Cuvelier et al., 2014; Kennedy et al., 2014; Pfister et al., 2014; Medema et al., 2015; Feng et al., 2016). In addition, *Nitrosospira* (AOB) was found in specimens *Aplysina aerophoba* from the Mediterranean Sea (Bayer et al., 2008a). Overall, these findings suggest that nitrification is an important microbial process in marine sponges. The appearance of abundant OTUs related to prokaryotic taxa known for nitrification in Vietnamese sponges further supports this idea.

Conclusion

In our present study we investigated the sponge-associated prokaryotic composition of 18 sponge species collected from the central coastal region of Vietnam. Our study highlights the prokaryotic diversity associated with Vietnamese sponges as well as the pattern of host-specificity among samples with replicates. The presence of OTUs significantly enriched in sponge microbiome database supports the general consensus that sponges host certain prokaryotic taxa that are not found or found only in a few samples of other environments. In addition, our study reveals the presence of prokaryotic taxa particularly known for nitrification, which indicates nitrification might be an important microbial process in sponge hosts.

Acknowledgements

This study was supported by BE-Basic Foundation-FES (No. F07.003.06), Vietnam Ministry of Science and Technology (No. ĐTĐLCN.17/14), MC-ITN-BluePharmTrain (No. 607786).

We would like to acknowledge Institute of Marine Biochemistry and Mientrung Institute for Scientific Research (VAST) for sample collection.

Data Availability

Illumina MiSeq raw sequence data can be accessed via the NCBI Sequence Read Archive with accession numbers SRS1815731 – SRS1815757.

The 18S rRNA and COI gene sequences can be accessed via GenBank under the accession numbers: KX894454 – KX894480 (18S rRNA genes) and KX894481 – KX894504 (COI genes).

Supplementary Information

Supplementary Table S1. Sample data, including sample name, identified taxonomy, sampling date and site, and higher taxonomic levels of collection for each sample.

Sample	Taxonomy	Date	Site	Coordinates	Class	Order	Family
AMC	*Amphimedon* sp. 1.	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Niphatidae*
AMQ	*Amphimedon* sp. 2	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Haplosclerida	*Niphatidae*
AXT.1	*Axinyssa* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Suberitida	*Halichondriidae*
AXT.2	*Axinyssa* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Suberitida	*Halichondriidae*
AXT.3	*Axinyssa* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Suberitida	*Halichondriidae*
AXT.4	*Axinyssa* sp.	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Suberitida	*Halichondriidae*
AXC	*Axos cliftoni*	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Tethyida	*Hemiasterellidae*
CIS	*Cinachyrella schulzei*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Spirophorida	*Tetillidae*
CLR.1	*Clathria reinwardti*	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Poecilosclerida	*Microcionidae*
CLR.2	*Clathria reinwardti*	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Poecilosclerida	*Microcionidae*
DAS.1	*Dactylospongia* sp.	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Dictyoceratida	*Thorectidae*
DAS.2	*Dactylospongia* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Dictyoceratida	*Thorectidae*
HAS	*Halichondria* sp.	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Suberitida	*Halichondriidae*
HAA.1	*Haliclona amboinensis*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Chalinidae*
HAA.2	*Haliclona amboinensis*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Chalinidae*
HAF	*Haliclona fascigera*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Chalinidae*
CRV	*Haplosclerida* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Haplosclerida	-
NIS	*Niphatidae* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Haplosclerida	*Niphatidae*
RHG	*Rhabdastrella globostellata*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Tetractinellida	*Ancorinidae*
SPV	*Spheciospongia* sp.	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Clionaida	*Clionaidae*

SPS.1	*Spirastrella* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Clionaida	*Spirastrellidae*
SPS.2	*Spirastrella* sp.	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Clionaida	*Spirastrellidae*
TES	*Tedania* sp.	Sep-2015	Hon Mun Island	109°18'08.1"E; 12°10'04.4"N	Demospongiae	Poecilosclerida	*Tedaniidae*
TEA	*Terpios aploos*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Hadromerida	*Suberitidae*
XES.1	*Xestospongia testudinaria*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Petrosiidae*
XES.2	*Xestospongia testudinaria*	May-2015	Lang Co Bay	108°07'10.5"E; 16°13'31.6"N	Demospongiae	Haplosclerida	*Petrosiidae*
XES.3	*Xestospongia testudinaria*	Aug-2015	Con Co Island	107°07'06.0"E; 17°04'50.2"N	Demospongiae	Haplosclerida	*Petrosiidae*

Supplementary Table S2. List of primers used in this study

Primer	Sequence (5'-3')	Target	Annealing temperature	Amplicon length (bp)	Use	Reference
EUKF	aacctggttgatcctgccagt	Eukaryotes 18S rRNA gene	55	1700-1800	Clone library	Medlin et al., 1998
EUKR	tgatccttctgcaggttcacctac	Eukaryotes 18S rRNA gene	55	1700-1800	Clone library	Medlin et al., 1998
jgLCO1490	titciaciaaycayaargayattgg	Mitochondrial COI gene	52	640	Clone library	Geller et al., 2013
jgHCO2198	taiiacytciggrtgiccraaraayca	Mitochondrial COI gene	52	640	Clone library	Geller et al., 2013
515F	gtgycagcmgccgcggtaa	V4 region of 16S rRNA gene	56	291	Illumina sequencing	Apprill et al., 2015
806R	ggactacnvgggtwtctaat	V4 region of 16S rRNA gene	56	291	Illumina sequencing	Apprill et al., 2015

Supplementary Table S3. List of barcodes, unitag sequences and accession numbers used in this study

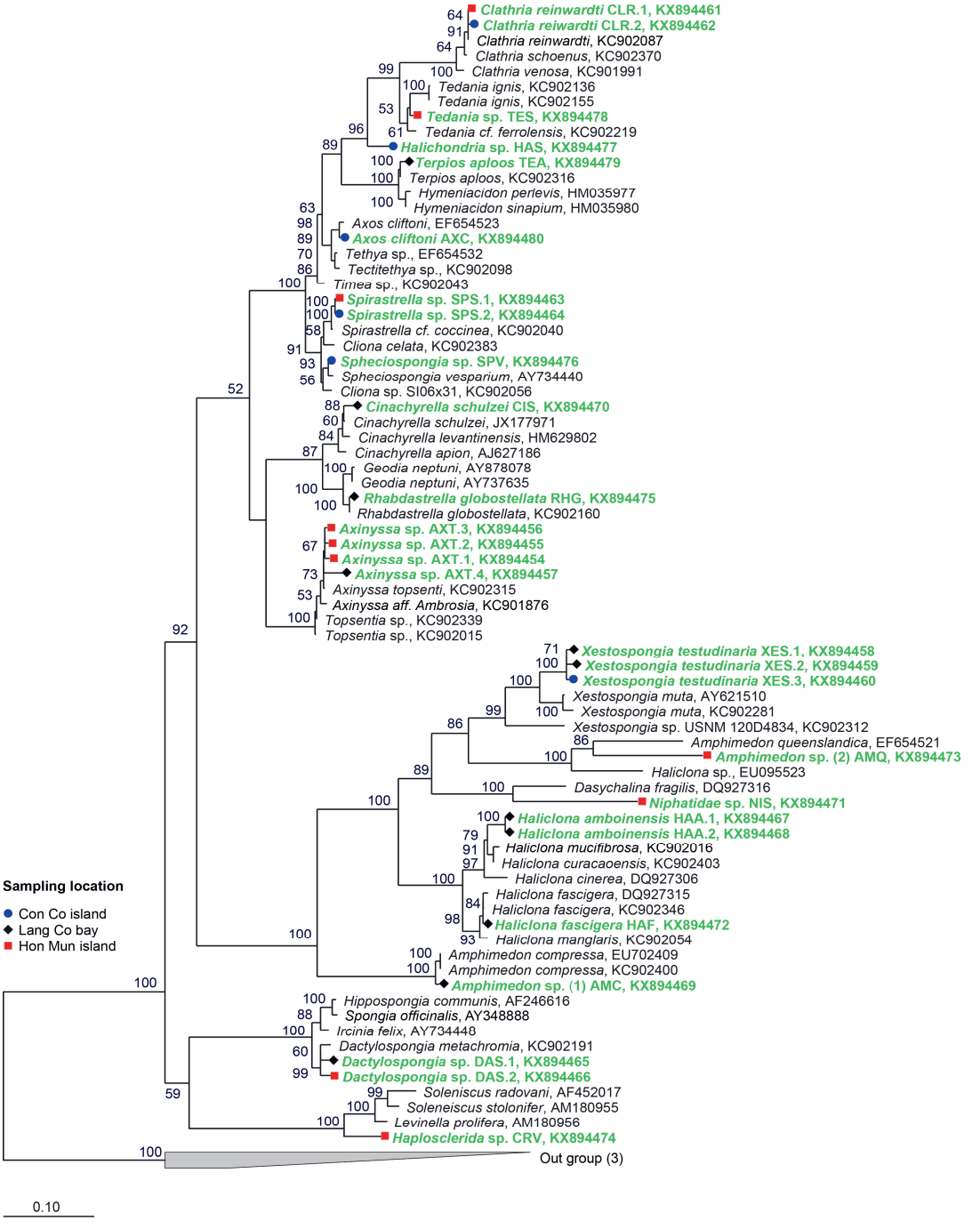
Sample	Barcode	UniTag1	UniTag2	Accession Number
AXT.1	CCGTCTGC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815746
AXT.2	TAATACGT	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815747
AXT.3	GGCCAGTA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815737
AXT.4	CTGAGTTC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815749
XES.1	TTCTGAAC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815757
XES.2	CTTCATGG	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815750
XES.3	CGCGCCAG	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815738
CLR.1	GCCTCATC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815736
CLR.2	TCAGGCGA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815744
SPS.1	CGTCTGAG	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815745
SPS.2	CGTCCTCC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815740
DAS.1	ATGTTCCA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815741
DAS.2	GGTCAGAT	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815751
HAA.1	GGAGTATG	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815734
HAA.2	TACTTATC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815733
AMC	ATCTCAGT	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815743
CIS	TGGTTGAC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815732
NIS	ATAAGGTC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815753
HAF	GGAGCGCA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815735
AMQ	GTCAACGT	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815731
CRV	CGACCGCG	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815739
RHG	CCAAGTCA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815754
SPV	GAGTTCAT	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815742
HAS	AATAAGGA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815748
TES	TCGTCGCC	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815752
TEA	CTGGATA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815756
AXC	AGAGATAA	GAGCCGTAGCCAGTCTGC	GCCGTGACCGTGACATCG	SRS1815755

Supplementary Table S4. Kruskal-Wallis analysis of the prokaryotic alpha diversity indices of sponge-species with duplicates. The Kruskal-Wallis test was performed using function *kruskal.test* within the FSA package in R. Significant differences are highlighted in bold.

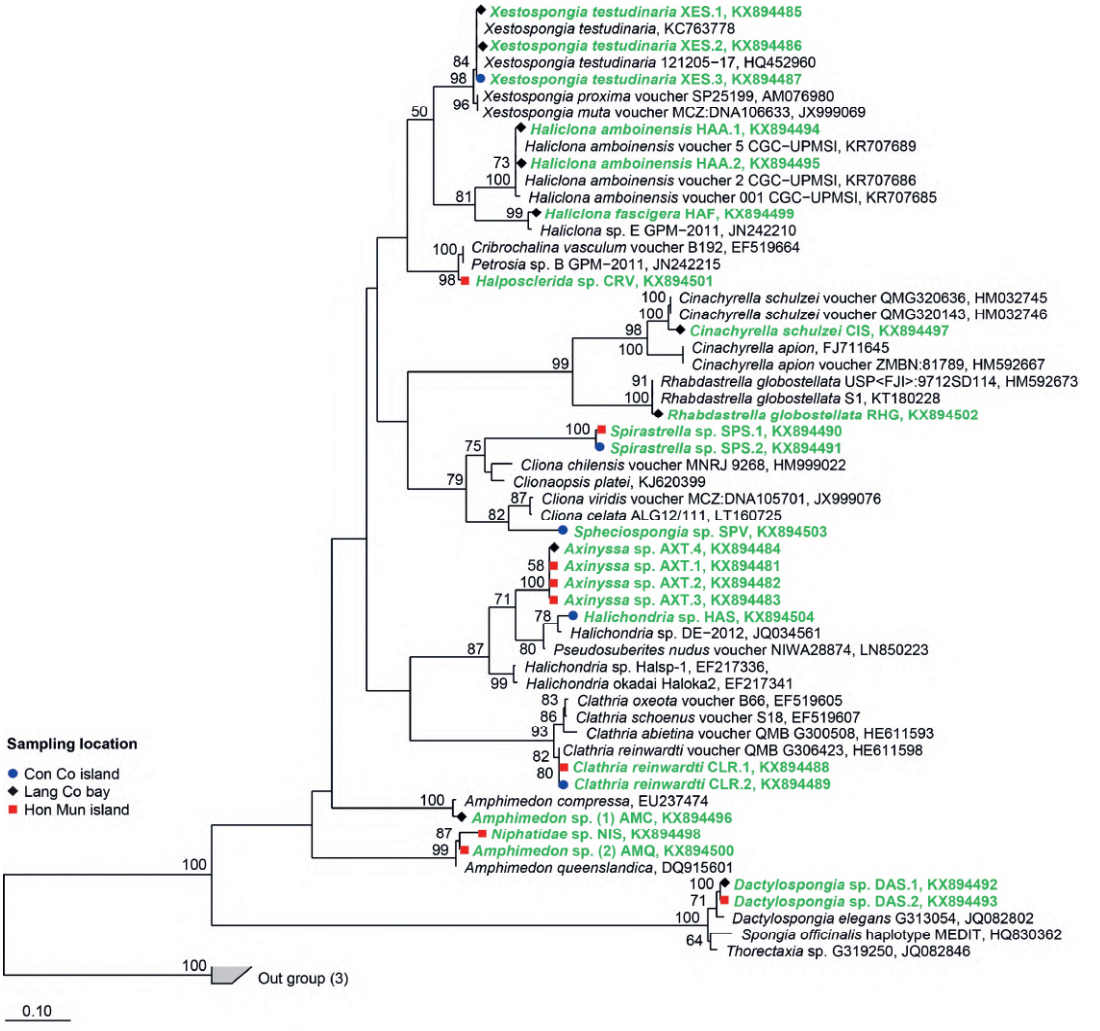
Index	**df**	***Kruskal-Wallis chi-squared***	***p-value***
Richness	5	11.837	**0.03709**
Shannon	5	11.967	**0.03525**
Evenness	5	13.087	**0.02257**
Inverse Simpson	5	12.742	**0.02592**

Supplementary Table S5. Multivariate analysis of the influence of host-identity on their prokaryotic communities for sponge-species with replicates. Multivariate analyses were performed using the functions *betadisper*, *permutest*, and the permutational multivariate analysis of variance function (*adonis)* of the vegan package in R. Significant differences are highlighted in bold.

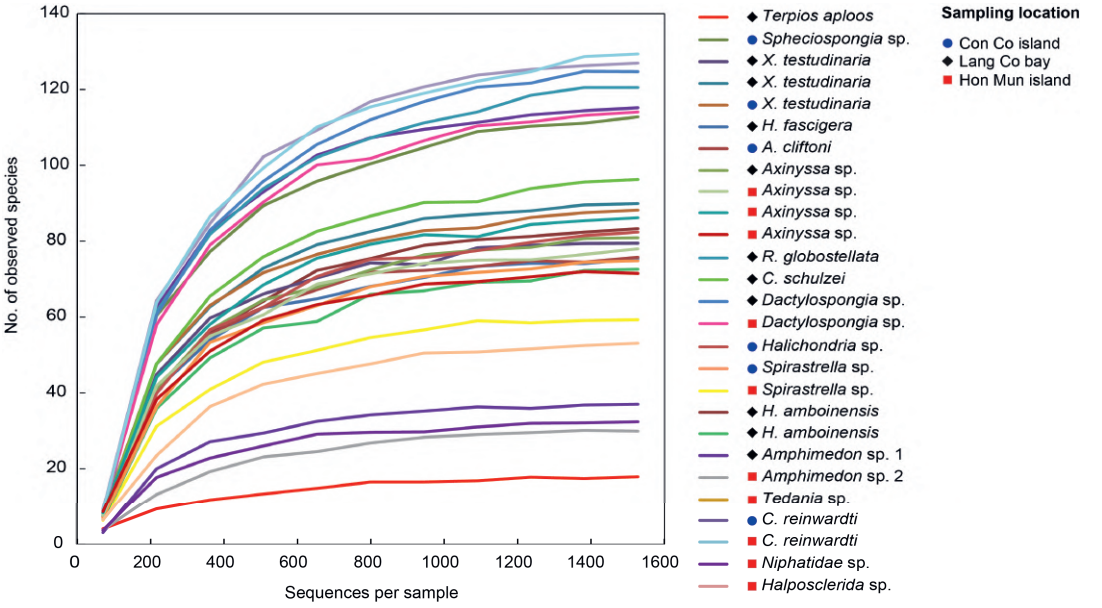
	Betadisper				
	df	***Sum Sq***	***Mean Sq***	***F-value***	***p-value***
Sponge-species group	5	0.044123	0.008825	2.4873	0.1019
Residuals	9	0.031931	0.003548		
Total	14	0.076054			
	Adonis				
	df	***Sum Sq***	***Mean Sq***	***R^2***	***p-value***
Sponge-species group	5	4.6035	0.92069	0.94	**0.000999**
Residuals	9	0.2936	0.03263	0.06	
Total	14	4.8971		1	



Supplementary Figure S1. Phylogenetic tree based on 18S rRNA gene sequences (>1700 bp sequences) of sponge specimens in this study (green bold font) and their closest sequences derived from NCBI (black). The sequences were aligned using MAFFT (v.7.222) with the FFT-NS-i strategy. The phylogenetic tree was constructed using RAxML version 7.2.6 with the GTRGAMMA model and 1000 bootstrap replicates. Bootstrap value < 50 are not shown.

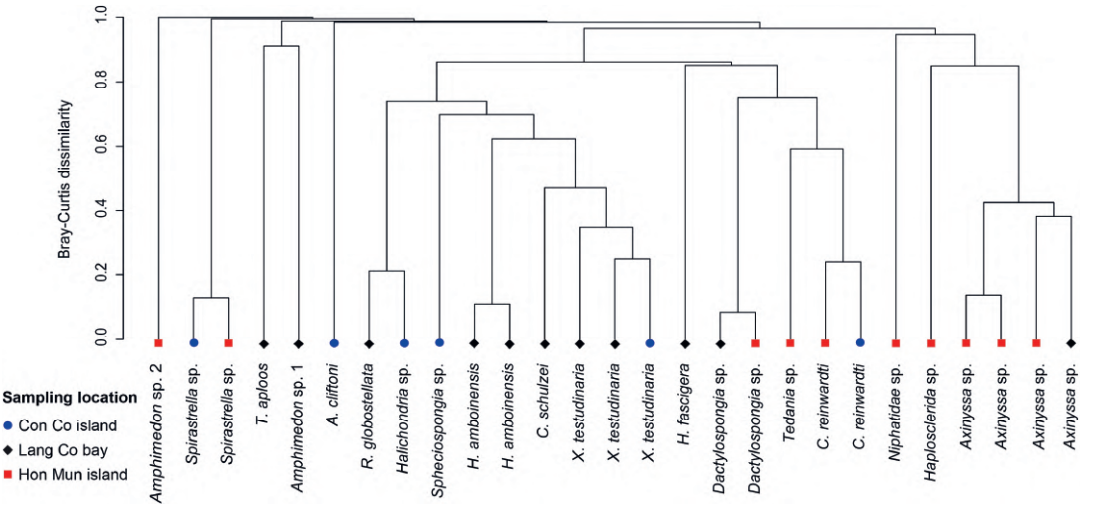


Supplementary Figure S2. Phylogenetic tree based on COI gene sequences (>600 bp sequences) of sponge specimens in this study (green bold font) and their closest sequences derived from NCBI (black). The sequences were aligned using MAFFT (v.7.222) with the FFT-NS-i strategy. Phylogenetic tree was constructed using RAxML version 7.2.6 with the GTRGAMMA model and 1000 bootstrap replicates. Bootstrap value < 50 are not shown.

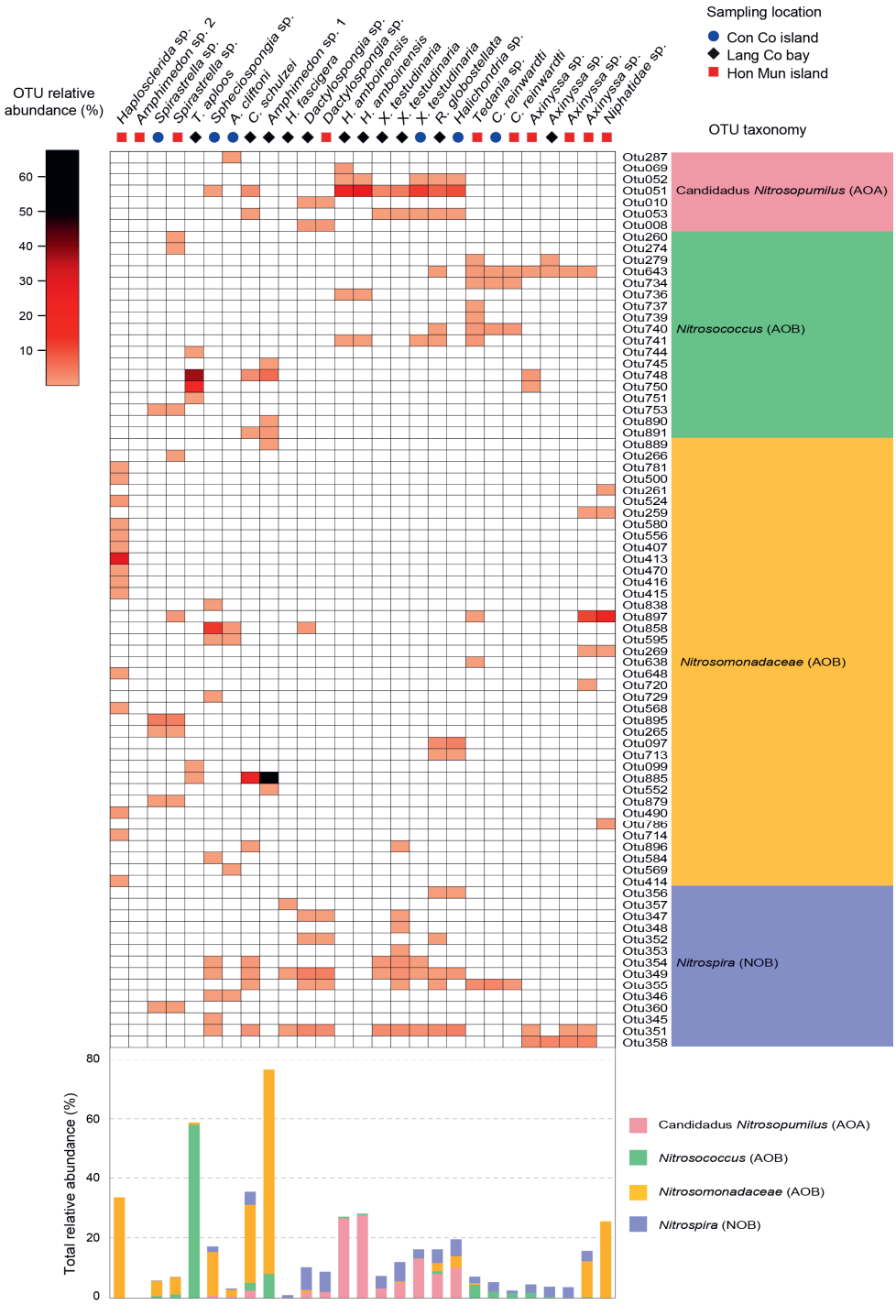


Supplementary Figure S3. Rarefaction curves indicating the average observed species for the different samples using QIIME script *alpha_rarefaction.py*.

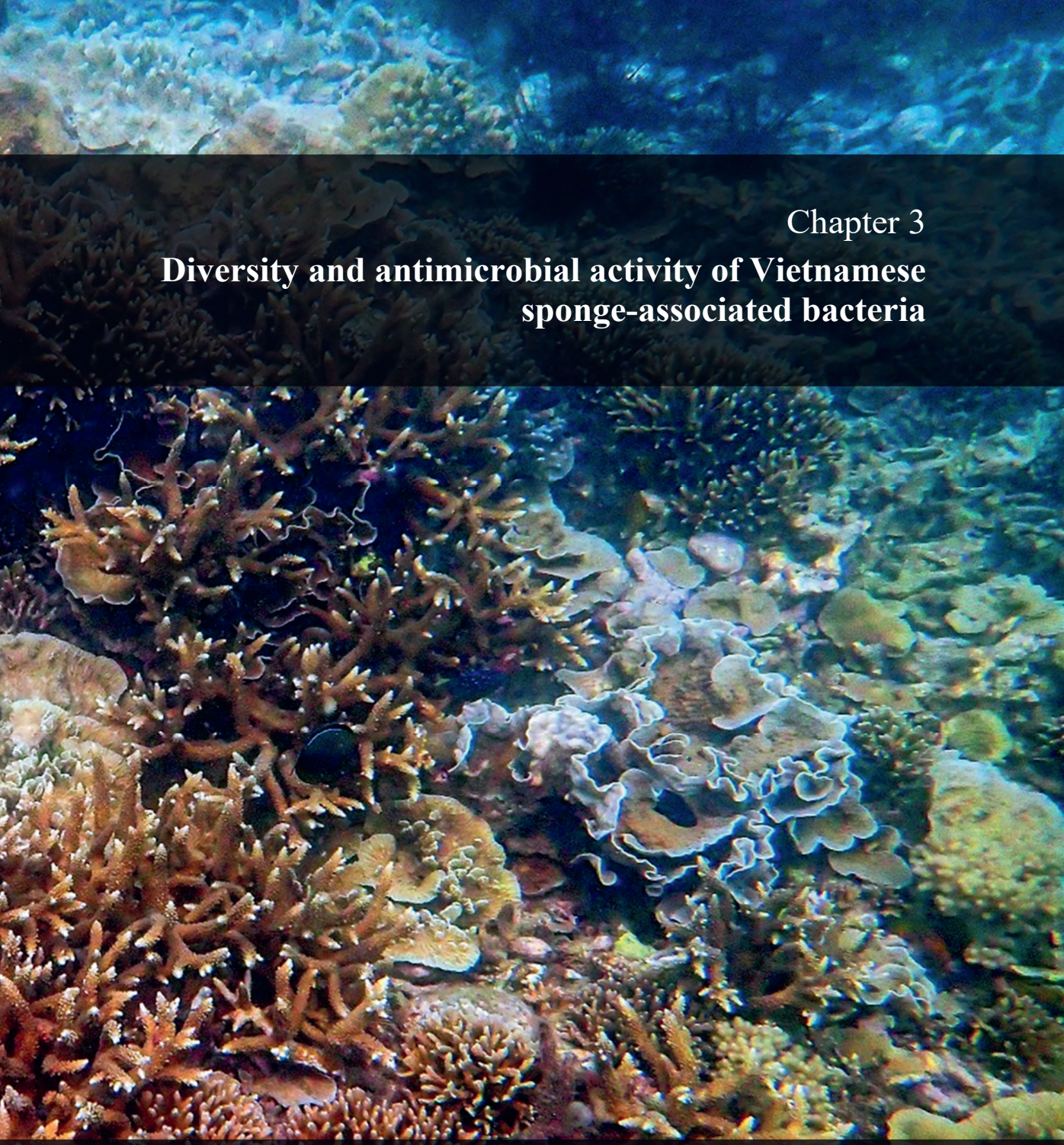
Supplementary Figure S4. Heatmap and bar chart of archaeal OTUs (phylum *Thaumarchaeota*) in different sponges. OTU taxonomy was assigned by NG-tax.



Supplementary Figure S5. Dendrogram displaying Bray-Curtis dissimilarity among sponge prokaryotic communities at OTU level. The hierarchical clustering based on Bray-Curtis similarity was calculated using the vegdist (method = "bray") function of the vegan package in R version 3.3.1.



Supplementary Figure S6. Heatmap and bar chart of OTUs belonging to known nitrification taxa in different sponges. OTU taxonomy was assigned by NG-tax.



Chapter 3

Diversity and antimicrobial activity of Vietnamese sponge-associated bacteria

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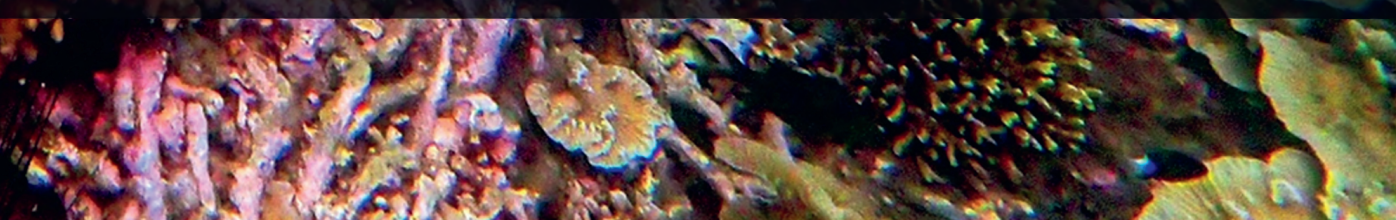
This chapter was published as Dat et al. (2021)
Marine Drugs 2021, 19(7):353. https://doi.org/10.3390/md19070353



Abstract

This study aimed to assess the diversity and antimicrobial activity of cultivable bacteria associated with Vietnamese sponges. In total, 460 bacterial isolates were obtained from 18 marine sponges. Of these, 58.3% belonged to *Proteobacteria*, 16.5% to *Actinobacteria*, 18.0% to *Firmicutes*, and 7.2% to *Bacteroidetes*. At the genus level, isolated strains belonged to 55 genera, of which several genera, such as *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces*, were the most predominant. Culture media influenced the cultivable bacterial composition, whereas, from different sponge species, similar cultivable bacteria were recovered. Interestingly, there was little overlap of bacterial composition associated with sponges when the taxa isolated were compared to cultivation-independent data. Subsequent antimicrobial assays showed that 90 isolated strains exhibited antimicrobial activity against at least one of seven indicator microorganisms. From the culture broth of the isolated strain with the strongest activity (*Bacillus* sp. M1_CRV_171), four secondary metabolites were isolated and identified, including cyclo(L-Pro-L-Tyr) (**1**), macrolactin A (**2**), macrolactin H (**3**), and 15,17-epoxy-16-hydroxy macrolactin A (4). Of these, compounds **2**–**4** exhibited antimicrobial activity against a broad spectrum of reference microorganisms.

Introduction

Antimicrobial resistance decreases the possibilities for prevention and treatment of infectious diseases caused by viruses, bacteria, parasites, and fungi. Factors listed as causes for the rising prevalence of antibiotic resistance include over-prescription of antibiotics both in hospitals and agriculture, poor infection control in hospitals and clinics, lack of hygiene, and poor sanitation (Cantón et al., 2013; Manyi-Loh et al., 2018; Collignon and Beggs, 2019; Malik and Bhattacharyya, 2019; Saleem et al., 2019). Thus, antibiotic resistance has considerable social and economic impact by the increase of morbidity and mortality of infectious diseases. Currently, it is estimated that approximately 700,000 people die every year from drug-resistant infections. By 2050, antibiotic resistance may attribute to 10 million deaths worldwide each year and trigger an economic loss of up to 100 trillion US dollars (O'Neill, 2014). While antibiotic resistance increases, only a limited number of new antibiotics have been discovered and approved for medical treatment (Taylor and Wright, 2008). Therefore, increasing efforts towards discovery and exploitation of novel antimicrobial compounds are urgently needed.

Modifications of existing drugs are often not effective enough to overcome the mutation rate of microbial pathogens and do not lead to the introduction of new classes of antimicrobial compounds (Projan, 2003). In addition, chemical synthesis and semi-synthesis approaches of new antimicrobial compounds and their analogues are hindered by the complexity of the molecules and low yield (Breitling and Takano, 2016; Kunakom and Eustáquio, 2019).

Therefore, the discovery of novel antimicrobial compounds from the biosphere remains an important avenue for finding our future antibiotics.

The terrestrial environment has been the main source of new antibiotics in recent decades. However, re-discovery of known compounds has limited the development of new drugs from the terrestrial environment for treating infectious diseases (Taylor and Wright, 2008). The marine environment encompasses several of the richest ecosystems on Earth, with an extreme diversity of life forms. However, its bioactive compounds have been largely unexplored (Hughes and Fenical, 2010; Montaser and Luesch, 2011; Choudhary et al., 2017). Of marine organisms, sponges (phylum Porifera) are considered the most promising source of bioactive natural products and contribute about 30% of the known marine natural products (Mehbub et al., 2014; Carroll et al., 2019). Furthermore, sponge-derived compounds exhibit a wide spectrum of biological properties, including antimicrobial, anticancer, anti-inflammatory, immunosuppressive, and neurosuppressive activity (Sipkema et al., 2005; Mehbub et al., 2014; Jaspars et al., 2016; Carroll et al., 2019). However, the low concentration of sponge-derived compounds in their tissues has led to issues with supply and halted preclinical and clinical studies of many promising therapeutic drug candidates (Proksch et al., 2003).

To date, of many sponge-derived secondary metabolites, the true origin is unknown. The secondary metabolites could be produced by the sponges, by their microbial symbionts, or by the cooperation between sponges and symbionts (Wagner-Döbler et al., 2002). Interestingly, several studies have highlighted that many bioactive compounds from sponges might be of bacterial origin due to the structural similarity of the molecules to compounds found in terrestrial microorganisms (Thoms and Schupp, 2005; Taylor et al., 2007; Fuerst, 2014). For example, it is now known that polybrominated biphenyl ether antibiotics isolated from the sponge *Dysidea herbacea* are actually produced by the endosymbiotic cyanobacterium *Oscillatoria spongeliae* (Newman and Hill, 2006). The antifungal peptide theopalauamide isolated from the marine sponge *Theonella swinhoei* has been found to be contained in a δ-proteobacterial symbiont (Schmidt et al., 1998). Furthermore, recent studies have confirmed that sponge-associated microorganisms are a valuable source of antimicrobial compounds with potent biological activity and diverse structural features, which make these microbial communities promising sources of novel antimicrobials (Laport et al., 2009; Fuerst, 2014; Juliana et al., 2014; Santos-Gandelman et al., 2014; Indraningrat et al., 2016). Thus, obtaining the sponge-associated bacterial producers of these compounds as pure cultures presents a way to overcome the supply issue.

A previous study based on cultivation-independent methods showed a high diversity of the prokaryotic communities associated with Vietnamese sponges (Dat et al., 2018). To explore the antimicrobial activity from sponge-associated bacteria, in this study, we aimed to isolate bacteria and screen the antimicrobial activity from 18 Vietnamese sponge species. The strain

(*Bacillus* sp. M1_CRV_171) with the highest antimicrobial activity was then subjected to isolation and purification of antimicrobial compounds.

Materials and Methods

Collection and identification of sponges

Sponge specimens were collected by Scuba diving from May to September 2015 from the central coastal region of Vietnam at 5–10 m depth and identified using molecular markers (18S rRNA and cytochrome oxidase I (COI) genes) in a previous study: *Axinyssa* sp., *Xestospongia testudinaria*, *Clathria reinwardti*, *Spirastrella* sp., *Dactylospongia* sp., *Haliclona amboinensis*, *Cinachyrella schulzei*, *Niphatidae* sp., *Haliclona fascigera*, *Amphimedon* sp. (1), *Amphimedon* sp. (2), *Haplosclerida* sp., *Rhabdastrella globostellata*, *Spheciospongia* sp., *Halichondria* sp., *Tedania* sp., *Terpios aploos*, and *Axos cliftoni* (Dat et al., 2018).

Isolation of bacteria from sponges

The sponge specimens were rinsed three times with sterile seawater to remove bacteria attached to the surface of sponges. The specimens were then further cleaned with a sterile scalpel in order to remove sediment and other organisms attached to the sponge. A piece of sponge specimen (~1 cm^3) was crushed with a sterile mortar and pestle and then homogenized in 10 volumes of sterile seawater. The cell suspension was serially diluted till 10^{-6} and subsequently plated onto seven different media: MA (0.5% peptone, 0.1% yeast extract, 1.5% agar) (modified from Weiner et al., 1985); M1 (1.0% starch, 0.4% yeast extract, 0.2% peptone, 1.5% agar) (modified from Mincer et al., 2002); R2A (0.05% yeast extract, 0.05% glucose, 0.05% peptone, 0.05% casein hydrolysate, 0.05% starch, 0.03% sodium pyruvate, 0.03% K_2HPO_4, 0.005% $MgSO_4$, 1.5% agar) (modified from Reasoner and Geldreich, 1985); SCA (0.5% starch, 0.002% casein, 0.1% KNO_3, 0.1% NaCl, 0.1% K_2HPO_4, 0.5 mL/L $MgSO_4$ 100 mM; 0.5 mL/L $FeSO_4$ 100 mM, 0.5 mL/L $CaCO_3$ 100 mM, 1.5% agar) (modified from Kuster and William, 1964); AIA (0.01% peptone, 0.001% L-asparagine, 0.4% sodium propionate, 0.005% K_2HPO_4, 0.001% $MgSO_4$, 0.001 g/L $FeSO_4$, 1 mL/L glycerol, 1.5% agar) (Sipkema et al., 2011); OLIGO (0.05% yeast extract, 0.05% tryptone, 0.01% sodium glycerolphosphate, 1.5% agar) (Olson et al., 2000); SWA (1.5% agar). Nalidixic acid and cycloheximide were supplemented into culture media at concentrations of 100 µg/L and 25 µg/L, respectively, to reduce growth of fast-growing Gram-negative bacteria and fungi. All culture media were prepared with sterile natural seawater at pH 7.8 and all plates were produced in triplicate and incubated for 3–5 days at 30 °C. The pure cultures were obtained by streaking on agar plates and were stored with 20% glycerol (*v*/*v*) at −80 °C at the Mientrung Institute for Scientific Research, Hue city, Thua Thien Hue province, Vietnam.

Screening for antimicrobial activity of the isolates

The isolates (n = 460) were incubated in M1 broth under aerobic conditions on a rotary shaker (150 rpm) at 30 °C until they reached the stationary phase, then the cultures were centrifuged at 14,000× *g* for 10 min. The cell-free supernatants were used for screening their antimicrobial activity.

Antimicrobial activity of the isolates was tested against seven indicator strains, including Gram positive bacteria: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, Gram negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, and the yeast *Candida albicans* ATCC 10231 using the agar well diffusion method. The indicator bacteria were grown in nutrient broth (NB), whereas the yeast was grown in potato dextrose agar (PDA). All indicator strains were grown aerobically on a rotary shaker (150 rpm) overnight and then the density of the strains in cultures was adjusted to an OD_{600} of 0.5. Subsequently, 100 µL of these growing cultures was spread on Mueller-Hinton (MH) agar (for bacteria) and RMPI agar supplemented with 2% glucose (for yeast). The agar wells were prepared by using a sterilized cork borer (6 mm in diameter). Antimicrobial activity of the isolates was examined by adding 100 µL of the cell-free supernatant of each isolate to the wells. For the negative controls, 100 µL of the respective sterile, uninoculated liquid media was added to the 6 mm wells. For positive controls, Ampicillin (10 µg), Kanamycin (30 µg), and Tetracycline (30 µg) were added to the 6 mm wells. The assay was conducted in triplicate for each of the indicator strains.

The agar plates were incubated at 35 °C for 24 h (for the indicator bacteria) and 28 °C for 48 h (for the yeast). After incubation, antimicrobial activity of isolates was determined based on the formation of inhibition zones around wells.

Identification of the isolates by 16S rRNA gene analysis

The glycerol stocks of picked isolates were regrown in the liquid media that were used for their initial isolation. The regrown isolates were identified by colony PCR. For cell lysis, the liquid cultures (2 mL) were centrifuged at 14,000× *g* for 10 min and the obtained pellets were suspended in 50 µL nuclease-free water. Subsequently, the cell suspension was stored at −20 °C for 2 h, followed by incubation at 98 °C for 10 min. The 16S rRNA gene of isolates was directly amplified with universal primers: 27f and 1492r (Lane et al., 1985) through the following PCR program: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 50 s, amplification at 72 °C for 1.5 min, and a final extension at 72 °C for 7 min. The 16S rRNA gene sequencing was carried with a ABI PRISM 3100® Genetic Analyzer (Applied Bioscience and Hitachi, Foster city, CA, USA) with primers 27f and 907r / 1492r (Lane, 1991) to facilitate alignment with Illumina MiSeq sequencing reads that were used to characterize the prokaryotic communities

of the sponges previously (Dat et al., 2018). The sequences were quality checked, and low-quality regions were removed from the sequence ends using BioEdit software v.7.2.6.1. The quality-checked sequences of the isolates were compared to available sequences in the NCBI GenBank using BLAST v.2.7.1+ (Altschul et al., 1990), with the algorithm megablast and the database nt (5 September 2018).

Alpha diversity indices (i.e., observed richness *S*, Shannon diversity index *H*, inverse Simpson *In*, Pielou's evenness *J*) were calculated in R v.3.0.3 with the vegan package (Oksanen et al., 2016; R Core Team, 2016). The relative abundance of the cultivable bacteria at the genus level from different sponge species and media was visualized with JColorGrid (Joachimiak et al., 2006).

To compare the sequences of the isolates to the cultivation-independent fraction, 16S rRNA gene sequences of the isolates in this study were blasted against Illumina MiSeq sequences of prokaryotic OTUs from the same sponge specimens described in the previous study (Dat et al., 2018) using a BLAST search (Altschul et al., 1990). A match was defined as sequences shared between the cultivation-dependent fraction and cultivation-independent fraction if their identity was 100%. The relative abundance of the shared OTUs from the cultivation fraction and cultivation-independent fraction was visualized with JColorGrid (Joachimiak et al., 2006).

Cultivation, extraction, and isolation of secondary metabolites

The strain *Bacillus* sp. M1_CRV_171 was cultured in a 250 mL flask containing 125 mL M1 broth at 30 °C for 48 h in a shaking incubator. The culture was transferred to a 5 L bioreactor (BioFlo 120, Eppendorf, Hamburg, Germany) containing 2.5 L M1 broth with an inoculum of 5% (*v*/*v*). The reactor was operated at 30 °C with agitation of 150 rpm for 48 h. Subsequently, the culture was used to inoculate a 100 L bioreactor (BioFlo 610, Eppendorf, Hamburg, Germany) containing 50 L M1 medium with an inoculum 5% (*v*/*v*). The reactor was operated at 30 °C with agitation of 150 rpm for 7 days. The culture broth (50 L) was extracted with ethyl acetate (25 L × 3 times) at room temperature. The ethyl acetate extract was concentrated under a reduced pressure to yield 11.0 g of crude residue. The residue was subjected to a silica gel chromatography column (CC) (Kiesel gel 60, 70–230 mesh, and 230–400 mesh, Merck, Germany) and eluted with a dichloromethane/methanol gradient (100/1 to 1/100, *v*/*v*) to give seven fractions VKB1-VKB7. The fraction VKB2 was chromatographed on an RP-18 column (30–50 μm, Fuji Silysia Chemical Ltd., Kasugai Aichi, Japan) and eluted with methanol/water (1/1, *v*/*v*) to obtain compound **2** (17.8 mg) and a subfraction VKB2.1. Compound **1** (6.3 mg) was obtained from fraction VKB2.1 by silica gel CC, eluted with dichloromethane/methanol (30/1, *v*/*v*). The fraction VKB3 was subjected to a silica gel CC and eluted with dichloromethane/methanol (30/1, *v*/*v*) to give two smaller fractions VKB3.1-VKB3.2. The fraction VKB3.1 was further separated on an RP-18 column and

eluted with methanol/water (1.5/1, *v*/*v*) to give compound **3** (3.7 mg). Compound **4** (3.4 mg) was purified from the fraction VKB3.2 on an RP-18 column eluted with methanol/water (1.5/1, *v*/*v*).

The electrospray ionization mass spectra (ESI-MS) of the compounds were recorded on a MicroQ-TOF III mass spectrometer (Bruker Daltonics, Bremen, Germany), and the NMR spectra (^{1}H NMR, ^{13}C NMR, HMBC, HSQC) of the compounds were recorded on a Bruker Avance III HD 500 FT NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) with TMS as an internal standard. The optical rotations were recorded on a JASCO P-2000 digital polarimeter (JASCO, Tokyo, Japan). The structures of the isolated compounds were elucidated by the examination of their ESI-MS, NMR spectra and compared with reported data.

Compound **1** (Cyclo(L-Pro-L-Tyr)): white solid; ESI-MS *m/z* 261.1 $[M+H]^+$; $[\alpha]_D^{24}$ = -17.5 (c 0.05, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ_H (ppm): δ_H 1.82 (2H, m, H-4), 2.11 (2H, m, H-5), 3.07 (2H, m, H-10), 3.37 (1H, m, H_a-3), 3.57 (1H, m, H_b-3), 4.07 (1H, m, H-6), 4.37 (1H, m, H-9), 6.72 (2H, d, *J* = 8.5 Hz, H-3', 5'), 7.05 (2H, d, *J* = 8.5 Hz, H-2', 6'); ^{13}C NMR (125 MHz, CD_3OD) δ_C (ppm): δ_C 22.7 (C-4), 29.3 (C-5), 37.6 (C-10), 45.9 (C-3), 57.9 (C-9), 60.0 (C-6), 116.2 (C-3', 5'), 127.6 (C-1'), 132.1 (C-2', 6'), 157.6 (C-4'), 166.9 (C-1), 170.8 (C-7).

Compound **2** (Macrolactin A): amorphous solid; ESI-MS *m/z* 403.2 $[M+H]^+$; $[\alpha]_D^{24}$ = -8.3 (c 0.10, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ_H (ppm): δ_H 1.27 (3H, d, *J* = 6.5 Hz, H-24), 1.52 (2H, m, H-21), 1.59 (1H, m, H_a-22), 1.63 (2H, m, H-14), 1.65 (1H, m, H_b-22), 2.12 (1H, m, H_a-20), 2.20 (1H, m, H_b-20), 2.34 (1H, m, H_a-12), 2.44 (2H, m, H-6), 2.50 (1H, m, H_b-12), 3.87 (1H, m, H-13), 4.28 (1H, m, H-7), 4.32 (1H, m, H-15), 5.02 (1H, m, H-23), 5.55 (1H, m, H-11), 5.56 (1H, m, H-2), 5.57 (1H, m, H-16), 5.66 (1H, m, H-19), 5.77 (1H, dd, *J* = 6.0, 15.0 Hz, H-8), 6.05 (1H, m, H-18), 6.10 (1H, m, H-17), 6.13 (1H, m, H-10), 6.19 (2H, m, H-5), 6.58 (1H, dd, *J* = 11.0, 15.0 Hz, H-9), 6.65 (1H, t, *J* = 11.5 Hz, H-3), 7.23 (1H, m, H-4); ^{13}C NMR (125 MHz, CD_3OD) δ_C (ppm): δ_C 20.1 (C-24), 25.6 (C-21), 32.9 (C-20), 36.1 (C-22), 36.4 (C-12), 42.8 (C-6), 43.9 (C-14), 69.2 (C-13), 69.8 (C-15), 72.2 (C-23), 72.3 (C-7), 117.9 (C-2), 125.9 (C-9), 128.4 (C-11), 130.2 (C-4), 131.2 (C-17), 131.3 (C-10), 131.7 (C-18), 135.1 (C-19), 135.2 (C-16), 137.5 (C-8), 142.1 (C-5), 144.9 (C-3), 168.0 (C-1).

Compound **3** (Macrolactin H): amorphous solid; ESI-MS *m/z* 377.3 $[M+H]^+$; $[\alpha]_D^{24}$ = -24.6 (c 0.05, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ_H (ppm): δ_H 1.27 (3H, d, *J* = 6.5 Hz, H-22), 1.40 (2H, m, H-19), 1.58 (1H, m, H_a-12), 1.59 (2H, m, H-14), 1.65 (1H, m, H_a-20), 2.08 (2H, m, H-18), 2.33 (1H, m, H_b-12), 2.45 (2H, m, H-6), 4.26 (1H, m, H-15), 4.28 (1H, m, H-21), 4.32 (1H, m, H-13), 4.98 (1H, m, H-7), 5.46 (1H, m, H-11), 5.55 (2H, m, H-2, 16), 5.59 (1H, m, H-4), 5.63 (1H, m, H-10), 5.65 (2H, m, H-3, 17), 5.76 (1H, m, H-8), 6.17 (1H, m, H-5),

6.75 (1H, m, H-9); ^{13}C NMR (125 MHz, CD_3OD) δ_C (ppm): δ_C 166.3 (C-1), 118.0 (C-2), 144.8 (C-3), 130.4 (C-4), 141.7 (C-5), 42.5 (C-6), 72.0 (C-7), 137.2 (C-8), 126.1 (C-9), 131.0 (C-10), 134.7 (C-11), 36.5 (C-12), 69.7 (C-13), 44.6 (C-14), 70.2 (C-15), 128.3 (C-16), 131.7 (C-17), 32.9 (C-18), 25.9 (C-19), 36.6 (C-20), 70.8 (C-21), 20.1 (C-22).

Compound **4** (15,17-epoxy-16-hydroxy macrolactin A): amorphous powder; ESI-MS *m/z* 419.3 $[M+H]^+$; $[\alpha]_D^{24}$ = -37.2 (c 0.01, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ_H (ppm): δ_H 1.28 (3H, d, J = 6.0 Hz, H-24), 1.40 (1H, m, H_a-14), 1.52 (2H, m, H-21), 1.67 (2H, m, H-22), 1.99 (1H, m, H_b-14), 2.05 (1H, m, H_a-20), 2.11 (1H, m, H_b-20), 2.13 (1H, m, H_a-12), 2.42 (1H, m, H_a-6), 2.51 (1H, m, H_b-6), 2.57 (1H, m, H_b-12), 2.94 (1H, t, J = 9.0 Hz, H-16), 3.46 (2H, m, H-13, 17), 3.54 (1H, m, H-15), 4.27 (1H, m, H-7), 4.98 (1H, m, H-23), 5.46 (1H, m, H-11), 5.54 (1H, d, J = 11.5 Hz, H-2), 5.64 (1H, m, H-8), 5.67 (1H, m, H-18), 5.70 (1H, m, H-19), 6.07 (1H, t, J = 11.0 Hz, H-10), 6.27 (1H, m, H-5), 6.56 (1H, dd, J = 11.0, 15.0 Hz, H-9), 6.67 (1H, t, J = 11.5 Hz, H-3), 7.25 (1H, m, H-4); ^{13}C NMR (125 MHz, CD_3OD) δ_C (ppm): δ_C 19.8 (C-24), 26.6 (C-21), 34.4 (C-20), 35.1 (C-12), 36.8 (C-22), 40.8 (C-14), 42.7 (C-6), 72.9 (C-23), 73.1 (C-7), 73.9 (C-15), 76.0 (C-13), 77.7 (C-16), 80.2 (C-17), 118.2 (C-2), 127.7 (C-9), 129.1 (C-18), 129.5 (C-11), 130.6 (C-4), 131.4 (C-10), 132.2 (C-19), 136.2 (C-8), 141.4 (C-5), 144.6 (C-3), 168.2 (C-1).

Antimicrobial activity of the secondary metabolites

Antimicrobial activities of the isolated secondary metabolites **1**–**4** were tested against a broad spectrum of reference microorganisms. Besides the seven reference microorganisms used for antimicrobial screening assays of isolated strains (see above), antimicrobial activity was evaluated against several microbial plant and aquaculture pathogens isolated in Vietnam: *P. putida* MISR 71218, *Rhodococcus* sp. MISR 16518, *B. cereus* MISR 12818, *Vibrio parahaemolyticus* MISR 21116, *V. vulnificus* MISR 20716, *V. alginolyticus* MISR 30816, *Aspergillus niger* MISR-11215, *Fusarium oxisporum* MISR 20415, and *Rhizoctonia solani* MISR 11115.

MICs of the secondary metabolites against reference microorganisms were determined using the broth microdilution method. Determination of MICs of the isolated compounds against the indicator bacteria was performed according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) discussion document E.Dis 5.1 (EUCAST, 2003). Briefly, the isolated compounds were dissolved in dimethyl sulfoxide (DMSO) and serially two-fold diluted in MH broth to a concentration range that was twice the desired final concentration (0.5–512 µg/mL) and obtained by adding an equal volume of indicator bacteria cell suspension (see below). 100 µL of the compound solutions were added to wells of 96-well plates. The indicator Bacteria were then incubated overnight in NB on a rotary shaker (150 rpm) at 37 °C, then the density of strains was adjusted to a McFarland standard of 0.5, which contains approximately 1.5×10^8 CFU/mL (range $1–2 \times 10^8$ CFU/mL).

The bacterial solution was subsequently diluted 150-fold in MH broth to reach a starting inoculum of 1×10^6 CFU/mL. Finally, 100 µL of this inoculum was added to wells containing 100 µL of the compound solution (see above) to obtain a final inoculum of 5×10^5 CFU/mL. The plates included growth control wells (inoculated in compound-free medium) and sterile (uninoculated) wells. The plate was incubated at 37 °C for 24 h, then the absorbance at 630 nm was measured using a microplate reader. The MIC was defined as the lowest concentration of a compound, at which there was no visible growth of indicator bacteria.

Determination of MICs of the isolated compounds against the yeast (*C. albicans*) was performed as recommended in the EUCAST definitive document E.DEF 7.3 (Arendrup et al., 2015a). The isolated compound solutions were diluted to the same concentrations (0.5–512 µg/mL) as the antibacterial assay in double strength RPMI 1640 broth (with L-glutamine and a pH indicator but without bicarbonate), supplemented with glucose to a final concentration of 2% (RPMI 2% G), similar to the above-described concentrations. The yeast strain was grown on PDA at 35 °C for 48 h, then colonies were suspended in 10 mL distilled water. The suspension was homogenized by vortexing for 15 s. Subsequently, the cell density was adjusted to the 0.5 McFarland standard, which contains approximately 1–5 × 10^6 CFU/mL by measuring the absorbance at a wavelength of 530 nm. The yeast solution was diluted 10-fold in sterile distilled water to obtain a starting inoculum of 1–5 × 10^5 CFU/mL, and 100 µL of this inoculum was then added to wells containing 100 µL of the compound solution (see above) to obtain a final inoculum of 0.5–2.5 × 10^5 CFU/mL. The plates included growth control wells (inoculated in compound-free medium) and sterile (uninoculated) wells. The plate was incubated at 35 °C for 24 h, then the absorbance at 530 nm was measured using a microplate reader. The MICs were defined as the lowest concentration of antifungal compounds, at which there was no visible growth of the indicator yeast.

Determination of MICs of the isolated compounds against the filamentous fungi was performed as recommended in the EUCAST definitive document E.DEF 9.3 (Arendrup et al., 2015b). For the filamentous fungi, the microdilution was performed as described for the yeast, with an exception for the preparation of the inoculum. Fungi were grown on PDA at 28 °C for 5 days, and the fungal colonies were covered with 10 mL distilled water supplemented with 0.1% Tween 20, and suspensions were made by scraping the surface with a sterile loop. Heavy particles in the suspension were allowed to settle for 15 to 20 min at room temperature. The suspension in the upper clear phase was transferred to sterile tubes and homogenized by vortexing for 15 s. The suspension was filtered using Whatman filter paper no. 1 with a pore size of 11 µm to remove large hyphal fragments. The optical density of the suspensions was recorded at 530 nm and then adjusted to transmittance of 80 to 85%. The number of colony-forming units was quantified by plating 100 µL of suspension on PDA and counting the colonies after incubating at 28 °C for 5 days. The inoculum concentrations used ranged from 2 to 5 × 10^6 CFU/mL. Each suspension was diluted (1:10) with sterile

distilled water to obtain the working inoculum concentration of 2 to 5 × 10^5 CFU/mL. An aliquot of 100 µL of this inoculum was then added to wells containing 100 µL of the compound solution (see above) to obtain a final inoculum of 1–2.5 × 10^5 CFU/mL. The plates included growth control wells (inoculated in compound-free medium) and sterile (uninoculated) wells. The plate was incubated at 28 °C for 48 h, and MICs of the antifungal compounds were determined visually as the lowest concentration, at which there was no visible growth of the fungi. The antibiotics ampicillin, kanamycin, tetracycline, and miconazole were used as positive controls.

Results

Diversity of sponge-associated cultivable bacteria

In total, agar plates with seven different media yielded 473 bacterial colonies from 18 sponge species (Supplementary Table S1). Of these, 460 bacterial colonies were regrown and identified by their 16S rRNA gene sequences. The isolated strains included four phyla: *Proteobacteria* (*α*-, *γ*- and *β-proteobacteria*), *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Figure 1A). Among them, the phylum *Proteobacteria* was the most predominant (58.3% of the isolated strains), followed by *Firmicutes* (18.0%) and *Actinobacteria* (16.5%), whereas the phylum *Bacteroidetes* represented 7.2% of the isolated strains. At the genus level, 55 genera were represented, of which the genera *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces* were the most predominant, each accounting for more than 5% of all isolated strains (Figures 1B and 2). The number of bacterial genera recovered from sponges ranged from 15 to 21. The composition of cultivable bacteria from different sponges was relatively similar at the genus level (Figure 2).

The culture media significantly affected the cultivable bacteria (*p*-values of 0.21 (Betadisper) and <0.001 (Adonis)) (Supplementary Table S2). Although the most abundant genera *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces* were isolated on all cultivation media, several genera were only isolated from nutrient-poor media, such as *Arthrobacter*, *Curtobacterium*, *Nonlabens*, *Tenacibaculum*, *Exiguobacterium*, *Anderseniella*, *Pseudoruegeria*, *Thalassobius*, and *Alcanivorax* (Figure 3). None of the isolated strains represented new species as, based on their 16S ribosomal RNA (rRNA) gene sequence, they were all 99–100% similar to known strains (Supplementary Table S5). The nutrient-rich media resulted in higher numbers of colonies and genera than nutrient-poor media (e.g., OLIGO, SWA) (Supplementary Table S1). M1 medium yielded the highest number of genera (38), followed by MA medium (35 genera), R2A (33 genera), SCA (30 genera), AIA (27 genera), and OLIGO (23 genera), and the lowest number of genera was observed with SWA, on which 17 genera were recovered (Figure 3). In addition, nutrient-rich media had significantly higher richness, Shannon diversity, and Inverse Simpson diversity, while nutrient-poor media (i.e., OLIGO, SWA) exhibited higher evenness (Tables S3 and S4). The

higher alpha diversity indices for rich media may be partially explained by the higher number of colonies obtained from those media (Supplementary Table S1).

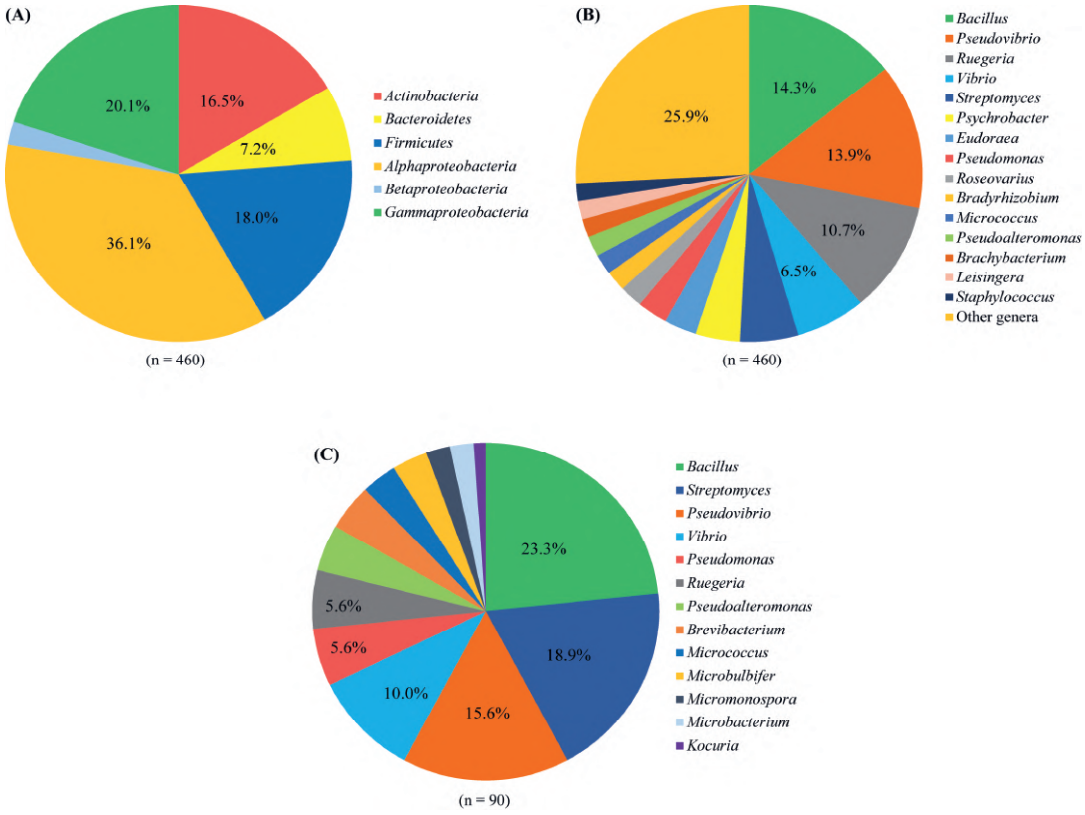


Figure 1. Composition of cultivable bacteria associated with sponges at the phylum level (at the class level for the phylum *Proteobacteria*) (**A**) and the genus level (**B**). Composition of bacteria with antimicrobial activity associated with sponges at the genus level (**C**).

The 16S rRNA gene sequences from the isolated strains were compared to sequences that were generated by direct Illumina MiSeq amplicon sequencing analysis of the same sponge samples (Dat et al., 2018). Six OTUs out of 921 OTUs detected by MiSeq sequences were also found by cultivation; however, their abundance was distinct for the two methods (Figure 4). For example, OTU909 (*Bacillus*), OTU337 (*Pseudovibrio*), OTU541 (*Ruegeria*), and OTU710 (*Pseudoalteromonas*) were cultivated from all sponge species; however, these OTUs were not detected or detected at very low relative abundance from the same sponge species by MiSeq analysis.

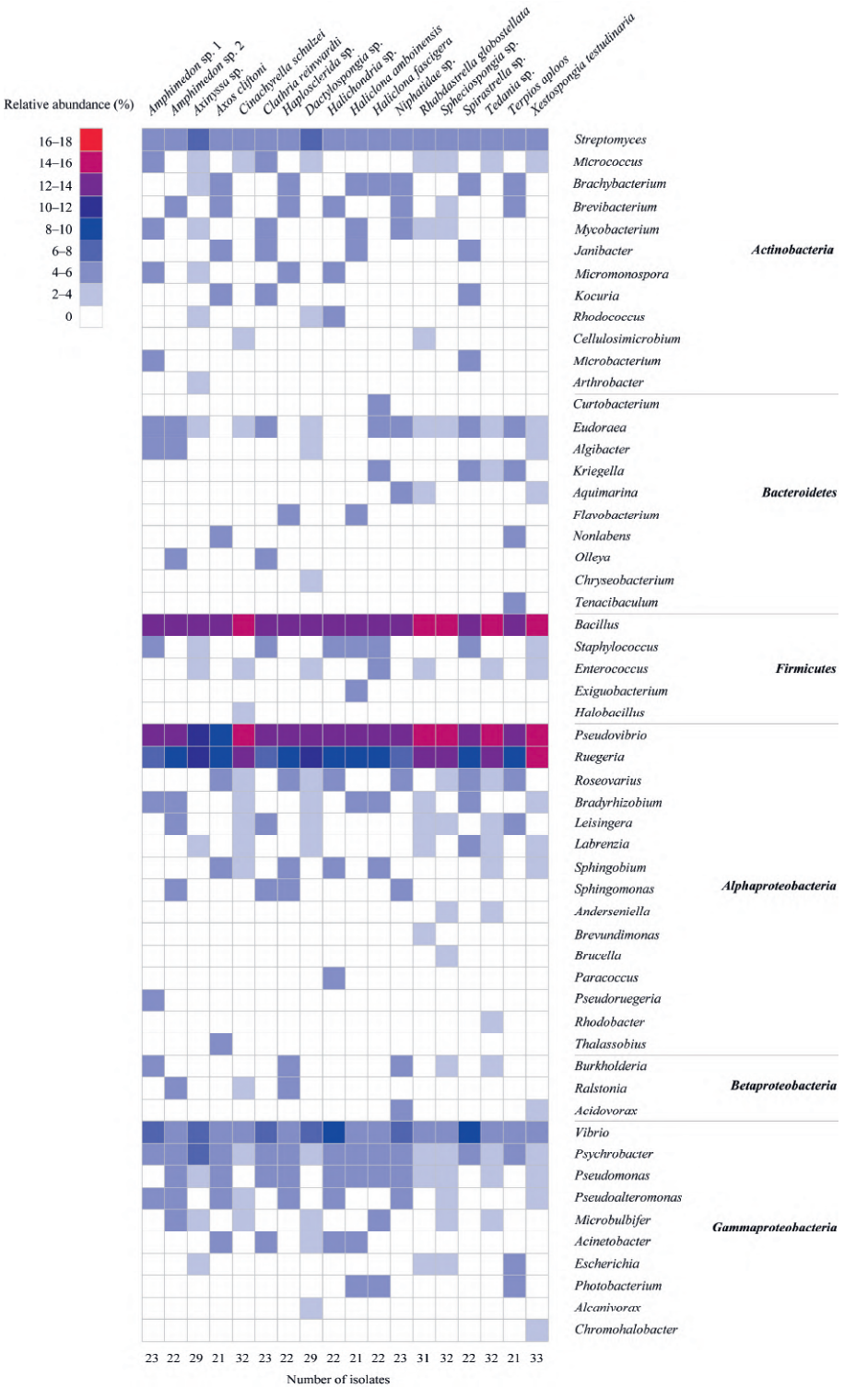


Figure 2. Heatmap of the composition and relative abundance of cultivable bacteria isolated from different sponge species at the genus level.

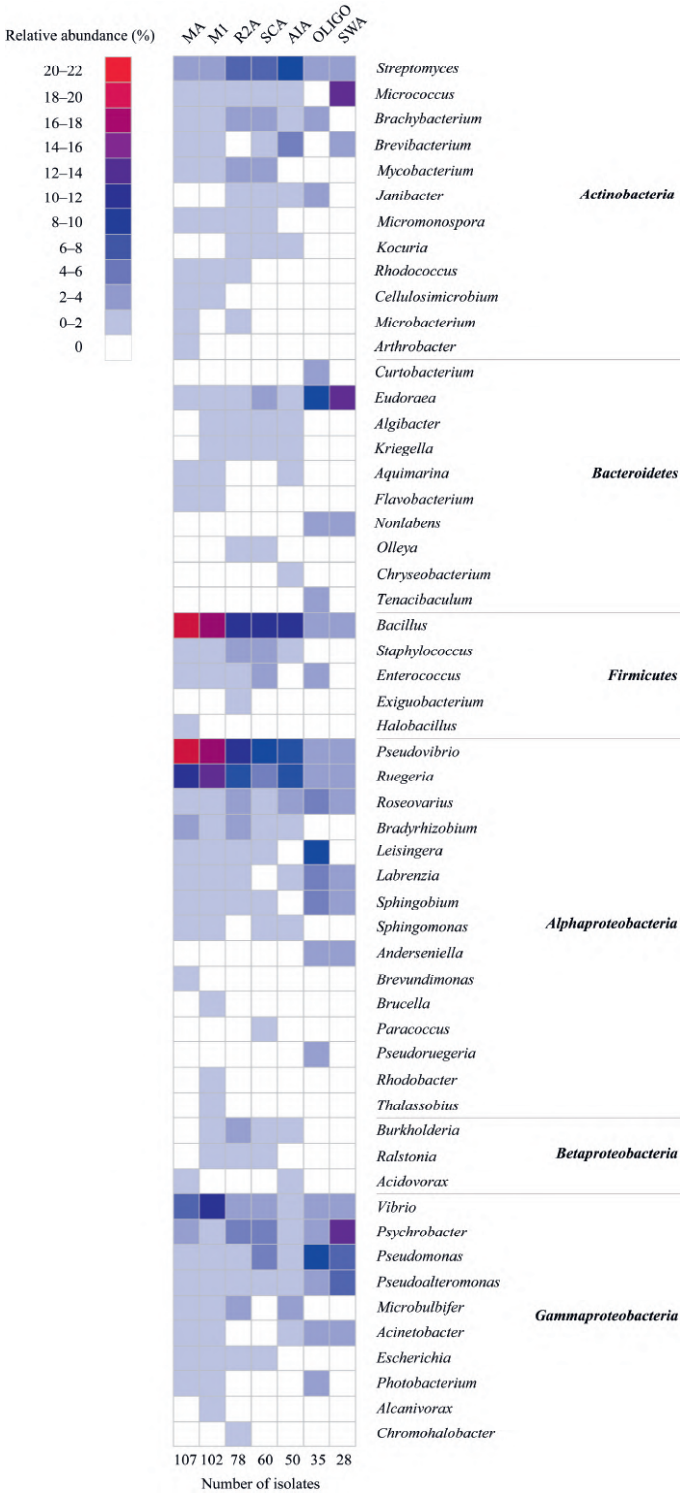


Figure 3. Heat map of the composition and relative abundance of cultivable bacteria isolated from different culture media at the genus level.

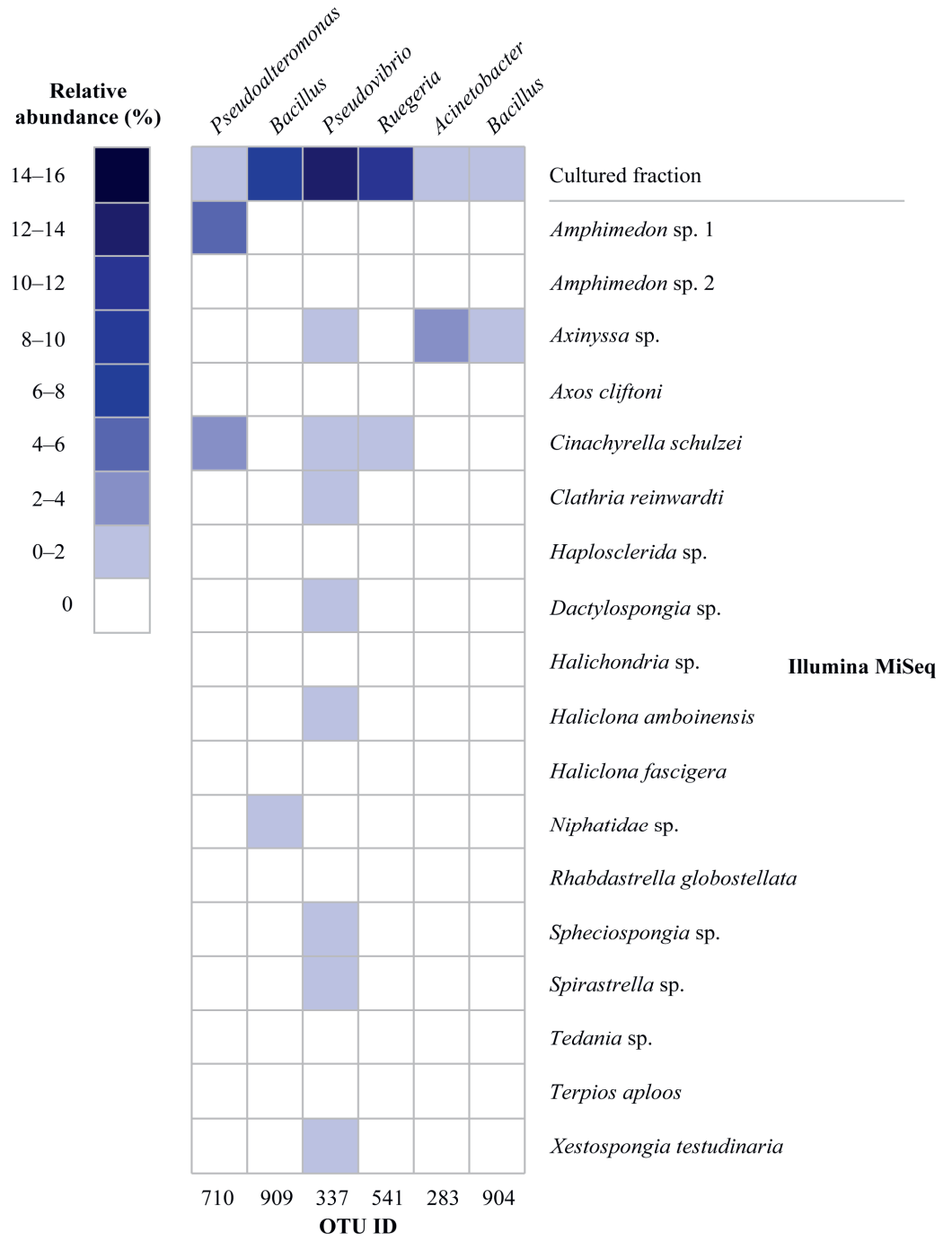


Figure 4. Heat map of relative abundance of shared OTUs recovered by cultivation and cultivation-independent approaches (MiSeq). The relative abundance of OTUs in the cultivable fraction was calculated for total sequences for all sponge species, whereas the relative abundance of OTUs in Illumina MiSeq data was calculated for each sponge species.

Antimicrobial activity of isolated strains

Cell-free supernatants from cultures of all isolated strains (n = 460) were screened for their antimicrobial activity against seven indicator microorganisms: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, *Salmonella enterica* ATCC 13076, and *Enterococcus faecalis* ATCC 29212. The cell-free culture supernatants of 90 isolated strains (nearly 20%) showed antimicrobial activity against one or more of the tested indicator microorganisms (Table 1). Among them, the cell-free culture supernatants of 57 isolated strains exhibited activity against one indicator strain, while 21 isolated strains exhibited activity against 2 indicator strains, 8 isolated strains exhibited activity against 3 indicator strains, 3 isolated strains exhibited activity against 4 indicator strains, and 1 isolated strain exhibited activity against 5 tested indicator strains. It was observed that the cell-free culture supernatants showed more antimicrobial activity towards Gram-positive bacteria (21 isolated strains against *S. aureus*, 26 isolated strains against *E. faecalis*, and 27 isolated strains against *B. subtilis*) than Gram-negative bacteria (16 isolated strains against *P. aeruginosa*, 17 isolated strains against *S. enterica*, and 20 isolated strains against *E. coli*), whereas the number of isolated strains exhibiting activity against yeast was the lowest (15 isolated strains). The identification of the active isolated strains revealed that they belonged to 13 genera, with *Bacillus*, *Streptomyces*, and *Pseudovibrio* being predominant with 23.3%, 18.9%, and 15.6%, respectively (Figure 1C).

Table 1. Antimicrobial activity of isolated bacterial strains.

Isolated Strains	Genus	Inhibition Zone Diameter (mm)						
		Gram-Negative			Gram-Positive			Yeast
		SE	EC	PA	SA	EF	BS	CA
MA_AMC_32	*Bacillus*	-	-	-	10 ± 2	-	-	-
MA_AMQ_66	*Bacillus*	-	7 ± 2	-	-	-	8 ± 3	-
MA_AXT_69	*Bacillus*	7 ± 2	-	-	-	-	-	-
MA_AXT_70	*Bacillus*	5 ± 2	-	8 ± 3	6 ± 3	-	8 ± 3	-
MA_AXC_75	*Bacillus*	-	7 ± 2	-	-	-	-	-
MA_CIS_78	*Bacillus*	-	-	-	-	-	-	6 ± 2
M1_CRV_171	*Bacillus*	-	5 ± 2	8 ± 2	7 ± 3	10 ± 3	-	6 ± 2
M1_DAS_199	*Bacillus*	-	-	12 ± 3	-	-	-	-
M1_HAA_234	*Bacillus*	-	-	-	-	5 ± 1	11 ± 3	7 ± 2
M1_HAA_246	*Bacillus*	-	-	-	6 ± 2	-	-	-
M1_NIS_274	*Bacillus*	5 ± 2	-	-	-	-	-	-
R2A_NIS_276	*Bacillus*	-	-	-	-	-	12 ± 3	-
R2A_RHG_312	*Bacillus*	-	-	-	5 ± 2	-	-	-
R2A_SPV_326	*Bacillus*	-	-	7 ± 2	-	5 ± 2	7 ± 2	-

R2A_SPV_338	*Bacillus*	-	4 ± 1	-	-	3 ± 1	-	6 ± 2
SCA_SPS_344	*Bacillus*	-	-	-	-	-	4 ± 1	-
SCA_TES_347	*Bacillus*	-	-	-	-	-	-	4 ± 1
AIA_TEA_438	*Bacillus*	-	-	-	-	4 ± 1	-	-
AIA_XES_454	*Bacillus*	-	-	15± 3	-	-	-	-
AIA_XES_458	*Bacillus*	12 ± 3	-	-	-	-	-	10 ± 3
M1_HAF_272	*Bacillus*	-	10 ± 3	-	-	-	8 ± 3	-
AIA_SPV_375	*Brevibacterium*	-	-	-	-	12 ± 3	-	-
AIA_HAS_264	*Brevibacterium*	-	-	-	-	-	4 ± 1	-
M1_AXC_175	*Brevibacterium*	-	-	-	-	6 ± 2	10 ± 3	-
MA_AMQ_34	*Brevibacterium*	-	8 ± 3	-	-	-	9 ± 2	-
R2A_AXC_194	*Kocuria*	10 ± 4	-	-	14 ± 4	-	-	7 ± 2
MA_AMC_87	*Microbacterium*	-	-	-	6 ± 2	-	-	-
R2A_SPS_90	*Microbacterium*	-	-	-	4 ± 1	-	-	-
M1_AXT_2	*Microbulbifer*	-	-	-	5 ± 1	-	-	-
R2A_DAS_4	*Microbulbifer*	-	-	3 ± 1	-	-	-	-
AIA_TES_7	*Microbulbifer*	-	12 ± 4	-	-	-	-	-
R2A_CIS_91	*Micrococcus*	-	-	-	-	-	-	9 ± 2
SCA_CLR_217	*Micrococcus*	8 ± 3	-	-	-	-	-	-
M1_AXT_88	*Micrococcus*	11 ± 3	-	-	-	-	-	-
R2A_CRV_10	*Micromonospora*	-	-	13 ± 3	-	-	8 ± 2	-
SCA_HAS_11	*Micromonospora*	-	-	-	6 ± 2	6 ± 2	-	4 ± 1
M1_AXC_17	*Pseudoalteromonas*	-	-	-	-	8 ± 3	-	-
MA_AMC_15	*Pseudoalteromonas*	6 ± 2	-	-	10 ± 4	8 ± 4	-	4 ± 2
R2A_CIS_18	*Pseudoalteromonas*	-	-	-	-	-	7 ± 2	-
SCA_CRV_19	*Pseudoalteromonas*	-	-	-	-	6 ± 1	-	-
MA_AMQ_98	*Pseudomonas*	-	-	6 ± 2	-	-	-	4 ± 1
M1_AXT_131	*Pseudomonas*	-	12 ± 4	-	-	-	-	-
R2A_AXC_259	*Pseudomonas*	-	-	-	-	-	6 ± 2	-
SCA_CLR_279	*Pseudomonas*	-	-	9 ± 3	-	-	-	-
AIA_HAA_313	*Pseudomonas*	-	11 ± 4	-	-	9 ± 2	14 ± 4	-
MA_AMC_93	*Pseudovibrio*	-	-	-	3 ± 1	-	-	-
MA_AMC_33	*Pseudovibrio*	-	-	6 ± 3	-	-	-	-
MA_AMQ_100	*Pseudovibrio*	-	-	-	-	12 ± 4	-	-
MA_AXT_177	*Pseudovibrio*	13 ± 4	-	-	10 ± 3	-	-	-
MA_AXC_181	*Pseudovibrio*	13 ± 4	-	-	-	-	-	-
MA_CIS_184	*Pseudovibrio*	11 ± 4	-	-	-	8 ± 2	10 ± 3	-

MA_CIS_186	*Pseudovibrio*	-	-	5 ± 1	-	-	-	-
MA_CIS_195	*Pseudovibrio*	-	-	-	-	-	7 ± 1	-
MA_CRV_231	*Pseudovibrio*	-	8 ± 3	-	-	-	-	-
M1_DAS_236	*Pseudovibrio*	-	13 ± 3	-	-	7 ± 2	10 ± 3	-
M1_HAA_265	*Pseudovibrio*	-	-	-	-	-	-	5 ± 1
R2A_RHG_301	*Pseudovibrio*	-	-	6 ± 2	-	-	-	-
SCA_TES_374	*Pseudovibrio*	8 ± 2	-	-	14 ± 4	-	-	-
AIA_TEA_401	*Pseudovibrio*	-	15 ± 4	-	-	-	-	-
MA_AMQ_136	*Ruegeria*	-	-	6 ± 2	-	-	-	-
MA_AXT_139	*Ruegeria*	4 ± 1	-	-	7 ± 2	7 ± 2	13 ± 4	-
MA_CIS_145	*Ruegeria*	-	13 ± 4	-	-	7 ± 2	-	-
M1_DAS_153	*Ruegeria*	3 ± 1	-	-	-	-	-	-
R2A_SPV_381	*Ruegeria*	-	6 ± 2	-	-	-	-	-
MA_AMC_38	*Streptomyces*	-	-	3 ± 1	-	8 ± 4	-	-
MA_AMQ_39	*Streptomyces*	7 ± 2	-	-	5 ± 2	-	-	-
M1_AXT_41	*Streptomyces*	-	13 ± 3	-	-	-	-	-
M1_CIS_51	*Streptomyces*	-	-	-	6 ± 2	-	10 ± 3	-
R2A_CLR_53	*Streptomyces*	-	4 ± 1	-	4 ± 2	-	-	-
R2A_DAS_58	*Streptomyces*	-	-	-	-	-	8 ± 3	-
R2A_HAS_60	*Streptomyces*	-	-	-	-	7 ± 2	-	-
SCA_HAF_63	*Streptomyces*	-	9 ± 3	-	-	-	-	-
SCA_RHG_65	*Streptomyces*	-	-	-	7 ± 2	-	-	-
AIA_SPV_83	*Streptomyces*	-	6 ± 2	-	-	-	-	-
AIA_SPS_85	*Streptomyces*	-	-	-	-	-	4 ± 2	-
AIA_TEA_127	*Streptomyces*	-	-	-	-	5 ± 2	-	-
AIA_TES_126	*Streptomyces*	-	-	-	-	-	14 ± 4	-
AIA_TES_125	*Streptomyces*	-	4 ±1	-	7 ± 2	-	-	-
OLIGO_XES_128	*Streptomyces*	3 ± 1	-	-	-	-	-	-
SWA_XES_129	*Streptomyces*	-	8 ± 3	-	-	-	10 ± 3	-
AIA_SPV_84	*Streptomyces*	-	-	-	-	-	10 ± 3	7 ± 2
MA_AMC_44	*Vibrio*	-	-	-	-	5 ± 3	-	6 ± 2
R2A_SPS_117	*Vibrio*	-	-	-	-	-	8 ± 3	-
MA_AMQ_46	*Vibrio*	-	-	-	10 ± 3	-	-	-
AIA_TEA_122	*Vibrio*	-	-	-	-	4 ± 2	-	-
M1_RHG_114	*Vibrio*	-	-	-	-	-	-	4 ± 1
M1_HAA_109	*Vibrio*	-	-	12 ± 3	-	-	-	-
MA_CIS_55	*Vibrio*	-	-	-	-	5 ± 1	-	-
SCA_TES_120	*Vibrio*	11 ± 3	-	-	-	17 ± 3	-	-
M1_CLR_86	*Vibrio*	-	-	13 ± 4	-	-	-	-

Ampicillin	18 ± 3	20 ± 4	-	25 ± 4	21 ± 3	23 ± 3	na
Kanamycin	12 ± 2	17 ± 3	-	19 ± 3	18 ± 2	19 ± 2	na
Tetracycline	10 ± 2	19 ± 3	-	22 ± 3	15 ± 2	21 ± 3	na
Miconazole	na	na	na	na	na	na	17 ± 2

SE—*Salmonella enterica* ATCC 13076; EC—*Escherichia coli* ATCC 25922; PA—*Pseudomonas aeruginosa* ATCC 27853; SA—*Staphylococcus aureus* ATCC 25923; EF—*Enterococcus faecalis* ATCC 29212; BS—*Bacillus subtilis* ATCC 6633; CA—*Candida albicans* ATCC 10231; "-": no inhibition; na: not applicable; values in table: mean ± SD of triplicate.

Isolation and identification of the compounds produced by *Bacillus* sp. M1_CRV_171

Well diffusion assays revealed that *Bacillus* sp. M1_CRV_171 was the only isolated strain to inhibit five reference strains, including Gram-negative bacteria, Gram-positive bacteria, and yeast. Furthermore, this isolated strain was also found in the same sponge by the cultivation-independent method (Dat et al., 2018), as the 16S rRNA gene sequence of this isolated strain was 100% identical with a bacterial OTU recovered from sponge tissue. Therefore, this isolated strain was subjected to isolation of secondary metabolites.

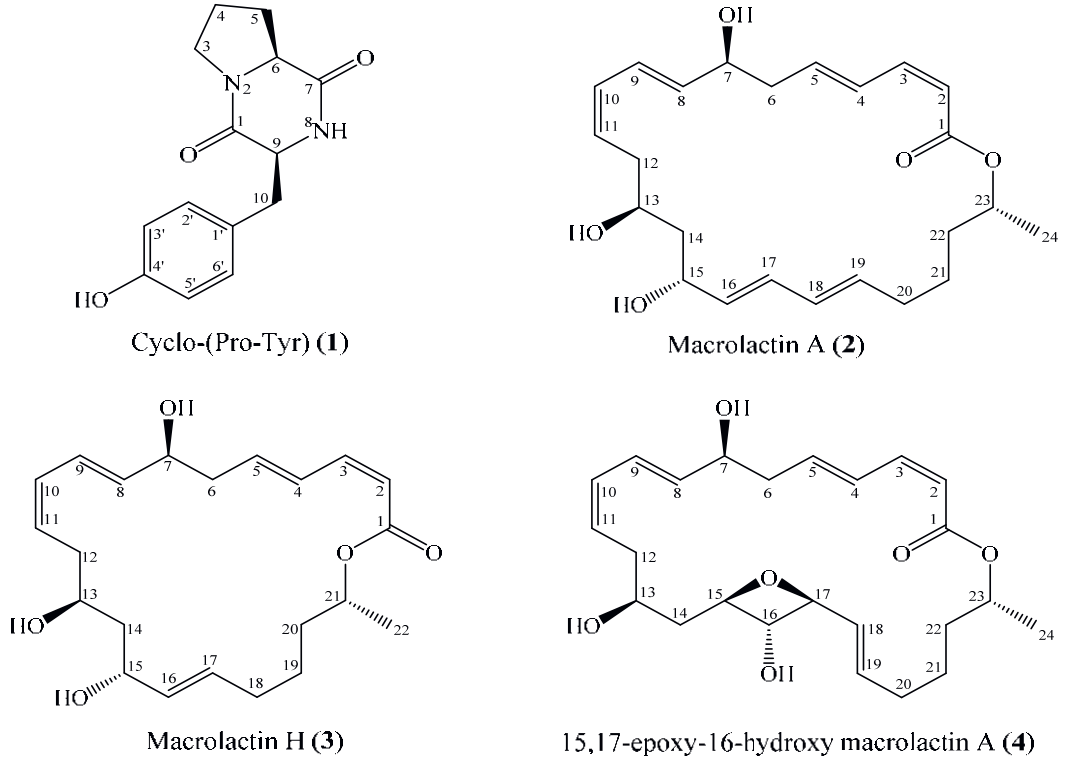


Figure 5. Chemical structures of isolated compounds (**1**–**4**)

Isolation and purification of ethyl acetate extracts from the culture broth of *Bacillus* sp. M1_CRV_171 led to the isolation of four compounds **1**–**4** (Figure 5). The structures of these compounds were elucidated by the examination of their ESI-MS and NMR (^{1}H NMR, ^{13}C NMR, HMBC, HSQC) spectra (Figures S1–S20) and comparison with reported data. The compounds were identified as cyclo(L-Pro-L-Tyr) (**1**) (Wattana-Amorn et al., 2016), macrolactin A (**2**) (Lee et al., 2004; Romero-Tabarez et al., 2006), macrolactin H (**3**) (Nagao et al., 2001), and 15,17-epoxy-16-hydroxy macrolactin A (**4**) (Mondol et al., 2011).

Antimicrobial activity of the isolated secondary metabolites

The four compounds isolated from the strain *Bacillus* sp. M1_CRV_171 were evaluated for their antimicrobial activity against a broad spectrum of microorganisms (Table 2).

Table 2. Antimicrobial activity of the isolated compounds **1**, **2**, **3**, and **4**.

Reference Microorganisms	MIC (µg/mL)							
	1	**2**	**3**	**4**	**Amp**	**Kan**	**Tet**	**Mic**
Gram negative bacteria								
E. coli ATCC 25922	-	64	16	32	8	16	4	na
S. enterica ATCC 13076	-	-	>256	>256	16	64	32	na
P. aeruginosa ATCC 27853	-	8	64	64	>256	>256	64	na
P. putida MISR 71218	-	128	128	128	>256	64	64	na
V. parahaemolyticus MISR 21116	>256	128	64	128	64	32	32	na
V. vulnificus MISR 20716	-	-	128	256	32	64	32	na
V. alginolyticus MISR 30816	-	-	256	128	>256	32	64	na
Gram positive bacteria								
E. faecalis ATCC 29212	-	-	>256	>256	8	128	32	na
S. aureus ATCC 25923	>256	16	32	64	16	16	4	na
B. subtilis ATCC 6633	-	128	64	64	8	16	32	na
B. cereus MISR 12818	-	128	64	64	32	16	64	na
Rhodococcus sp. MISR 16518	-	32	64	128	16	8	32	na
Fungi								
C. albicans ATCC 10231	-	128	64	64	na	na	na	32
A. niger MISR 11215	-	64	64	64	na	na	na	8
F. oxisporum MISR 20415	>256	64	64	64	na	na	na	32
R. solani MISR 11115	>256	64	64	64	na	na	na	32

Amp—ampicillin; Kan—kanamycin; Tet—tetracycline; Mic—miconazole; "-": no activity; na: not applicable.

Compound **1** was inactive against most of the reference strains, except for *S. aureus*, *Vibrio parahaemolyticus*, *Fusarium oxisporum*, and *Ralstonia solani*, with minimum inhibitory

concentrations (MICs) > 256 µg/mL. Compounds **2**–**4** exhibited antimicrobial activity against a wide spectrum of reference strains. Compound **2** exhibited antimicrobial activity against *P. aeruginosa*, *S. aureus*, and *Rhodococcus* sp., with MICs of 8, 16, and 32 µg/mL, respectively. Notably, antimicrobial activity against *P. aeruginosa* of compound **2** was comparable to the positive controls tetracycline (MIC > 64 µg/mL), kanamycin (MIC > 256 µg/mL), and ampicillin (MIC > 256 µg/mL). Furthermore, compound **2** exhibited antimicrobial activity against a wide spectrum of tested microorganisms, with MICs ranging from 64 to >256 µg/mL, while it was inactive against *S. enterica*, *E. faecalis*, *Vibrio vulnificus*, and *Vibrio alginolyticus*. Compound **3** showed antimicrobial activity against *E. coli* and *S. aureus*, with MIC values of 16 µg/mL and 32 µg/mL, respectively, which was comparable to the positive controls (ampicillin, kanamycin, and tetracycline), with MIC values ranging from 4 to 16 µg/mL. Compound **3** also exhibited antimicrobial activity against other tested microorganisms, with MICs ranging from 64 to >256 µg/mL. For compound **4**, the strongest activity was found against *E. coli*, with a MIC value of 32 µg/mL. For other tested microorganisms, compound **4** showed antimicrobial activity with MICs ≥ 64 µg/mL.

Discussion

Cultivable bacteria from Vietnamese sponges

It is well known that bacteria associated with marine sponges are phylogenetically diverse and an important source of bioactive compounds, including antimicrobial compounds (Laport et al., 2009; Fuerst, 2014; Juliana et al., 2014; Santos-Gandelman et al., 2014; Indraningrat et al., 2016). The present study aimed to isolate bacteria associated with Vietnamese sponges, which have only been studied very little, and screen for antimicrobial activity of the isolated strains. The 16S rRNA gene sequence analysis showed that cultivable bacteria from the Vietnamese sponges belonged to four phyla, which is much lower compared to the 15 phyla that were detected by direct Illumina MiSeq amplicon sequencing analysis of the same sponge samples (Dat et al., 2018). This discrepancy has also been observed in previous investigations of sponge-associated bacteria using both cultivation-dependent and -independent approaches (Li et al., 2011a; Sipkema et al., 2011; Hardoim and Costa, 2014; Montalvo et al., 2014; Versluis et al., 2017). The failure of obtaining the diverse bacteria associated with sponges by cultivation can be partially explained by the failure to mimic the natural growth conditions of sponge-associated bacteria (e.g., host-symbiont interactions, substrates required for growth) in the laboratory. Recent developments in reconstructing composite genomes of currently uncultivable bacteria out of metagenomes have provided clues on growth substrates of some sponge-associated bacteria, e.g., predicted consumption of carnitine, spermidine, and sulfated polysaccharides (Slaby et al., 2017; Taylor et al., 2021), compounds that are hardly ever included in bacterial cultivation media. In addition, extensive cultivation efforts by using alternative techniques (e.g., cultivation media and growth condition diversification, liquid culture, floating filter, diffusion chamber

cultivation) have improved the cultivability of sponge-associated bacteria and should be used in more bacterial cultivation experiments with sponges (Sipkema et al., 2011; Steinert et al., 2014; Esteves et al., 2016; Versluis et al., 2017).

At the genus level, cultivable bacteria from Vietnamese sponges were predominated by genera such as *Bacillus*, *Pseudovibrio*, and *Ruegeria*, which are commonly isolated from marine sponges (Hentschel et al., 2001; Webster and Hill, 2001; Enticknap et al., 2006; Muscholl-Silberhorn et al., 2008; O' Halloran et al., 2011; Sipkema et al., 2011; Montalvo et al., 2014; Esteves et al., 2016). A cultivable *Pseudovibrio* sp. has also been found in sponge larvae and may thus represent a bacterium that is vertically transmitted in sponges (Enticknap et al., 2006). Despite their predominance in our cultivation experiment, these genera were not detected, or were detected at very low relative abundance, in the same sponge specimens by direct Illumina MiSeq amplicon sequencing analysis (Dat et al., 2018). It is possible that these bacteria may be selected for cultivation experiments as they may grow quickly in rich-nutrient media and outcompete other slow-growing species, although they are not abundant in the original inoculum (Garland et al., 2001). In addition, several common genera, such as *Pseudovibrio*, *Bacillus*, *Vibrio*, and *Ruegeria* grew well on different cultivation media, suggesting that these genera can adapt well to different cultivation media and conditions. Recent genome studies have reported that genomes of these genera contain the genetic machinery encoding for a versatile metabolism and harbor diverse genomic features linked to symbiosis and lifestyles allowing for host switching (Steil et al., 2003; Bondarev et al., 2013; Ian et al., 2014; Alex and Antunes, 2015b; Tian et al., 2016; Belbahri et al., 2017; Alex and Antunes, 2018; Brito et al., 2018; Díez-Vives et al., 2018; Hernández-González et al., 2018; Lin et al., 2018; Romano, 2018; Versluis et al., 2018).

Antimicrobial activity of sponge-associated bacteria

Marine sponges are sessile organisms, and their defense mechanism against predators (e.g., bacteria, eukaryotic organisms, viruses) is mainly based on the production of a diverse range of secondary metabolite products, allowing efficient chemical protection (Pawlik, 2011; Rohde et al., 2015; Helber et al., 2018). Increasing evidence has shown that many bioactive compounds, including antimicrobial compounds (Laport et al., 2009; Fuerst, 2014; Juliana et al., 2014; Santos-Gandelman et al., 2014; Indraningrat et al., 2016), from sponges are produced by their microbial symbionts (Flowers et al., 1998; Schmidt et al., 1998; Haygood et al., 1999; Faulkner et al., 2000; Hildebrand et al., 2004; Piel et al., 2004; Newman and Hill, 2006). The antimicrobial screening assay of cultured bacteria in this study showed that approximately 20% of the isolated strains exhibited antimicrobial activity. It was observed that strains showed more activity against Gram-positive bacteria than Gram-negative bacteria. The difference in membrane structure between Gram-positive and Gram-negative bacteria could partially explain their different sensitivity to antimicrobial agents. The outer membrane of Gram-negative bacteria is covered by a layer of lipopolysaccharide, which

protects them from antibiotics attacking the peptidoglycan layer (Page, 2012). Among the isolated strains with antimicrobial activity, *Bacillus*, *Streptomyces*, and *Pseudovibrio* were the most frequently obtained. These genera have been reported as major producers of bioactive compounds with antimicrobial activity in sponges (Indraningrat et al., 2016). Genome analyses of sponge-derived isolated bacteria from these genera have reported that they contain a large number of secondary metabolite biosynthesis gene clusters (Ian et al., 2014; Belbahri et al., 2017; Naughton et al., 2017; Harwood et al., 2018; Romano, 2018; Versluis et al., 2018; Xu et al., 2019). One *Bacillus* isolated strain (M1_CRV_171) inhibited the growth of Gram-positive and Gram-negative bacteria and fungi. The genus *Bacillus* produces versatile antimicrobials with diverse structures (e.g., polyketides, ribosomal peptides, non-ribosomal peptides, lipopeptides, macrolactones, polypeptides, isocoumarins) (Mondol et al., 2013; Sumi et al., 2014; Caulier et al., 2019). A large-scale analysis of 1566 genomes of *Bacillus* strains resulted in identifying nearly 20,000 biosynthetic gene clusters (BGCs) (Grubbs et al., 2017). Of these, a large number of BGCs encode the enzymes required for the production of unknown compounds, indicating *Bacillus* as a prolific source of secondary metabolites. It is estimated that at least 4–8% of the genome of *Bacillus* is devoted to synthesizing antimicrobial compounds (Kunst et al., 1997; Stein, 2005; Chen et al., 2007).

Among the antimicrobial isolated strains, the strain *Bacillus* sp. M1_CRV_171 is one of the cultured strains that was found in the cultivation-independent bacterial community analysis (OTU909). Therefore, this isolated strain was subjected to investigation of its secondary metabolites. From culture broth of *Bacillus* sp. M1_CRV_171, one cyclic dipeptide, cyclo(L-pro-L-tyr) (**1**), was isolated and purified. This cyclic dipeptide has been reported to be produced by different bacteria (i.e., *Bacillus*, *Pseudomonas*, *Ruegeria*, *Psychrobacter*) associated with different sponges (Jayatilake et al., 1996; De Rosa et al., 2003; Mitova et al., 2004; Huayue et al., 2008; Sawangwong et al., 2008; Abbamondi et al., 2014), indicating that it may play a role in the sponge holobiont. The antimicrobial assays in our study showed that cyclo(L-pro-L-tyr) exhibited antimicrobial activity against *S. aureus*, *V. parahaemolyticus*, *F. oxisporum*, and *R. solani* and showed no activity against the other tested microorganisms. This confirms previous bioactivity investigations of cyclo(L-pro-L-tyr), which reported that this cyclic dipeptide exhibits antimicrobial activity against several bacteria and plant pathogenic fungi, such as *S. epidermis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *V. cholerae* (Kumar et al., 2014), *Micrococcus luteus*, *S. aureus*, *S. enterica*, *E. coli*, and *Fusarium* sp. (Smaoui et al., 2012), *Xanthomonas axonopodis pv. citri*, and *Ralstonia solanacearum* (Wattana-Amorn et al., 2016), *F. oxysporum* and *Penicillium* sp. (Sansinenea et al., 2016), *A. niger* (Yonezawa et al., 2011), *Phytophthora infestans*, and *Plasmopara viticola* (Cimmino et al., 2014; Puopolo et al., 2014). However, cyclic dipeptides, including cyclo(L-pro-L-tyr), have gained more attention because of their role as quorum sensing (QS) signal molecules (Holden et al., 1999; Degrassi et al., 2002; González and Keshavan, 2006; Ryan and Dow, 2008; Wang et al., 2010; Li et al., 2011b; Ortiz-Castro

et al., 2011; Teasdale et al., 2011). It has been reported that sponge-associated bacteria, as well as cyclic dipeptides isolated from sponge-associated bacteria, are involved in QS by activating acyl homoserine lactones (AHLs) bioreporters (Abbamondi et al., 2014). AHLs are QS signal molecules mainly produced by Gram-negative bacteria and may directly bind to transcription factors (e.g., LuxR) to regulate gene expression (Fuqua et al., 2001; Smith et al., 2006), indicating that these bacteria and cyclic dipeptides may interact with AHL producers and play a certain role in QS. Interestingly, previous studies have demonstrated the production of AHLs by both sponges and their associated bacteria (Taylor et al., 2004a; Bin Saidin et al., 2016; Bose et al., 2017; Britstein et al., 2017), and these compounds may thus be involved in communication in the sponge holobiont.

Furthermore, three macrolides **2**–**4** were isolated from culture broth of *Bacillus* sp. M1_CRV_171. Macrolide compounds are widely used as antibiotics in clinics (e.g., erythromycin) and display different biological activities, including modulating inflammation (Kanoh and Rubin, 2010). Macrolactins are a large group of macrolide antibiotics with a 22- to 24-member lactone ring mainly discovered in marine microorganisms, especially in the genus *Bacillus* (Mondol et al., 2013; Karpiński, 2019). Although numerous macrolide compounds have been isolated and identified from sponges and their symbionts (Karpiński, 2019), this is the first study to report the isolation of macrolactins from sponge-associated bacteria. Macrolactin A (**2**) has been frequently isolated from different bacteria (Nagao et al., 2001; Lee et al., 2004; Romero-Tabarez et al., 2006; Lu et al., 2008; Kim et al., 2011; Tareq et al., 2013; Li et al., 2016a), whereas macrolactin H (**3**) and 15, 17-epoxy-16-hydroxy macrolactin A (**4**) have been rarely isolated (Nagao et al., 2001; Mondol et al., 2011). Previous bioassays revealed that macrolactin A exhibited antimicrobial activity against Gram-negative bacteria (i.e., *E. coli*), Gram-positive bacteria (i.e., *S. aureus*, methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *B. subtilis*, *E. faecalis,* and vancomycin-resistant enterococci), as well as fungi (i.e., *Botrytis cinerea, A. niger*, *B. cinerea*, *Colletotrichum acutatum*, *R. solani*, *S. cerevisiae*, *C. albicans, Pestalotiopsis theae*, and *C. gloeosporioides*) (Nagao et al., 2001; Lee et al., 2004; Romero-Tabarez et al., 2006; Lu et al., 2008; Kim et al., 2011; Tareq et al., 2013; Li et al., 2016a). The antimicrobial assays in our studies showed similar results, in which macrolactin A exhibited antimicrobial activity against a broad spectrum of reference microorganisms. Furthermore, antibacterial activity of macrolactin A against *Vibrio* spp. was reported for the first time in this study. The biological properties of macrolactin H (**3**) and 15, 17-Epoxy-16-hydroxy macrolactin A (**4**) are less well known. The isolation and antimicrobial activity of these two compounds have been reported once by (Mondol et al., 2011) and (Nagao et al., 2001), respectively. Macrolactin H was reported to exhibit antimicrobial activity against *S. aureus* (MIC = 10 μg/mL) and *B. subtilis* (MIC = 60 μg/mL) (Nagao et al., 2001), whereas 15, 17-epoxy-16-hydroxy macrolactin A was reported to exhibit antimicrobial activity against *B. subtilis*, *E. coli*, and *S. cerevisiae* with the same MICs of 0.16 μM (Mondol et al., 2011). Although macrolactins show

antimicrobial activity against a broad spectrum of microorganisms, including Gram-negative and Gram-positive bacteria and fungi, the mode of action of macrolactins is not understood. However, several recent studies indicate that some macrolactin compounds, such as 7-O-Malonyl Macrolactin A, induce disruption of cell division (Romero-Tabarez et al., 2006) and macrolactin N inhibits bacterial peptide deformylase (Kim et al., 2011), whereas bamemacrolactin C affects fungal mycelial morphology, the cell wall, and protein expression by interrupting the oxidative phosphorylation (Li et al., 2016a). In addition, Zotchev et al. (2006) speculate that the antibacterial activity of macrolactins could be due to the inhibition of the H^+-transporting two-sector ATPase, which is essential for viability of bacterial cells.

Conclusion

This study yielded cultivable bacteria from 18 Vietnamese sponge species and represented four bacterial phyla and 55 genera. The cultivable bacteria were predominantly members of the genera *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces*. A big gap was observed between bacterial communities detected by cultivation-dependent and cultivation-independent approaches. Nevertheless, antimicrobial assays showed that 90 cultivable bacteria in this study exhibited antimicrobial activity against a large number of indicator microorganisms. From *Bacillus* sp. M1_CRV_171, the isolated strain with the most versatile antimicrobial activity, four known compounds with antimicrobial activity against a wide spectrum of indicator microorganisms were isolated: the cyclic dipeptide, cyclo(L-pro-L-tyr) and three macrolactins. Although these results confirm that many sponge-associated bacterial isolates have antimicrobial activity, they also stress the risks of rediscovery of compounds based on isolates obtained through traditional techniques. Therefore, the uncultivated majority of the bacteria should be targeted to get access to the full arsenal of bioactive molecules from sponges.

Acknowledgements

This research was funded by the Vietnam Ministry of Science and Technology, grant number ĐTĐLCN.17/14; BE-Basic Foundation-FES grant from the Dutch Ministry of Economic Affairs, grant number: F07.003.06; and MC-ITN-BluePharmTrain funded through the European Union Programme FP7/2007-2013/ under REA grant agreement n° 607786.

Data Availability

The 16S rRNA gene sequences of isolates are available in the NCBI database under accession numbers: MN703812–MN704271.

Supplementary Information

Supplementary Table S1. Total number of colonies and genera from sponge samples and from media

Sample ID	Sponge sample	Culture media							Total colonies per sample	Number of genera per sample
		MA	M1	R2A	SCA	AIA	OLIGO	SWA		
AMC	*Amphimedon* sp. 1	20	3	0	0	0	2	0	**25**	**15**
AMQ	*Amphimedon* sp. 2	17	3	2	0	0	0	0	**22**	**16**
AXT	*Axinyssa* sp.	22	6	1	0	0	0	0	**29**	**19**
AXC	*Axos cliftoni*	11	6	3	0	0	2	0	**22**	**16**
CIS	*Cinachyrella schulzei*	20	6	5	2	0	0	0	**33**	**21**
CLR	*Clathria reinwardti*	9	4	4	6	0	0	0	**23**	**15**
CRV	*Haplosclerida* sp.	4	6	7	5	0	0	0	**22**	**16**
DAS	*Dactylospongia* sp.	0	17	6	2	4	0	0	**29**	**20**
HAS	*Halichondria* sp.	0	10	4	5	2	2	0	**23**	**16**
HAA	*Haliclona amboinensis*	3	11	4	3	2	0	2	**25**	**16**
HAF	*Haliclona fascigera*	0	11	3	4	0	4	0	**22**	**16**
NIS	*Niphatidae* sp.	2	8	4	3	3	3	0	**23**	**14**
RHG	*Rhabdastrella globostellata*	2	9	11	4	2	3	2	**33**	**18**
SPV	*Spheciospongia* sp.	0	2	15	3	5	5	2	**32**	**21**
SPS	*Spirastrella* sp.	0	0	8	6	3	3	2	**22**	**15**
TES	*Tedania* sp.	0	2	0	17	5	4	5	**33**	**19**
TEA	*Terpios aploos*	0	0	0	2	10	4	5	**21**	**16**
XES	*Xestospongia testudinaria*	0	0	2	0	14	6	12	**34**	**19**
Total colonies per medium		**110**	**104**	**79**	**62**	**50**	**38**	**30**	**473**	
Total genera per medium		**35**	**38**	**33**	**30**	**27**	**23**	**17**		**55**

Supplementary Table S2. Betadisper and Adonis analyses of cultivable bacteria isolated from different culture media types

	Betadisper				
	df	***Sum Sq***	***Mean Sq***	***F***	***p-value***
Culture media types	6	0.040115	0.0066858	1.7053	0.2068
Residuals	14	0.054889	0.0039206		
Total	20	0.076054			
	Adonis				
	df	***Sum Sq***	***Mean Sq***	***R^2***	***p-value***
Culture media types	6	1.7159	0.28598	0.46294	**0.001**
Residuals	14	1.9907	0.14219	0.53706	
Total	20	3.7066		1	

Supplementary Table S3. Alpha diversity indexes of cultivable bacteria isolated from culture media

Media	**S**	**H**	**J**	**In**
MA	17.0±1.0	2.75±0.06	0.970±0.002	14.14±0.94
M1	17.0±1.0	2.75±0.06	0.972±0.001	14.42±0.86
R2A	17.7±1.5	2.84±0.09	0.989±0.002	16.51±1.54
SCA	16.0±1.0	2.75±0.06	0.993±0.003	15.34±0.80
AIA	13.0±1.0	2.55±0.07	0.994±0.002	12.60±0.87
OLIGO	10.7±1.5	2.35±0.15	0.997±0.001	10.52±1.52
SWA	8.0±1.0	2.06±0.13	0.995±0.001	7.84±1.00

Supplementary Table S4. Kruskal-Wallis chi-squared of alpha diversity indexes of cultivable bacteria isolated from culture media

Index	**df**	***Kruskal-Wallis chi-squared***	***p-value***
Richness (*S*)	6	17.083	**0.0090**
Shannon (*H*)	6	16.639	**0.0107**
Evenness (*J*)	6	18.085	**0.0060**
Inverse Simpson (*In*)	6	17.522	**0.0075**

Supplementary Table S5. List of isolated strains from sponges

Isolate ID	Closest relative	Accession number	Identity (%)
MA_AMQ_1	*Microbulbifer variabilis* strain Ni-2088 16S ribosomal RNA, partial sequence	NR_041021.1	100
M1_AXT_2	*Microbulbifer variabilis* gene for 16S rRNA, partial sequence	AB266055.1	100
M1_CIS_3	*Microbulbifer* sp. NBRC 101763 gene for 16S rRNA, partial sequence	AB681553.1	100
R2A_DAS_4	*Microbulbifer variabilis* strain HNS025 16S ribosomal RNA gene, partial sequence	JN128259.1	100
R2A_HAF_5	*Microbulbifer* sp. HB09007 16S ribosomal RNA gene, partial sequence	FJ796077.1	100
AIA_SPV_6	*Microbulbifer* sp. CUA-860 16S ribosomal RNA gene, partial sequence	KJ732924.1	100
AIA_TES_7	*Microbulbifer variabilis* strain Mcap_H38 16S ribosomal RNA gene, partial sequence	KP640585.1	100
MA_AMC_8	*Micromonospora* sp. 1G68 16S ribosomal RNA gene, partial sequence	DQ994713.1	100
M1_AXT_9	*Micromonospora* sp. R1 16S ribosomal RNA gene, partial sequence	EU714258.1	100
R2A_CRV_10	*Micromonospora* sp. 173307 16S ribosomal RNA gene, partial sequence	EF544151.1	100
SCA_HAS_11	*Micromonospora* sp. S16-05 gene for 16S ribosomal RNA, partial sequence	LC011674.1	100
MA_HAA_12	*Photobacterium* sp. VibC-Oc-011 16S ribosomal RNA gene, partial sequence	KF577081.1	100
M1_HAF_13	*Photobacterium gaetbulicola* strain MMSZ2016-2 16S ribosomal RNA gene, partial sequence	KY174898.1	100
OLIGO_TEA_14	*Photobacterium* sp. VibC-Oc-011 16S ribosomal RNA gene, partial sequence	KF577081.1	100
MA_AMC_15	*Pseudoalteromonas* sp. strain E704-6 16S ribosomal RNA gene, partial sequence	MF975627.1	100
M1_AMQ_16	*Pseudoalteromonas* sp. PL85 gene for 16S ribosomal RNA, partial sequence	LC373532.1	100
M1_AXC_17	*Pseudoalteromonas* sp. strain E704-6 16S ribosomal RNA gene, partial sequence	MF975627.1	100
R2A_CIS_18	*Pseudoalteromonas* sp. PL85 gene for 16S ribosomal RNA, partial sequence	LC373532.1	100
SCA_CRV_19	*Pseudoalteromonas* sp. strain E707-9 16S ribosomal RNA gene, partial sequence	MF975632.1	100
AIA_HAS_20	*Pseudoalteromonas* sp. strain Bu15_18 16S ribosomal RNA gene, partial sequence	KY671152.1	100

OLIGO_NIS_21	*Pseudoalteromonas* sp. PL85 gene for 16S ribosomal RNA, partial sequence	LC373532.1	100
SWA_SPV_22	*Pseudoalteromonas* sp. An2 partial 16S rRNA gene, isolate An2	AJ551143.1	100
SWA_XES_23	*Pseudoalteromonas* sp. PL85 gene for 16S ribosomal RNA, partial sequence	LC373532.1	100
MA_AXT_24	*Enterococcus faecium* strain 12 16S ribosomal RNA gene, partial sequence	MH236313.1	99
MA_CIS_25	*Enterococcus faecalis* strain SLDL-211 16S ribosomal RNA gene, partial sequence	MH779826.1	99
M1_DAS_26	*Enterococcus faecalis* strain ZX2-1 16S ribosomal RNA gene, partial sequence	MG694661.1	100
R2A_HAF_27	*Enterococcus faecalis* strain ZX2-1 16S ribosomal RNA gene, partial sequence	MG694661.1	99
SCA_RHG_28	*Enterococcus faecalis* strain A3-1 16S ribosomal RNA gene, partial sequence	MH385351.1	99
SCA_TES_29	*Enterococcus faecium* strain HPRTGL206 16S ribosomal RNA gene, partial sequence	MH393916.1	99
MA_AXT_30	*Brachybacterium paraconglomeratum* strain SL-88 16S ribosomal RNA gene, partial sequence	KU894797.1	99
MA_AMC_31	*Microbacterium* sp. strain MB30 16S ribosomal RNA gene, partial sequence	MH699187.1	99
MA_AMC_32	*Bacillus* sp. CF9 16S ribosomal RNA gene, partial sequence	KP126830.1	99
MA_AMC_33	*Pseudovibrio* sp. strain S42 16S ribosomal RNA gene, partial sequence	KX989360.1	99
MA_AMQ_34	*Brevibacterium* sp. DN213_3F7 16S ribosomal RNA gene, partial sequence	KP769419.1	99
MA_AMC_35	*Bacillus amyloliquefaciens* strain B-4 chromosome, complete genome	CP031424.1	100
MA_AMC_36	*Bacillus amyloliquefaciens* strain B-4 chromosome, complete genome	CP031424.1	100
OLIGO_XES_37	*Enterococcus faecalis* strain A3-1 16S ribosomal RNA gene, partial sequence	MH385351.1	99
MA_AMC_38	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
MA_AMQ_39	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	100
MA_AXT_40	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
M1_AXT_41	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
MA_AXT_42	*Rhodococcus* sp. strain A23 16S ribosomal RNA gene, partial sequence	MH688762.1	100
M1_DAS_43	*Rhodococcus qingshengii* strain B2 16S ribosomal RNA gene, partial sequence	KJ028076.1	100

MA_AMC_44	*Vibrio* sp. strain PrV0032 16S ribosomal RNA gene, partial sequence	MF948961.1	100
MA_AMC_45	*Vibrio sp.* strain PrV0032 16S ribosomal RNA gene, partial sequence	MF948961.1	100
MA_AMQ_46	*Vibrio harveyi* strain DZ141003 16S ribosomal RNA gene, partial sequence	KU245722.1	100
MA_AXT_47	*Vibrio parahaemolyticus* strain HH101313 16S ribosomal RNA gene, partial sequence	MG386398.1	100
MA_AXT_48	*Vibrio mediterranei* partial 16S rRNA gene, strain CAIM 1601	HF541964.1	100
MA_AXC_49	*Vibrio* sp. strain InS-286 16S ribosomal RNA gene, partial sequence	MF359517.1	99
M1_AXC_50	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
M1_CIS_51	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
R2A_CIS_52	*Streptomyces* sp. PN1018 16S ribosomal RNA gene, partial sequence	KF287177.1	100
R2A_CLR_53	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
MA_CIS_54	*Vibrio harveyi* strain DZ141003 16S ribosomal RNA gene, partial sequence	KU245722.1	100
MA_CIS_55	*Vibrio harveyi* strain vh10 16S ribosomal RNA gene, partial sequence	KJ700303.1	100
MA_AMC_56	*Micrococcus luteus* strain CGAPGPBBS-084 16S ribosomal RNA gene, partial sequence	KY495217.1	100
R2A_CRV_57	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
R2A_DAS_58	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
R2A_DAS_59	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
R2A_HAS_60	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
MA_CLR_61	*Vibrio harveyi* strain vh10 16S ribosomal RNA gene, partial sequence	KJ700303.1	100
SCA_HAA_62	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
SCA_HAF_63	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
SCA_NIS_64	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
SCA_RHG_65	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	100
MA_AMQ_66	*Bacillus* sp. V3X 16S ribosomal RNA gene, partial sequence	HQ727961.1	100

MA_AMQ_67	*Bacillus* sp. A5-11 16S ribosomal RNA gene, partial sequence	JX134465.1	100
MA_AMQ_68	*Bacillus* sp. (in: Bacteria) MH50 gene for 16S ribosomal RNA, partial sequence	LC373525.1	100
MA_AXT_69	*Bacillus* sp. (in: Bacteria) strain LPOC3 16S ribosomal RNA gene, partial sequence	MH412687.1	100
MA_AXT_70	*Bacillus* sp. (in: Bacteria) MH50 gene for 16S ribosomal RNA, partial sequence	LC373525.1	100
MA_AXT_71	*Bacillus* sp. (in: Bacteria) MH50 gene for 16S ribosomal RNA, partial sequence	LC373525.1	99
MA_AXT_72	*Bacillus* sp. (in: Bacteria) MH50 gene for 16S ribosomal RNA, partial sequence	LC373525.1	99
MA_AXC_73	*Bacillus pumilus* strain PPL-SSC2 16S ribosomal RNA gene, partial sequence	KM226935.1	99
MA_AXC_74	*Bacillus aryabhattai* strain DN67_5C7 16S ribosomal RNA gene, partial sequence	KP769436.1	100
MA_AXC_75	*Bacillus aryabhattai* strain DL 1 16S ribosomal RNA gene, partial sequence	MH321604.1	100
MA_CIS_76	*Bacillus xiamenensis* strain AKU6 16S ribosomal RNA gene, partial sequence	MF173074.1	100
MA_CIS_77	*Bacillus megaterium* strain As23 16S ribosomal RNA gene, partial sequence	KC633281.1	100
MA_CIS_78	*Bacillus algicola* strain HMF4132 16S ribosomal RNA gene, partial sequence	KT984000.1	100
MA_CIS_79	*Bacillus horikoshii* strain DSM 8719 16S ribosomal RNA, partial sequence	NR_040852.1	99
MA_CIS_80	*Bacillus* sp. (in: Bacteria) strain DE024 16S ribosomal RNA gene, partial sequence	KY860719.1	100
MA_CLR_81	*Bacillus sp*. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	100
SCA_RHG_82	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	99
AIA_SPV_83	*Streptomyces* sp. strain 762 16S ribosomal RNA gene, partial sequence	MG654687.1	99
AIA_SPV_84	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	99
AIA_SPS_85	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	99
M1_CLR_86	*Vibrio alginolyticus* strain HH101307 16S ribosomal RNA gene, partial sequence	MG386392.1	99
MA_AMC_87	*Microbacterium* sp. 8 SY-2016 partial 16S rRNA gene, strain 8	LT009507.1	100
M1_AXT_88	*Micrococcus luteus* strain Amic_3 16S ribosomal RNA gene, partial sequence	KX223364.1	100
SCA_HAS_89	*Paracoccus* sp. strain KSTI85 16S ribosomal RNA gene, partial sequence	KX989451.1	100

R2A_SPS_90	*Microbacterium* sp. 8 SY-2016 partial 16S rRNA gene, strain 8	LT009507.1	100
R2A_CIS_91	*Micrococcus luteus* strain PZ-51 16S ribosomal RNA gene, partial sequence	KX108885.1	100
MA_AMC_92	*Pseudovibrio* sp. strain S42 16S ribosomal RNA gene, partial sequence	KX989360.1	100
MA_AMC_93	*Pseudovibrio* sp. strain S42 16S ribosomal RNA gene, partial sequence	KX989360.1	100
MA_AMQ_94	*Pseudovibrio* sp. strain S42 16S ribosomal RNA gene, partial sequence	KX989360.1	100
MA_AXT_95	*Arthrobacter agilis* strain OAct554 16S ribosomal RNA gene, partial sequence	KJ812397.1	100
MA_CRV_96	*Flavobacterium johnsoniae* strain A3 16S ribosomal RNA gene, partial sequence	EU860081.1	100
M1_HAA_97	*Flavobacterium johnsoniae* strain A3 16S ribosomal RNA gene, partial sequence	EU860081.1	100
MA_AMQ_98	*Pseudomonas* sp. strain SAM89 16S ribosomal RNA gene, partial sequence	KX670902.1	99
MA_NIS_99	*Acidovorax* sp. DQS-01 16S ribosomal RNA gene, partial sequence	KP126996.1	99
MA_AMQ_100	*Pseudovibrio* sp. Pb3 16S ribosomal RNA gene, partial sequence	HQ647018.1	99
MA_AMQ_101	*Pseudovibrio* sp. strain S42 16S ribosomal RNA gene, partial sequence	KX989360.1	99
MA_AXT_102	*Pseudovibrio* sp. 2011SOCNI42 16S ribosomal RNA gene, partial sequence	KF582882.1	100
MA_AMC_103	*Psychrobacter celer* strain MT3 16S ribosomal RNA gene, partial sequence	MH213234.1	99
M1_CRV_104	*Vibrio harveyi* strain DZ141003 16S ribosomal RNA gene, partial sequence	KU245722.1	100
M1_DAS_105	*Vibrio parahaemolyticus* strain AP323 16S ribosomal RNA gene, partial sequence	MG575446.1	99
M1_DAS_106	*Vibrio* sp. strain InS-159 16S ribosomal RNA gene, partial sequence	MF359408.1	99
M1_HAS_107	*Vibrio* sp. strain PrV0032 16S ribosomal RNA gene, partial sequence	MF948961.1	100
M1_HAS_108	*Vibrio* sp. strain PrV0032 16S ribosomal RNA gene, partial sequence	MF948961.1	100
M1_HAA_109	*Vibrio harveyi* strain HQB1367 16S ribosomal RNA gene, partial sequence	MH044640.1	100
M1_HAF_110	*Vibrio harveyi* strain DZ141003 16S ribosomal RNA gene, partial sequence	KU245722.1	100
M1_NIS_111	*Vibrio harveyi* strain DZ141003 16S ribosomal RNA gene, partial sequence	KU245722.1	100
M1_NIS_112	*Vibrio alginolyticus* strain HH101307 16S ribosomal RNA gene, partial sequence	MG386392.1	99

M1_RHG_113	*Vibrio alginolyticus* strain HH101307 16S ribosomal RNA gene, partial sequence	MG386392.1	100
M1_RHG_114	*Vibrio harveyi* strain vh10 16S ribosomal RNA gene, partial sequence	KJ700303.1	100
M1_SPV_115	*Vibrio harveyi* strain vh10 16S ribosomal RNA gene, partial sequence	KJ700303.1	100
R2A_SPV_116	*Vibrio mediterranei* partial 16S rRNA gene, strain CAIM 1601	HF541964.1	100
R2A_SPS_117	*Vibrio mediterranei* partial 16S rRNA gene, strain CAIM 1601	HF541964.1	99
R2A_XES_118	*Chromohalobacter* sp. 582s-4 16S ribosomal RNA gene, partial sequence	GU371671.1	99
R2A_SPS_119	*Vibrio mediterranei* partial 16S rRNA gene, strain CAIM 1601	HF541964.1	99
SCA_TES_120	*Vibrio shilonii* strain NIOSSK079#72 16S ribosomal RNA gene, partial sequence	KY604834.1	99
SCA_TES_121	*Vibrio alginolyticus* strain hq-V214 16S ribosomal RNA gene, partial sequence	MH553019.1	100
AIA_TEA_122	*Vibrio alginolyticus* strain hq-V214 16S ribosomal RNA gene, partial sequence	MH553019.1	100
OLIGO_XES_123	*Vibrio alginolyticus* strain hq-V214 16S ribosomal RNA gene, partial sequence	MH553019.1	100
SWA_XES_124	*Vibrio alginolyticus* strain CX-73 16S ribosomal RNA gene, partial sequence	MH368393.1	100
AIA_TES_125	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	99
AIA_TES_126	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	100
AIA_TEA_127	*Streptomyces* sp. PN1018 16S ribosomal RNA gene, partial sequence	KF287177.1	100
OLIGO_XES_128	*Streptomyces* sp. PN1018 16S ribosomal RNA gene, partial sequence	KF287177.1	100
SWA_XES_129	*Streptomyces* sp. A01111 16S ribosomal RNA gene, partial sequence	KU382716.1	100
M1_DAS_130	*Alcanivorax marinus* strain NIOSSD020#62 16S ribosomal RNA gene, partial sequence	KY616238.1	100
M1_AXT_131	*Pseudomonas mendocina* strain PRM6 16S ribosomal RNA gene, partial sequence	JN544146.1	99
M1_SPV_132	*Brucella melitensis* strain AUH2 16S ribosomal RNA gene, partial sequence	EF187230.1	100
MA_AMC_133	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	100
MA_AMC_134	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	100
M1_AXC_135	*Thalassobius aestuarii* strain JC2049 16S ribosomal RNA gene, partial sequence	AY442178.2	99

MA_AMQ_136	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
MA_AMQ_137	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	99
MA_AXT_138	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
MA_AXT_139	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	99
MA_AXT_140	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	100
OLIGO_AMC_141	*Pseudoruegeria sabulilitoris* strain GJMS-35 16S ribosomal RNA, partial sequence	NR_134142.1	99
MA_AXC_142	*Ruegeria* sp. AD4 16S ribosomal RNA gene, partial sequence	KT731244.1	99
MA_AXC_143	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
MA_CIS_144	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
MA_CIS_145	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
MA_CIS_146	*Ruegeria* sp. AU103_Ted2_MB partial 16S rRNA gene, isolate AU103_Ted2_MB	LN909258.1	100
MA_CIS_147	*Ruegeria* sp. AU448_Ted1_MB20SE partial 16S rRNA gene, isolate AU448_Ted1_MB20SE	LN909189.1	100
MA_CLR_148	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	100
MA_CLR_149	*Ruegeria* sp. AU103_Ted2_MB partial 16S rRNA gene, isolate AU103_Ted2_MB	LN909258.1	99
M1_CRV_150	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
M1_CRV_151	*Ruegeria* sp. AU103_Ted2_MB partial 16S rRNA gene, isolate AU103_Ted2_MB	LN909258.1	100
MA_AXC_152	*Roseovarius scapharcae* strain MA4-5 16S ribosomal RNA, partial sequence	NR_145897.1	99
M1_DAS_153	*Ruegeria* sp. 2011SOCNI17 16S ribosomal RNA gene, partial sequence	KF582894.1	99
MA_AXT_154	*Labrenzia* sp. strain 1334-139 16S ribosomal RNA gene, partial sequence	KY770164.1	99
M1_DAS_155	*Ruegeria* sp. AU267A partial 16S rRNA gene, isolate AU267A	LN878642.1	99
M1_DAS_156	*Ruegeria atlantica* strain 58303 16S ribosomal RNA gene, partial sequence	KX418531.1	100
M1_HAS_157	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	100
M1_HAS_158	*Ruegeria* sp. strain EL46 16S ribosomal RNA gene, partial sequence	MF461362.1	99

M1_HAA_159	*Ruegeria* sp. R214E7 16S ribosomal RNA gene, partial sequence	FJ357642.1	100
M1_CIS_160	*Labrenzia* sp. strain 1334-139 16S ribosomal RNA gene, partial sequence	KY770164.1	99
M1_HAA_161	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	100
R2A_DAS_162	*Labrenzia* sp. strain 1334-139 16S ribosomal RNA gene, partial sequence	KY770164.1	99
R2A_AXC_163	*Janibacter melonis* strain IHBB 11057 16S ribosomal RNA gene, partial sequence	KR085938.1	99
M1_AXC_164	*Brachybacterium* sp. strain BAB-2786 16S ribosomal RNA gene, partial sequence	MF188116.1	99
MA_AMQ_165	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
M1_CIS_166	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
M1_CIS_167	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
R2A_CRV_168	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
MA_CLR_169	*Bacillus* sp. (in: Bacteria) MH50 gene for 16S ribosomal RNA, partial sequence	LC373525.1	99
MA_CLR_170	*Bacillus* sp. (in: Bacteria) strain LPOC3 16S ribosomal RNA gene, partial sequence	MH412687.1	100
M1_CRV_171	*Bacillus* sp. (in: Bacteria) strain LPOC3 16S ribosomal RNA gene, partial sequence	MH412687.1	100
R2A_CRV_172	*Brachybacterium* sp. strain BAB-2786 16S ribosomal RNA gene, partial sequence	MF188116.1	99
M1_CRV_173	*Bacillus* sp. (in: Bacteria) strain LPOC3 16S ribosomal RNA gene, partial sequence	MH412687.1	99
M1_CRV_174	*Bacillus* sp. (in: Bacteria) strain LPOC3 16S ribosomal RNA gene, partial sequence	MH412687.1	99
M1_AXC_175	*Brevibacterium* sp. EP11 partial 16S rRNA gene, strain EP11	AM398220.1	99
SCA_CRV_176	*Brevibacterium* sp. EP11 partial 16S rRNA gene, strain EP11	AM398220.1	100
MA_AXT_177	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
R2A_HAS_178	*Rhodococcus qingshengii* strain B2 16S ribosomal RNA gene, partial sequence	KJ028076.1	100
MA_AXT_179	*Pseudovibrio* sp. AB113 16S ribosomal RNA gene, partial sequence	KP319346.1	100
MA_RHG_180	*Brevundimonas vesicularis* strain G2-1-80 16S ribosomal RNA gene, partial sequence	KC494336.1	99
MA_AXC_181	*Pseudovibrio* sp. 4G02 16S ribosomal RNA gene, partial sequence	KP319311.1	100

MA_AXC_182	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
MA_AMQ_183	*Psychrobacter* sp. 228(130zx) 16S rRNA gene, strain 228(130zx)	AM403661.1	99
MA_CIS_184	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
MA_CIS_185	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
MA_CIS_186	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	99
MA_CIS_187	*Pseudovibrio* sp. AB111 16S ribosomal RNA gene, partial sequence	KP319345.1	100
MA_AXT_188	*Escherichia coli* strain PYK21 16S ribosomal RNA gene, partial sequence	MF582348.1	100
M1_TES_189	*Rhodobacter* sp. TUT3735 gene for 16S rRNA, partial sequence	AB251411.1	100
OLIGO_SPV_190	*Anderseniella* sp. strain Gy4 16S ribosomal RNA gene, partial sequence	KY885189.1	99
M1_HAF_191	*Ruegeria* sp. EK38B gene for 16S ribosomal RNA, partial sequence	LC053425.1	99
M1_HAF_192	*Ruegeria* sp. EK38B gene for 16S ribosomal RNA, partial sequence	LC053425.1	99
MA_AXC_193	*Acinetobacter radioresistens* strain DSSKY-A-001 chromosome, complete genome	CP027365.1	100
R2A_AXC_194	*Kocuria rhizophila* strain F2 16S ribosomal RNA gene, partial sequence	KM577162.1	99
MA_CIS_195	*Pseudomonas* sp. Snoq 117.2 16S ribosomal RNA gene, partial sequence	KF597277.1	99
MA_AMC_196	*Staphylococcus epidermidis* strain CSF41498 chromosome, complete genome	CP030246.1	99
M1_CLR_197	*Acinetobacter radioresistens* strain DSSKY-A-001 chromosome, complete genome	CP027365.1	99
M1_AXT_198	*Mycobacterium* sp. NB01 16S ribosomal RNA, partial sequence	AY188086.1	99
M1_DAS_199	*Bacillus* sp. NCIM5373 16S ribosomal RNA gene, partial sequence	KT291159.1	99
OLIGO_AXC_200	*Nonlabens arenilitoris* strain DN166_3E9 16S ribosomal RNA gene, partial sequence	KP769429.1	100
MA_AXT_201	*Psychrobacter celer* strain MT3 16S ribosomal RNA gene, partial sequence	MH213234.1	99
MA_AXT_202	*Psychrobacter celer* strain MT3 16S ribosomal RNA gene, partial sequence	MH213234.1	99
R2A_DAS_203	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
M1_DAS_204	*Bacillus* sp. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	100

MA_CLR_205	*Pseudovibrio* sp. 8H04 16S ribosomal RNA gene, partial sequence	KP319343.1	99
MA_CLR_206	*Pseudovibrio* sp. MaPt6 16S ribosomal RNA gene, partial sequence	JX436420.1	100
MA_AMC_207	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	99
M1_DAS_208	*Bacillus megaterium* strain As23 16S ribosomal RNA gene, partial sequence	KC633281.1	100
MA_AMQ_209	*Bradyrhizobium viridifuturi* strain SEMIA 690 16S ribosomal RNA, partial sequence	NR_145860.1	100
MA_AXT_210	*Staphylococcus* sp. M_Sw_oHS_07/11_10_1(2) 16S ribosomal RNA gene, partial sequence	KF777597.1	100
MA_CIS_211	*Bradyrhizobium viridifuturi* strain SEMIA 690 16S ribosomal RNA, partial sequence	NR_145860.1	99
MA_CLR_212	*Pseudovibrio* sp. 4G02 16S ribosomal RNA gene, partial sequence	KP319311.1	100
M1_AXC_213	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	99
M1_NIS_214	*Ruegeria* sp. CECT 5091 16S ribosomal RNA gene, partial sequence	MH023307.1	99
M1_NIS_215	*Ruegeria atlantica* strain 2 DIS 4B 16S ribosomal RNA gene, partial sequence	MK224686.1	99
R2A_CIS_216	*Psychrobacter celer* strain MT3 16S ribosomal RNA gene, partial sequence	MH213234.1	99
SCA_CLR_217	*Micrococcus luteus* strain CGAPGPBBS-084 16S ribosomal RNA gene, partial sequence	KY495217.1	100
R2A_CLR_218	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
M1_RHG_219	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	100
SCA_CLR_220	*Janibacter melonis* strain IHBB 11057 16S ribosomal RNA gene, partial sequence	KR085938.1	100
M1_DAS_221	*Bacillus aryabhattai* strain DN67_5C7 16S ribosomal RNA gene, partial sequence	KP769436.1	100
M1_HAS_222	*Bacillus aryabhattai* strain DN67_5C7 16S ribosomal RNA gene, partial sequence	KP769436.1	100
R2A_HAA_223	*Brachybacterium* sp. strain BAB-2786 16S ribosomal RNA gene, partial sequence	MF188116.1	100
R2A_CRV_224	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
M1_HAS_225	*Bacillus pumilus* strain PPL-SSC2 16S ribosomal RNA gene, partial sequence	KM226935.1	99
R2A_CLR_226	*Mycobacterium septicum* strain D13 16S ribosomal RNA gene, partial sequence	AY772166.1	99
M1_HAS_227	*Bacillus stratosphericus* strain 907R 16S ribosomal RNA gene, partial sequence	MF083091.1	100

R2A_CLR_228	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
SCA_DAS_229	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
SCA_HAF_230	*Brachybacterium* sp. V589 16S ribosomal RNA gene, partial sequence	AF324202.2	100
MA_CRV_231	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
MA_CRV_232	*Pseudovibrio* sp. 1D08 16S ribosomal RNA gene, partial sequence	KP319277.1	100
MA_CRV_233	*Pseudovibrio* sp. 7H04 16S ribosomal RNA gene, partial sequence	KP319334.1	99
M1_HAA_234	*Bacillus* sp. FS2 16S ribosomal RNA gene, partial sequence	GU084156.1	100
M1_DAS_235	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
M1_DAS_236	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	99
M1_DAS_237	*Pseudovibrio* sp. 1D08 16S ribosomal RNA gene, partial sequence	KP319277.1	100
M1_DAS_238	*Pseudovibrio* sp. 8H06 16S ribosomal RNA gene, partial sequence	KP319344.1	100
SWA_TES_239	*Anderseniella* sp. strain Gy4 16S ribosomal RNA gene, partial sequence	KY885189.1	99
M1_CLR_240	*Staphylococcus saprophyticus* strain P45L1B 16S ribosomal RNA gene, partial sequence	MH748281.1	100
MA_AXC_241	*Sphingobium* sp. RAC03 chromosome, complete genome	CP016456.1	99
R2A_DAS_242	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
M1_HAA_243	*Bacillus horikoshii* strain DSM 8719 16S ribosomal RNA, partial sequence	NR_040852.1	99
M1_CIS_244	*Sphingobium* sp. RAC03 chromosome, complete genome	CP016456.1	99
R2A_CRV_245	*Sphingobium sp.* RAC03 chromosome, complete genome	CP016456.1	99
M1_HAA_246	*Bacterium* BAB-662 16S ribosomal RNA gene, partial sequence	KF913655.1	100
M1_RHG_247	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	99
M1_HAS_248	*Pseudovibrio* sp. 8H04 16S ribosomal RNA gene, partial sequence	KP319343.1	100
M1_HAF_249	*Bacillus* sp. A5-11 16S ribosomal RNA gene, partial sequence	JX134465.1	100
M1_HAF_250	*Bacillus algicola strain HMF4132 16S ribosomal RNA gene, partial sequence*	KT984000.1	100

M1_HAS_251	*Pseudovibrio* sp. 8G09 16S ribosomal RNA gene, partial sequence	KP319341.1	100
SCA_NIS_252	*Brachybacterium* sp. strain BAB-2786 16S ribosomal RNA gene, partial sequence	MF188116.1	100
SCA_HAS_253	*Sphingobium sp.* RAC03 chromosome, complete genome	CP016456.1	100
OLIGO_RHG_254	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
R2A_HAS_255	*Psychrobacter* sp. RKEM 1523 16S ribosomal RNA gene, partial sequence	KU198810.1	100
SCA_HAS_256	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
AIA_DAS_257	*Acinetobacter radioresistens* strain DSSKY-A-001 chromosome, complete genome	CP027365.1	100
OLIGO_HAS_258	*Acinetobacter radioresistens* strain DSSKY-A-001 chromosome, complete genome	CP027365.1	100
R2A_AXC_259	*Pseudomonas* sp. C127 16S ribosomal RNA gene, partial sequence	DQ005892.1	99
OLIGO_SPV_260	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
SCA_HAA_261	*Psychrobacter* sp. RKEM 1523 16S ribosomal RNA gene, partial sequence	KU198810.1	100
AIA_HAA_262	*Janibacter melonis* strain IHBB 11057 16S ribosomal RNA gene, partial sequence	KR085938.1	100
M1_HAS_263	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	99
AIA_HAS_264	*Brevibacterium linens* strain Bv7 16S ribosomal RNA gene, partial sequence	MH493690.1	100
M1_HAA_265	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	100
M1_HAA_266	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	100
M1_HAA_267	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	100
M1_HAF_268	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	100
M1_HAF_269	*Pseudovibrio* sp. 8H04 16S ribosomal RNA gene, partial sequence	KP319343.1	99
M1_HAF_270	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	99
M1_NIS_271	*Pseudovibrio* sp. 3H01 16S ribosomal RNA gene, partial sequence	KP319304.1	100
M1_HAF_272	*Bacillus* sp. (in: Bacteria) strain DE024 16S ribosomal RNA gene, partial sequence	KY860719.1	100
R2A_RHG_273	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	100

M1_NIS_274	*Bacillus* sp. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	100
R2A_NIS_275	*Bacillus* sp. 42-7 16S ribosomal RNA gene, partial sequence	FJ607051.1	100
R2A_NIS_276	Uncultured *Bacillus* sp. clone ACH-S-9 16S ribosomal RNA gene, partial sequence	KM873141.1	99
SCA_HAF_277	Uncultured *Psychrobacter* sp. clone CI45 16S ribosomal RNA gene, partial sequence	FJ695565.1	99
SCA_NIS_278	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
SCA_CLR_279	*Pseudomonas* sp. strain Po_C2_3 16S ribosomal RNA gene, partial sequence	KY653040.1	100
SCA_CRV_280	*Pseudomonas sp.* CJ11064 16S ribosomal RNA gene, partial sequence	AF500211.1	99
AIA_NIS_281	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
AIA_RHG_282	*Psychrobacter celer* strain z1099 16S ribosomal RNA gene, partial sequence	KY582846.1	100
OLIGO_TES_283	*Leisingera methylohalidivorans* DSM 14336 strain MB2 16S ribosomal RNA, complete sequence	NR_121711.2	99
R2A_RHG_284	*Bacillus* sp. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	100
SCA_HAS_285	*Pseudomonas* sp. strain Po_C2_3 16S ribosomal RNA gene, partial sequence	KY653040.1	100
AIA_SPV_286	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
M1_NIS_287	*Pseudovibrio* sp. BC118 16S ribosomal RNA gene, partial sequence	KP319347.1	100
M1_NIS_288	*Pseudovibrio axinellae* strain Ad2 16S ribosomal RNA, partial sequence	NR_118255.1	99
M1_RHG_289	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	100
M1_RHG_290	*Pseudovibrio* sp. 7H02 16S ribosomal RNA gene, partial sequence	KP319333.1	99
R2A_RHG_291	*Pseudovibrio axinellae* strain Ad2 16S ribosomal RNA, partial sequence	NR_118255.1	99
R2A_RHG_292	*Pseudovibrio axinellae* strain Ad2 16S ribosomal RNA, partial sequence	NR_118255.1	99
MA_NIS_293	*Aquimarina megaterium* XH134 16S ribosomal RNA, partial sequence	NR_118560.1	100
M1_DAS_294	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	99
M1_HAA_295	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	99
R2A_RHG_296	*Bacillus* sp. (in: Bacteria) strain DE024 16S ribosomal RNA gene, partial sequence	KY860719.1	100

R2A_HAF_297	Uncultured *Bradyrhizobium* sp. clone PSC8 16S ribosomal RNA gene, partial sequence	GU293167.1	100
OLIGO_TEA_298	*Tenacibaculum* sp. MOLA 512 partial 16S rRNA gene, culture collection MOLA:512	AM990737.1	100
AIA_SPS_299	*Brachybacterium paraconglomeratum* strain CM22 16S ribosomal RNA gene, partial sequence	EU660345.1	99
R2A_RHG_300	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	100
R2A_RHG_301	*Pseudovibrio* sp. DN206_4B7 16S ribosomal RNA gene, partial sequence	KP769445.1	99
OLIGO_TEA_302	*Brachybacterium* sp. strain BAB-2786 16S ribosomal RNA gene, partial sequence	MF188116.1	100
AIA_NIS_303	*Brevibacterium aurantiacum* strain SMQ-1335 chromosome, complete genome	CP017150.1	100
OLIGO_SPV_304	*Psychrobacter celer* strain B_IV_3L25 16S ribosomal RNA gene, partial sequence	JF710994.1	100
R2A_RHG_305	*Ruegeria atlantica* strain 2 DIS 4B 16S ribosomal RNA gene, partial sequence	MK224686.1	99
R2A_SPV_306	*Ruegeria* sp. ws1-3 16S ribosomal RNA gene, partial sequence	JF719277.1	100
R2A_SPV_307	*Ruegeria atlantica* strain 2 DIS 4B 16S ribosomal RNA gene, partial sequence	MK224686.1	99
OLIGO_HAF_308	*Sphingobium* sp. RAC03 chromosome, complete genome	CP016456.1	100
M1_RHG_309	*Aquimarina megaterium* XH134 16S ribosomal RNA, partial sequence	NR_118560.1	100
R2A_RHG_310	*Bacillus megaterium* strain H-1 16S ribosomal RNA gene, partial sequence	KT273285.1	100
OLIGO_TES_311	*Sphingobium* sp. SA2 16S ribosomal RNA gene, partial sequence	KJ767657.1	99
R2A_RHG_312	*Bacillus* sp. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	99
AIA_HAA_313	*Pseudomonas* sp. strain Po_C2_3 16S ribosomal RNA gene, partial sequence	KY653040.1	99
OLIGO_SPS_314	*Roseovarius* sp. EF1C-B42 16S ribosomal RNA gene, partial sequence	KC545259.1	99
R2A_SPV_315	*Pseudovibrio* sp. DN206_4B7 16S ribosomal RNA gene, partial sequence	KP769445.1	99
R2A_SPV_316	*Pseudovibrio* sp. AB113 16S ribosomal RNA gene, partial sequence	KP319346.1	100
R2A_SPV_317	Uncultured *Pseudovibrio* sp. clone 0307_BHM1_39 16S ribosomal RNA gene, partial sequence	JQ515611.1	99
R2A_SPV_318	Uncultured *Pseudovibrio* sp. clone 0307_BHM1_39 16S ribosomal RNA gene, partial sequence	JQ515611.1	99
OLIGO_HAF_319	*Pseudomonas stutzeri* Hiro-3 gene for 16S ribosomal RNA, partial sequence	LC339941.1	99

R2A_RHG_320	*Bacillus horikoshii* strain DSM 8719 16S ribosomal RNA, partial sequence	NR_040852.1	99
MA_AMQ_321	*Sphingomonas* sp. DN81_6F7 16S ribosomal RNA gene, partial sequence	KP769438.1	99
R2A_SPV_322	*Pseudovibrio* sp. 8H04 16S ribosomal RNA gene, partial sequence	KP319343.1	99
SWA_SPS_323	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
SWA_TES_324	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
R2A_SPV_325	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	100
R2A_SPV_326	*Bacillus* sp. DN88_4G3 16S ribosomal RNA gene, partial sequence	KP769435.1	100
OLIGO_NIS_327	*Pseudomonas* sp. strain Po_C2_3 16S ribosomal RNA gene, partial sequence	KY653040.1	100
OLIGO_TES_328	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
SWA_TEA_329	*Nonlabens arenilitoris* strain DN166_3E9 16S ribosomal RNA gene, partial sequence	KP769429.1	100
SWA_TEA_330	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
SWA_XES_331	*Psychrobacter celer* strain DN193_4B9 16S ribosomal RNA gene, partial sequence	KP769422.1	100
R2A_SPV_332	*Bacillus* sp. Ar1 16S ribosomal RNA gene, partial sequence	EU009208.1	99
OLIGO_RHG_333	*Pseudomonas* sp. strain Po_C2_3 16S ribosomal RNA gene, partial sequence	KY653040.1	100
AIA_XES_334	*Aquimarina megaterium* XH134 16S ribosomal RNA, partial sequence	NR_118560.1	99
OLIGO_SPS_335	*Janibacter melonis* strain IHBB 11057 16S ribosomal RNA gene, partial sequence	KR085938.1	100
SCA_CLR_336	*Kocuria rhizophila* strain RW13 16S ribosomal RNA gene, partial sequence	MH715224.1	99
R2A_SPV_337	*Bacillus horikoshii* strain DSM 8719 16S ribosomal RNA, partial sequence	NR_040852.1	99
R2A_SPV_338	*Bacillus horikoshii* strain DSM 8719 16S ribosomal RNA, partial sequence	NR_040852.1	99
SCA_SPV_339	*Bacillus stratosphericus* strain PgKB21 16S ribosomal RNA gene, partial sequence	MF979092.1	100
SCA_SPS_340	*Bacillus aerophilus* strain IHBB 11116 16S ribosomal RNA gene, partial sequence	KR085933.1	100
R2A_HAA_341	*Mycobacterium* sp. strain TM-B39 16S ribosomal RNA gene, partial sequence	MH698707.1	99
SCA_SPS_342	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	100

AIA_XES_343	*Bradyrhizobium* sp. strain MA108 16S ribosomal RNA gene, partial sequence	MH699180.1	99
SCA_SPS_344	*Bacillus pumilus* strain MB1 NIOT 2010 16S ribosomal RNA gene, partial sequence	HQ858057.1	99
SCA_SPS_345	*Bacillus altitudinis* strain CF13 16S ribosomal RNA gene, partial sequence	JX438703.1	100
SCA_TES_346	*Bacillus algicola* strain F12 16S ribosomal RNA, partial sequence	NR_029077.1	99
SCA_TES_347	*Bacillus algicola* strain HMF4132 16S ribosomal RNA gene, partial sequence	KT984000.1	100
SCA_TES_348	*Bacillus algicola* strain HMF4132 16S ribosomal RNA gene, partial sequence	KT984000.1	100
M1_CLR_349	*Sphingomonas* sp. DN81_6F7 16S ribosomal RNA gene, partial sequence	KP769438.1	100
SCA_CRV_350	*Sphingomonas* sp. DN81_6F7 16S ribosomal RNA gene, partial sequence	KP769438.1	100
R2A_SPS_351	*Pseudovibrio* sp. DN206_4B7 16S ribosomal RNA gene, partial sequence	KP769445.1	99
R2A_SPS_352	*Pseudovibrio* sp. DN206_4B7 16S ribosomal RNA gene, partial sequence	KP769445.1	99
M1_AMC_353	*Algibacter lectus* strain KMM 3902 16S ribosomal RNA, partial sequence	NR_025696.1	99
R2A_AMQ_354	*Algibacter lectus* strain KMM 3902 16S ribosomal RNA, partial sequence	NR_025696.1	99
M1_HAF_355	*Kriegella aquimaris* strain KMM 3942 16S ribosomal RNA gene, partial sequence	EU246690.1	99
R2A_SPS_356	*Kriegella aquimaris* strain KMM 3942 16S ribosomal RNA gene, partial sequence	EU246690.1	99
R2A_AMQ_357	*Olleya namhaensis* strain WT-MY15 16S ribosomal RNA, partial sequence	NR_109551.1	100
SCA_CLR_358	*Olleya namhaensis* strain WT-MY15 16S ribosomal RNA, partial sequence	NR_109551.1	100
SCA_DAS_359	*Algibacter lectus* strain KMM 3902 16S ribosomal RNA, partial sequence	NR_025696.1	100
AIA_XES_360	*Algibacter lectus* strain KMM 3902 16S ribosomal RNA, partial sequence	NR_025696.1	100
SCA_TES_361	*Kriegella aquimaris* strain KMM 3942 16S ribosomal RNA gene, partial sequence	EU246690.1	99
AIA_TEA_362	*Kriegella aquimaris* strain KMM 3942 16S ribosomal RNA gene, partial sequence	EU246690.1	99
R2A_NIS_363	*Mycobacterium* sp. strain DL90 16S ribosomal RNA gene, partial sequence	MH290160.3	99
SCA_RHG_364	*Mycobacterium* sp. strain DL90 16S ribosomal RNA gene, partial sequence	MH290160.3	100
SCA_TES_365	*Bacillus* sp. ADB_BD072 16S ribosomal RNA gene, partial sequence	KX027018.1	99

AIA_DAS_366	*Micrococcus yunnanensis* strain BAE13 16S ribosomal RNA gene, partial sequence	KP717416.1	99
SCA_SPS_367	*Pseudovibrio* sp. 8H04 16S ribosomal RNA gene, partial sequence	KP319343.1	99
SCA_TES_368	*Pseudovibrio* sp. AB113 16S ribosomal RNA gene, partial sequence	KP319346.1	100
R2A_HAS_369	*Staphylococcus warneri* strain FP12 16S ribosomal RNA gene, partial sequence	MH037141.1	100
R2A_HAA_370	*Staphylococcus saprophyticus* strain P45L1B 16S ribosomal RNA gene, partial sequence	MH748281.1	100
SCA_HAF_371	*Staphylococcus saprophyticus* strain P45L1B 16S ribosomal RNA gene, partial sequence	MH748281.1	100
SWA_XES_372	*Sphingobium* sp. strain dr-6 16S ribosomal RNA gene, partial sequence	MH173287.1	100
AIA_NIS_373	*Sphingobium* sp. strain dr-6 16S ribosomal RNA gene, partial sequence	MH173287.1	99
SCA_TES_374	*Pseudovibrio* sp. 8G10 16S ribosomal RNA gene, partial sequence	KP319342.1	100
AIA_SPV_375	*Brevibacterium linens* strain Bv7 16S ribosomal RNA gene, partial sequence	MH493690.1	100
OLIGO_TEA_376	*Leisingera aquimarina* strain DN172_5F6 16S ribosomal RNA gene, partial sequence	KP769430.1	100
SCA_SPV_377	*Mycobacterium* sp. strain 7402 16S ribosomal RNA gene, partial sequence	KX607208.1	100
AIA_TES_378	*Bacillus oryzaecorticis* strain KN-23 16S ribosomal RNA gene, partial sequence	KU933480.1	99
SWA_TEA_379	*Brevibacterium linens* strain Bv7 16S ribosomal RNA gene, partial sequence	MH493690.1	100
SWA_TEA_380	*Roseovarius albus* strain CECT 7450 16S ribosomal RNA, partial sequence	NR_134162.1	99
R2A_SPV_381	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
MA_AMC_382	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
AIA_RHG_383	*Labrenzia* sp. strain 1334-139 16S ribosomal RNA gene, partial sequence	KY770164.1	99
OLIGO_SPS_384	*Labrenzia* sp. A-3-20 16S ribosomal RNA gene, partial sequence	KT583494.1	99
R2A_SPS_385	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	100
R2A_SPS_386	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
SCA_TES_387	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
SCA_TES_388	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99

SCA_TES_389	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
SCA_TES_390	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
AIA_TEA_391	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
OLIGO_TES_392	*Labrenzia* sp. A-3-20 16S ribosomal RNA gene, partial sequence	KT583494.1	99
SWA_XES_393	*Labrenzia* sp. A-3-20 16S ribosomal RNA gene, partial sequence	KT583494.1	99
M1_AMQ_394	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
R2A_AXT_395	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
SCA_TES_396	*Pseudovibrio* sp. Mexcell6.3B 16S ribosomal RNA gene, partial sequence	JX436424.1	100
SCA_TES_397	*Pseudovibrio* sp. Mexcell6.3B 16S ribosomal RNA gene, partial sequence	JX436424.1	100
SCA_TES_398	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	99
SCA_TEA_399	*Pseudovibrio* sp. Mexcell6.3B 16S ribosomal RNA gene, partial sequence	JX436424.1	100
AIA_TEA_400	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100
AIA_TEA_401	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100
AIA_XES_402	*Pseudovibrio* sp. 2011SOCNI2 16S ribosomal RNA gene, partial sequence	KF582856.1	100
AIA_XES_403	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100
SCA_CIS_404	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
SCA_CLR_405	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
AIA_DAS_406	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
OLIGO_HAF_407	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
OLIGO_NIS_408	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
OLIGO_RHG_409	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
OLIGO_SPV_410	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
AIA_XES_411	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100

AIA_TEA_412	*Ruegeria* sp. DN71_7G3 16S ribosomal RNA gene, partial sequence	KP769439.1	99
OLIGO_XES_413	*Pseudovibrio sp.* 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100
AIA_XES_414	*Ruegeria* sp. 2011SOCNI17 16S ribosomal RNA gene, partial sequence	KF582894.1	100
AIA_XES_415	*Ruegeria* sp. 2011SOCNI17 16S ribosomal RNA gene, partial sequence	KF582894.1	100
SWA_RHG_416	*Micrococcus luteus* strain FC1737 16S ribosomal RNA gene, partial sequence	MH665979.1	100
SWA_SPV_417	*Micrococcaceae bacterium* HW-2 16S ribosomal RNA gene, partial sequence	KM885169.1	100
SWA_TES_418	*Micrococcus luteus* strain SSW15 16S ribosomal RNA gene, partial sequence	KU922513.1	100
SWA_XES_419	*Pseudovibrio* sp. 2011SOCNI18 16S ribosomal RNA gene, partial sequence	KF582862.1	100
SWA_XES_420	*Micrococcus* sp. strain T3211-1-1 16S ribosomal RNA gene, partial sequence	MG254804.1	100
AIA_XES_421	*Ruegeria* sp. 2011SOCNI17 16S ribosomal RNA gene, partial sequence	KF582894.1	100
OLIGO_XES_422	*Ruegeria* sp. 2011SOCNI17 16S ribosomal RNA gene, partial sequence	KF582894.1	100
SWA_SPS_423	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
SWA_TES_424	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
SWA_TEA_425	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
SWA_XES_426	*Eudoraea chungangensis* strain CAU 1296 16S ribosomal RNA, partial sequence	NR_1482991	99
MA_CIS_427	*Cellulosimicrobium cellulans* partial 16S rRNA gene, strain PrF	HG000003.1	99
M1_RHG_428	*Cellulosimicrobium cellulans* partial 16S rRNA gene, strain PrF	HG000003.1	99
SCA_SPS_429	*Staphylococcus* sp. 29-3pA 16S ribosomal RNA gene, partial sequence	KU060115.1	99
OLIGO_SPV_430	*Pseudomonas plecoglossicida* strain SLr02 16S ribosomal RNA gene, partial sequence	MG755757.1	100
M1_AMC_431	*Burkholderia* sp. A-3 gene for 16S ribosomal RNA, partial sequence	AB694974.1	99
R2A_CRV_432	*Burkholderia* sp. strain MR5 16S ribosomal RNA gene, partial sequence	MG674315.1	100
R2A_NIS_433	*Burkholderia* sp. strain JGSB06 16S ribosomal RNA gene, partial sequence	MF285777.1	100
AIA_DAS_434	Uncultured *Chryseobacterium* sp. clone 6 16S ribosomal RNA gene, partial sequence	EU026431.1	99

M1_AMQ_435	*Ralstonia* sp. strain CWS_Control 16S ribosomal RNA gene, partial sequence	KX025096.1	99
R2A_CIS_436	Uncultured *Ralstonia* sp. clone EC34BC01 16S ribosomal RNA gene, partial sequence	JN032362.1	99
SCA_SPV_437	*Burkholderia* sp. strain JGSB06 16S ribosomal RNA gene, partial sequence	MF285777.1	100
AIA_TEA_438	*Bacillus* sp. strain MML5312 16S ribosomal RNA gene, partial sequence	MF687522.1	99
AIA_TES_439	*Burkholderia* sp. strain MR5 16S ribosomal RNA gene, partial sequence	MG674315.1	100
AIA_SPS_440	*Kocuria rosea* partial 16S rRNA gene, strain PrD	HG000002.1	100
SWA_HAA_441	*Acinetobacter indicus* strain SGAir0564 chromosome, complete genome	CP024620.1	100
R2A_HAA_442	*Exiguobacterium mexicanum* strain MSSRFS9 16S ribosomal RNA gene, partial sequence	KF471138.1	99
AIA_XES_443	*Staphylococcus saprophyticus* strain FDAARGOS_168 chromosome, complete genome	CP014113.2	100
OLIGO_HAF_444	*Curtobacterium* sp. strain B4 16S ribosomal RNA gene, partial sequence	MG234436.1	100
SWA_TES_445	*Pseudomonas stutzeri* partial 16S rRNA gene, strain 24a75	AJ312229.1	99
AIA_XES_446	*Acidovorax* sp. DQS-01 16S ribosomal RNA gene, partial sequence	KP126996.1	100
M1_RHG_447	*Escherichia coli* strain F3-1-9 16S ribosomal RNA gene, partial sequence	KX349997.1	100
R2A_SPV_448	*Escherichia coli* strain F3-1-9 16S ribosomal RNA gene, partial sequence	KX349997.1	100
SCA_TEA_449	*Escherichia coli* strain 2452 chromosome, complete genome	CP031833.1	100
SWA_XES_450	*Ruegeria atlantica* strain DN83_2B6 16S ribosomal RNA gene, partial sequence	KP769432.1	99
AIA_TEA_451	*Bacillus* sp. (in: Bacteria) strain CL42 16S ribosomal RNA gene, partial sequence	MH605371.1	100
MA_CIS_452	*Halobacillus* sp. strain Q-13 16S ribosomal RNA gene, partial sequence	MF035455.1	100
AIA_TEA_453	Uncultured *Bacillus* sp. clone TOPO4 16S ribosomal RNA gene, partial sequence	KY963640.1	99
AIA_XES_454	*Bacillus* sp. strain pc-3w 16S ribosomal RNA gene, partial sequence	MF993476.1	100
SCA_CRV_455	*Ralstonia pickettii* strain VIT-SRM1 16S ribosomal RNA gene, partial sequence	KJ716446.1	99
AIA_XES_456	*Bacillus altitudinis* strain TR38T1 16S ribosomal RNA gene, partial sequence	KF737162.1	100
SWA_XES_457	*Pseudomonas* sp. BBAL-02d 16S ribosomal RNA gene, partial sequence	FJ217180.1	99

AIA_XES_458	*Bacillus* sp. (in: Bacteria) strain T16-2-1 16S ribosomal RNA gene, partial sequence	MG254766.1	100
OLIGO_XES_459	*Bacillus* sp. (in: Bacteria) strain Firmi-75 16S ribosomal RNA gene, partial sequence	MH683164.1	99
SWA_XES_460	*Bacillus infantis* NRRL B-14911, complete genome	CP006643.1	100

Supplementary Table S6. ^{1}H and ^{13}C NMR data of Cyclo-(L-Pro-L-Tyr) (**1**)

Position	$^{\#}\delta_C$[a]	δ_C[a,b]	δ_H[a,c] mult (J = Hz)
1	165.6	166.9	-
2	-	-	-
3	44.5	45.9	3.37 m, 3.57 m
4	21.3	22.7	1.82 (2H, m)
5	28.0	29.3	2.11 (2H, m)
6	58.7	60.0	4.07 (m)
7	169.4	170.8	-
8	-	-	-
9	56.5	57.9	4.37 (m)
10	36.3	37.6	3.07 (2H, m)
1′	126.4	127.6	-
2′, 6′	130.7	132.1	7.05 (2H, d, 8.5)
3′, 5′	114.8	116.2	6.72 (2H, d, 8.5)
4′	156.3	157.6	-

Measured in [a]CD_3OD, [b]125 MHz, [c] 500 MHz, $^{\#}\delta_C$ of Cyclo-(L-Pro-L-Tyr) (Quyen et al., 2015)

Supplementary Table S7. ^{1}H and ^{13}C NMR data of macrolactin A (**2**)

Position	$^{\#}\delta_C{}^{a}$	$\delta_C{}^{a,b}$	$\delta_H{}^{a,c}$ mult (J = Hz)
1	166.83	168.0	
2	118.15	117.9	5.56 (m)
3	145.12	144.9	6.65 (t, 11.5)
4	130.42	130.2	7.23 (m)
5	142.34	142.1	6.19 (m)
6	43.02	42.8	2.44 (2H, m)
7	72.37	72.3	4.28 (m)
8	137.71	137.5	5.77 (dd, 6.0, 15.0)
9	126.10	125.9	6.58 (dd, 11.0, 15.0)
10	131.56	131.3	6.13 (m)
11	128.54	128.4	5.55 (m)
12	36.67	36.4	2.34 (m), 2.50 (m)
13	69.37	69.2	3.87 (m)
14	44.07	43.9	1.63 (2H, m)
15	69.97	69.8	4.32 (m)
16	135.40	135.2	5.57 (m)
17	131.36	131.2	6.10 (m)
18	131.87	131.7	6.05 (m)
19	135.29	135.1	5.66 (m)
20	25.81	25.6	1.52 (2H, m)
21	33.14	32.9	2.12 (m), 2.20 (m)
22	36.17	36.0	1.59 (m), 1.65 (m)
23	72.49	72.2	5.02 (m)
24	20.27	20.1	1.27 (3H, d, 6.5)

Measured in [a] CD_3OD, [b] 500 MHz, [c] 125 MHz, $^{\#}\delta_C$ of macrolactin A (Lee et al., 2004)

Supplementary Table S8. ^{1}H and ^{13}C NMR data of macrolactin H (**3**)

Position	$^{\#}\delta_C$ [d]	δ_C [a,b]	δ_H [a,c] mult (J = Hz)
1	166.3	166.3	-
2	117.9	118.0	5.55 (m)
3	143.7	144.8	6.65 (m)
4	130.1	130.4	5.59 (m)
5	139.9	141.7	6.17 (m)
6	41.5	42.5	2.45 (2H, m)
7	70.6	72.0	4.98 (m)
8	135.9	137.2	5.76 (m)
9	126.0	126.1	6.75 (m)
10	130.7	131.0	5.63 (m)
11	134.2	134.7	5.46 (m)
12	36.1	36.5	1.58 (m), 2.23 (m)
13	69.8	69.7	4.32 (m)
14	41.5	44.6	1.59 (2H, m)
15	70.6	70.2	4.26 (m)
16	128.1	128.3	5.55 (m)
17	130.4	131.7	5.65 (m)
18	32.1	32.9	2.08 (2H, m)
19	24.7	25.9	1.40 (2H, m)
20	35.0	36.6	1.65 (2H, m)
21	70.8	70.8	4.28 (m)
22	19.6	20.1	1.27 (3H, d, 6.5)

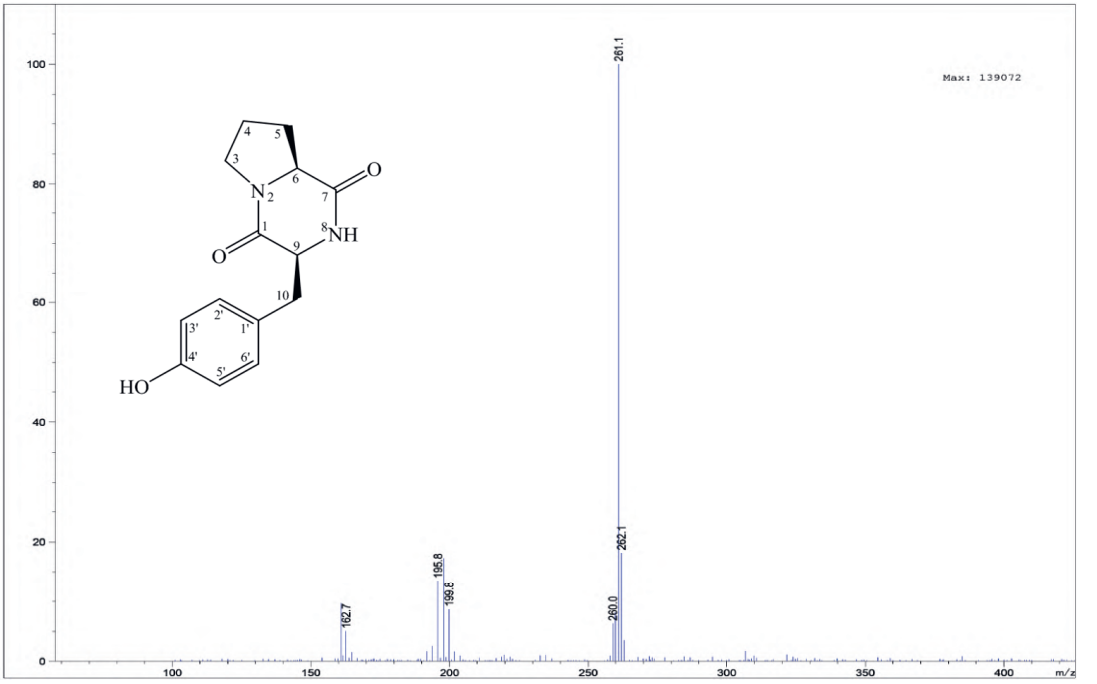
Measured in [a] CD_3OD, [b] 125 MHz, [c] 500 MHz, [d] Benzen-d_6, $^{\#}\delta_C$ of macrolactin H (Nagao et al., 2001)

Supplementary Table S9. ^{1}H and ^{13}C NMR data of 15,17-epoxy-16-hydroxy macrolactin A (**4**)

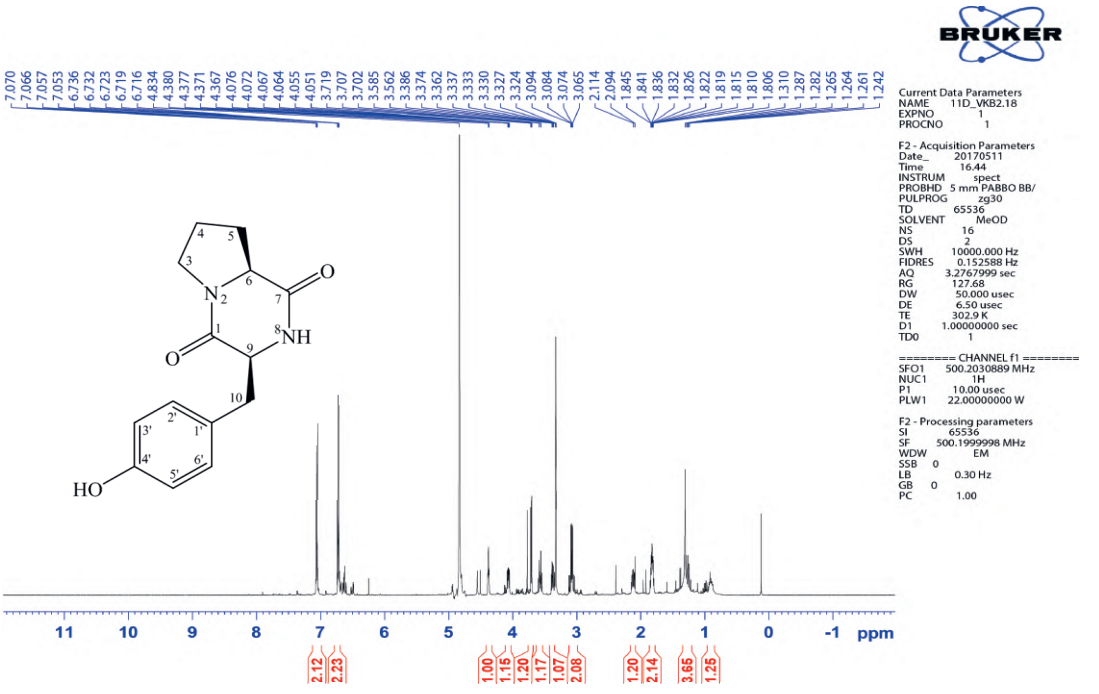
Position	$^{\#}\delta_C$ [a,d]	δ_C [a,b]	δ_H [a,c] mult (*J* = Hz)
1	168.3	168.2	-
2	118.3	118.2	5.54 (d, 11.5)
3	144.8	144.6	6.67 (t, 11.5)
4	130.7	130.6	7.25 (m)
5	141.6	141.4	6.27 (m)
6	42.9	42.7	2.51 (m), 2.42 (m)
7	73.2	73.1	4.27 (m)
8	136.4	136.2	5.64 (m)
9	127.9	127.7	6.56 (dd, 11.0, 15.0)
10	131.5	131.4	6.07 (t, 11.0)
11	129.7	129.5	5.46 (m)
12	35.3	35.1	2.57 (m), 2.13 (m)
13	76.1	76.0	3.46 (m)
14	40.9	40.8	1.99 (m), 1.40 (m)
15	74.0	73.9	3.54 (m)
16	77.8	77.7	2.94 (t, 9.0)
17	80.3	80.2	3.46 (m)
18	129.2	129.1	5.67 (m)
19	132.3	132.2	5.70 (m)
20	34.6	34.4	2.11 (m), 2.05 (m)
21	26.8	26.6	1.52 (2H, m)
22	37.0	36.8	1.67 (2H, m)
23	73.1	72.9	4.98 (m)
24	20.0	19.8	1.28 (3H, d, 6.0)

Measured in [a] CD_3OD, [b] 125 MHz, [c] 500 MHz, $^{\#}\delta_C$ of 15,17-epoxy-16-hydroxy macrolactin A (Mondol et al., 2011)

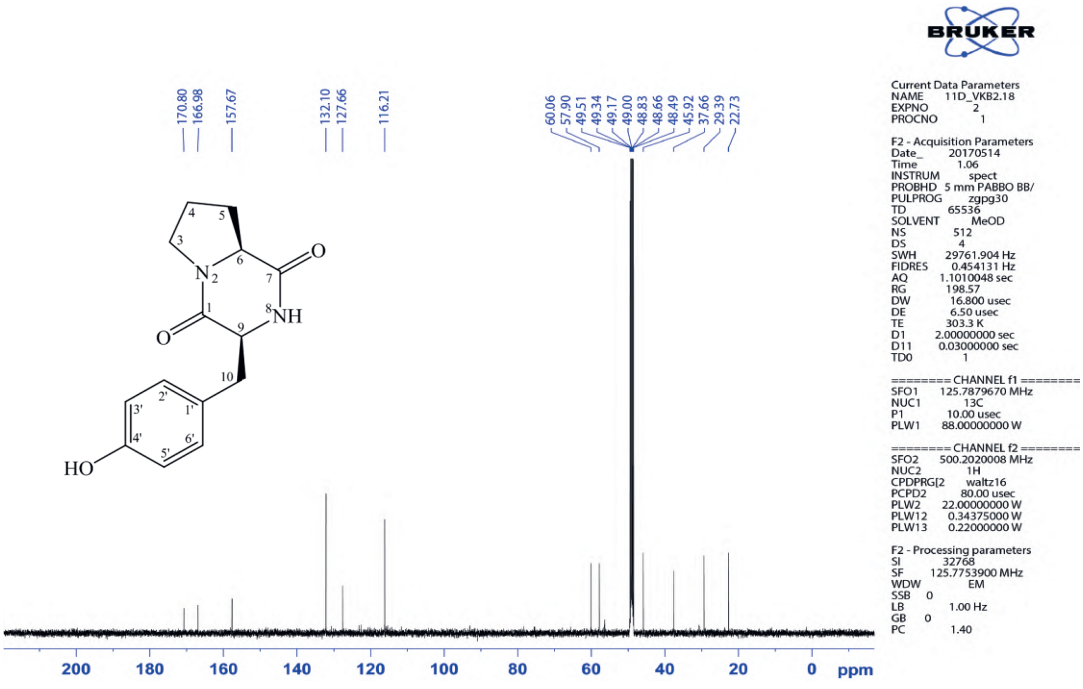
Spectra of the isolated compounds



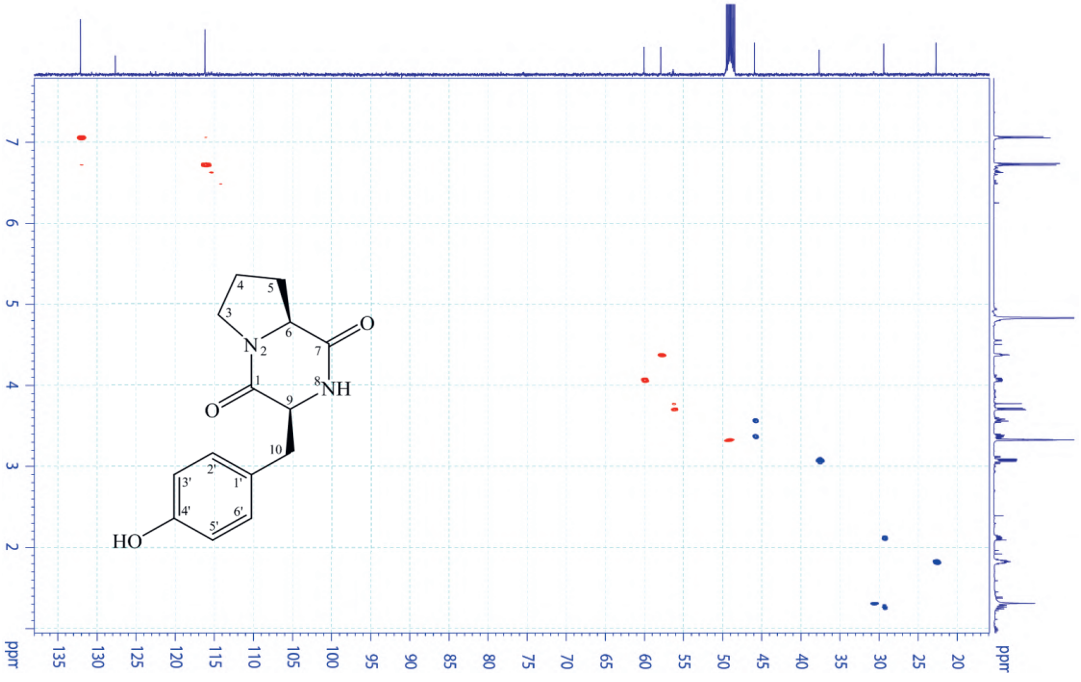
Supplementary Figure S1. ESI-MS spectrum of Cyclo-(L-Pro-L-Tyr) (**1**)



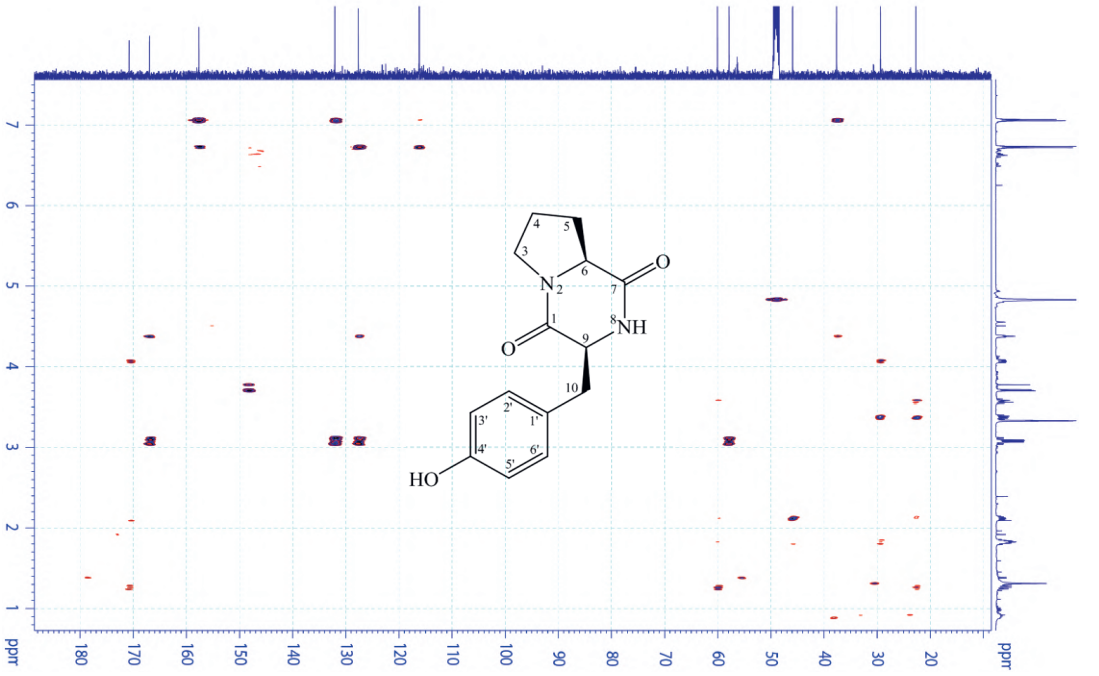
Supplementary Figure S2. ^{1}H NMR spectrum of Cyclo-(L-Pro-L-Tyr) (**1**)



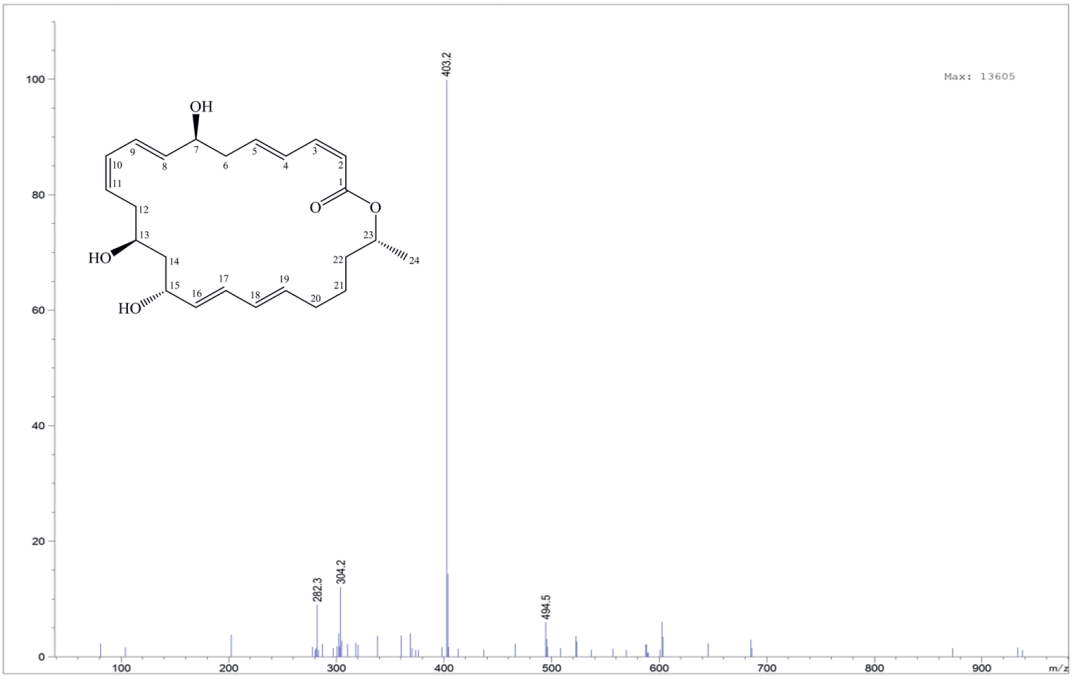
Supplementary Figure S3. ^{13}C NMR spectrum of Cyclo-(L-Pro-L-Tyr) (**1**)



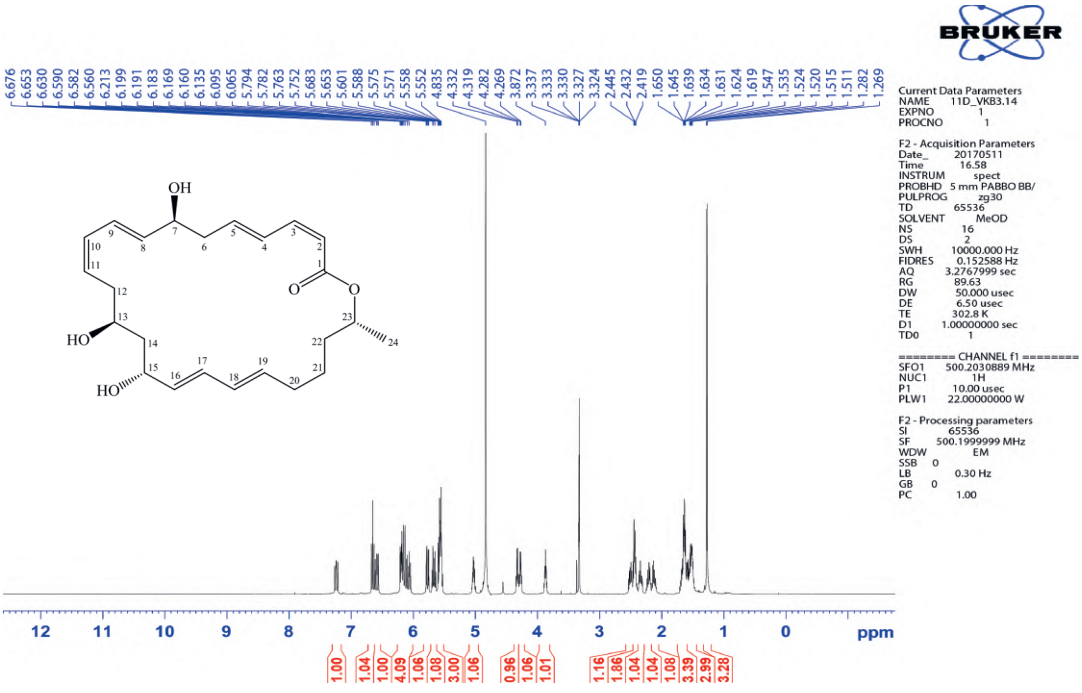
Supplementary Figure S4. HSQC spectrum of Cyclo-(L-Pro-L-Tyr) (**1**)



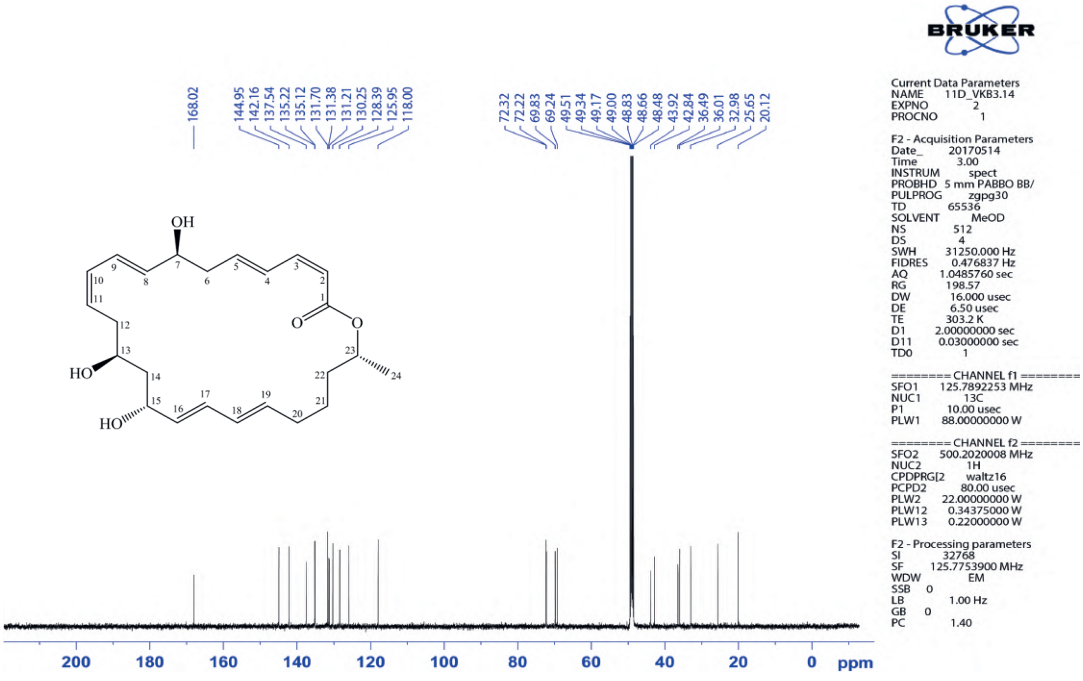
Supplementary Figure S5. HMBC spectrum of Cyclo-(L-Pro-L-Tyr) (**1**)



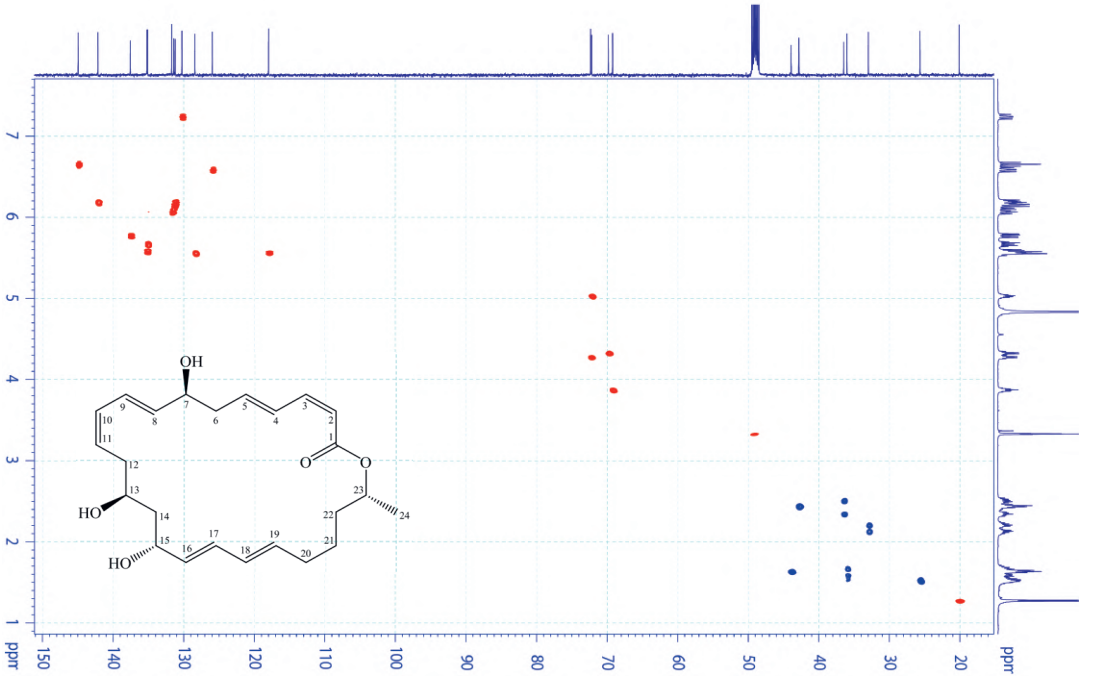
Supplementary Figure S6. ESI-MS spectrum of macrolactin A (**2**)



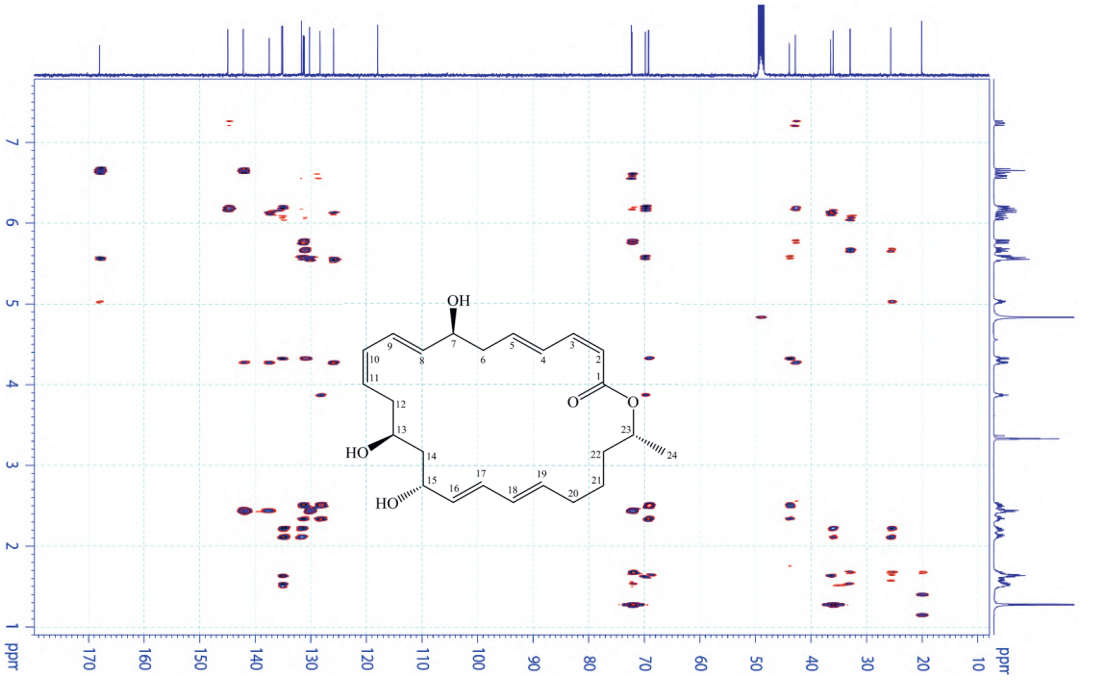
Supplementary Figure S7. ^{1}H NMR spectrum of macrolactin A (**2**)



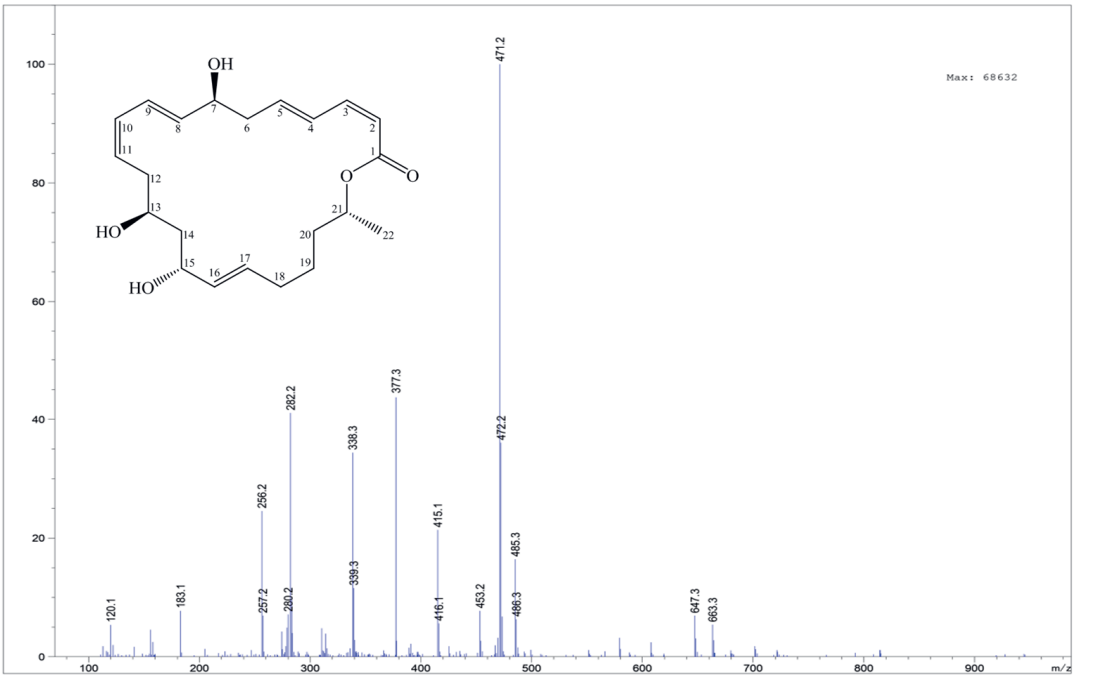
Supplementary Figure S8. ^{13}C NMR spectrum of macrolactin A (**2**)



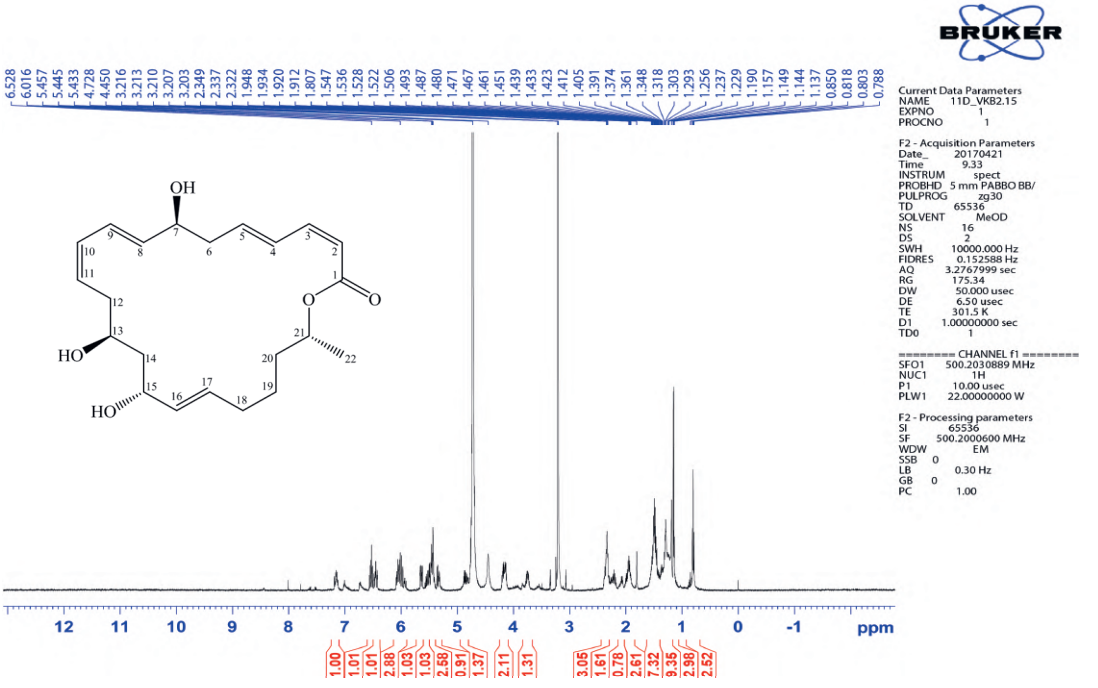
Supplementary Figure S9. HSQC spectrum of macrolactin A (**2**)



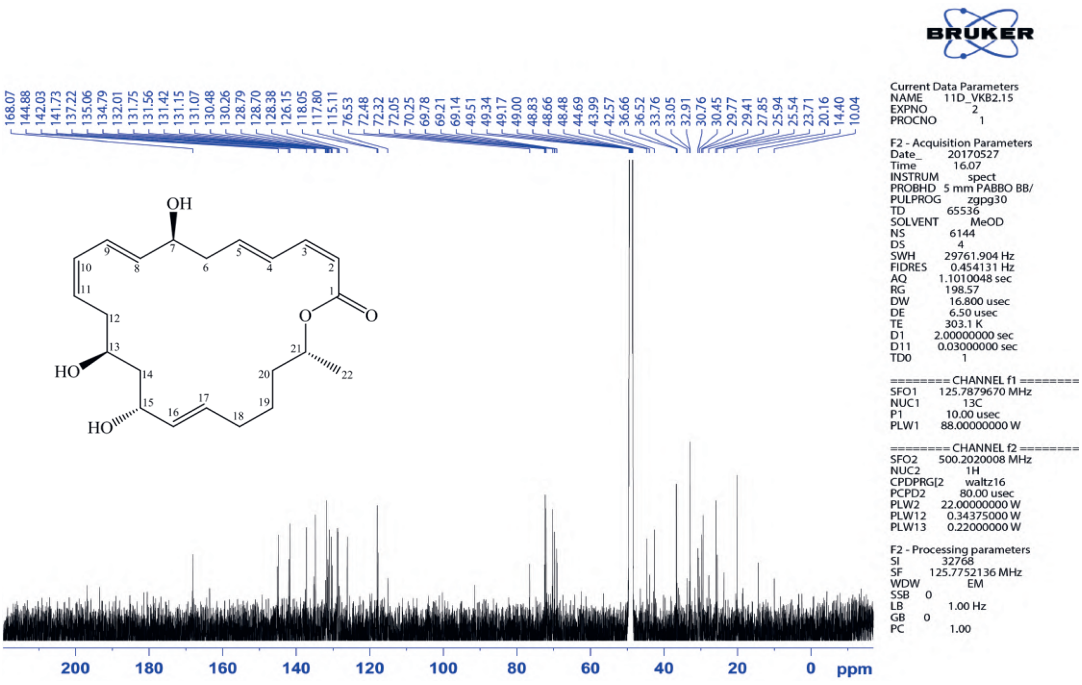
Supplementary Figure S10. HMBC spectrum of macrolactin A (**2**)



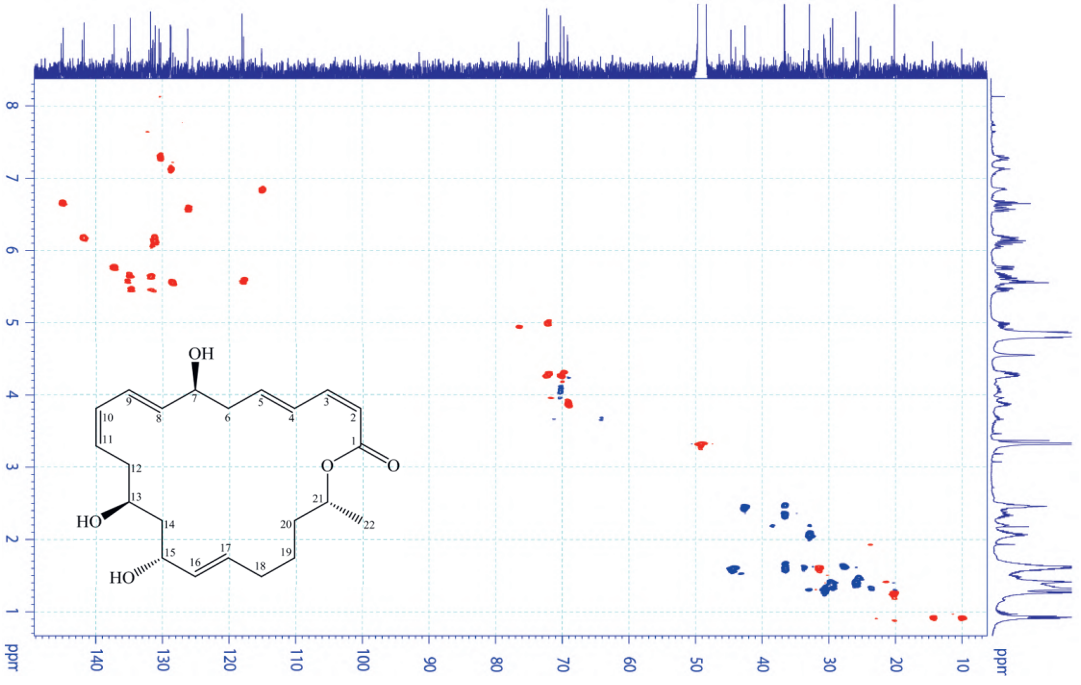
Supplementary Figure S11. ESI-MS spectrum of macrolactin H (**3**)



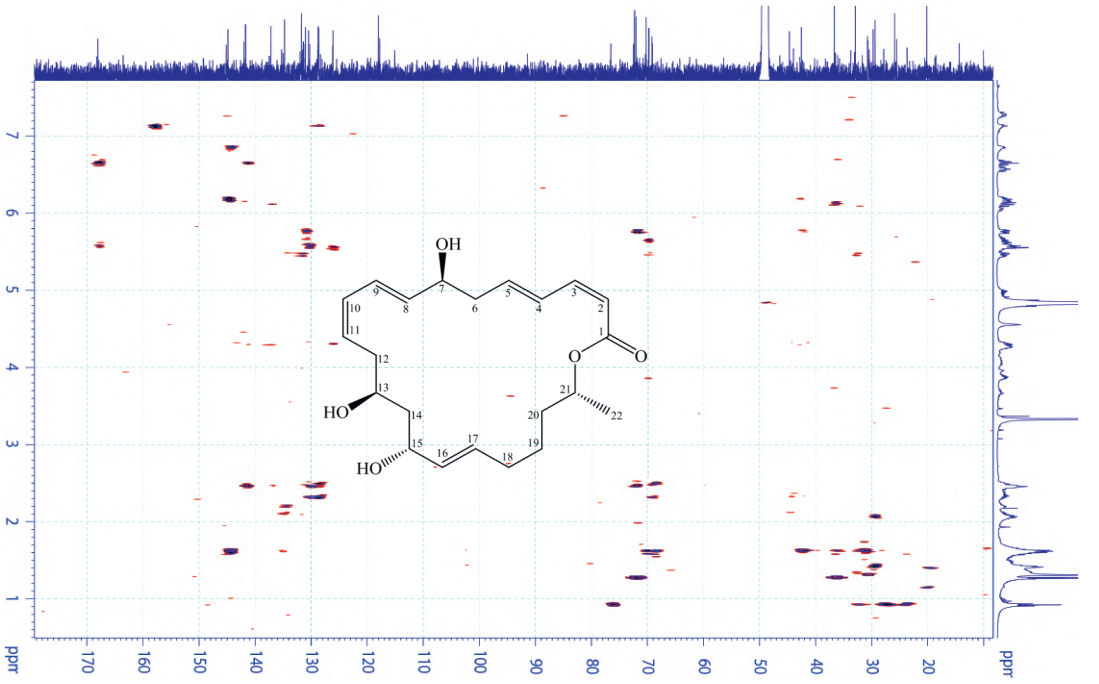
Supplementary Figure S12. ^{1}H NMR spectrum of macrolactin H (**3**)



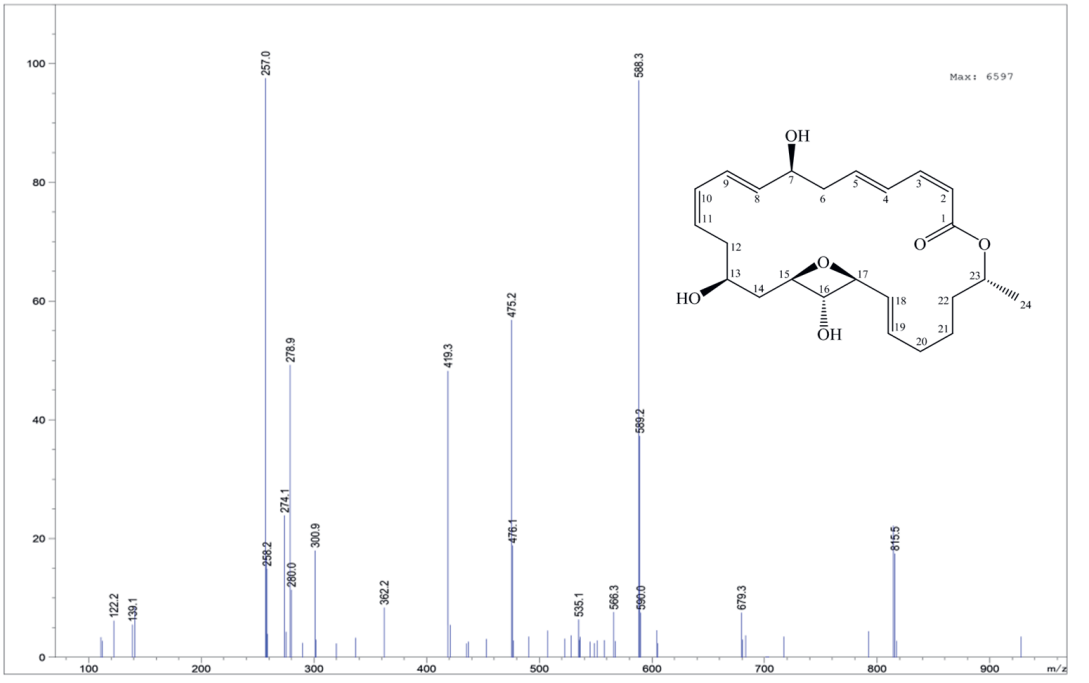
Supplementary Figure S13. ^{13}C NMR spectrum of macrolactin H (**3**)



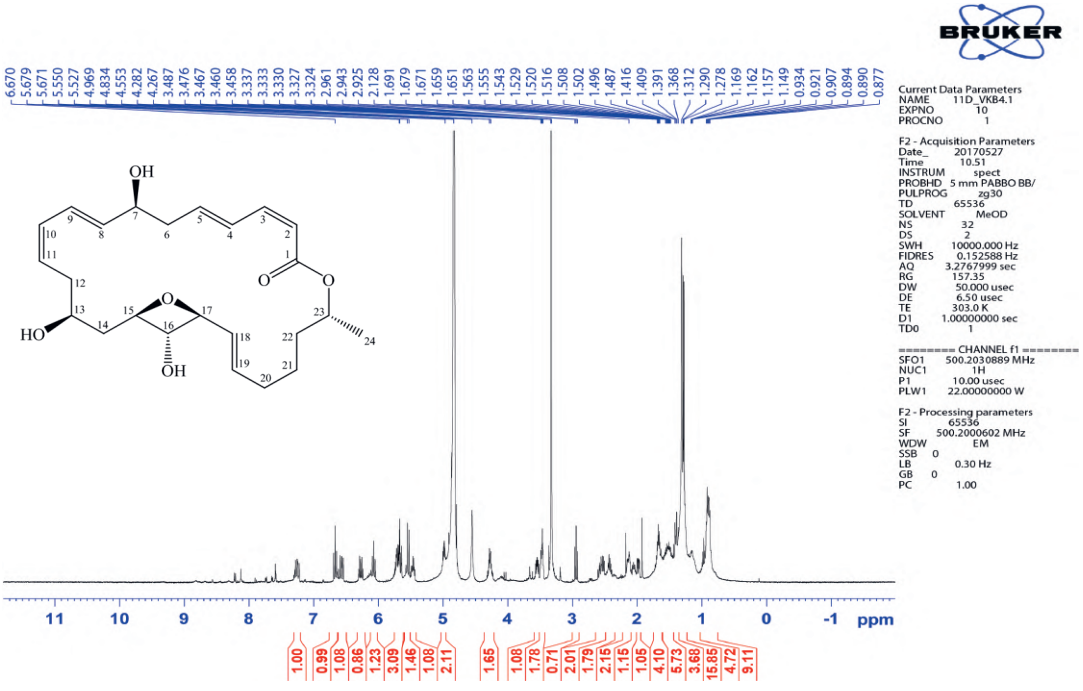
Supplementary Figure S14. HSQC spectrum of macrolactin H (**3**)



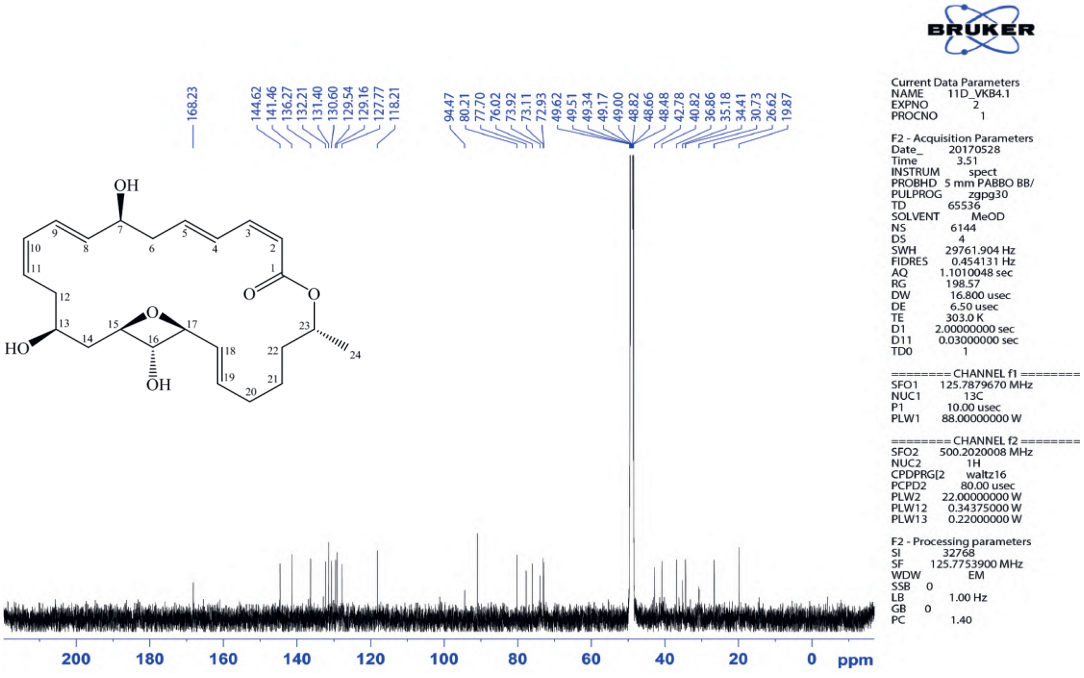
Supplementary Figure S15. HMBC spectrum of macrolactin H (**3**)



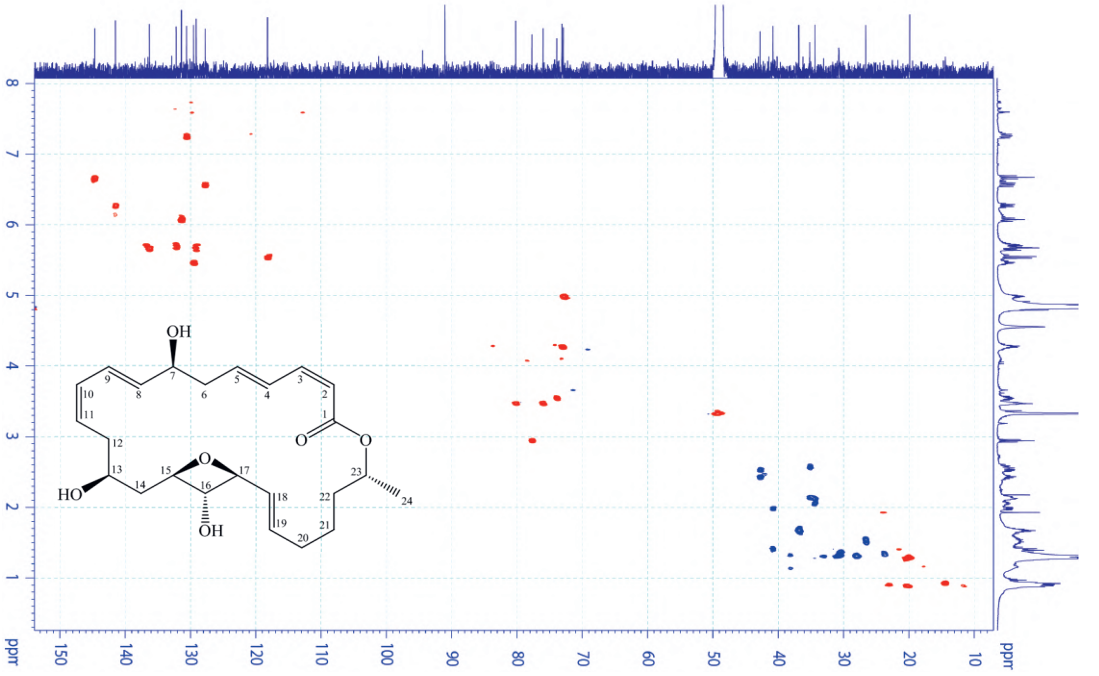
Supplementary Figure S16. ESI-MS spectrum of 15,17-epoxy-16-hydroxy macrolactin A (**4**)



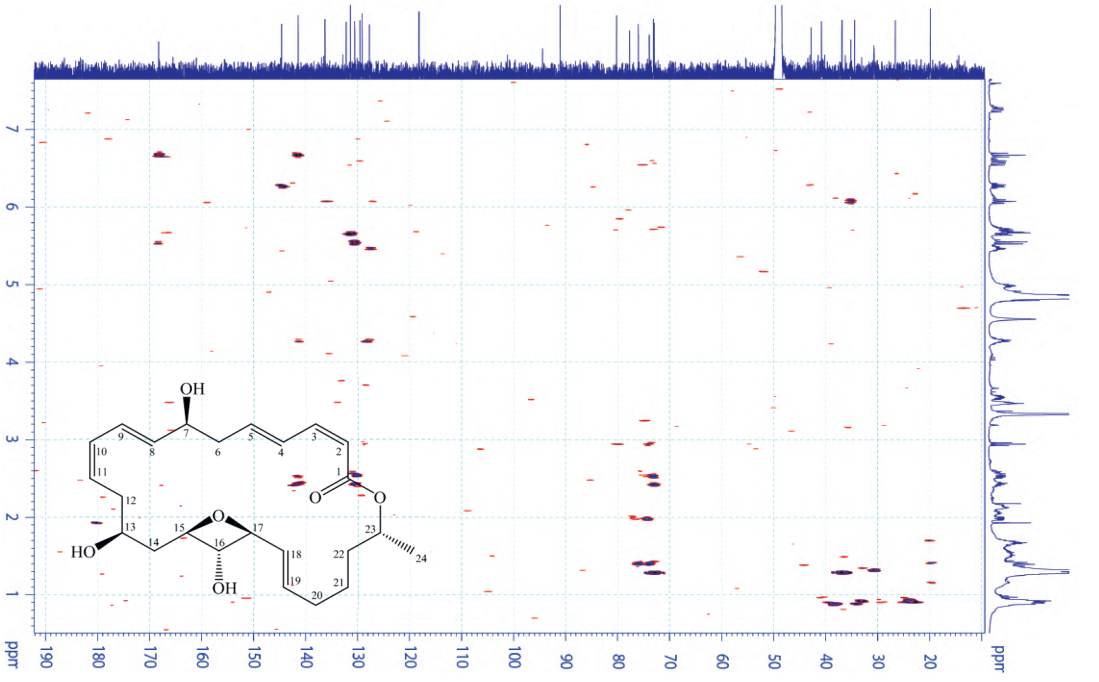
Supplementary Figure S17. ^{1}H NMR spectrum of 15,17-epoxy-16-hydroxy macrolactin A (**4**)



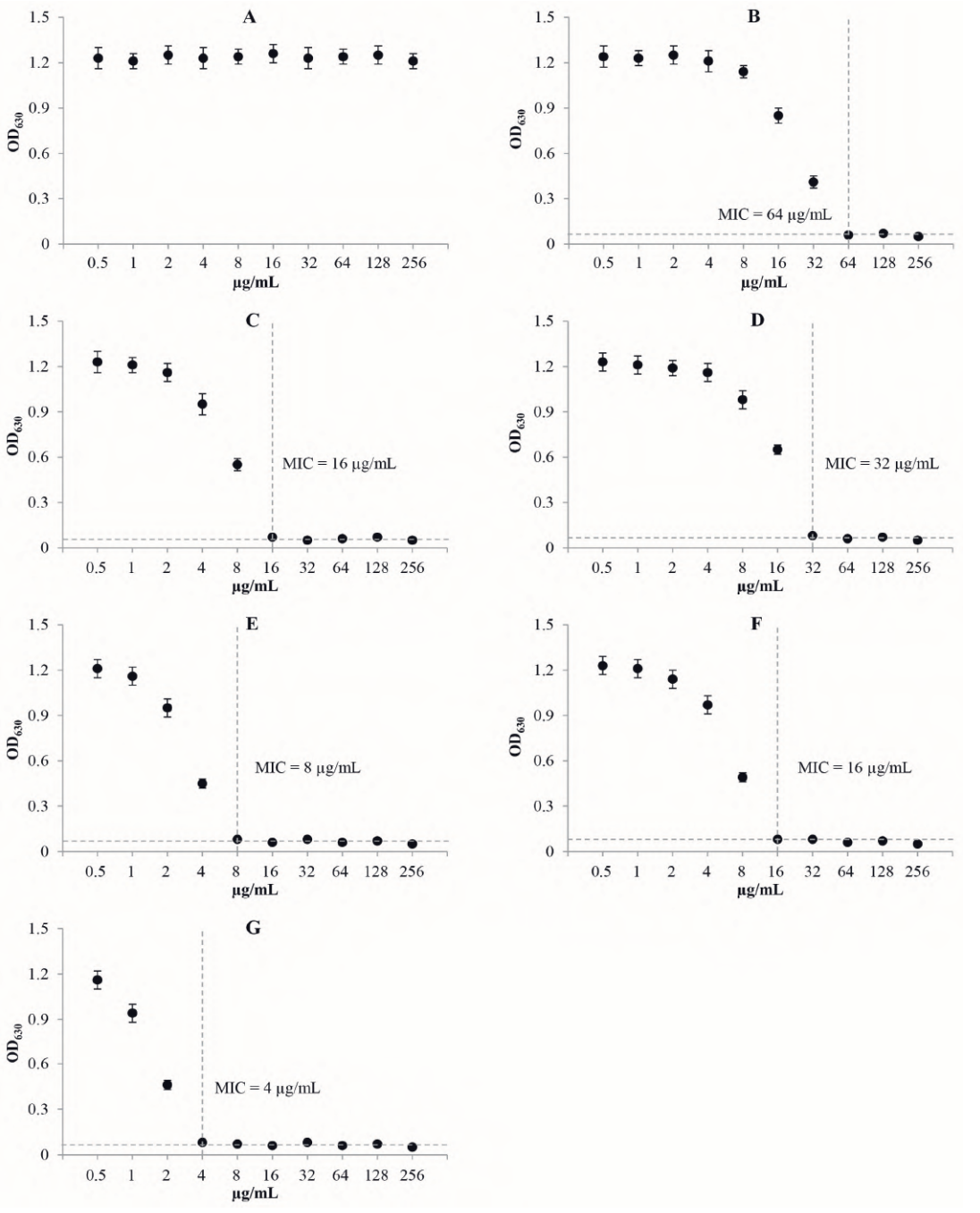
Supplementary Figure S18. ^{13}C NMR spectrum of 15,17-epoxy-16-hydroxy macrolactin A (**4**)



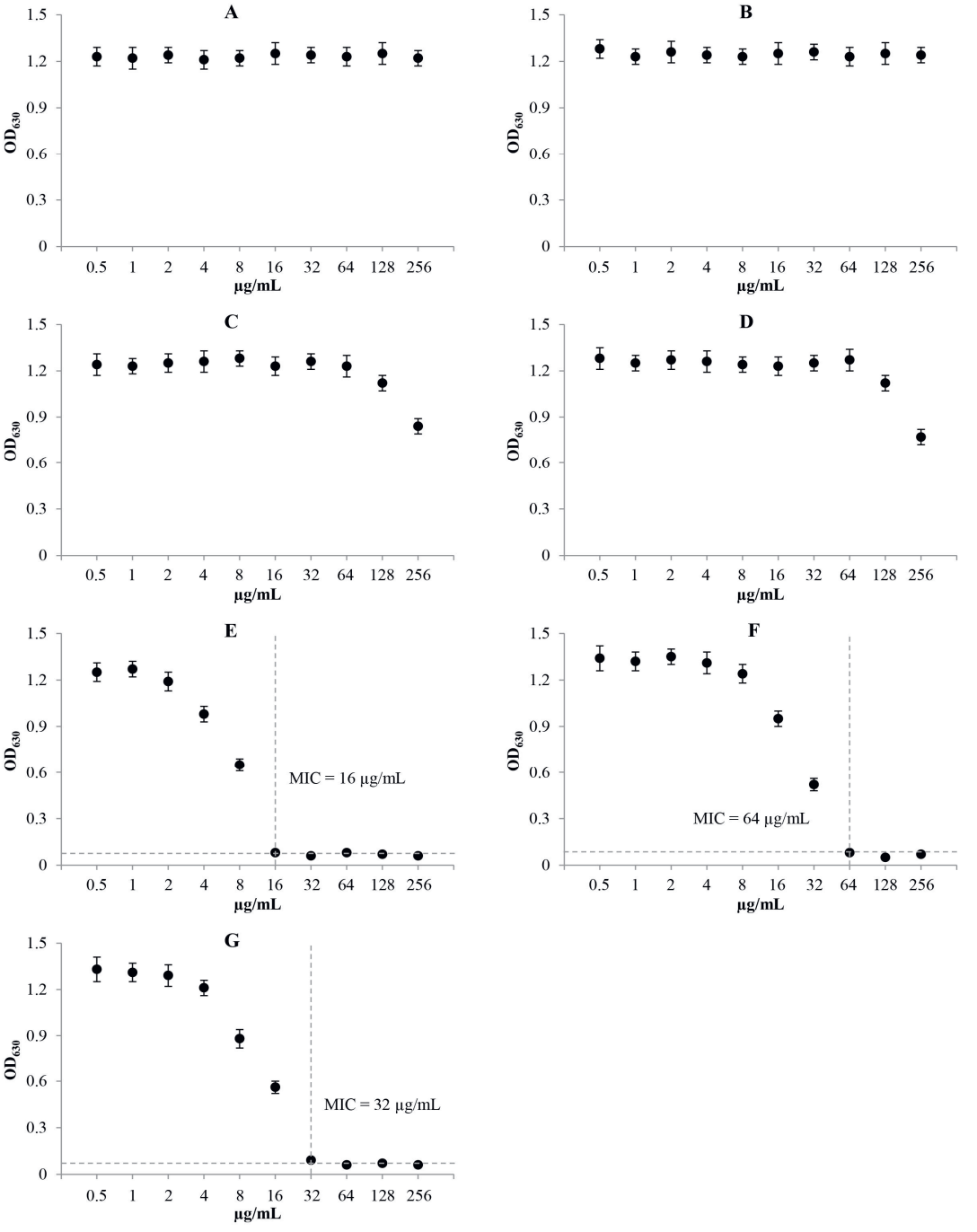
Supplementary Figure S19. HSQC spectrum of 15,17-epoxy-16-hydroxy macrolactin A (**4**)



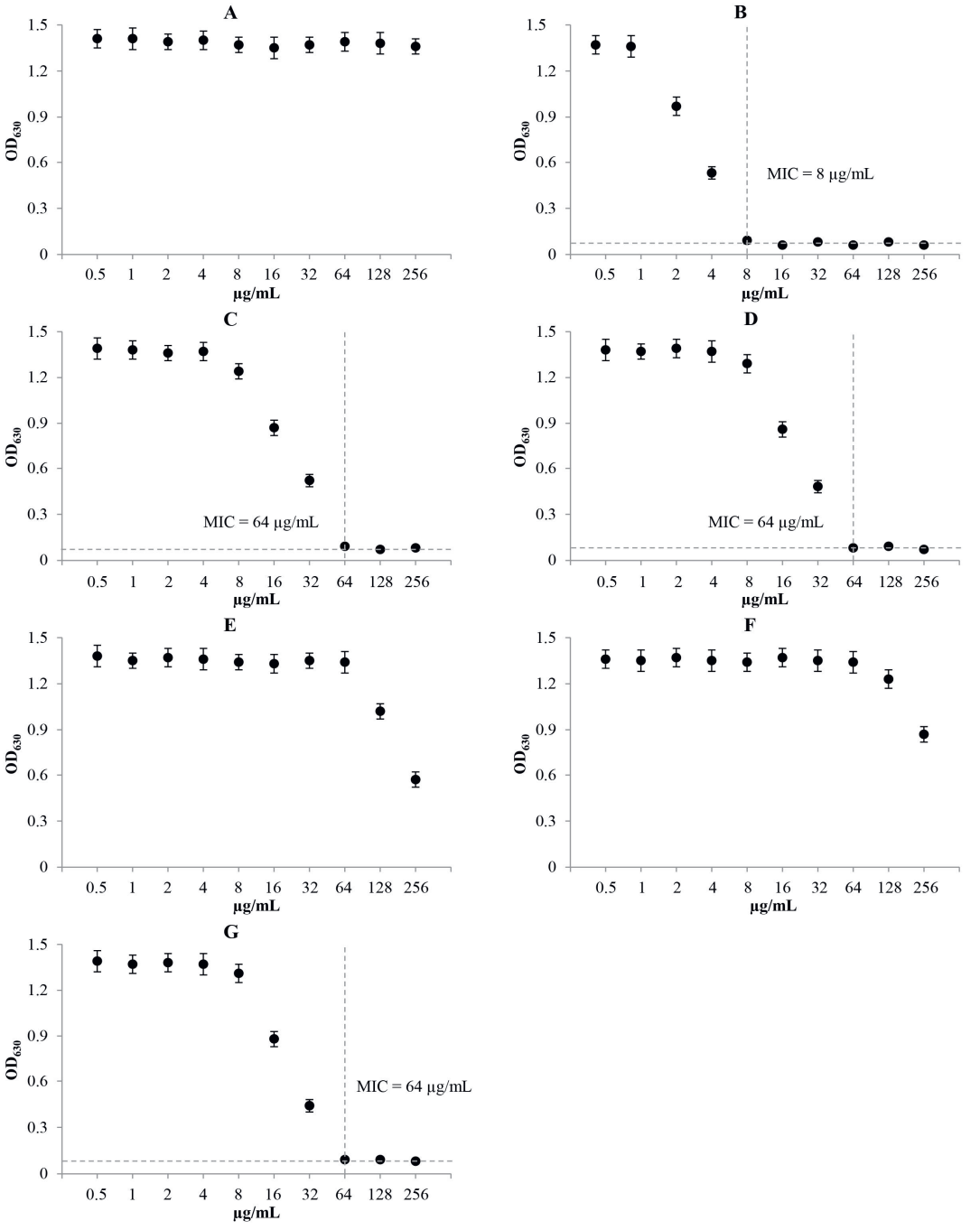
Supplementary Figure S20. HMBC spectrum of 15,17-epoxy-16-hydroxy macrolactin A (**4**)



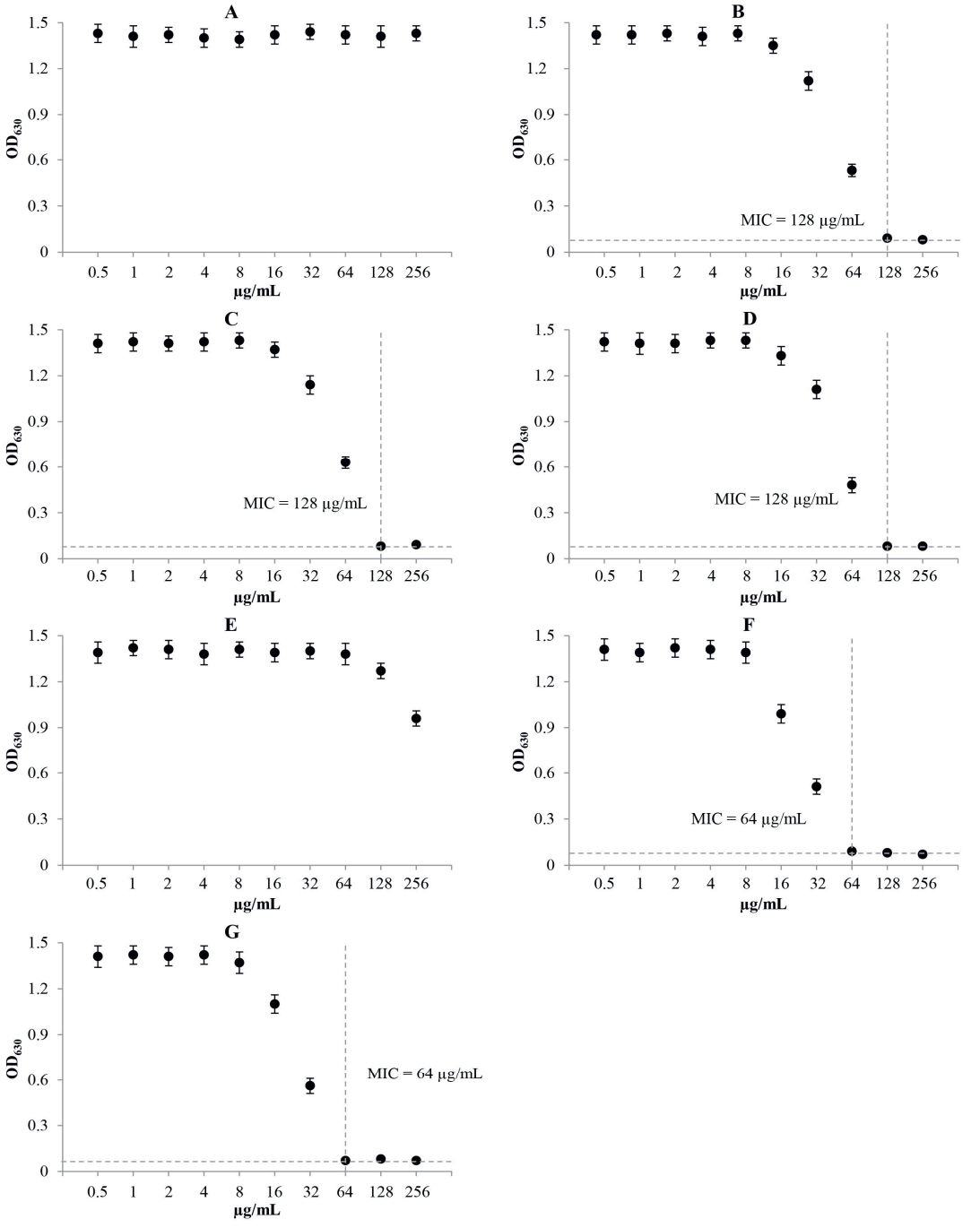
Supplementary Figure S21. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *E. coli* ATCC 25922



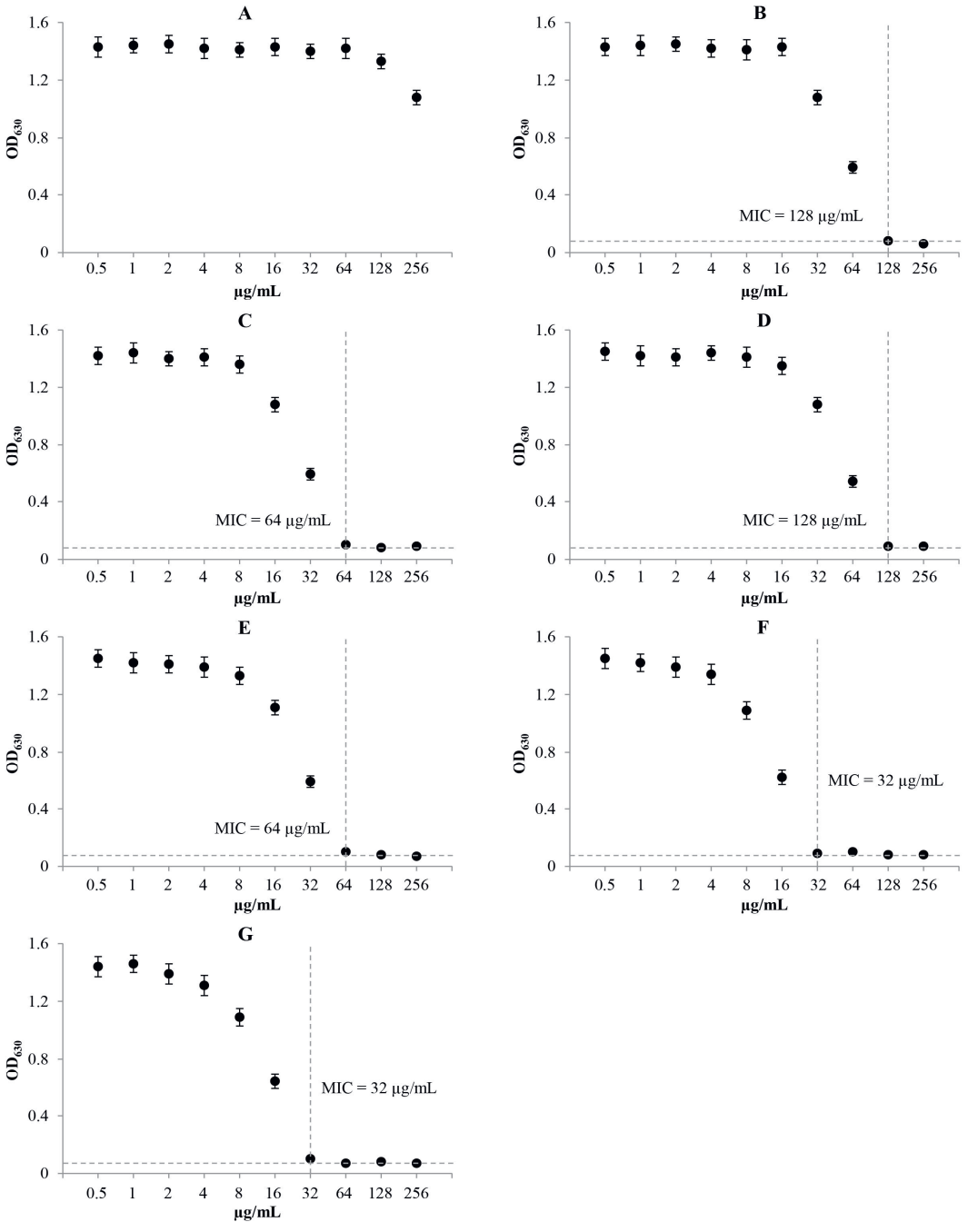
Supplementary Figure S22. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *S. enterica* ATCC 13076



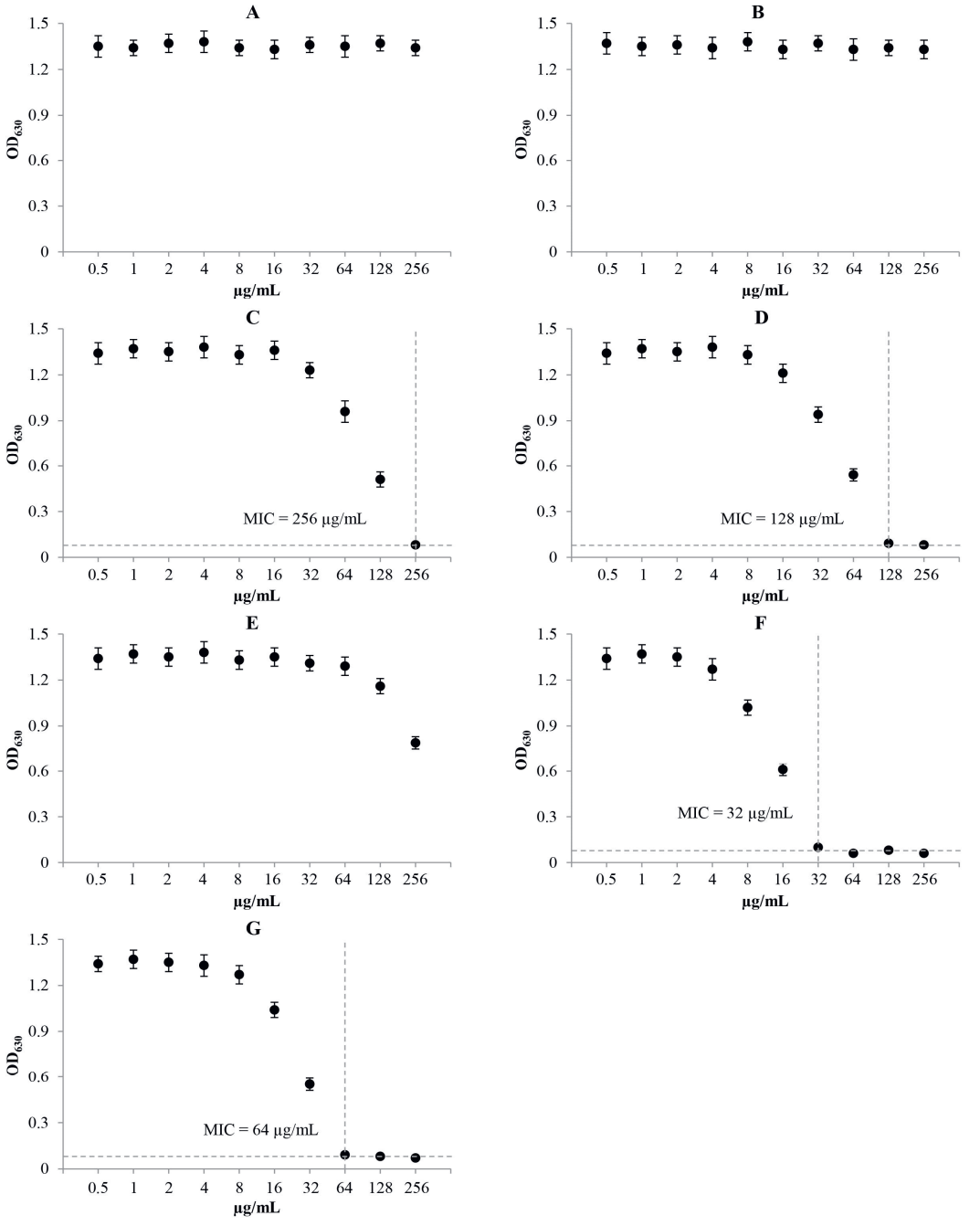
Supplementary Figure S23. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *P. aeruginosa* ATCC 27853



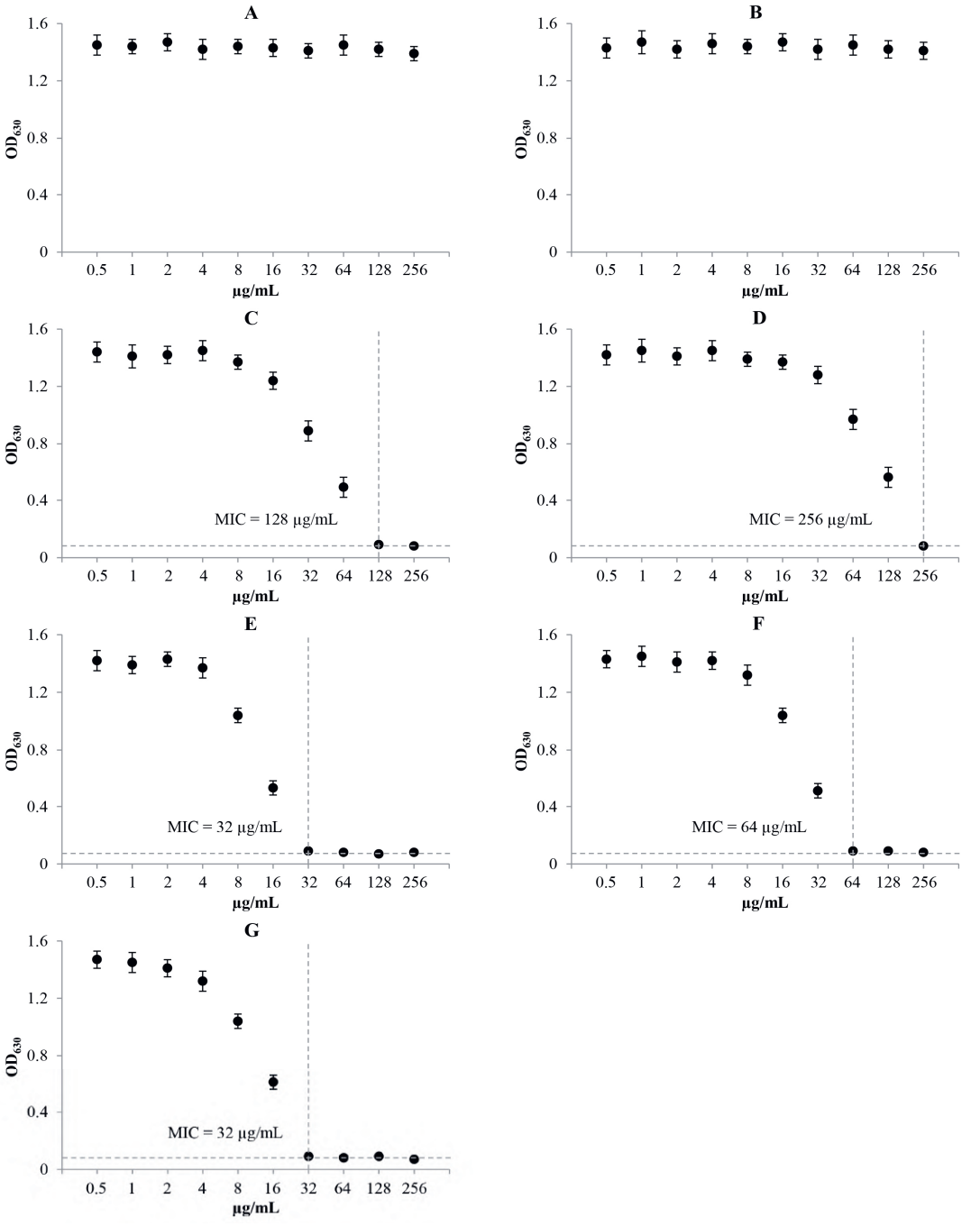
Supplementary Figure S24. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *P. putida* MISR 71218



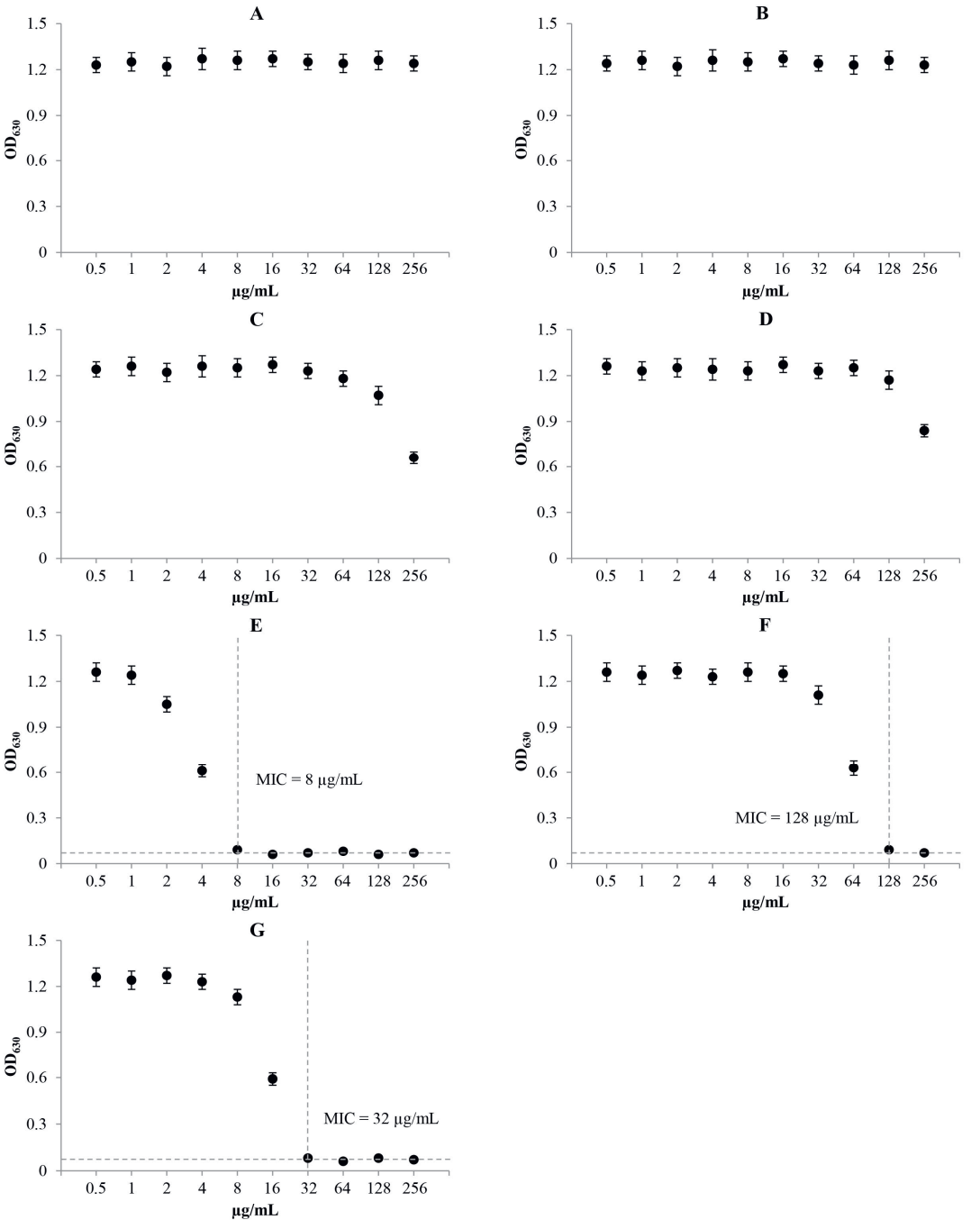
Supplementary Figure S25. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *V. parahaemolyticus* MISR 21116



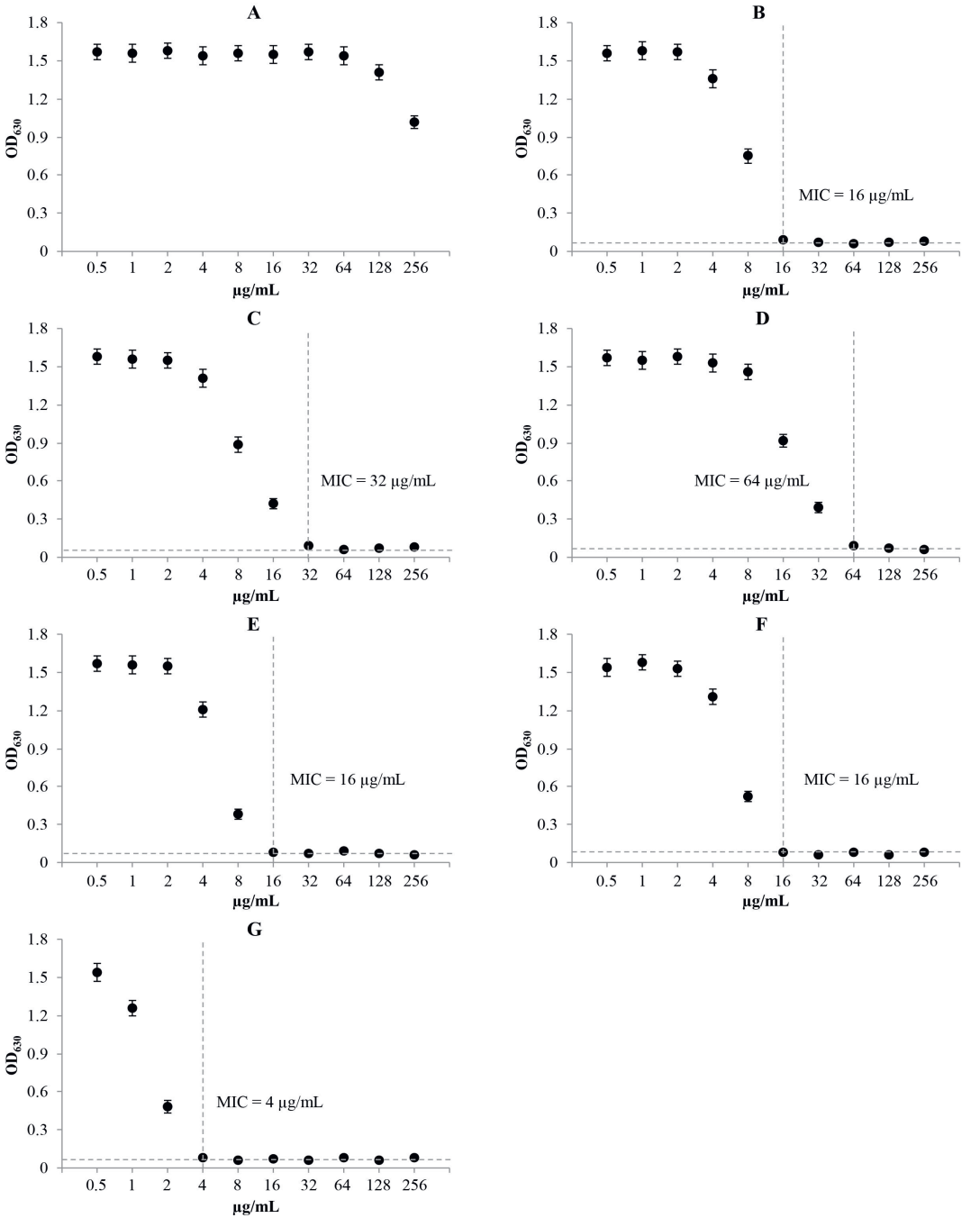
Supplementary Figure S26. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *V. alginolyticus* MISR 30816



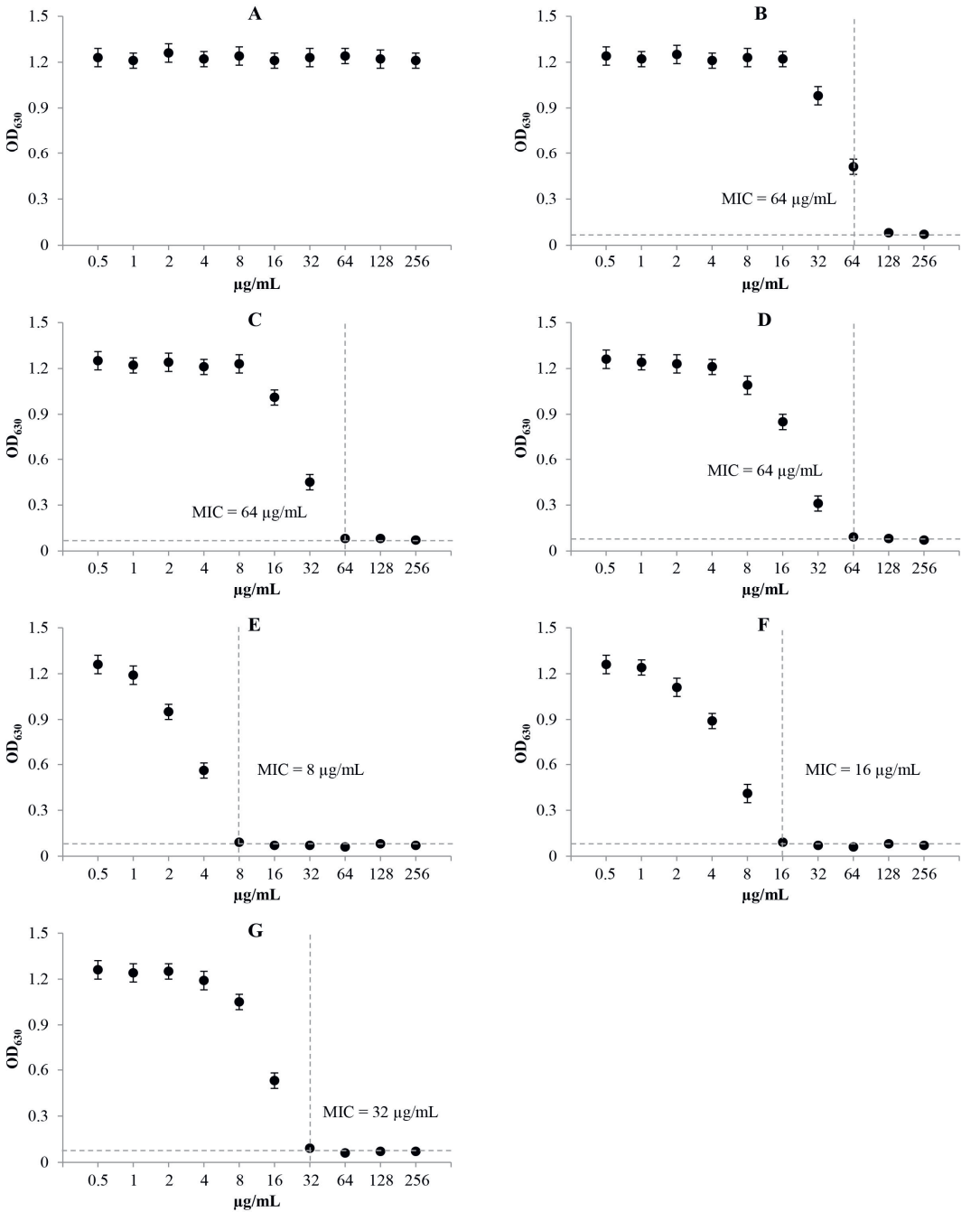
Supplementary Supplementary Figure S27. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *V. vulnificus* MISR 20716



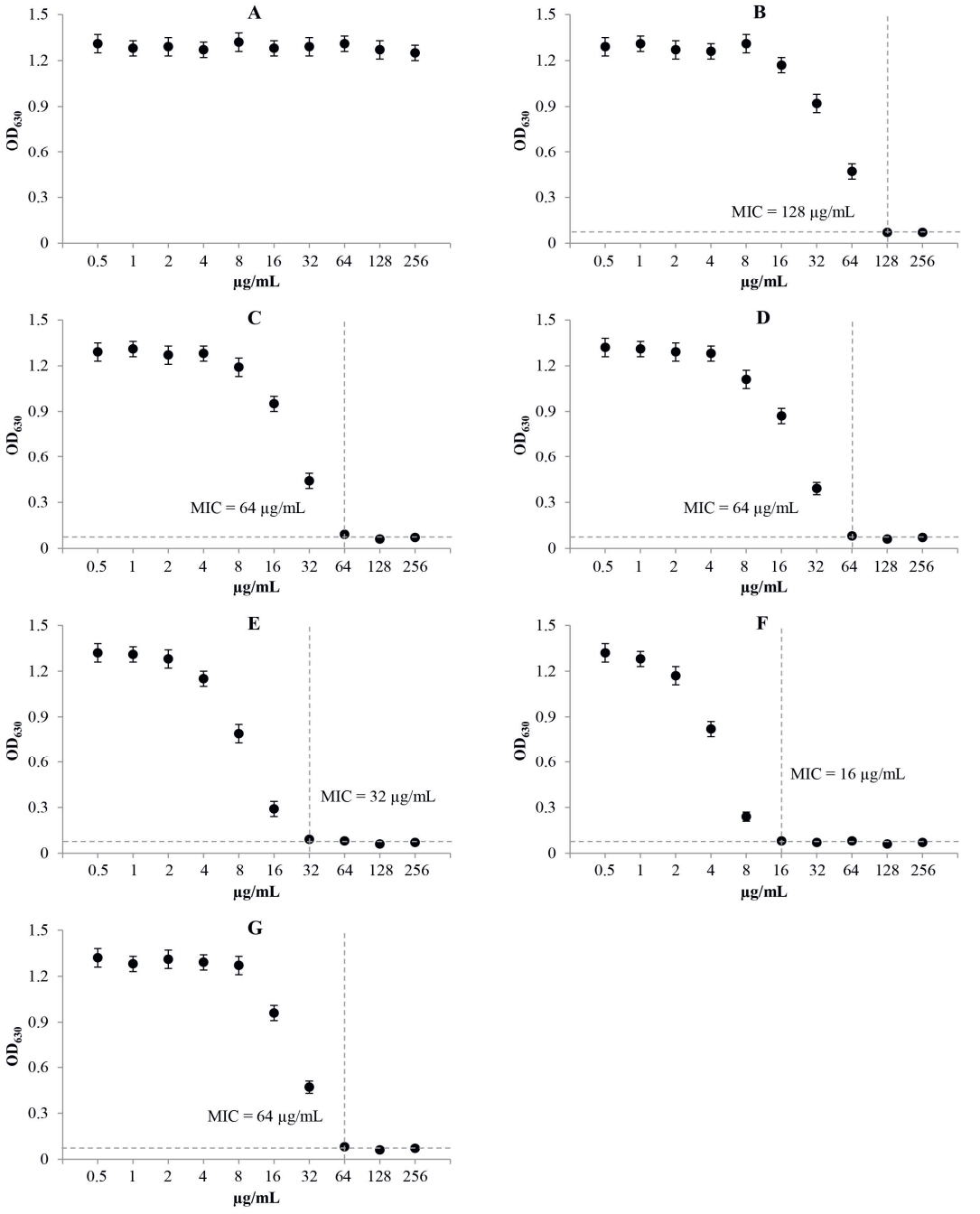
Supplementary Figure S28. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *E. faecalis* ATCC 29212



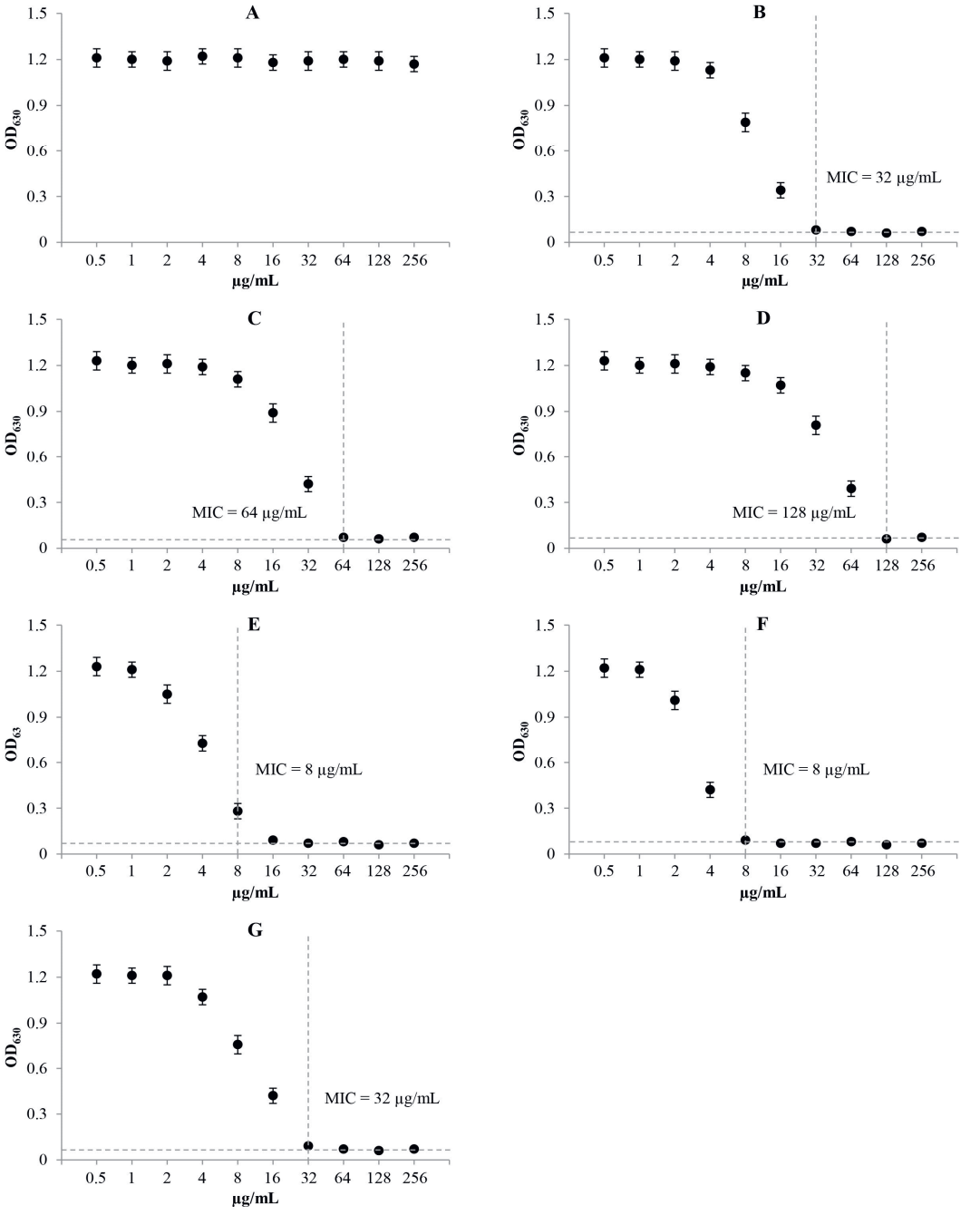
Supplementary Figure S29. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *S. aureus* ATCC 25923



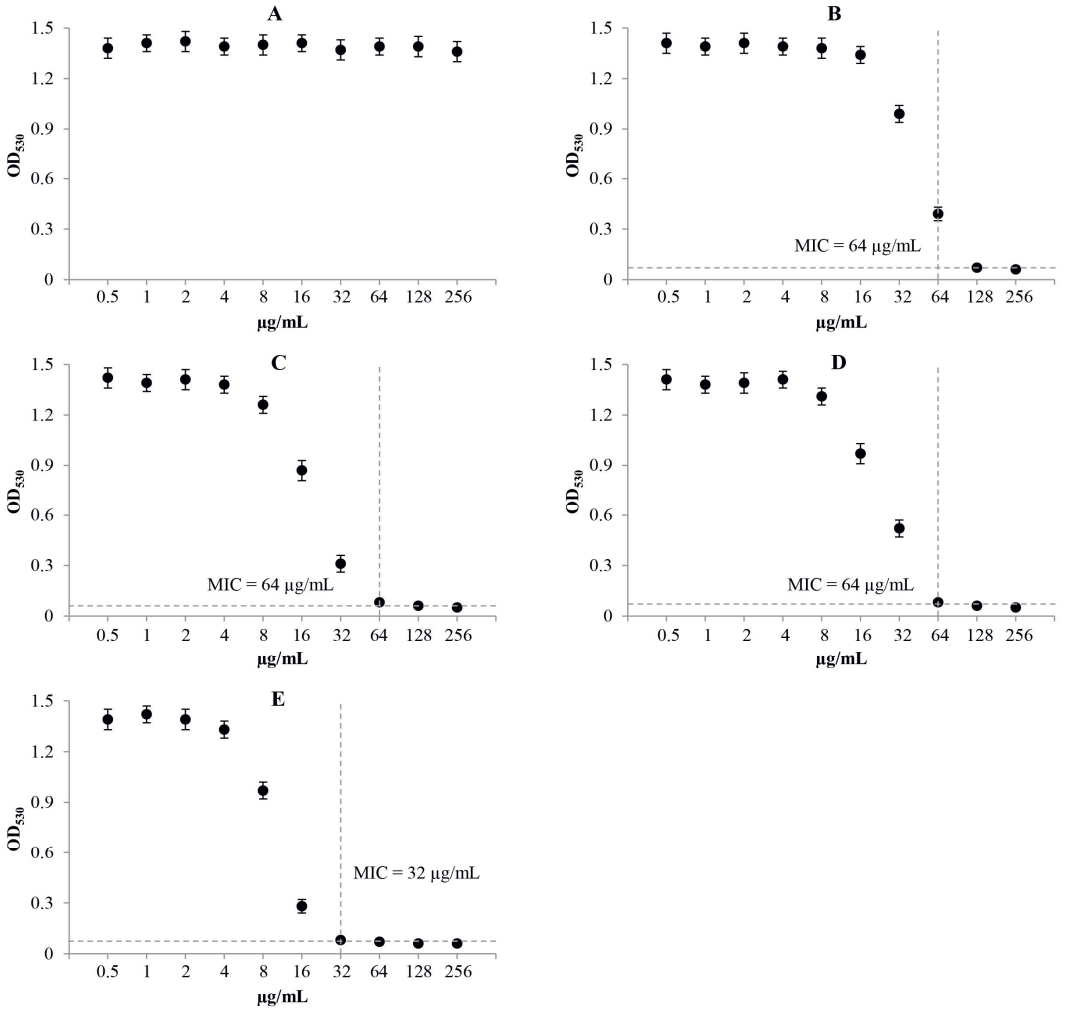
Supplementary Figure S30. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *B. subtilis* ATCC 6633



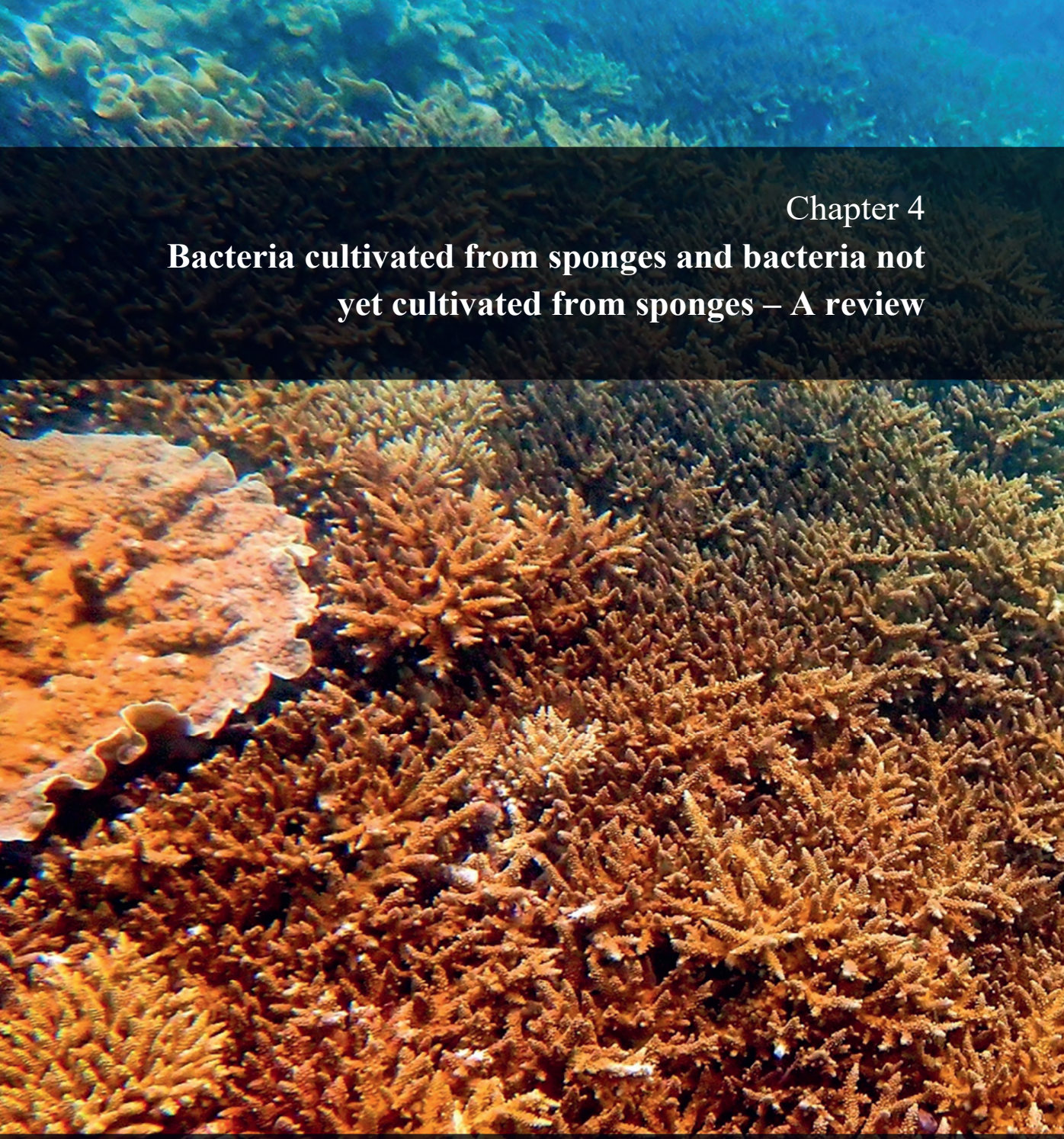
Supplementary Figure S31. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *B. cereus* MISR 12818



Supplementary Figure S32. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Ampicillin (**E**), Kanamycin (**F**) and Tetracycline (**G**) on the growth of *Rhodococcus* sp. MISR 16518



Supplementary Figure S33. Effects of compounds Cyclo-(L-Pro-L-Tyr) (**A**), macrolactin A (**B**), macrolactin H (**C**), 15,17-epoxy-16-hydroxy macrolactin A (**D**), Miconazole (**E**) on the growth of *C. albicans* ATCC 10231



Chapter 4

Bacteria cultivated from sponges and bacteria not yet cultivated from sponges – A review

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Nguyen Thi Kim Cuc, Hauke Smidt, Detmer Sipkema

This chapter was published as Dat et al. (2021)
Front. Microbiol. 2021, 12. https://doi.org/10.3389/fmicb.2021.737925

Abstract

The application of high-throughput microbial community profiling as well as 'omics' approaches unveiled high diversity and host-specificity of bacteria associated with marine sponges, which are renowned for their wide range of bioactive natural products. However, exploration and exploitation of bioactive compounds from sponge-associated bacteria have been limited because the majority of the bacteria remains recalcitrant to cultivation. In this review, we i) discuss recent/novel cultivation techniques that have been used to isolate sponge-associated bacteria, ii) provide an overview of bacteria isolated from sponges until 2017 and the associated culture conditions and identify the bacteria not yet cultured from sponges, and iii) outline promising cultivation strategies for cultivating the uncultivated majority of bacteria from sponges in the future. Despite intensive cultivation attempts, the diversity of bacteria obtained through cultivation remains much lower than that seen through cultivation-independent methods, which is particularly noticeable for those taxa that were previously marked as 'sponge-specific' and 'sponge-enriched'. This poses an urgent need for more efficient cultivation methods. Refining cultivation media and conditions based on information obtained from metagenomic datasets and cultivation under simulated natural conditions are the most promising strategies to isolate the most wanted sponge-associated bacteria.

Introduction

As the most ancient of multi-cellular metazoans (Feuda et al., 2017), marine sponges represent an ecologically important and highly diverse component of marine benthic communities (Wulff, 2006; Bell, 2008; Maldonado et al., 2012; de Goeij et al., 2013). While sponges exhibit a relatively simple body plan, the different sponge cell layers (Hentschel et al., 2012) provide unique ecological niches for a wide range of different symbionts, such as archaea, bacteria, and micro- and macroeukaryotes (Taylor et al., 2007; Duris et al., 2011; Lattig and Martín, 2011; Li et al., 2011a; Hentschel et al., 2012; Thomas et al., 2016). Microorganisms can constitute up to one-third of the sponge's biomass (Hentschel et al., 2006) and perform diverse metabolic functions in the holobiont, including nitrogen, carbon, sulfur, and phosphorus cycling (Zhang et al., 2015; Li et al., 2016b; Pita et al., 2018). Sponge-associated microorganisms also produce secondary metabolites that contribute to the defence of the host against predation, fouling, and diseases (Hentschel et al., 2006; Taylor et al., 2007; Pawlik, 2011; Hentschel et al., 2012).

During the last decade, especially high-throughput sequencing provided a wealth of information with respect to the composition, host specificity and spatio-temporal dynamics of sponge-associated microbial communities (Schmitt et al., 2012; White et al., 2012; Cleary et al., 2013; Steinert et al., 2017; Dat et al., 2018). To date, more than 60 bacterial phyla,

including newly discovered candidate phyla that lack any cultured representative, have been reported from sponges (Thomas et al., 2016; Moitinho-Silva et al., 2017b; Taylor et al., 2021). Although molecular methods have provided a lot of information about the diversity and composition of bacteria associated with sponges, studies on the cultivation of pure bacterial strains from sponges have not lost their relevance. Phenotypic characteristics can only be comprehensively characterised based on pure cultures. Omics-based approaches (e.g., metagenomics, metatranscriptomics, metaproteomics, and metabolomics) are highly relevant to make predictions of the lifestyle of currently uncultivated bacteria, but bacterial isolates are needed for the verification of such predictions (Gutleben et al., 2018). Furthermore, omics-based approaches have limited predictive power for truly novel or unexpected physiological functions of not-yet-cultured bacteria since functional predictions mainly rely on available well-annotated genomes from cultured microorganisms. Thus, a large fraction of detected genes cannot be unequivocally assigned to any function and/or metabolic pathway (Overmann et al., 2017).

Sponge-associated bacteria are also a prolific and rich source of natural products, which include pharmacologically valuable compounds (Abdelmohsen et al., 2014; Indraningrat et al., 2016; Brinkmann et al., 2017; Fehmida et al., 2017; Zhang et al., 2017b). Unfortunately, despite their tremendous pharmaceutical and biotechnological potential, we still have been unable to access most of these secondary metabolites. This is mainly due to lack of cultivation success for most sponge-associated bacteria as only a minor fraction of the bacterial community (0.1 to 14%) has been successfully cultured in the laboratory (Olson et al., 2000; Webster and Hill, 2001; Sipkema et al., 2011; Fuerst, 2014; Montalvo et al., 2014). Artificial growth media and culture conditions are often not quite right to mimic the natural conditions (e.g., physicochemical properties in the sponge, the interactions of bacteria in a community as well as between bacteria and sponge) required for microbial growth (Alain and Querellou, 2009). The development of novel cultivation techniques based on knowledge about interactions between sponge cells and bacteria (or between different bacteria) can enhance the cultivability of previously uncultured bacteria from sponges as well as stimulate the production of bioactive compounds (Romano et al., 2018).

Previous reviews related to sponges and their symbionts (e.g., Taylor et al., 2007; Hentschel et al., 2012; Simister et al., 2012b; Moitinho-Silva et al., 2017b) focus on insights related to diversity, evolution, ecology, biotechnological potential as well as interactions between sponges and their symbionts, whereas a comprehensive review on the cultivability of sponge-associated bacteria is lacking. In this review, we i) discuss cultivation techniques that have been used to cultivate sponge-associated bacteria, ii) evaluate and analyze the cultivable bacterial diversity from sponges and the associated culture conditions, and iii) highlight promising cultivation strategies for cultivating currently uncultivable bacteria from sponges.

Cultivation techniques used for isolation of sponge-associated bacteria

Agar plate-based cultivation

The traditional cultivation of bacteria using agar plates was introduced by Robert Koch in 1881, and is still the most popularly used method until today. Consequently, agar plate-based cultivation has also been the most commonly used method for the cultivation of sponge-associated bacteria to date. A wide variety of growth media and culture conditions (e.g., with respect to oxygen, temperature, etc.) have been tested to provide the right growth conditions and enhance the cultivability of sponge-associated bacteria on agar plates (Sipkema et al., 2011; Öztürk et al., 2013; Lavy et al., 2014; Montalvo et al., 2014; Matobole et al., 2017; Indraningrat et al., 2019; Gutleben et al., 2020; Dat et al., 2021a). For example, Sipkema et al. (2011) used a diverse set of 19 culture media for cultivating bacteria from *Haliclona* (Gellius) sp. In addition, different supplements (e.g., antibiotics, sponge extracts, siderophores, and bacterial signal molecules) were added to culture media to improve the recovery of bacteria from this sponge. Members of phyla/classes that are generally less or not successfully cultured (e.g., *Deltaproteobacteria, Planctomycetes, Verrucomicrobia*) were also isolated, and the fraction of cultivable bacteria from the sponge represented 14% of the bacterial species detected by cultivation-independent means. In another study, we used physiological information of the sponge and genomic information of the associated bacteria to design specific culture media and conditions for bacterial taxa present in the sponge. They isolated 59 Operational Taxonomic Units (OTUs: clusters of 16S rRNA gene sequences within a percent sequence similarity threshold, typically 97%) from the sponge *Theonella swinhoei*, of which 22 OTUs were identified as novel species based on 97% 16S rRNA gene sequence identity. The addition of specific components to culture media can also increase the cultivability of sponge-associated bacteria. For example, the addition of sodium pyruvate, catalase (Olson et al., 2000), alpha-butyrolactone (Selvin et al., 2009), crude sponge extracts (Webster et al., 2001a; Sfanos et al., 2005; Selvin et al., 2009; Abdelmohsen et al., 2010; Sipkema et al., 2011; Zeng et al., 2013; Abdelmohsen et al., 2014; Steinert et al., 2014; Esteves et al., 2016) or sponge skeleton (Kaboré et al., 2019) to culture media enhanced the cultivability of sponge symbionts. Notably, Kaboré et al. (2019) showed that the addition of spongin-based sponge skeleton and autoclaved aqueous filtrate of sponge skeleton to culture media significantly improved the growth of *Gemmata* spp. (*Planctomycetes*). Antibiotics have been often used in media targeting *Actinomycetes* to inhibit the growth of other, fast-growing bacteria (Montalvo et al., 2005; Li and Liu, 2006; Zhang et al., 2006; Abdelmohsen et al., 2010; Vicente et al., 2013; Montalvo et al., 2014). In addition, the use of a light spectrum and intensity similar to the natural conditions was shown to be important to improve the cultivability of sponge-associated cyanobacteria and other light-harvesting bacteria (Pagliara and Caroppo, 2011; Izumi et al., 2013; Lavy et al., 2014; Keren et al., 2015; Gutleben et al., 2020).

Although the number of studies applying a range of culture media and conditions is still limited (many studies only use media with high organic carbon concentrations), the strategy of using a large variety of media and conditions generally results in improved cultivability of sponge-associated bacteria. In addition, it is believed that the use of a wide range of culture media and conditions may also be beneficial for secondary metabolite production (Bader et al., 2010), and is likely to yield a higher chemical diversity of metabolites (Fuerst, 2014). However, although a diversified cultivation strategy is easy to design, it is laborious and time-consuming, and requires considerable consumables (e.g., media, materials).

Other solid substrates

Apart from traditional cultivation relying on agar, other solid matrix-based approaches have been introduced. For example, Zengler et al. (2002) developed a microcapsule-based cultivation approach to encapsulate single cells in combination with parallel microbial cultivation under low nutrient conditions. Single encapsulated cells that are able to grow under these conditions form microcolonies within the microcapsules (Zengler et al., 2002; Zengler et al., 2005). Gerardo Toledo et al. (2012) applied this method to cultivate bacteria from the sponge *Mycale armata*. Furthermore, the isolates were de-replicated via Fourier transform infrared (FT/IR) spectroscopy. This study showed that 42% of the isolated strains obtained by using the microcapsule-based cultivation were novel (< 98% 16S rRNA gene identity) as compared to 7% using the traditional agar plate isolation technique (Gerardo Toledo et al., 2012). Furthermore, the use of FT/IR spectroscopy to de-replicate isolates before identification reduced the time and cost investment to obtain novel isolates.

Floating filter cultivation is an alternative strategy that has been applied for the cultivation of bacteria from sponges. This approach was for the first time used to isolate bacteria from sponges by Sipkema et al. (2011), who used polycarbonate filters floating on top of a liquid medium in order to mimic the inner structures of the filter-feeding sponge. By applying this approach, Sipkema et al. (2011) recovered 23 OTUs that were neither isolated on agar plates nor in liquid cultures. Esteves et al. (2016) also used this approach for the cultivation of bacteria from *Cymbastela concentrica and Scopalina* sp. Although low bacterial numbers and richness were observed on the floating filter, the study identified three floating filter-specific OTUs (Esteves et al., 2016).

Liquid cultures

Although agar-based cultivation is popular to recover bacterial isolates, many 'interesting' bacteria seem incapable of growing at the solid-air interface. Therefore, liquid culture techniques are commonly used as an alternative approach to cultivate bacteria. In addition, liquid cultures may be more effective than agar-based cultures for the cultivation of abundant, but slow-growing bacteria from environmental samples (Reinhold et al., 1988; Cartwright et al., 1994; Kataoka et al., 1996). To date, liquid cultivation has been successfully used as an

enrichment technique for isolation of the most abundant seawater bacterium, *Pelagibacter ubique*, as well as anaerobic bacteria and photosynthetic bacteria (Beck, 1971; van Niel, 1971; Connon and Giovannoni, 2002; Takenaka et al., 2013; Ňancucheo et al., 2016; Vekeman et al., 2017).

Liquid cultures have been applied in several studies aiming at the cultivation of sponge symbionts (Ishikawa et al., 2003; Huang et al., 2011; Sipkema et al., 2011; Izumi et al., 2013; Öztürk et al., 2013; Esteves et al., 2016). Sipkema et al. (2011) used liquid cultures to isolate bacteria from the sponge *Haliclona* (Gellius) sp. and recovered ten OTUs that were exclusively obtained from liquid cultures and not on agar-based media or floating filters. In another study, Izumi et al. (2013) used liquid culture to enrich and isolate members of the phylum *Planctomycetes* from the sponge *Niphates* sp. Liquid enrichment cultivation led to the isolation of 17 *Planctomycetes* strains, including novel lineages (< 91% 16S rRNA gene sequence identity). Ishikawa et al. (2003) isolated six novel halophilic and alkaliphilic lactic acid bacterial strains (*Marinilactibacillus psychrotolerans*) from an unidentified sponge using liquid enrichment cultures. Furthermore, Huang et al. (2011) successfully isolated 139 2-haloacid degrading bacteria from the sponge *Hymeniacidon perlevis* using liquid enrichment cultures.

Other liquid-based culture methods (i.e., liquid-solid media cultivation and liquid Winogradsky columns) have been applied by Gutleben et al. (2020) in order to isolate sponge-associated bacteria. For liquid-solid media cultivation, bacteria were incubated in a liquid medium and subsequently transferred to petri dishes containing the same medium solidified with gelrite and covered with the same liquid growth medium. In the case of 'liquid' Winogradsky columns, bacteria were incubated in the liquid phase in 25 mL glass culture tubes that were present above artificial sediment composed of silica sand and crystalline cellulose as a carbon source. The columns were closed with metal caps and aluminum foil to allow oxygen diffusion and then incubated at room temperature under natural light conditions for 130 days. By applying these methods, Gutleben et al. (2020) recovered many bacteria that were not obtained from agar plates, including novel *Planctomycetes*, and many bacteria were cultivated solely by one of the cultivation approaches.

Co-culture, community culture, culture *in situ,* culture in simulated natural conditions
In natural environments, microorganisms often occur in complex networks with other organisms. For example, many obligate symbionts are extremely difficult to culture and isolate under laboratory conditions because they have co-evolved with their host, which leads to complex nutritional requirements or other growth conditions that are still unknown or hard to mimic *in vitro* (Taylor et al., 2007). It has been shown that co-culture can be a fruitful strategy for cultivation of recalcitrant microorganisms, and co-culture has yielded a number

of previously uncultured bacteria, for example, microorganisms from marine sediments (Doesburg et al., 2006; D'Onofrio et al., 2010). A few recent studies have used co-cultivation to isolate bacteria associated with sponges and to stimulate the production of bioactive compounds that bacteria often fail to produce in pure cultures (Dashti et al., 2014; Adnani et al., 2015; Adnani et al., 2017; Knobloch et al., 2019; Hifnawy et al., 2020).

Bollmann et al. (2007) developed a diffusion-chamber-based cultivation approach, which allows microorganisms to grow in their natural environment by *in situ* cultivation. Microorganisms are incubated in a diffusion chamber, which is sealed off from the environment by membranes. The membranes are impermeable for microorganisms, but allow diffusion of molecules from the environment (e.g., nutrients, the "natural" sponge metabolome, and other signaling molecules) to the diffusion chamber, thereby giving the microorganisms access to metabolites and other molecules from their natural environment (Bollmann et al., 2007). Application of this method to cultivate sponge-associated bacteria was first reported by Steinert et al. (2014). A cell suspension of homogenized sponge tissue was mixed with growth media containing low, medium, or high nutrient concentrations and then poured into the diffusion chambers. Fully assembled diffusion chambers were inserted into the tissue of the sponge *Rhabdastrella globostellata* and incubated *in situ* for four weeks. After this incubation, the enrichment cultures from the diffusion chamber were further isolated on agar plates. By applying this method, the authors isolated 15 bacterial species that were not previously cultured. Thus, the study showed that the diffusion chamber method is one of the potential methods to enrich and subsequently isolate novel sponge-associated bacteria. However, it should be noted that not all sponge species are suitable for applying this method, as *Stylissa massa* rejected the growth chamber by retracting its tissue where the chamber had been inserted. In addition, encrusting sponges may be too small to fit the diffusion chamber. Therefore, modified diffusion growth chambers (e.g., smaller versions) are desirable.

Similarly, the I-tip developed by Jung et al. (2014) uses a yellow pipette tip as the basic element for *in situ* cultivation. It allows bacteria to enter and natural chemical compounds to diffuse into the tip, thereby allowing the bacteria to grow on a solid support in their natural environment. This device was applied for cultivation of bacteria and fungi from sponges from Lake Baikal and resulted in the recovery of a substantial fraction of the microbial diversity compared to standard agar plate cultivation at both genus and phylum levels.

Knobloch et al. (2019) used a co-cultivation approach to enhance the cultivability of sponge-associated bacteria in a multi-chamber device where *Halichondria panicea* explants and microbes were separated by a membrane. In brief, the bottom chamber of a microfiltration apparatus was filled with marine agar and the chambers were inoculated with sponge-derived bacterial suspension. These chambers were separated from the top part of the apparatus

holding the sponge explants through a membrane. The devices were clamped together and placed in a seawater aquarium for ten weeks. By applying this co-cultivation technique, (Knobloch et al., 2019) showed that bacterial classes, such as *Spirochaetia*, *Fusobacteriia*, *Delta-* and *Epsilonproteobacteria*, and *Clostridia* were enriched compared to standard agar plate cultivation.

In summary, the above-mentioned cultivation techniques often result in substantially improving the cultivability of sponge-associated bacteria or at least in capturing different bacteria than with more traditional methods. However, these non-standard cultivation approaches have been only scarcely applied for the cultivation of bacteria from sponges and may be important to not retrieve the same opportunistic bacteria over and over again from sponges.

Cultivable bacterial diversity from sponges and impact of culture conditions

Composition of cultivable bacteria associated with sponges

In order to investigate the diversity of the cultivable bacteria associated with sponges, a total of 4915 16S rRNA gene sequences of cultured bacteria from sponges were selected based on published papers updated till 2017, retrieved from NCBI Genbank, and then re-classified based on the Silva database (v128) using the mothur pipeline (Schloss et al., 2009). Based on these collected 16S rRNA gene sequences of bacteria cultured from sponges, representatives of 11 bacterial phyla were retrieved, including *Proteobacteria*, *Actinobacteria, Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Acidobacteria*, *Lentisphaerae*, *Chloroflexi*, and *Chlorobi* (Figure 1A). However, cultivable bacteria from sponges were dominated by four phyla: *Proteobacteria* (53.8%), *Actinobacteria* (23.4%), *Firmicutes* (16.0%), and *Bacteroidetes* (5.0%). Each of the remaining phyla accounted for less than 1% of the sequences. Almost all cultivable *Proteobacteria* belonged to two classes: *Alphaproteobacteria* (29.1%) and *Gammaproteobacteria* (24.0%).

These results show that extensive cultivation efforts have resulted in the isolation of a large phylogenetic diversity of sponge-associated bacteria. However, it is still far away from capturing the diversity of bacteria in sponges as described by culture-independent approaches. More than 60 bacterial phyla and candidate phyla have been detected from sponges by culture-independent methods, and predominant phyla include *Chloroflexi, Acidobacteria*, and *Cyanobacteria* (Thomas et al., 2016; Moitinho-Silva et al., 2017b) that are rarely obtained in culture. At the genus level, the most cultured genera include *Pseudovibrio* (14.3%), *Bacillus* (10.2%), *Streptomyces* (9.1%), followed by *Vibrio* (5.9%) and *Ruegeria* (4.9%) (Figure 1B). However, use of cultivation-independent approaches has

indicated that these genera were not prevalent in the original sponge samples, leading to the assumption that cultivation selects for opportunistic "weedy" bacteria that grow quickly in nutrient-rich media and outcompete slow-growing species that are more abundant in the original sample (Garland et al., 2001). Indeed, previous investigations where the bacterial species from the same sponge sample were studied by cultivation-dependent and cultivation-independent methods have shown little overlap (Li et al., 2011a; Sipkema et al., 2011; Hardoim and Costa, 2014; Montalvo et al., 2014; Versluis et al., 2017).

Impact of isolation methods and culture conditions on the cultivable bacteria retrieved

For this review, information about culture conditions (e.g., isolation method, culture media, temperature) of strains was also retrieved from corresponding published papers for investigating the impact of culture conditions on the composition of the cultivable bacteria from those sponges. It is important to note that the dataset is not balanced and that there are large differences in the frequency at which certain methods have been used. This means that, in most cases, we have restricted ourselves to observational reporting of the data and not used statistics as the data distribution did not allow meaningful statistics.

With respect to isolation methods, we only considered the following main methods: agar plates, diffusion growth chambers, floating filters, and liquid cultures (representing 4398 out of the total of 4915 isolates). Based on the collected data, agar plate-based cultivation has been the most popular method for the isolation of bacteria from sponges (used to isolate 89.1% of the cultivable bacteria), whereas bacteria that were isolated using the remaining methods, i.e. diffusion-growth chamber, liquid culture, and floating filter only accounted for 4.7%, 3.7%, and 2.5%, respectively. The use of different isolation methods has resulted in the recovery of different cultivable bacterial communities (Figure 2A & 3A). A total of 265 genera was only isolated by one method: 236 genera only on agar plates, six only in diffusion growth chambers, ten only on floating filters, and 15 only in liquid culture (Figure 2A). Furthermore, several of the most abundant genera were isolated by only one method, such as *Salinispora, Nocardiopsis*, *Endozoicomonas*, *Pseudoalteromonas* (agar plate), *Cohaesibacter, Neiella* (diffusion-chamber), *Croceibacter, Halioglobus* (floating filter), and *Marinilactibacillus* (liquid cultures) (Figure 3A). Other genera were not only, but preferentially isolated through one of the methods. For example, cultivable bacteria on agar plates were dominated by *Streptomyces*, *Rhodococcus*, and *Shewanella*, whereas diffusion-chamber-based isolates were dominated by *Terribacillus, Thalassospira, Aestuariibacter*, and *Agarivorans*. Predominant floating filter-based isolates belonged to the genera *Lutimonas, Algibacter, Sphingorhabdus, Roseovarius*, *Roseobacter*, *Tateyamaria*, *Tistrella*, *Desulfovibrio*, and *Haliea,* and isolates from liquid cultures by *Tepidibacter, Blastopirellula*, *Rhodopirellula*, *Roseimaritima*, *Paracocccus*, *Erythrobacter*, *Yangia*, and *Acinetobacter* (Figure 3A). Other bacterial genera isolated from sponges were always obtained at high frequencies, no matter which cultivation method was used: *Bacillus*, *Pseudovibrio*, *Labrenzia*, *Ruegeria*, and *Vibrio*.

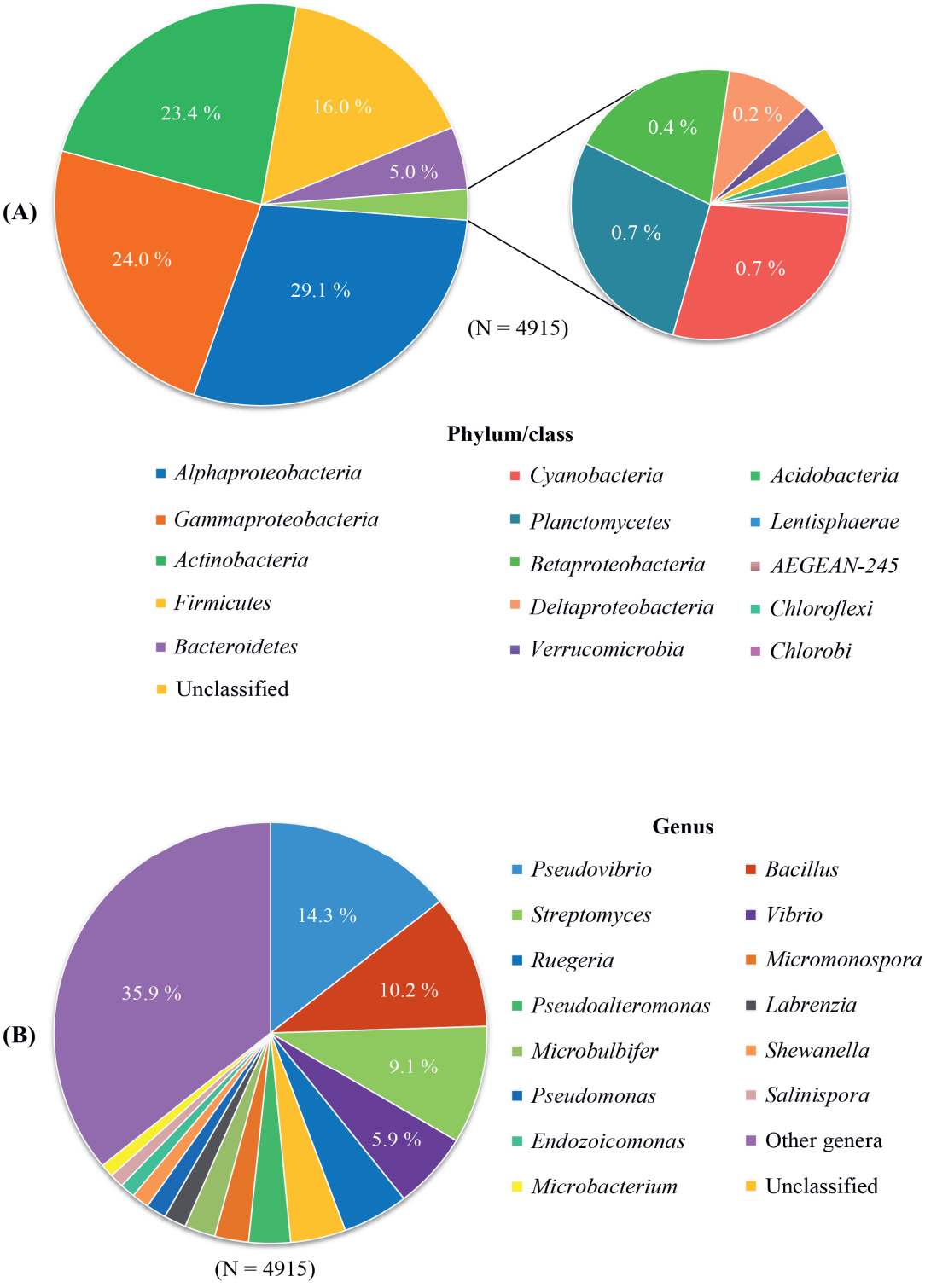

Figure 1. Composition of cultured bacteria associated with sponge at phylum/class level (A) and genus level (B).

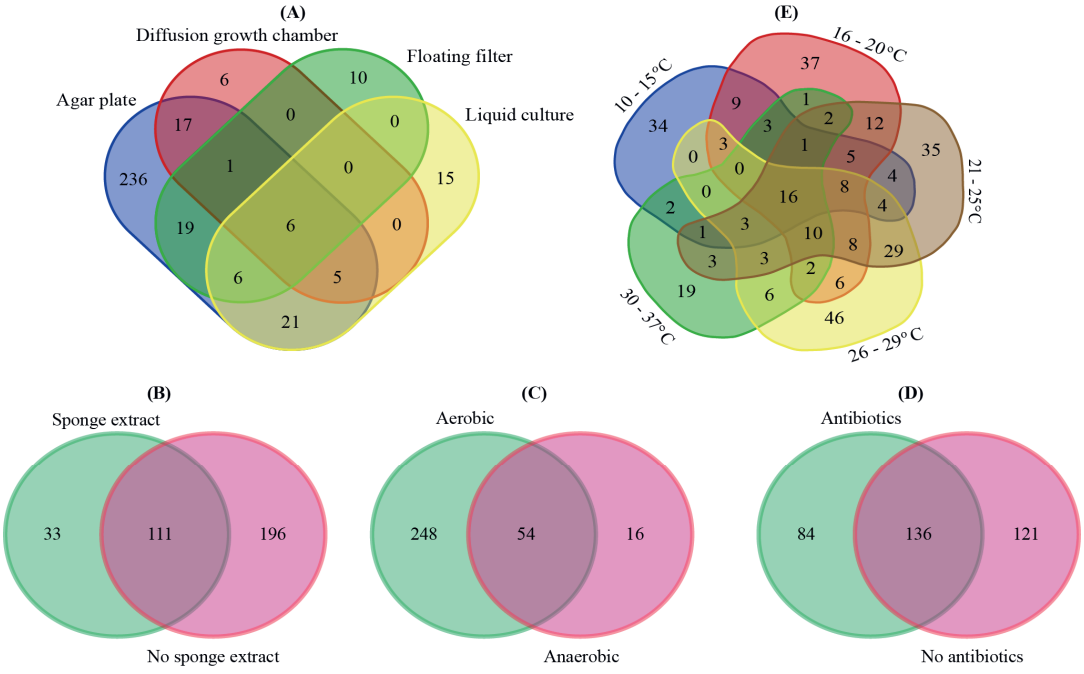

Figure 2. Venn diagram of sponge-associated bacteria cultured under different conditions: isolation methods (A), with/without of sponge extracts in culture media (B), with/without oxygen (C), with/without antibiotics in culture media (D), and temperatures (E) at genus level. The numbers in the figure indicate the number of genera.

We also specifically considered studies that added sponge extract to culture media to provide the "natural nutrients" that sponge-associated bacteria may be exposed to (Li and Liu, 2006; Selvin et al., 2009; Sun et al., 2010; Sipkema et al., 2011; Steinert et al., 2014; Esteves et al., 2016). From the isolates obtained from media with (11.8%) and without (88.3%) sponge extract, 33 bacterial genera were only isolated from media with sponge extract, 196 bacterial genera only from media without sponge extract, and 111 genera from media both with and without sponge extract (Figure 2B). Generally, the relative abundance of the most frequently isolated genera was quite similar with and without sponge extract (Figure 3B). However, some genera were predominantly isolated from media either with or without sponge extracts, such as *Salinispora*, *Mycobacterium*, *Microbacterium*, *Rhodococcus* and *Erythrobacter* (with sponge extract), and *Micromonospora*, *Microbulbifer*, *Endozoicomonas*, *Pseudoalteromonas*, *Shewanella*, and *Alteromonas* (without sponge extract) (Figure 3B).

Similarly, cultivation with (95.7% of the isolates) or without oxygen (4.3%) led (as could be expected) to different bacterial recovery rates. Fifty-four genera were isolated both using oxic and anoxic conditions, whereas 248 genera were only isolated under oxic conditions and 16 genera only under anoxic conditions (Figure 2C). The most abundant genera isolated

under oxic conditions belonged to the phylum *Actinobacteria* and the class *Gammaproteobacteria*. On the other hand, several genera such as *Mycobacterium*, *Microbacterium* (*Actinobacteria*), *Tepidibacter* (*Firmicutes*), *Roseobacter, Roseovarius, Tateyamaria* (*Alphaproteobacteria*), *Desulfovibrio* (*Deltaproteobacteria*), and *Photobacterium* and *Colwellia* (*Gammaproteobacteria*) were relatively more often obtained using anoxic conditions (Figure 3C).

Furthermore, the addition of antibiotics to culture media affected the composition of the isolated bacteria. In the presence of antibiotics, 84 bacterial genera were isolated that were not obtained from media without antibiotics. Similarly, cultivation without antibiotics resulted in the isolation of 121 bacterial genera that were not isolated from media with antibiotics (Figure 2D). Genera from the phylum *Actinobacteria* were isolated at higher frequencies from media supplemented with antibiotics, whereas media without antibiotics generally enhanced the recovery of genera from the class *Gammaproteobacteria* (Figure 3D).

The use of different incubation temperatures led to the isolation of different bacterial genera: 34 genera were only isolated at a temperature ranging from 10–15°C, 37 genera only at 16–20°C, 35 genera only at 21–25°C, 46 genera only at 26–29°C, and 19 genera only at 30–37°C, whereas relatively lower numbers of genera were isolated at multiple temperature ranges (Figure 2E). Except for a limited number of genera, such as *Bacillus*, *Pseudovibrio*, *Ruegeria*, and *Vibrio* that were isolated at a wide range of temperatures, other bacterial genera were primarily recovered at a more restricted temperature range. For example, genera belonging to the phylum *Actinobacteria* tended to grow best at a range of 21–37ºC, whereas many genera of *Alphaproteobacteria* grew better at lower temperatures (Figure 3E).

The impact of medium composition can be assessed for many different medium components. In this review, we chose to investigate the impact of the amount of organic carbon in culture media on the isolated bacterial diversity from sponges. Several genera were isolated for the entire range of carbon concentrations from culture media containing low organic carbon concentrations to media with high organic carbon concentrations. These genera include *Bacillus, Pseudovibrio, Ruegeria, Vibrio*, *Pseudomonas* and *Streptomyces* (Figure 4). In contrast, other genera were particularly recovered from seawater without additional carbon sources, such as *Micromonospora, Labrenzia, Cohaesibacter*, and *Microbulbifer*. Yet other genera, mainly belonging to phyla/classes *Cyanobacteria*, *Alphaproteobacteria*, *Planctomycetes*, *Bacteroidetes*, and *Verrucomicrobia* were relatively more often retrieved from media with lower organic carbon concentrations (≤ 5.0 g/L), while many genera belonging to phyla/classes *Actinobacteria*, *Firmicutes*, and *Gammaproteobacteria* grew better in media containing higher organic carbon concentrations (> 5 g/L) (Figure 4).

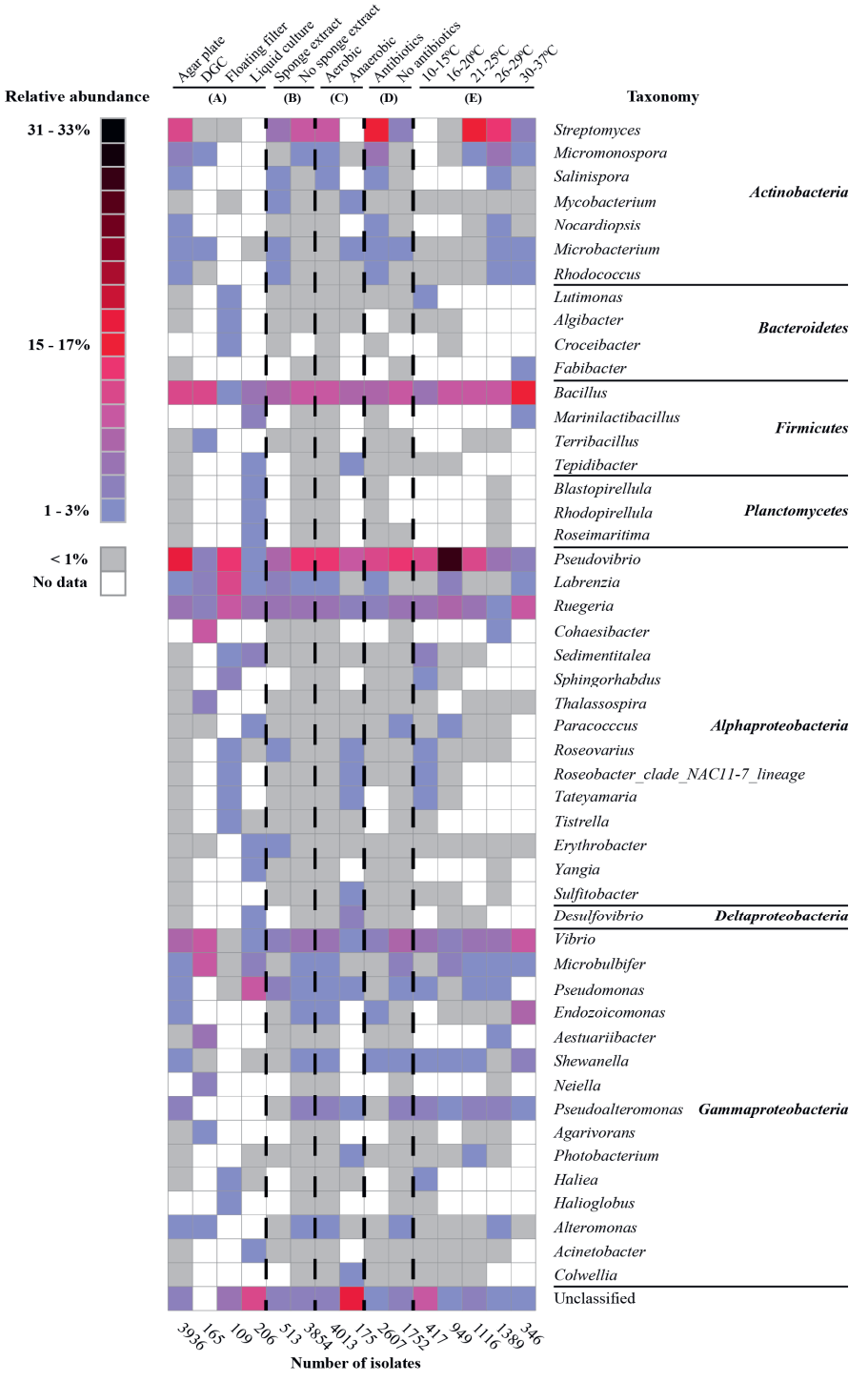

Figure 3. Heat map of the most abundant genera (50 genera) under different cultivation conditions. The percentages in the heatmap were calculated for each of the categories (sum for each column is 100%).

Overall, our meta-analysis showed that isolation methods, culture conditions, and growth medium composition substantially influence the composition of the cultivable bacterial community from sponges. Although this result of differentially recovered bacteria may in part be the result of the severe imbalance in the data in that some methods and media are overrepresented, and that isolation from a given sample was only done by a subset of methods and media, it may provide leads for future experiments targeting particular members of the sponge-associated microbiota. Although agar plate-based experiments may be the most straightforward method to obtain pure cultures, the growth of single strains poorly reflects the situation in the mesohyl, especially for high-microbial-abundance sponges where a large number of different bacterial species is densely packed together. Thus, unless the nature and relevance of interactions between sponge cells and bacteria (or between different bacteria) are known and deducted how these interactions can be compensated for by supplying specific media components, agar plate-based assays are unlikely to result in the recovery of the uncultivated majority of sponge symbionts. Supplying a sponge extract may alleviate some of these issues (e.g., Li and Liu, 2006; Selvin et al., 2009; Sun et al., 2010; Sipkema et al., 2011; Steinert et al., 2014; Esteves et al., 2016); however, the often used sponge extracts (e.g., organic sponge extract, aqueous sponge extract) may not be the best choice to capture primary metabolites that may be exchanged between the sponge host and bacteria. In addition, the concentrations used may be too low to sustain sufficient bacterial cell divisions to obtain visible colonies. On the other hand, in many attempts to isolate bacteria from sponges, carbon (and other nutrients) concentrations are much higher than those expected to prevail inside the sponge tissue and are likely to select for bacteria such as *Pseudovibrio*, *Bacillus*, *Ruegeria, Vibrio*, *Pseudomonas* and *Streptomyces* spp.. One aspect that we did not consider for this review is the duration of cultivation experiments. We know little about generation times of bacteria inside the sponge tissue, but dividing bacteria are not commonly seen on electron microscopic images (Gloeckner et al., 2014), which suggests that division rates in the natural environment are relatively low. As such, long cultivation experiments spanning several weeks or months may be required to successfully isolate sponge-associated bacteria. Having said that, the duration of cultivation experiments was often not accurately reported for individual isolates, and therefore the impact of cultivation duration could not be properly assessed.

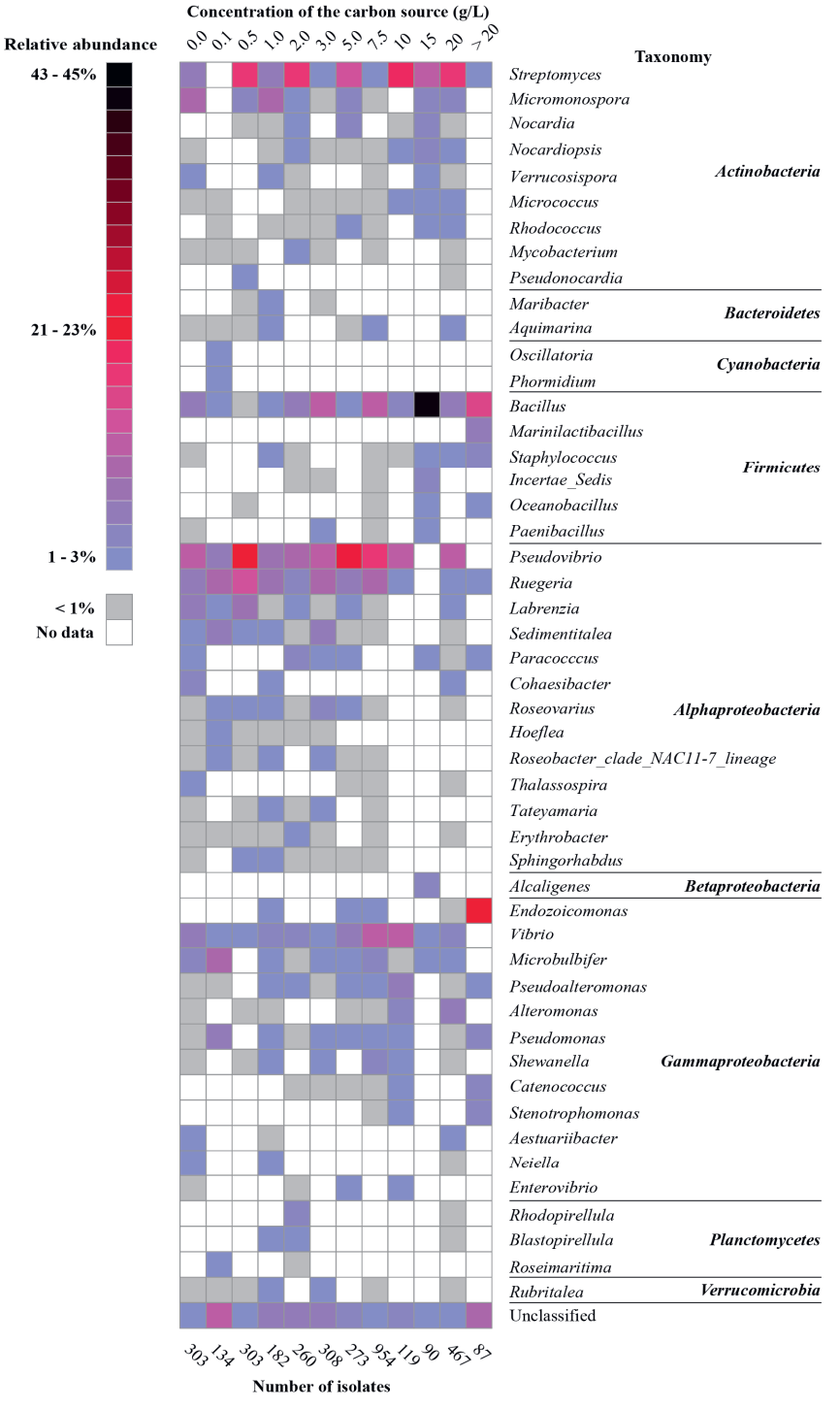

Figure 4. Heat map of the most abundant genera (50 genera) based on carbon amount in culture media. The percentage was calculated for each of the categories (sum for each column is 100%).

Sponge-specific (SC) and sponge-coral-specific (SCC) cultivable bacteria

Cultivation-independent sponge-associated bacterial profiling studies have revealed that sponges host bacterial communities distinct from communities in the surrounding seawater (Naim et al., 2014; Alex and Antunes, 2015a; Rodríguez-Marconi et al., 2015; Weigel and Erwin, 2015; Steinert et al., 2016). Initial studies based on electron microscopy have shown the existence of three groups of sponge-associated microbes: i) abundant microbes in the sponge mesohyl, ii) a small number of intracellular microbes, and iii) transient microbes from the surrounding seawater (Vacelet, 1975; Wilkinson, 1978b). In addition, microscopic studies of sponge larvae and embryos, and subsequent molecular studies have shown that many sponge-associated bacteria can be acquired vertically and/or horizontally (Wilkinson et al., 1981; Ereskovsky et al., 2005; Enticknap et al., 2006; de Caralt et al., 2007; Schmitt et al., 2007; Sharp et al., 2007; Lee et al., 2009; Sipkema et al., 2015). Recent sponge microbiology studies based on phylogenetic analyses of the bacterial 16S rRNA genes retrieved from sponges revealed the existence of sponge-specific bacteria, i.e. bacteria that are highly enriched in sponges compared to other environments where they are rarely present (Hentschel et al., 2002; Taylor et al., 2007; Simister et al., 2012b). Simister et al. (2012a) published a comprehensive phylogenetic study based on near-complete 16S rRNA gene sequences of sponge-associated microorganisms and identified that 27% of 7546 sponge-derived 16S rRNA gene sequences belonged to sponge-specific clusters (SCs) or sponge-coral-specific clusters (SCCs), the latter comprising sequences highly enriched in both sponges and corals. This comprehensive phylogeny included 205 bacterial SC(C)s.

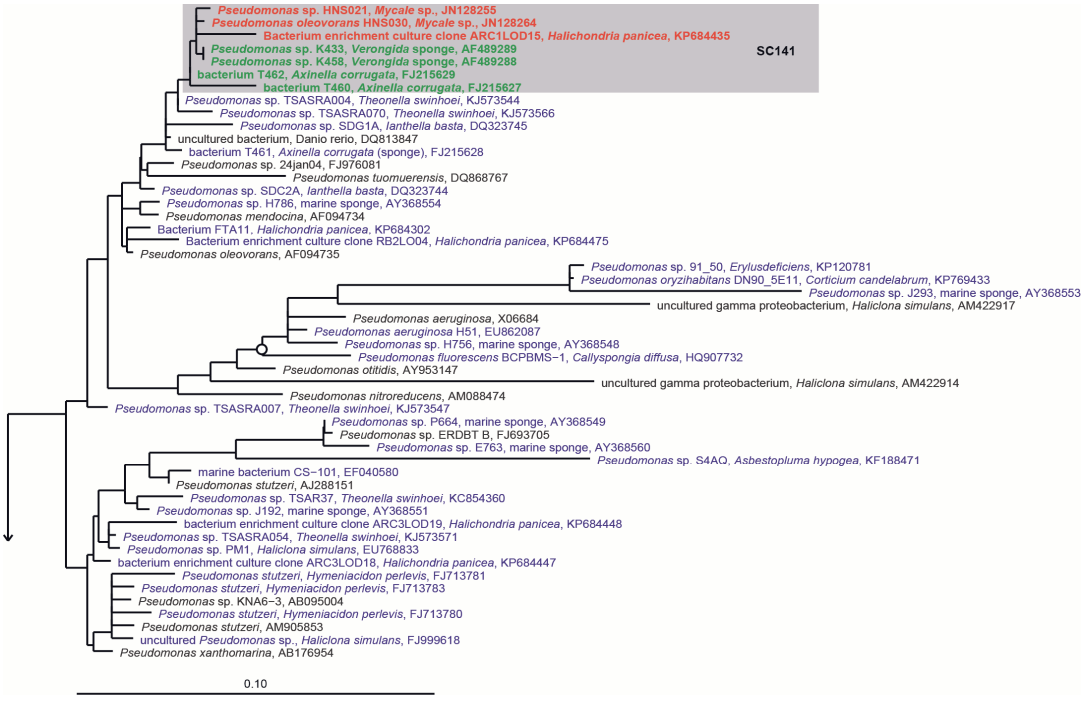

Figure 5. 16S rRNA gene-based phylogeny of sponge-specific cluster SC141 within the

Gammaproteobacteria. Filled circles indicate bootstrap support of ≥ 90%, and open circles represent bootstrap support of ≥ 75%. SC indicates sponge-specific cluster, and SCC indicates sponge- and coral-specific cluster. Blue letters indicate cultured bacteria from sponges, bold green letters indicate cultured bacteria belonging to known SC(S)s by (Simister et al. (2012b)), and bold red letters indicate newly cultured bacteria added into known SC(C)s.

In order to determine which SC(C)s have cultured representatives, we integrated the 16S rRNA gene sequences of bacteria cultured from sponges from published papers (until 2017) into the phylogenetic tree constructed by Simister et al. (2012a). Briefly, the 4915 sequences collected for this review as well as the sequences from the phylogenetic tree from Simister *et al.* were aligned and then the new sequences collected for this review were added to the previous phylogeny using the RAxML algorithm (see File S1). Based on our phylogeny, 201 sequences from cultured sponge-derived bacteria were clustered with 37 of the SC(C)s proposed by Simister et al. (2012a) (Supplementary Table S1 & Figures S1-S11). These cultured isolates within SC(C)s belong to the phyla/classes *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*, *Actinobacteria, Bacteroidetes, Firmicutes*, and *Cyanobacteria*. The majority of cultivable bacteria that belong to SC(C) represent frequently cultured genera, such as *Pseudovibrio*, *Shewanella*, *Vibrio*, and *Bacillus* (Supplementary Table S1). Although the isolation of most bacteria belonging to SC(C) was achieved using agar plates, liquid culture and floating filter cultivation also contributed to the isolation of seven and two bacteria belonging to SC(C)s, respectively. The use of antibiotics as well as sponge extracts in culture media in some cases also allowed to isolate additional bacteria from SC(C)s. Forty-one bacterial strains belonging to SC(C)s were retrieved from culture media supplemented with antibiotics, whereas 19 bacterial strains belonging to SC(C)s were retrieved from culture media supplemented with sponge extract.

Notably, some SC(C)s contain only sequences obtained from bacterial isolates and were not found by cultivation-independent means (SC11, SC13, SC14, SC55, SC58, SC59, SC60, SC61, SC78, SC84, SC85, SC93, SC108, SC126, SC127, SC128, SC133, SC138, SC139, SC141) (Figure 5 & Supplementary Figure S1-S11). These clusters mainly represent isolates from the genera *Bacillus, Pseudovibrio, Ruegeria, Nocardiopsis, Brevibacterium, Pseudomonas*, *Rheinheimera*, *Vibrio*, *Pseudoalteromonas, Aquimarina*, and *Erythrobacter*, which are usually absent or found at very low abundances by cultivation-independent methods (Li et al., 2011a; Sipkema et al., 2011; Hardoim and Costa, 2014; Montalvo et al., 2014; Versluis et al., 2017). This implies that, although representatives from these clusters have been isolated from multiple sponge species, they are most likely present as part of the rare biosphere or as spores (for some *Firmicutes* and *Actinobacteria*) in sponges. However, the fact they are part of the rare biosphere does not exclude that these bacteria may have specific relationships with sponges, as they have been selectively isolated from sponges and not (yet) from other environments. For example, for sponge-associated *Pseudovibrio* spp.

making part of SC84 and SC85, it has been found that their genomes are enriched in SEL1 and tetratricopeptide repeats type III, IV, and VI secretion systems, which have been implicated in host colonisation (Versluis et al., 2018). On the other hand, it has become obvious that bacteria from these genera are not abundant in sponges and they are unlikely to have a substantial quantitative role in sponge holobiont metabolism. The isolates that make part of other SC(C)s including sequences from both uncultivated and cultivated bacteria (SC26, SC27, SC28, SC56, SC62, SC86, SC94, SC112, SC130, SC132, SC137, SC149, SCC6, SCC7, SCC18, SCC28, SCC31) (Figure 6 & Supplementary Figure S1-S11) are more likely to represent true sponge symbionts. These clusters belong to a range of different taxa, including TK85 (*Acidobacteria*), *Candidatus* Branchiomonas (*Betaproteobacteria*), the Pir4 lineage (*Planctomycetes*), *Flavobacteriaceae* and *Fabibacter* (*Bacteroidetes*), *Oscillatoria* (*Cyanobacteria*), *Clostridiaceae* (*Firmicutes*), *Rhodospirillales*, *Erythrobacter* and *Tistlia* (*Alphaproteobacteria*), *Alteromonadales*, BD1-7 clade, *Enterobacteriaceae*, *Endozoicomonas* and *Stenotrophomonas* (*Gammaproteobacteria*). These taxa are often found at a high relative abundance in sponges by cultivation-independent methods, which implies that these bacteria may play important roles in the sponge holobiont, which makes them particularly interesting in the study of sponge-symbiont relationships. However, based on the current data of successful isolation of representatives of sponge-specific bacteria, it is not straightforward which conditions or media are recipes for success in isolating sponge-specific bacteria. Understanding the sponge-symbiont and symbiont-symbiont interactions and the molecules that mediate these interactions may be keys to develop more successful protocols for cultivation of sponge-specific bacteria.

Sponge-enriched cultivable bacteria

Recent deep sequencing studies revealed that some sequences that make part of SC(C)s are also found in seawater and sediment samples, albeit at very low relative abundances (Webster et al., 2010; Taylor et al., 2013; Thomas et al., 2016). Therefore, these clusters can no longer be strictly called “sponge-specific”, but are better described as “sponge-enriched” (Taylor et al., 2013; Thomas et al., 2016). In order to examine which cultivable bacteria can be classified as “sponge-enriched”, the collected 16S rRNA gene sequences of cultivable bacteria in this review were blasted against 64,424 ASVs that were extracted from the sponge Earth Microbiome Project (EMP) database (https://github.com/amnona/SpongeEMP). In total, 3636 out of 4915 16S rRNA gene sequences of cultivable bacteria from sponges were 100% similar to 577 ASVs from the sponge Earth Microbiome Project (EMP) database. However, the majority of the cultivable bacteria from sponges were found to be significantly enriched in seawater and sediment samples, and only 180 cultivable bacteria (21 ASVs) were significantly enriched in sponges (Supplementary Table S2). As such, comparing the 16S rRNA gene sequences from sponge isolates with the larger sponge EMP project dataset of shorter sequences confirms that a substantial number of cultivable bacteria from sponges are probably not true sponge symbionts (e.g., bacteria from the outer surface of sponges,

transient bacteria from the surrounding seawater) because they are enriched in the environment rather than in sponges.

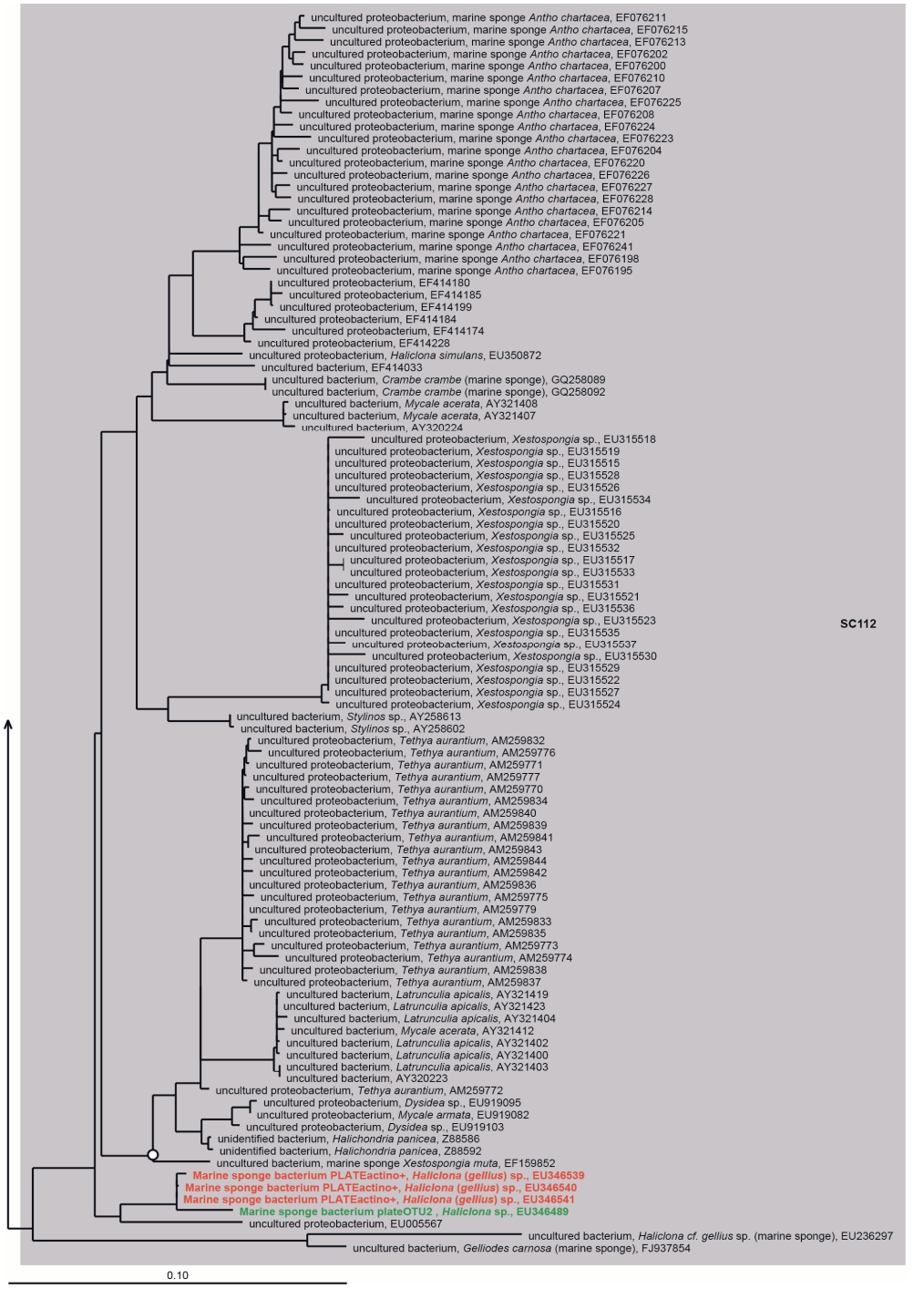

Figure 6. 16S rRNA gene-based phylogeny of sponge-specific cluster SC112 within the *Betaproteobacteria*. Details are as provided for Supplementary Figure S5.

Promising strategies and new methods for cultivation of yet uncultured sponge-associated bacteria in the future

As mentioned above, extensive cultivation efforts have led to a number of successes in the cultivation of sponge-associated bacteria. However, the large majority of sponge-associated bacteria has remained uncultivable up to now. Therefore, innovative strategies are needed.

Modification of the preparation of culture media

One of the major reasons for the poor cultivability of sponge-associated bacteria in the laboratory is that we might not know the required nutrients to support the growth of sponge-associated bacteria. Some low hanging fruits may be picked by small modifications in the preparation of cultivation media. Chemical reactions between medium components during autoclaving, for instance, between phosphate and sugars, proteins or agar have been found to produce compounds that inhibit the growth of bacteria (e.g., hydrogen peroxide) (Finkelstein and Lankford, 1957; Nakashima et al., 2010; Tanaka et al., 2014; Kawasaki and Kamagata, 2017). The addition of hydrogen peroxide scavengers such as catalase and pyruvate to culture media were shown to remove hydrogen peroxide and yield higher colony counts (Kawasaki and Kamagata, 2017). Furthermore, replacing agar with alternative gelling agents (e.g., gellan gum) in solid media is expected to improve the cultivability of some sponge-associated bacteria. Several studies on soil, freshwater sediment, and seawater have shown that numbers of viable counts and colonies increase on substrates solidified with gellan gum compared to media solidified with agar (Shungu et al., 1983; Lin and Casida, 1984; Harris, 1985; Rule and Alexander, 1986; Janssen et al., 2002; Tamaki et al., 2005; Tamaki et al., 2009; Rygaard et al., 2017), resulting in the isolation of previously uncultured bacteria.

Development of culture media based on 'omics'-derived information

Although some bacteria may be 'rescued' by more fine-tuned media preparation, it remains mostly a black-box approach. The development of "omics" approaches can provide more functional information about sponge microbiota, e.g. presence or absence of metabolic pathways and gene expression levels, which may be used to design new omics-inspired culture media (Gutleben et al., 2018 and references therein). For instance, genome analyses revealed that *Poribacteria* has the genomic repertoire for autotrophic CO_2-fixation through the Wood-Ljungdahl pathway (Kamke et al., 2014). The same was found for another well-known sponge symbiont, the sponge-specific archaeon *Cenarchaeum symbiosum, for which it* has been shown that it uses ammonia as an energy source and carbon dioxide as a carbon source (Hallam et al., 2006), a carbon source rarely used to isolate bacteria from sponges. The genome of a sponge-specific uncultured delta-proteobacterium was found to possess a glutathione porter that allows for growth on glutathione as the sole sulphur source, which can be exploited in cultivation experiments. In addition, its genome encodes a tetracycline resistance protein (Liu et al., 2011) and points towards the incorporation of tetracycline in

the medium to limit the growth of non-target bacteria. Furthermore, genomes of *Aplysina aerophoba*-associated bacteria revealed the nutritional specialization of two symbiont groups. The first group, including members of SAR202, Sva0996, OM1, TK85, *Nitrospinae*, *Desulfurellaceae*, *Rhodospirillaceae*, and *Alphaproteobacteria*, has genomes that are enriched in genes related to carnitine metabolism, while genomes of the second group, including members of *Albidovulum*, *Poribacteria*, *Spirochaetaceae*, *Caldilineaceae*, and *Chloroflexi* are enriched in genes related to sulphated polysaccharide metabolism. Both are abundant molecules of the sponge extracellular matrix, and therefore it is hypothesized that the sponge symbionts feed on the sponge cells that are shed as part of the cell turnover, and on components of the sponge extracellular matrix (Slaby et al., 2017). Thus, a growing body of literature points towards testing autotrophic growth conditions and the application of a range of sponge host-derived molecules as carbon sources.

Innovative cultivation methods

A number of innovations in bacterial cultivation have been developed, and although they have occasionally been applied for the isolation of bacteria from sponges, their potential is far from exhausted. A series of *in situ* cultivation methods, such as diffusion chamber cultivation (Steinert et al., 2014), I-tip (Jung et al., 2014), iCHIP (Nichols et al., 2010), hollow-fiber membrane chamber (HFMC) (Aoi et al., 2009), single-colony co-cultivation (Tanaka and Benno, 2014), substrate membrane system (Svenning et al., 2003), cultivation trap (Gavrish et al., 2008), and cultivation in agar spheres (Ben-Dov et al., 2009), have only scarcely been used for the isolation of sponge-associated bacteria, but may be used to overcome the limitations of pure cultures.

In addition, cultivable bacteria from sponges are usually species that are present at low relative abundances in the sponge mesohyl, whereas the most abundant bacteria detected in sponges by culture-independent methods still have not been cultured (Li et al., 2011a; Sipkema et al., 2011; Hardoim and Costa, 2014; Montalvo et al., 2014; Versluis et al., 2017). It is likely that fast-growing microbes compete for resources with uncultured slow-growing species. As such, it may be important to separate single microbial cells from complex microbial communities, e.g. through dilution-to-extinction, microfluidics, flow cytometry, micromanipulation, and compartmentalization to grow targeted bacteria physically separated from each other (Ben-Dov et al., 2009; Ishii et al., 2010; Ma et al., 2014; Pivetal et al., 2014; Jiang et al., 2016; Zhou et al., 2018; Benítez et al., 2021), while perhaps retaining possibilities for interactions using the cultivation setups listed earlier in this paragraph. These methods have led to the isolation of previously uncultured bacterial species of clades SAR11 (Alphaproteobacteria), OM43 (Betaproteobacteria), SAR92 (Gammaproteobacteria), and OM60/OM241 (γ subclass) (Connon and Giovannoni, 2002; Simu and Hagström, 2004; Stingl et al., 2007; Song et al., 2009) that are numerically abundant in their environment.

The last point that deserves attention is the dereplication of bacterial isolates. Every cultivation study may result in the isolation of hundreds or even thousands of colonies/cultures. However, the majority of the strains retrieved have been isolated before or are replicate colonies of the same strain. Therefore, the use of dereplication technologies to rapidly screen for replicate isolates is important to save time and resources. Several dereplication tools have been used and shown their effectiveness in rapid differentiation and identification of bacteria in cultivation studies, such as matrix-assisted desorption/ionization-time-of-light mass spectrometry (MALDI-TOF MS) (Dieckmann et al., 2005; Dieckmann et al., 2008; Lagier et al., 2012; Pfleiderer et al., 2013; Dubourg et al., 2014; Strejcek et al., 2018) and Fourier transform-infrared (FT-IR) spectroscopy (Kirschner et al., 2001; Oberreuter et al., 2002; Ngo-Thi et al., 2003; Naumann, 2006; Rebuffo et al., 2006; Beekes et al., 2007). The specificity of these techniques is rather high and may allow to differentiate bacteria down to the subspecies or strain level (Naumann, 2006; Beekes et al., 2007). As such, these tools may allow quick identification of 'usual suspects' among the sponge-associated isolates, such as *Pseudovibrio*, *Bacillus* and *Ruegeria* and direct efforts towards the yet uncultured bacteria.

Conclusion

Although the majority of bacteria from sponges have remained resistant to cultivation in the laboratory, the accumulative cultivation efforts of many researchers have resulted in the cultivation of bacteria from eleven bacterial phyla. These isolates are dominated by the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* and the genera *Pseudovibrio*, *Bacillus*, *Streptomyces*, *Vibrio*, and *Ruegeria*. Furthermore, among the isolates are representatives of 21 sponge-enriched bacteria as defined by the sponge EMP (Moitinho-Silva et al., 2017b) and 37 SC(C)s as defined by Simister et al. (2012b). However, this also implies that 168 SC(C)s have no representative isolate. Meta-analysis of culture conditions and growth media used suggests a substantial impact of these on the taxa isolated, most obvious for cultivation temperature and carbon concentration present in the media. In conclusion, the cultivation of especially the most abundant sponge symbionts is still far from realised because of our limited understanding of the complex sponge-symbiont interactions and translating them to pseudo natural growth conditions for these symbionts in the laboratory. A next level of cultivation strategies will be needed to recover those bacteria in isolation as well. This would also be a major step forward to the application of bioactive compounds found in sponges.

Acknowledgements

This study was supported by a grant from Vietnam Ministry of Science and Technology (ĐTĐLCN.17/14), a BE-Basic Foundation-FES grant from the Dutch Ministry of Economic

Affairs (F07.003.06), and the MC-ITN-BluePharmTrain funded through the European Union Programme FP7/2007-2013/ under REA grant agreement n° 607786.

Supplementary Information

Data collection

In this review, we collected 4915 16S ribosomal RNA (rRNA) gene sequences of cultivable bacteria associated with sponges from the NCBI database via published papers. Firstly, published papers reporting cultivable bacteria from sponges were retreived from literature databases, including PubMed, Scopus, Web of Science, Google Scholar, using a query containing keywords related to cultivable bacteria from sponges: "(sponge OR porifera) AND (16S rRNA OR bacteria) AND (cultivation OR cultivable OR culture)" (updated to 2017). From the papers, accession numbers of cultivable bacteria from sponges were collected and used to retrieve the 16S rRNA gene sequences from the NCBI database. Corresponding data concerning culture conditions and culture media was also collected from published papers and/or from authors of papers. The metadata of sponge-associated culture bacteria can be accessed at the FigShare online Repository (https://doi.org/10.6084/m9.figshare.13489998.v1). For taxonomy, all 16S rRNA gene sequences of cultivable bacteria were re-classified based on the Silva database (v128) using the script *classify.seqs* in the Mothur pipeline with default parameters (Schloss et al., 2009).

Heat map and Venn diagram

In order to investigate the influence of carbon content (amount) in cultivation media on the composition of isolated sponge-associated bacteria, the amount of carbon in cultivation media was roughly estimated based on the amount of substrates containing carbon in culture media. For media using only seawater and agar for cultivation, we assumed that the amount of carbon in the media was zero. A heatmap of the 50 most abundant genera based on the amount of carbon and culture conditions was plotted using JColorGrid v.1.860 (Joachimiak et al., 2006). Venn diagrams of sponge-associated bacteria recovered under different cultivation conditions were created at genus level using the online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Identification of additional sequences as part of known sponge-specific and sponge coral-specific clusters

The 16S rRNA gene sequences of cultured bacteria in our dataset and the 16S rRNA gene sequences from (Simister et al., 2012) were grouped per phylum. For each phylum, the sequences were aligned using the online Silva aligner (https://www.arb-silva.de/aligner/) with the global alignment mode (Pruesse et al., 2012). The aligned 16S rRNA gene sequences of cultured bacteria were added to available phylogenetic trees constructed by Simister et al. (2012b) using RAxML version 8.2 (Stamatakis, 2014). The additional sequences were

identified as part of known SC/SCC clusters if their positions in the tree were inside known SC/SCCs.

Sponge-enriched cultured bacteria

In brief, the 16S rRNA gene sequences of cultured bacteria from our dataset (n = 4915) were subjected to a BLAST search (Altschul et al., 1990) against a curated sponge microbiome database, containing 64,424 high quality deblurred subOTU sequences that were extracted from the sponge Earth Microbiome Project (EMP) database (https://github.com/amnona/SpongeEMP). The curated spongeEMP BLAST database and additional information describing the database creation can be accessed here: https://github.com/marinemoleco/spongeEMP_BLASTdb. The sponge microbiome project subOTU sequences with 100% similarity to the 4915 bacterial isolate sequences were uploaded to the spongeEMP online server (www.spongeemp.com) in order to identify isolates that are significantly enriched in sponge specimens (for the category "host-associated" in the field env_package) using ranksum_p (relative frequency–based ranksum test) or binomial_p (presence/absence binomial test) as described by (Moitinho-Silva et al., 2017).

Supplementary Table S1. Sponge-associated cultivable bacteria belonging to SC(C) clusters

Accesion number	SC(C)s	Phylum/ class	Genus
EF198904	SC11	*Actinobacteria*	*Nocardiopsis*
EF198903	SC11	*Actinobacteria*	*Nocardiopsis*
EF198900	SC11	*Actinobacteria*	*Nocardiopsis*
EU873251	SC11	*Actinobacteria*	*Nocardiopsis*
EU554278	SC11	*Actinobacteria*	*Nocardiopsis*
EU554275	SC11	*Actinobacteria*	*Nocardiopsis*
FJ596490	SC13	*Actinobacteria*	*Brevibacterium*
FJ596452	SC13	*Actinobacteria*	-
FJ596451	SC13	*Actinobacteria*	-
FJ596420	SC13	*Actinobacteria*	*Brevibacterium*
FJ596548	SC14	*Actinobacteria*	*Brevibacterium*
FJ596537	SC14	*Actinobacteria*	*Brevibacterium*
GQ153943	SC14	*Actinobacteria*	*Brevibacterium*
EU346604	SC26	*Bacteroidetes*	-
EU346568	SC26	*Bacteroidetes*	*Gaetbulibacter*
EU346429	SC26	*Bacteroidetes*	*Gaetbulibacter*
AM990722	SC27	*Bacteroidetes*	-

KF282348	SC27	*Bacteroidetes*	-
FM180520	SC28	*Bacteroidetes*	*Aquimarina*
FJ348470	SC28	*Bacteroidetes*	*Aquimarina*
KF282352	SC28	*Bacteroidetes*	*Aquimarina*
FJ392545	SC55	*Cyanobacteria*	*Oscillatoria*
EF537057	SC55	*Cyanobacteria*	*Oscillatoria*
EF537058	SC55	*Cyanobacteria*	*Oscillatoria*
EF537059	SC55	*Cyanobacteria*	*Oscillatoria*
EF537060	SC55	*Cyanobacteria*	*Oscillatoria*
EF537061	SC55	*Cyanobacteria*	*Oscillatoria*
EF537062	SC55	*Cyanobacteria*	*Oscillatoria*
EF537054	SC56	*Cyanobacteria*	*Oscillatoria*
EF537055	SC56	*Cyanobacteria*	*Oscillatoria*
EF537056	SC56	*Cyanobacteria*	*Oscillatoria*
FJ596448	SC58	*Firmicutes*	*Bacillus*
FJ596439	SC58	*Firmicutes*	*Bacillus*
FJ596384	SC58	*Firmicutes*	*Bacillus*
KJ372507	SC58	*Firmicutes*	*Bacillus*
EU199239	SC59	*Firmicutes*	-
DQ274117	SC59	*Firmicutes*	*Bacillus*
DQ274116	SC59	*Firmicutes*	*Falsibacillus*
EU862089	SC60	*Firmicutes*	*Bacillus*
FJ596414	SC60	*Firmicutes*	*Bacillus*
FJ596413	SC60	*Firmicutes*	*Falsibacillus*
JX575224	SC60	*Firmicutes*	*Bacillus*
DQ888874	SC60	*Firmicutes*	*Bacillus*
DQ888882	SC60	*Firmicutes*	*Bacillus*
EU199230	SC61	*Firmicutes*	-
FJ596505	SC61	*Firmicutes*	-
FJ596485	SC61	*Firmicutes*	-
DQ903972	SC61	*Firmicutes*	*Bacillus*
KP684471	SC62	*Firmicutes*	-
DQ227660	SC78	*Alphaproteobacteria*	-
EF040555	SC78	*Alphaproteobacteria*	*Ruegeria*
EF040554	SC78	*Alphaproteobacteria*	*Ruegeria*
EF040543	SC78	*Alphaproteobacteria*	*Ruegeria*
EU346642	SC84	*Alphaproteobacteria*	*Pseudovibrio*

EU346493	SC84	*Alphaproteobacteria*	*Pseudovibrio*
FJ596464	SC84	*Alphaproteobacteria*	*Pseudovibrio*
FJ596368	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647081	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647070	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647064	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647057	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647053	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647045	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647039	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647038	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647036	SC84	*Alphaproteobacteria*	*Pseudovibrio*
HQ647031	SC84	*Alphaproteobacteria*	*Pseudovibrio*
EU346547	SC84	*Alphaproteobacteria*	*Pseudovibrio*
EU346538	SC84	*Alphaproteobacteria*	*Pseudovibrio*
KP684314	SC84	*Alphaproteobacteria*	*Pseudovibrio*
KP319339	SC84	*Alphaproteobacteria*	*Pseudovibrio*
KP319303	SC84	*Alphaproteobacteria*	*Pseudovibrio*
LN878392	SC84	*Alphaproteobacteria*	*Pseudovibrio*
LN878393	SC84	*Alphaproteobacteria*	*Pseudovibrio*
LN878600	SC84	*Alphaproteobacteria*	*Pseudovibrio*
EU346554	SC85	*Alphaproteobacteria*	*Pseudovibrio*
EU346546	SC85	*Alphaproteobacteria*	*Pseudovibrio*
EU346483	SC85	*Alphaproteobacteria*	*Pseudovibrio*
DQ888857	SC85	*Alphaproteobacteria*	*Pseudovibrio*
DQ888843	SC85	*Alphaproteobacteria*	*Pseudovibrio*
DQ888838	SC85	*Alphaproteobacteria*	*Pseudovibrio*
DQ888830	SC85	*Alphaproteobacteria*	*Pseudovibrio*
DQ888829	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647088	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647083	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647074	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647073	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647072	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647069	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647054	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HQ647051	SC85	*Alphaproteobacteria*	*Pseudovibrio*

JX575241	SC85	*Alphaproteobacteria*	*Pseudovibrio*
EU346585	SC85	*Alphaproteobacteria*	*Pseudovibrio*
EU346577	SC85	*Alphaproteobacteria*	*Pseudovibrio*
EU346543	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KF582876	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KF582850	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HE818274	SC85	*Alphaproteobacteria*	*Pseudovibrio*
HE818278	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319326	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319316	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319314	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319311	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319307	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319306	SC85	*Alphaproteobacteria*	*Pseudovibrio*
KP319299	SC85	*Alphaproteobacteria*	*Pseudovibrio*
LN878487	SC85	*Alphaproteobacteria*	*Pseudovibrio*
AY372908	SC86	*Alphaproteobacteria*	*Pseudovibrio*
AY372915	SC86	*Alphaproteobacteria*	*Pseudovibrio*
EU346594	SC93	*Alphaproteobacteria*	*Erythrobacter*
AY367756	SC93	*Alphaproteobacteria*	*Erythrobacter*
AJ849370	SC93	*Alphaproteobacteria*	*Erythrobacter*
FM180516	SC94	*Alphaproteobacteria*	-
AY371429	SC108	*Alphaproteobacteria*	*Tistlia*
DQ869302	SC108	*Alphaproteobacteria*	*Tistlia*
DQ167235	SC108	*Alphaproteobacteria*	*Tistlia*
EU346489	SC112	*Betaproteobacteria*	*Candidatus*_Branchiomonas
EU346541	SC112	*Betaproteobacteria*	*Candidatus*_Branchiomonas
EU346540	SC112	*Betaproteobacteria*	*Candidatus*_Branchiomonas
EU346539	SC112	*Betaproteobacteria*	*Candidatus*_Branchiomonas
FJ596536	SC126	*Gammaproteobacteria*	-
FJ596528	SC126	*Gammaproteobacteria*	*Pseudoalteromonas*
FJ596371	SC126	*Gammaproteobacteria*	-
FJ596367	SC126	*Gammaproteobacteria*	*Pseudoalteromonas*
FJ596342	SC126	*Gammaproteobacteria*	*Pseudoalteromonas*
AY241442	SC127	*Gammaproteobacteria*	*Vibrio*
AY241441	SC127	*Gammaproteobacteria*	*Vibrio*
AY241439	SC127	*Gammaproteobacteria*	*Vibrio*

AY241438	SC127	*Gammaproteobacteria*	*Vibrio*
AY241435	SC127	*Gammaproteobacteria*	*Vibrio*
FJ596487	SC127	*Gammaproteobacteria*	*Vibrio*
FJ596459	SC127	*Gammaproteobacteria*	*Vibrio*
FJ596424	SC127	*Gammaproteobacteria*	*Vibrio*
FJ596343	SC127	*Gammaproteobacteria*	*Vibrio*
EF040571	SC128	*Gammaproteobacteria*	*Escherichia-Shigella*
EF040564	SC128	*Gammaproteobacteria*	*Escherichia-Shigella*
EF040551	SC128	*Gammaproteobacteria*	-
EF040537	SC128	*Gammaproteobacteria*	*Escherichia-Shigella*
EF040567	SC128	*Gammaproteobacteria*	-
EF114194	SC130	*Gammaproteobacteria*	-
AY368567	SC133	*Gammaproteobacteria*	*Rheinheimera*
EF450321	SC133	*Gammaproteobacteria*	*Rheinheimera*
EF450318	SC133	*Gammaproteobacteria*	*Rheinheimera*
EU346557	SC137	*Gammaproteobacteria*	*BD1-7_clade*
EU346468	SC138	*Gammaproteobacteria*	*BD1-7_clade*
EU346467	SC138	*Gammaproteobacteria*	*BD1-7_clade*
AY371439	SC138	*Gammaproteobacteria*	*BD1-7_clade*
AY371442	SC139	*Gammaproteobacteria*	*Endozoicomonas*
AY371441	SC139	*Gammaproteobacteria*	*Endozoicomonas*
EF620871	SC139	*Gammaproteobacteria*	*Endozoicomonas*
AF489288	SC141	*Gammaproteobacteria*	*Pseudomonas*
AF489289	SC141	*Gammaproteobacteria*	*Pseudomonas*
FJ215629	SC141	*Gammaproteobacteria*	*Pseudomonas*
FJ215627	SC141	*Gammaproteobacteria*	*Pseudomonas*
JN128264	SC141	*Gammaproteobacteria*	*Pseudomonas*
JN128255	SC141	*Gammaproteobacteria*	*Pseudomonas*
KP684435	SC141	*Gammaproteobacteria*	*Pseudomonas*
EF114176	SC149	*Gammaproteobacteria*	*Stenotrophomonas*
JN615424	SC149	*Gammaproteobacteria*	*Stenotrophomonas*
DQ994722	SCC6	*Acidobacteria*	-
EF629874	SCC7	*Bacteroidetes*	*Fabibacter*
EF629869	SCC7	*Bacteroidetes*	*Fabibacter*
EF629864	SCC7	*Bacteroidetes*	*Fabibacter*
EF629858	SCC7	*Bacteroidetes*	*Fabibacter*
EF629855	SCC7	*Bacteroidetes*	*Fabibacter*

EF629831	SCC7	*Bacteroidetes*	*Fabibacter*
JF443756	SCC18	*Planctomycetes*	*Pir4_lineage*
EU346463	SCC28	*Alphaproteobacteria*	-
AY369986	SCC31	*Gammaproteobacteria*	*Shewanella*
AY371432	SCC31	*Gammaproteobacteria*	-
AY371431	SCC31	*Gammaproteobacteria*	*Paraferrimonas*
AY948366	SCC31	*Gammaproteobacteria*	*Shewanella*
DQ167234	SCC31	*Gammaproteobacteria*	*Shewanella*
DQ180743	SCC31	*Gammaproteobacteria*	*Shewanella*
JX575276	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372448	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372447	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372446	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372445	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372444	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372443	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372442	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372441	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372440	SCC31	*Gammaproteobacteria*	-
KJ372439	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372438	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372437	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372436	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372435	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372434	SCC31	*Gammaproteobacteria*	*Shewanella*
KJ372433	SCC31	*Gammaproteobacteria*	*Shewanella*
HE818163	SCC31	*Gammaproteobacteria*	*Shewanella*
HE818167	SCC31	*Gammaproteobacteria*	*Shewanella*
HE818174	SCC31	*Gammaproteobacteria*	*Shewanella*
HE818216	SCC31	*Gammaproteobacteria*	*Shewanella*
KP684413	SCC31	*Gammaproteobacteria*	*Shewanella*
KP684406	SCC31	*Gammaproteobacteria*	*Shewanella*
KP684297	SCC31	*Gammaproteobacteria*	*Shewanella*
EF629566	SCC31, SC132	*Gammaproteobacteria*	*Shewanella*
EF629564	SCC31, SC132	*Gammaproteobacteria*	*Shewanella*
EF629559	SCC31, SC132	*Gammaproteobacteria*	*Shewanella*

Supplementary Table S2. Cultivable bacteria enriched in sponge

Accession number	OTU	p_value	Category	Phylum/ class	Genus
KC854357	OTU93	binomial_p=0.071110, ranksum_p=0.000005	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
AB012864	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AF295099	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY364592	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY372904	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY372911	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097237	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097238	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097239	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097240	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097241	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097242	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097243	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097244	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097245	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097246	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097247	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097248	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097249	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097250	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*

KC854357	OTU93	binomial_p=0.071110, ranksum_p=0.000005	Sponge-enriched	Gammaproteobacteria	Endozoicomonas
AB012864	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AF295099	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY364592	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY372904	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY372911	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097237	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097238	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097239	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097240	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097241	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097242	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097243	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097244	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097245	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097246	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097247	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097248	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097249	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097250	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097251	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097252	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097253	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097254	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*

DQ097256	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097257	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097259	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097260	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097261	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097262	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ097263	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ156536	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ227655	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ227656	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ227657	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ227658	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399712	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399713	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399714	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399715	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399717	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399719	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399720	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399721	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399722	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399723	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399724	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ399725	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*

DQ399726	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ869304	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
DQ874974	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF040574	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF040575	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF040579	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF414057	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF414061	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF513634	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF513636	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF620867	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
EF629879	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215561	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215563	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215564	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215565	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215567	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215568	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215569	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215577	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215578	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215580	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215583	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FJ215619	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*

FJ215620	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
FM180515	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
GQ169725	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
JN128253	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
JN615421	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
JN615432	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
JN615449	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KC854396	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282364	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282365	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282368	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282369	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282374	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282424	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282442	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282463	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282533	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282544	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KF282576	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KJ372497	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KJ372498	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KJ372499	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
KJ573545	OTU1146	binomial_p=0.244977, ranksum_p=0.025374	Sponge-enriched	*Alphaproteobacteria*	*Pseudovibrio*
AY368536	OTU12079	binomial_p=0.312694, ranksum_p=0.087930	Sponge-enriched	*Alphaproteobacteria*	*Paracocccus*

AB695088	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
AB695089	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
AM990755	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
AY370008	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372449	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372450	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372451	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372452	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372453	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372454	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372455	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372456	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
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KJ372464	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
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KJ372478	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KJ372479	OTU14946	binomial_p=0.084680, ranksum_p=0.000019	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
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DQ167235	OTU1500	binomial_p=0.169591, ranksum_p=0.002361	Sponge-enriched	*Alphaproteobacteria*	*Tistlia*
DQ869302	OTU1500	binomial_p=0.169591, ranksum_p=0.002361	Sponge-enriched	*Alphaproteobacteria*	*Tistlia*
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HE818343	OTU17006	binomial_p=0.183246, ranksum_p=0.003901	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
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HE818167	OTU5891	binomial_p=0.103132, ranksum_p=0.000077	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
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HE818216	OTU5891	binomial_p=0.103132, ranksum_p=0.000077	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KP684297	OTU5891	binomial_p=0.103132, ranksum_p=0.000077	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KP684406	OTU5891	binomial_p=0.103132, ranksum_p=0.000077	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KP684413	OTU5891	binomial_p=0.103132, ranksum_p=0.000077	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
AB205011	OTU6081	binomial_p=0.060665, ranksum_p=0.000002	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
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FJ215566	OTU6081	binomial_p=0.060665, ranksum_p=0.000002	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
KF282425	OTU6081	binomial_p=0.060665, ranksum_p=0.000002	Sponge-enriched	*Gammaproteobacteria*	*Endozoicomonas*
AY367748	OTU6229	binomial_p=0.299246, ranksum_p=0.082665	Sponge-enriched	*Actinobacteria*	*Corynebacterium_1*
AY371431	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Paraferrimonas*
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KJ372436	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
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KJ372438	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372440	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Unclassified*
KJ372441	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372442	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372443	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372444	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
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KJ372446	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372447	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*
KJ372448	OTU6659	binomial_p=0.274222, ranksum_p=0.043864	Sponge-enriched	*Gammaproteobacteria*	*Shewanella*

Supplementary Figure S1. 16S rRNA gene-based phylogeny of sponge-associated *Acidobacteria*. Filled circles indicate bootstrap support of ≥ 90%, and open circles represent bootstrap support of ≥ 75%. SC indicates sponge-specific cluster, and SCC indicates sponge- and coral-specific cluster. Blue letters indicate cultured bacteria from sponges, bold green letters indicate cultured bacteria belonging to known SC(C)s by Simister et al. (2012b), and bold red letters indicate newly cultured bacteria added into known SC(C)s.

Supplementary Figure S2. 16S rRNA gene-based phylogeny of sponge-associated *Actinobacteria*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S3. 16S rRNA gene-based phylogeny of sponge-associated *Alphaproteobacteria*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S4. 16S rRNA gene-based phylogeny of sponge-associated *Bacteroidetes*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S5. 16S rRNA gene-based phylogeny of sponge-associated *Betaproteobacteria*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S6. 16S rRNA gene-based phylogeny of sponge-associated *Chloroflexi*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S7. 16S rRNA gene-based phylogeny of sponge-associated *Cyanobacteria*. Details are as provided for Supplementary Figure S1.

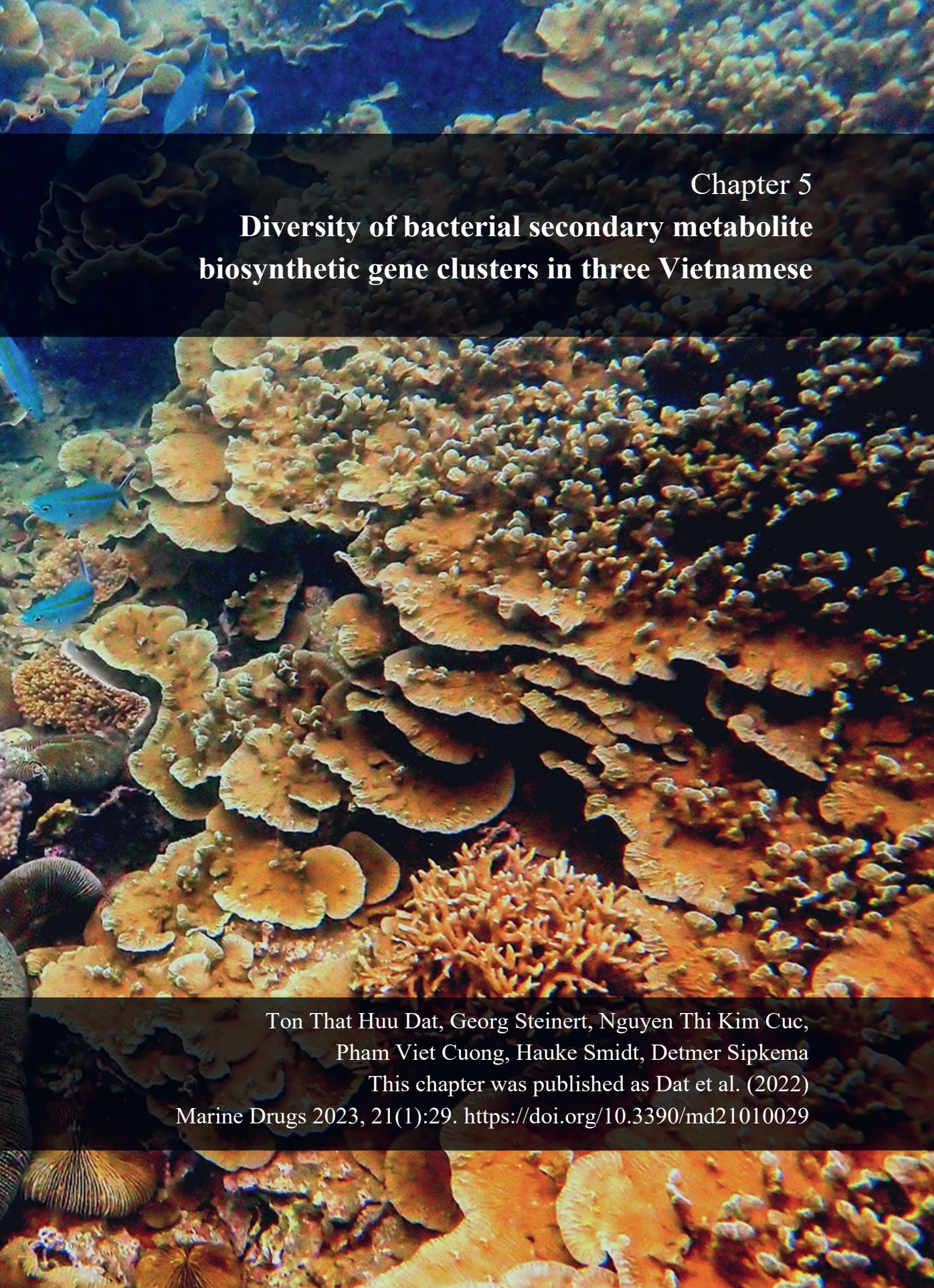
Supplementary Figure S8. 16S rRNA gene-based phylogeny of sponge-associated *Deltaproteobacteria*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S9. 16S rRNA gene-based phylogeny of sponge-associated *Firmicutes*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S10. 16S rRNA gene-based phylogeny of sponge-associated *Gammaproteobacteria*. Details are as provided for Supplementary Figure S1.

Supplementary Figure S11. 16S rRNA gene-based phylogeny of sponge-associated *Caldithrix/Deferribacteres*, *Chlamydiae*, *Halanaerobiales*, *Lentisphaerae*, NPL-UP2, OP3, *Planctomycetes*, "*Poribacteria*", *Verrucomicrobia* organisms and SAUL (sponge-associated unidentified lineage). These taxa are affiliated with the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum. Details are as provided for Supplementary Figure S1.

Note: Supplementary Figure S1-S11 are available via the following link: https://www.frontiersin.org/articles/10.3389/fmicb.2021.737925/full#supplementary-material



Chapter 5

Diversity of bacterial secondary metabolite biosynthetic gene clusters in three Vietnamese

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This chapter was published as Dat et al. (2022)
Marine Drugs 2023, 21(1):29. https://doi.org/10.3390/md21010029

Abstract

Recent reviews have reinforced that sponge-associated bacteria are a valuable source of structurally diverse secondary metabolites with potent biological properties, which make these microbial communities promising sources of new drug candidates. However, overall diversity of secondary metabolite biosynthetic potential present in bacteria is difficult to access due to the fact that the majority of bacteria are not readily cultured in the laboratory. Thus, use of cultivation-independent approaches may allow accessing "silent" and "cryptic" secondary metabolite biosynthetic gene clusters present in bacteria that cannot yet be cultured. In the present study, we investigated diversity of secondary metabolite biosynthetic gene clusters (BGCs) in metagenomes of bacterial communities associated to three sponge species: *Clathria reinwardti*, *Rhabdastrella globostellata*, and *Spheciospongia* sp. The results reveal that the three metagenomes contain a high number of predicted BGCs, ranging from 282 to 463 BGCs per metagenome. The types of BGCs were diverse and represented 12 different cluster types. Clusters predicted to encode fatty acid synthases and polyketide synthases (PKS) were the most dominant BGC types, followed by clusters encoding synthesis of terpenes and bacteriocins. Based on BGC sequence similarity analysis, 363 gene cluster families (GCFs) were identified. Interestingly, no GCFs were assigned to pathways responsible for the production of known compounds, implying that the clusters detected might be responsible for production of several novel compounds. The KS gene sequences from PKS clusters were used to predict the taxonomic origin of the clusters involved. The KS sequences were related to 12 bacterial phyla with *Actinobacteria*, *Proteobacteria*, and *Firmicutes* as the most predominant. At the genus level, the KSs were most related to those found in the genera *Mycolicibacterium*, *Mycobacterium*, *Burkholderia*, and *Streptomyces*. Phylogenetic analysis of KS sequences resulted in detection of two known 'sponge-specific' BGCs, i.e., *SupA* and *SwfA* as well as a new 'sponge-specific' cluster related to fatty acid synthesis in the phylum *Candidatus* Poribacteria composed only by KS sequences of the three sponge-associated bacterial communities assessed here.

Introduction

Bacterial secondary metabolites, also referred to as natural products or specialized metabolites, are an important source of biologically-active compounds for healthcare and agriculture applications (Butler et al., 2014; Katz and Baltz, 2016; Newman and Cragg, 2016; Singh et al., 2017). These valuable secondary metabolites represent a group of low-molecular weight structurally diverse compounds including polyketides (PKs), non-ribosomal peptides (NRPs), ribosomally synthesized and post-translationally modified peptides (RiPPs), terpenoids, saccharides, and a plethora of hybrids. The genes responsible for the biosynthesis of secondary metabolites are physically co-located in a biosynthetic gene cluster (BGC) with a size from a few to more than 100 kb (Osbourn, 2010; Smanski et al., 2016).

In bacteria, non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) gene clusters are two main clusters responsible for the biosynthesis of secondary metabolites (Weissman, 2015). Polyketides are typically produced by PKS assembly lines that are composed of different functional modules. Each module is responsible for the incorporation and tailoring of a two-carbon unit into the final pathway product (Sánchez et al., 2006). The minimal domain of a PKS module consists of an acyltransferase (AT), which selects and loads coenzyme-A (CoA) thioester-derived extender units; an acyl carrier protein (ACP), which functions as site for the covalent tethering of both the growing product chain and AT-selected extender units; and a ketosynthase (KS) domain, required for catalysis of carbon-carbon bond formation between the downstream product chain and the upstream extender unit (Robbins et al., 2016). Non-ribosomal peptide synthetases (NRPS) also form multienzyme assembly lines that are similar to those of PKS, and produce peptide-containing compounds. In the NRPS module, an adenylation (A) domain activates an amino acid, and loads it onto a peptidyl carrier protein (PCP). Subsequently, a condensation (C) domain generates an amide bond with the growing chain tethered to the upstream PCP domain (Miller and Gulick, 2016a). Interestingly, many bacteria have been found to possess the hybrid NRPS-PKS enzymes (Masschelein et al., 2013; Mizuno et al., 2013; Komaki et al., 2014; Komaki et al., 2018; Rischer et al., 2018) and show that both PKS and NRPS function simultaneously in the same assembly line, but the products synthesized by most of the hybrid PKS/NRPS enzymes are still unknown. With the advances in metagenome sequencing, numerous uncharacterized BGCs have been found in uncultivated bacteria, which are regarded as an untapped source for the discovery of new drugs (Storey et al., 2020; Nguyen et al., 2021; Rego et al., 2021; Van Goethem et al., 2021).

Marine sponges are well known to be a particularly rich source of structurally unique secondary metabolites with a broad spectrum of biological properties (Mehbub et al., 2014; Mehbub et al., 2016; Calcabrini et al., 2017), many of which are probably produced by their associated bacteria. For example, it is now known that polybrominated biphenyl ether antibiotics isolated from the sponge *Dysidea herbacea* are actually produced by the endosymbiotic cyanobacterium *Oscillatoria spongeliae* (Newman and Hill, 2006). Also, the antifungal peptide theopalauamide isolated from the marine sponge *Theonella swinhoei* has been found to be contained in a δ-proteobacterial symbiont (Schmidt et al., 1998). Subsequent metagenomic analysis of sponge microbiomes has revealed the bacterial origin of many polyketide and modified peptide secondary metabolites (Wakimoto et al., 2014; Wilson et al., 2014; Ueoka et al., 2015; Freeman et al., 2016; Nakashima et al., 2016; Lackner et al., 2017a; Mori et al., 2018; Tianero et al., 2019; Storey et al., 2020; Loureiro et al., 2022). Although the chemical role of many symbiont-derived compounds in the sponge holobionts remains unclear, accumulated evidence shows that symbiont-derived secondary metabolites play a role in chemical defense against predators, biofouling and competition for space (Aguila-Ramírez et al., 2014; Balskus, 2014; Wakimoto et al., 2014; Wilson et al., 2014; Pita

et al., 2018; Tianero et al., 2019). These biological properties make these sponge-associated microbial communities promising sources of new drug candidates (Fuerst, 2014; Juliana et al., 2014; Santos-Gandelman et al., 2014; Indraningrat et al., 2016; Zhang et al., 2017b). However, overall diversity of secondary metabolite biosynthetic potential present in sponge-associated bacteria is difficult to access because the large majority of these bacteria are not readily cultured in laboratory environment (Dat et al., 2021b). Thus, use of cultivation-independent approaches (e.g., metagenomics, cloning, heterologous expression) for investigation of the chemistry of sponge symbiotic bacteria may allow access to secondary metabolite biosynthetic gene clusters of currently uncultivable symbiotic bacteria of sponges.

We have previously investigated the bacterial and archaeal diversity of the 18 marine sponge species and found that particularly three species *Spheciospongia* sp., *Rhabdastrella globostellata* and *Clathria reinwardti* harbored diverse communities of bacteria (Dat et al., 2018). Furthermore, recent chemical investigations have revealed diverse secondary metabolites derived from these sponge species, including *C. reinwardti* (Goud et al., 2005; Tai et al., 2021; Trang et al., 2022b), *R. globostellata* (Bourguet-Kondracki et al., 2000; Tasdemir et al., 2002; Clement et al., 2006; Fouad et al., 2006; Hirashima et al., 2010; Li et al., 2010; Trang et al., 2022a), and *Spheciospongia* spp. (Costantino et al., 2005; 2008; Whitson et al., 2008; Eltamany et al., 2015; Tawfike et al., 2019). However, it has remained unclear whether/which sponge-associated bacteria are responsible for the production of these natural products. Here, we investigate the diversity of secondary metabolite biosynthetic gene clusters in metagenomes of the bacterial communities associated with these three sponge species.

Materials and Methods

Collection and identification of sponges

Sponge specimens of *Spheciospongia* sp., *Rhabdastrella globostellata* and *Clathria reinwardti* were collected by Scuba diving from May to September 2015 from the central coastal region of Vietnam at 5 - 10 m depth and identified using molecular markers (18S rRNA and COI genes) in a previous study (Dat et al., 2018).

Enrichment of sponge-associated bacteria

The specimens were rinsed three times with sterile artificial seawater to remove any debris attached to the sponge. Then the specimens were further cleaned with a sterile scalpel in order to remove sediment and other organisms more strongly attached to the sponges. Sponge-associated bacterial cells were enriched by stepwise centrifugation as described by (Abe et al., 2012). In brief, the sponge tissue was cut into small pieces and ground in TEN buffer (3.5% sodium chloride, 10 mM tris-hydroxymethyl-aminomethane, 50 mM ethylenediaminetetraacetic acid, pH 8.5) with a sterilized mortar and pestle. The cell

suspensions were then filtered through a large nylon mesh (20 µm) to further remove sediment particles and clumps of cells. Then the sponge cells and bacteria were separated by step-wise centrifugation. The cell suspension was first centrifuged at 500 g for 5 min at 4°C. The supernatant was then transferred to another tube and centrifuged at 1,000 g for 15 min at 4°C. Next, the supernatant was transferred to another tube and centrifuged at 3,000 g for 15 min at 4°C. Finally, the supernatant was then transferred to another tube and centrifuged at 8,000 g for 15 min at 4°C. The pellet was washed twice with TEN buffer and centrifuged at 8,000 g for 20 min at 4°C. This cell pellet was used for metagenomic DNA extraction.

Metagenomic DNA extraction, sequencing and assembly

Metagenomic DNA of sponge-associated bacteria was extracted using the ZymoBeadTM Genomic DNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The concentration of the extracted DNA was determined with a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and its integrity was examined by gel electrophoresis on a 1% (w/v) agarose gel. Metagenomic DNA was sequenced on an Illumina HiSeq2500 platform at BaseClear BV (Leiden, The Netherlands). Quality control of FASTQ files was done with FastQC v.0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw reads were checked and the adapters, low-quality reads, artifacts and PhiX contamination were removed using the command bbduk.sh of the BBmap v.34 (https://sourceforge.net/projects/bbmap/). Reads with a low Phred quality score (< 30) and reads shorter than 70 base pairs were filtered out. Next, reads were coverage-normalized with the command bbnorm.sh of BBMap v.34 at default settings. Reads were then assembled with SPAdes v.3.5.0 (Bankevich et al., 2012). The non-normalized Illumina reads were mapped to the contigs with Bowtie2 v.2.2.2 at default settings (Langmead and Salzberg, 2012). The resulting SAM files were converted to BAM, sorted and indexed with SAMtools v.0.1.18 (Li et al., 2009). HTSeq v.0.6.1 was used to calculate the average coverage of each contig (Anders et al., 2015). Contigs longer than 1000 base pairs were used for further analysis. Open reading frames (ORFs) were called with Prodigal v.2.6.1 with -m and -p meta options enabled (Hyatt et al., 2010).

Prediction of secondary metabolite gene biosynthetic clusters and ketosynthase phylogeny

The predicted protein-coding genes were subjected to a search for secondary metabolite biosynthetic gene clusters (BGCs) using antiSMASH v.4.1.0 (bacterial version). The KnownClusterBlast function in antiSMASH was enabled to identify BGCs that are responsible for the biosynthesis of known compounds in the metagenomes of the sponge-associated bacteria. The predicted biosynthetic gene clusters were also searched for known biosynthetic pathways using the Natural product domain seeker (NaPdoS) (http://napdos.ucsd.edu/). Biosynthetic gene cluster similarity networks and gene cluster

families were generated using BiG-SCAPE with default settings. The networks were visualized using Cytoscape v. 3.6.0 (https://cytoscape.org) (Shannon et al., 2003).

For ketosynthase (KS) phylogeny, KS domain fragments of the biosynthetic gene clusters were extracted and trimmed using NaPdoS (http://napdos.ucsd.edu/) (Ziemert et al., 2012). The KS sequences were used to search for homologous KS sequences in the NCBI Genbank database using *blastp* (http://www.ncbi.nlm.nih.gov/) against the *nr* database (accessed on 5 November 2020). KS sequences with a short length < 200 amino acids were excluded from the phylogenetic analysis. The KS sequences (from this study, their three most similar sequences, and sequences of known biosynthetic pathways from NaPdoS server) were aligned using the MAFFT (v.7.504) program with the FFT-NS-i strategy (Katoh and Standley, 2013). Poorly aligned positions and non-conserved regions were removed from the alignments using the trimAl v.1.2 (Capella-Gutiérrez et al., 2009). The ketosynthase phylogeny was created using MEGA version 11.0.11 (Tamura et al., 2021) with the neighbor-joining (NJ) method, the Poison model and 1,000 bootstrap replicates. The phylogeny was visualized using iTOL v.5 (Letunic and Bork, 2021) via the online server (https://itol.embl.de/). Taxonomy of the bacteria harbouring the KS sequences were predicted based on the most homologous KS sequences (the first hit) by a BLAST search of the KS sequences against the *refseq_protein* database (v.2018) at NCBI using *blastp* (http://www.ncbi.nlm.nih.gov/).

Results

Metagenomes of the sponge-associated bacteria

The metagenomes of bacteria associated with the three sponge species *Spheciospongia* sp., *Rhabdastrella globostellata* and *Clathria reinwardti* were sequenced. The numbers of paired-end reads obtained from the samples were in a range of 30,414,932 to 66,835,559 with average G+C content between 61% and 62 % (Supplementary Table S1). Quality filtering of the reads resulted in a range of 27,783,369 to 62,722,556 high-quality reads that were used in downstream analyses. Assembling reads per sample generated 607,208, 466,098 and 504,262 contigs for the metagenomes of *C. reinwardti*, *R. globostellata*, and *Spheciospongia* sp., respectively. The N50 values of the contigs ranged from 21,342 to 56,925 and the number of protein-coding sequences ranged from 782,060 to 903,985 for the three samples.

Diversity of secondary metabolite biosynthetic gene clusters from the metagenomes of the sponge-associated bacteria

For the prediction of secondary metabolite biosynthetic gene clusters (BGCs) from the metagenomes from sponge-associated bacteria, only contigs longer than 1,000 bp were analyzed. These yielded a total of 1,116 BGCs as identified by antiSMASH (Tables S2-S4). The number of BGCs identified per metagenome ranged from 282 to 463 (Table 1). Diverse

types of BGCs were observed with 12 different cluster types. Cf fatty acid and type I PKS were the most dominant BGC types with 86-164 and 83-125 clusters per sample, respectively, followed by terpenes (58-97 clusters per sample), and bacteriocins (12-16 clusters per sample), whereas the remaining cluster types only accounted for less than 11 clusters per sample. Surprisingly, not a single NRPS cluster was detected from metagenomes of bacteria associated with three sponge species and interestingly, the use of the *KnownClusterBlast* function in antiSMASH suggested that none the identified BGCs was encoding for biosynthetic pathways of known compounds.

Table 1. Secondary metabolite biosynthetic gene clusters identified in the metagenomes of the sponge-associated bacteria by antiSMASH

Gene cluster type	*C. reinwardti*	*R. globostellata*	*Spheciospongia* sp.
Type I PKS	83	112	125
Type II PKS	1	1	1
Type III PKS	3	6	5
Trans-AT type I PKS	2	0	2
Trans-AT PKS	0	2	0
Terpene	58	66	97
Bacteriocin	12	16	14
Aryl polyene	8	10	9
Ladderane	3	4	2
Phosphonate	5	5	6
Lantipeptide	2	1	3
Cf_fatty_acid	86	119	164
Others	19	29	35
Number of clusters	**282**	**371**	**463**
Number of contigs ≥ 1000	**93,883**	**118,632**	**154,114**

To identify orthologous clusters among BGCs across the different sponge metagenomes, we reconstructed a BGC sequence similarity network (Figure 1). The analysis of BGC sequence similarity showed that the connected components corresponded to 363 gene cluster families (GCFs), including 86 GCFs of type I PKSs, 5 GCFs of other PKSs, 63 GCFs of terpenes, 8 GCFs of ribosomally synthesized and post-translationally modified peptides (RiPPs), and 201 GCFs of other BGCs including cf fatty acid (Figure 1). Several larger networks were composed of multiple GCFs, because these networks could not be resolved into single GCF networks using default similarity cutoffs.

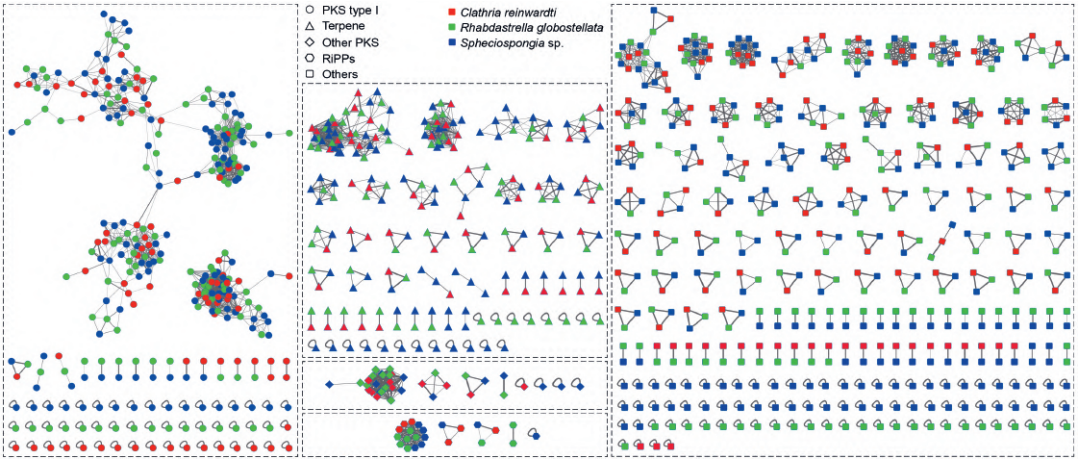


Figure 1. BGC sequence similarity network of the BGCs identified from the metagenomes of the sponge-associated bacteria. Each node represents one BGC identified by antiSMASH

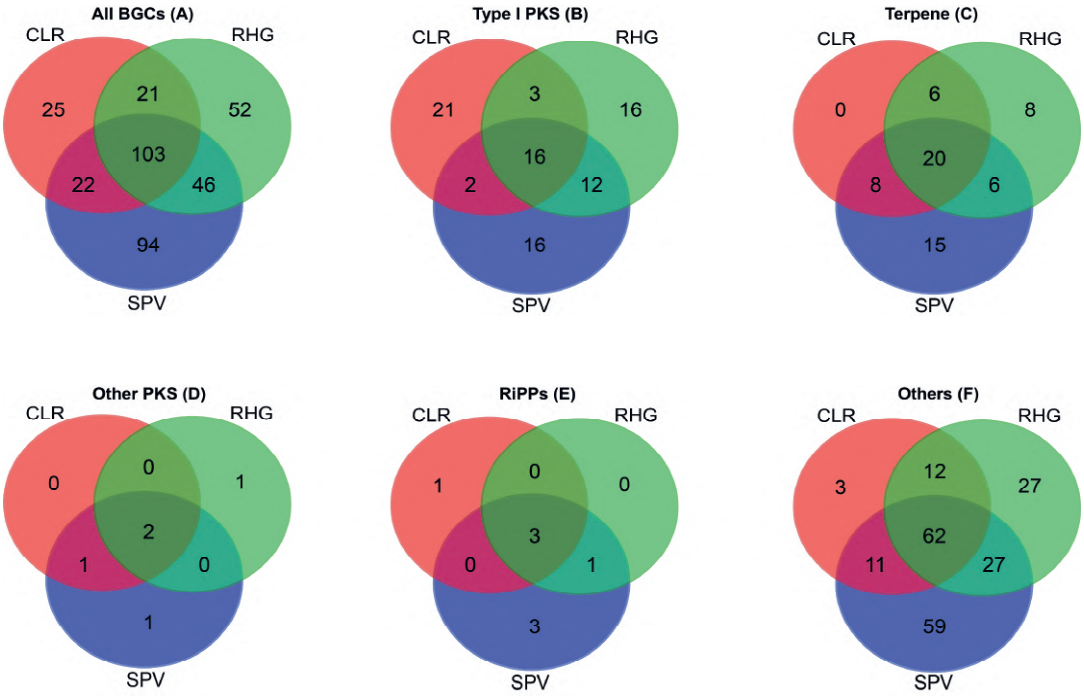


Figure 2. Venn diagrams of the identified GCFs from the metagenomes of three sponge-associated bacteria. CLR - *Clathria reinwardti*, RHG - *Rhabdastrella globostellata*, and SPV- *Spheciospongia* sp.

In general, approximately one third (103) of GCFs were found in all specimens, 89 GCFs in two specimens, and 171 GCFs only in individual specimens (singletons) (Figure 2A). It appears that Type I PKSs are more often unique to a sponge species/sample, while terpenes, other PKS and RiPPs are more often shared between species/samples (Figures 2B-2E).

Comparison of the BGCs identified with BGCs that encode known compounds in the MIBiG reference database (Minimum Information about a Biosynthetic Gene cluster) (Medema et al., 2015) showed that no GCFs from the metagenomes were assigned to pathways responsible for the production of known compounds (Tables S2-S4).

Ketosynthase phylogeny of BGCs

The ketosynthase (KS) domain is the most conserved catalytic domain of the PKS gene cluster and involved in the tailoring the polyketide by catalyzing chain condensation. Therefore, the KS sequences extracted from the identified BGCs were used for phylogenetic analysis (Figure 3). The KS sequences had low identity (21% - 66%) to the KS sequences of the characterized pathways from the NaPDos database (Tables S5-S7) as could be expected as BGCs from our study did not encode known pathways. Also, many KS sequences of the identified BGCs from the metagenomes showed low identities (30 - 70%) to KS sequences from NCBI GenBank (Tables S8-S13).

Phylogenetic analysis of KS sequences showed that most of the KS sequences of the cf_fatty acid BGCs identified from the sponge bacteria metagenomes clustered together with previously found KS sequences from fatty acid biosynthesis gene clusters retrieved from NCBI GenBank (Figure 3B). Several KS sequences from fatty acid BGCs predicted from the sponge-associated bacteria metagenomes had KS sequences from fatty acid BGCs from *Candidatus* Poribacteria metagenome-assembled genomes as best hit and formed a phylogenetically distant clade from other KS sequences from fatty acid BGCs derived from other sources (Figure 3B). KS sequences from the aryl polyene and ladderane BGCs identified from the metagenomes formed subclusters within clades of KS sequences belonging to fatty acid synthesis pathways as retrieved from NCBI Genbank. For PKS BGCs obtained from the sponge-associated bacteria metagenomes in the present study, only a few KS sequences were placed in the same clades as reference non-sponge KS sequences retrieved from NCBI GenBank and NaPDos, and the large majority of KS sequences had best hit with sponge-derived KS sequences and formed two separate clades that were phylogenetically distant to non-sponge-derived KS sequences (*supA* and *swfA*) (Figure 3C-D).

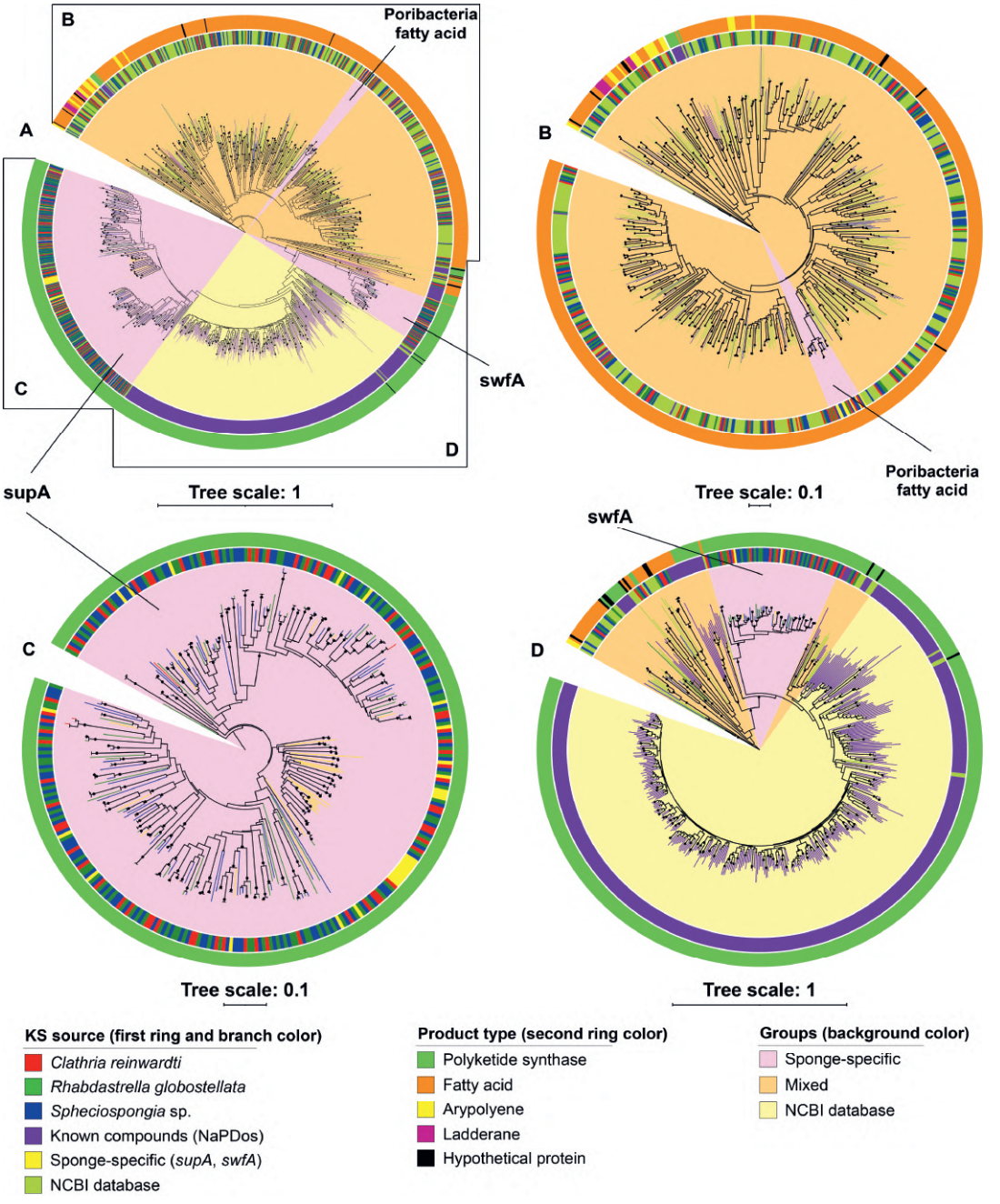


Figure 3. The phylogeny of the KS sequences from the identified BGCs from sponge-associated bacterial metagenomes including KS sequences from the NaPDoS database and NCBI GenBank (A) and its subtrees (B-D).

In addition, we used the BLASTX searches of the KS sequences to predict the bacterial taxa harbouring these sequences and they were predicted to be related to KS sequences from

diverse bacteria represented by 12 phyla, including *Actinomycetes*, *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Acidobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Nitrospirae*, *Verrucomicrobia*, *Spirochaetes*, *Aquificae*, *Nitrospinae* and *Ignavibacteriae* (Figure 4A, Tables S11-S13). Among them, *Actinobacteria* was the most predominant phylum (46.2% of the KS sequences), followed by the classes of *Proteobacteria* (3.5 - 11.7%) and the phylum *Firmicutes* (9.1%). Each of remaining phyla accounted for less than 5%. At the genus level, the KS sequences of the identified BGCs from the metagenomes were linked to 142 genera (Figure 4B). Of these, the genera *Mycolicibacterium*, *Mycobacterium*, *Burkholderia*, and *Streptomyces* were most dominant and accounted for 20.1, 15.2, 6.7, and 5.1% of the total KS sequences, respectively.

Except for the KS sequences retrieved from fatty acid BGCs, which were found from a wide range of bacterial phyla (Supplementary Figure S1), KS sequences from other BGC types were non-uniformly distributed across sponge-associated bacterial taxa (Figures S2-S4). The KS sequences retrieved from PKS BGCs were predominantly related to those from *Actinobacteria* (80% of the PKS KS sequences), particularly the genera *Mycolicibacterium* (37%), *Mycobacterium* (28%), *Burkholderia* (12%), *Streptomyces* (7%), and *Sciscionella* (5%) (Supplementary Figure S2). On the other hand, the KS sequences from aryl propylene BGCs were predominantly related to those from *Proteobacteria* (> 80% of the sequences), including *Betaproteobacteria* (45%), *Gammaproteobacteria* (26%), and *Deltaproteobacteria* (11%) with *Verminephrobacter*, *Cellvibrio*, *Syntrophus*, *Cupriavidus*, *Lechevalieria* and *Thiobacillus* as main genera (Supplementary Figure S3). In case of ladderane BGCs, the KS sequences were mainly related to only two classes of *Proteobacteria* (*Alpha-*, and *Delta-*) and three genera *Tropicibacter*, *Sandaracinus* and *Pseudovibrio* (Supplementary Figure S4).

Discussion

Diversity of secondary metabolite biosynthetic gene clusters from the metagenomes of sponge-associated bacteria

Gene clusters related to putative fatty acid synthesis is the most predominant BGC type identified from the metagenomes of sponge-associated bacteria in our study. This is expected because fatty acids are essential components in cell membranes of bacteria. Of particular interesting is the detection of a separate cluster of KS genes related to fatty acid synthase from *Candidatus* Poribacteria, a sponge-specific bacterial candidate phylum. Members of the phylum *Candidatus* Poribacteria were not detected in the same sponge specimens by 16S rRNA amplicon sequencing (Dat et al., 2018). However, this discrepancy between 16S rRNA sequencing and metagenomics has frequently been observed and the lack of *Candidatus* Poribacteria in the PCR amplicon-based study may be attributed to a bias of the specificity of primers (Podell et al., 2019). Except for central roles in the integrity of cell membranes

and energy storage (Schweizer and Hofmann, 2004), many fatty acids are recently suggested to be potential antimicrobial, antifouling, and antivirulence agents (Desbois and Smith, 2010; Yoon et al., 2018; Kumar et al., 2020). Several reports have also shown fatty acids to enhance the antibacterial activities of antibiotics (Hess Donavon et al., 2014; Chan et al., 2015; Casillas-Vargas et al., 2021; Kim et al., 2021; Park et al., 2022).

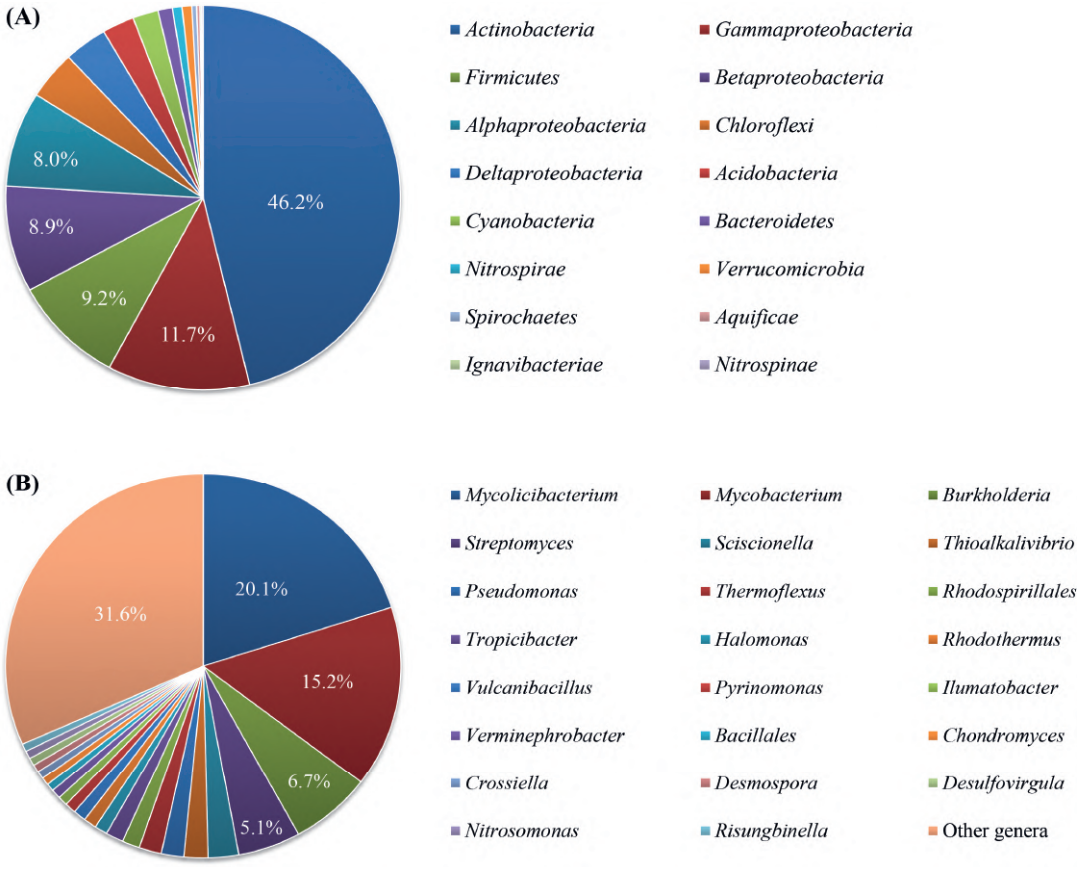


Figure 4. Taxonomy of the bacteria harboring KS sequences most identical to KS sequences (the best hit) identified from the metagenomes of the sponge-associated bacteria at (A) the phylum (or class level for *Proteobacteria*) and (B) genus level

PKSs are among the most dominant BGC types in the metagenomes of sponge-associated bacteria. Of these, PKS type I is most dominant, while only a few PKS type II and type III clusters were detected in the metagenomes. Almost all type I PKSs detected in the metagenomes of sponge-associated bacteria in our study were the non-iterative type I PKSs. Previous investigations have revealed that the distribution of PKSs appears to be origin-dependent. The non-iterative type I PKSs are often found in bacteria, whereas iterative type I PKSs are mainly found in fungi (Staunton and Weissman, 2001; Cox, 2007; Hertweck, 2009). Bacterial type I PKSs are particularly attractive targets for exploration and

exploitation of their bioactive compounds, because they are defined by a modular organization, remarkable versatility, and amenability for pathway engineering (Chen and Du, 2016). Several antibiotics derived from these multicatalytic enzymes include macrolides (erythromycin), polyenes (nystatin) as well as linear polyketides (tautomycetin) (Rawlings, 2001; Fjærvik and Zotchev, 2005; Choi et al., 2007). In our study type I PKSs from the sponge-associated bacteria metagenomes had a best BLAST hit to clusters encoding for the production of diverse bioactive compounds, such as puwainaphycins, ajudazol, polycyclic tetramate macrolactams, glycopeptidolipid, epothilone, phenalamide, crocacin, curacin, microsclerodermins, nostophycin, jamaicamide, chondrochloren, gephyronic acid, cylindrospermopsin, soraphen, amphotericin, reveromycin, stigmatellin, candicidin, myxalamid, gulmirecin, heat-stable antifungal factor, myxothiazol, tautomycin and ECO-02301 (a new class of antifungal agent) (Tables S14-S16). Notably, several *trans*-AT PKSs were also detected in the sponge-associated bacteria metagenomes. *Trans*-AT PKSs lack integrated AT domains. Instead, a free-standing AT domain acts in *trans* to load acyl building blocks into the assembly line (Nguyen et al., 2008). Nearly 40% of all bacterial modular PKSs belong to the *trans*-AT type, suggesting that the polyketides of these enzymes constitute a major natural product class (O'Brien et al., 2014). Indeed, several drugs which are used as antibiotics were identified as *trans*-AT PKS products (e.g., mupirocin, streptogramins) (El-Sayed et al., 2003; Mast et al., 2011). Many compounds from sponges and their symbionts are known to be biosynthesized by *trans*-AT PKS pathways (e.g., diffusomycin, psymberin, onnamide A, calyculin A, swinhoeiamide A, swinhodile A, clavosine A, geoetricin A, misakinolide A, oocydin A, mycalamide A) (Piel et al., 2004; Helfrich and Piel, 2016; Helfrich et al., 2021). The *trans*-AT PKS gene clusters detected in the sponge-associated bacteria metagenomes in our study had a best BLAST hit to gene clusters involved in the production of puwainaphycins and epothilones (Tables S14-S16). However, none of the clusters in our dataset could be reliable assigned to a cluster encoding for the production of a known compounds (MiBIG database). These findings imply that the type I PKSs detected in our study are likely involved in the biosynthesis of currently unknown compounds.

Type II PKSs are found exclusively in bacteria and are responsible for producing aromatic polyketides such as the antibiotics actinorhodin, tetracycline or doxorubicin (Hertweck et al., 2007). Although none of the type II PKSs detected in the metagenomes of sponge-associated bacteria in our study could be confidently assigned to gene clusters encoding known compounds, some BGCs had best BLAST hits related to clusters encoding compounds belonging to the class angucyclines (lomaiviticin) and benzoisochromanequinones (frenolicin) (Tables S14-S16). Type III PKSs are relatively small proteins that are mainly involved in the production of important plant compounds such as flavonoids and stilbenes (phenolic), but they are also found in bacteria and fungi (Moore and Hopke, 2001). All type III PKSs detected in the metagenomes of sponge-associated bacteria in our study had the

cluster encoding for the proteins producing the alkylresorcinols as best BLAST hit (Tables S14-S16). Alkylresorcinols are synthesized by plants, fungi, and bacteria, and have diverse biological activities including antioxidant, antibacterial, cytotoxic, and signaling properties (Stasiuk and Kozubek, 2010; Kikuchi et al., 2017; Martins et al., 2019).

Terpene BGCs were the third most predominant among BGCs predicted from the three metagenomes. Originally, terpenes were considered to be plant and fungal products. However, extensive bacterial genome analysis has shown that terpene synthase genes are also widespread in bacteria (Cane and Ikeda, 2012; Yamada et al., 2015; Dickschat, 2016). Recent genome analyses also detected BGCs of terpenes in genomes of sponge-associated bacteria (Matobole et al., 2017; Jackson et al., 2018; Karimi et al., 2019) and several terpenes have been isolated from sponges and their associated symbionts (Gross and König, 2006; Thomas et al., 2010b). Terpenes are among targets for the drug developments because they exhibit various biological properties, such as antimicrobial, anticancer and anti-respiratory activities (Thomas et al., 2010b). Notably, the terpene gene clusters detected in the sponge-associated bacteria metagenomes in our study were, again, not closely related to gene clusters associated to known compounds. Best BLAST hits were clusters encoding the synthesis of diverse known terpenes, such as hopene, thalianol, lupeol, sioxanthin, tirucalla, brasilicardin A, clavaric acid, squalestatin, isorenieratene, arabidiol, baruol and astaxanthin dideoxyglycoside (Tables S14-S16). Therefore, the terpene gene clusters are likely involved in the biosynthesis of similar, but still different terpenes.

Several RiPP BGC types were detected in the metagenome of sponge-associated bacteria in our study, including 12-16 bacteriocin BGCs and 1-3 lantipeptide BGCs per metagenome. Bacteriocins are ribosomally synthetized peptides or proteins that act as antimicrobial compounds against other Gram-positive and Gram-negative bacteria (Yang et al., 2014; Simons et al., 2020). Genome analyses have detected BGCs of bacteriocins in sponge-associated bacteria (Jackson et al., 2018; Karimi et al., 2019; Zhou et al., 2019). Interestingly, Phelan et al. (2013) isolated a new bacteriocin (subtilomycin) from *Bacillus subtilis* MMA7 associated with the sponge *Haliclona simulans* and found that the subtilomycin biosynthetic cluster is widespread among *B. subtilis* strains isolated from different shallow and deep water marine sponges. However, the generally large discrepancy between bacteria isolated from sponges and the dominant bacteria present in sponges (Dat et al., 2021b) is also translated to biosynthetic gene clusters present in isolates from sponges and those present in metagenomes from sponges. Indeed, no BGC related to subtilomycin biosynthesis was observed and, in fact, none of the bacteriocin gene clusters detected in the sponge-associated bacteria metagenomes was closely related to clusters encoding the proteins that catalyze biosynthesis of known bacteriocins. Best BLAST hits were obtained for clusters related to the production of bacteriocins, such as patellins, goadsporin, aeruginosamide, microcyclamide, trichamide, pheganomycin, patellamides, tenuecyclamides, SRO15-3108. Lantipeptides are widely

distributed among bacteria (Yu et al., 2013; Schorn et al., 2016; Belknap et al., 2020) and were previously found in genomes of sponge-associated bacteria (Harjes et al., 2014; Ian et al., 2014; Jackson et al., 2018; Guerrero-Garzón et al., 2020; Matroodi et al., 2020). A number of known lantipeptides have shown activity against microbial pathogens. Of these, several lantipeptides have been in pre-clinical trials as antibiotics for treatment of *Clostridium difficile* infections (Grasemann et al., 2007; Donadio et al., 2010; Mathur et al., 2013) and other Gram-positive bacterial infections, such as microbisporicin NAI-107, mutacin 1140, duramycin, NVB302, and lactacins 3147 α and β (Ghobrial et al., 2010; Jabés et al., 2011; Piper et al., 2012). The best BLAST hits of lantipeptide BGCs were related to actagardine, SRO15-3108 and labyrinthopeptins biosynthesis, but with only low identity scores.

Interestingly, no NRPS-like BGCs were detected in three sponge bacterial metagenomes in the present study. This may be attributed to several reasons. Firstly, an investigation of PKS and NRPS from microbes associated with *Discodermia dissoluta* from three metagenomic libraries (i.e., whole sponge tissue, and fractions enriched for unicellular or filamentous bacteria) revealed that NRPS sequences were only detected in the fraction enriched by filamentous bacteria, where no NRPS sequences were found in whole sponge tissue and the unicellular-enriched fraction (Schirmer et al., 2005). During the enrichment for bacteria in our study prior to DNA extraction and metagenomic sequencing, most filamentous bacteria (if present) were probably lost and hence their BGCs not included in our dataset. In addition, some NRPS BGCs may be difficult to recover by next-generation sequencing approaches (e.g., from low GC or high AT species) due to bias of sequencing or assembly methods (Quail et al., 2012; Ghurye et al., 2016; Sato et al., 2019; Gunasekera et al., 2021; Lapidus and Korobeynikov, 2021).

Previous chemical investigations have reported diverse natural products isolated from three sponges, including *C. reinwardti* (Goud et al., 2005; Tai et al., 2021; Trang et al., 2022b), *R. globostellata* (Bourguet-Kondracki et al., 2000; Tasdemir et al., 2002; Clement et al., 2006; Fouad et al., 2006; Hirashima et al., 2010; Li et al., 2010; Trang et al., 2022a), and *Spheciospongia* spp. (Costantino et al., 2005; 2008; Whitson et al., 2008; Eltamany et al., 2015; Tawfike et al., 2019). The natural products from the reported sponge species and their associated cultivated bacteria included mainly terpenes and lipids, and a few peptide, aromatic, phenolic, and indole compounds, which are products resultsing from the main BGC classes (terpenes, PKSs, fatty acids, RiPPs) detected in sponge metagenomes in our study. The decadal challenge is now to link the striking novelty in sponge and other environmental BGCs to the natural products they produce.

Predicted taxa of the secondary metabolite biosynthetic gene clusters

Previous extensive investigations of BGCs in bacterial genomes revealed non-uniform distribution of BGCs in bacterial taxa. The phyla known to possess a high number of BGCs in their genomes include *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Cyanobacteria* (Tracanna et al., 2017). Since the large majority of the BGCs in our sponge-derived metagenomes contained KS sequences (cf_fatty acids, PKSs, aryl polyenes and ladderanes), the KS sequences can be used to identify the major taxa that harbour BCGs in sponge-associated bacteria. Unsurprisingly, genera of the phylum *Actinobacteria*, such as *Mycolicibacterium*, *Mycobacterium*, and *Streptomyces* were most dominant as predicted hosts of the KS sequences. Apart from *Streptomyces* and *Bacillus*, which are rich sources of natural products (Zhao and Kuipers, 2016), other bacterial taxa are receiving increased attention as novel target genera with a high biosynthetic potential, including *Pseudomonas*, *Clostridium*, *Burkholderia*, *Pseudonocardia*, *Photorhabdus*, *Xenorhabdus*, *Chitinophaga*, *Herpetosiphon*, and *Planctomyces* (Challinor and Bode, 2015; Subramani and Sipkema, 2019). Particularly in sponges, the genus "*Candidatus* Entotheonella" has been recognized for their unusually rich and varied arsenal of BGCs (Mori et al., 2018). In our study, *Streptomyces*, *Pseudomonas* and *Burkholderia* were among the prominent genera to which KS sequences were assigned, but "*Candidatus* Enthotheonella" was not identified, which corroborates with its absence in these three sponge species based on 16S rRNA gene analysis (Dat et al., 2018). In addition, other genera, such as *Pseudovibrio* and *Bacillus* that are frequently isolated from sponges and linked to a high prevalence of antimicrobial activity (Indraningrat et al., 2016), were not identified here based on cultivation-independent means.

Generally, the predicted taxa of bacteria harbouring the BGCs in the present study corresponded to 16S rRNA gene analysis in a previous study at the phylum level, but their relative abundances are diffferent (Dat et al., 2018). This is likely due to different frequencies of BGCs among bacteria taxa (Tracanna et al., 2017; Loureiro et al., 2022).

Putative 'sponge-specific' clusters

It is well known that marine sponges harbor specific microbial communities (Simister et al., 2012b). Interestingly, the KS sequence analysis also revealed the presence of several 'sponge-specific' PKS groups. In a systematic analysis of 150 KS sequences from metagenomic DNA from 20 different demosponges through PCR screenings of KS genes, Fieseler et al. (2007) detected a cluster of sponge-specific PKS sequences, termed "symbiont ubiquitous type I PKS" (*Sup*). In another study, Sala et al. (2013) detected another sponge-specific KS group, named "sponge (symbiont) widespread fatty acid synthase" (*Swf*), in several high-microbial-abundance sponges. Notably, both *SupA* and *SwfA* are involved in the synthesis of methyl branched fatty acids (Hochmuth et al., 2010; Sala et al., 2013). Phylogenetic reconstruction of KS sequences derived from the metagenomes of sponge-associated bacteria in our study showed three monophyletic sponge-specific clusters that

were phylogenetically distant from KS sequences from other sources. Two sponge-specific KS groups were affiliated to the known sponge-specific KS groups, i.e., *SupA* and *SwfA*.

The third sponge-specific KS group is involved in fatty acids synthesis in *Candidatus* Poribacteria. This sponge-specific KS group includes KS sequences from our study and KS sequences retrieved from *Candidatus* Poribacteria metagenome-assembled genomes (Podell et al., 2019). Borchert et al. (2016) investigated the diversity of NRPS and PKS genes in microbiome of three deep-sea sponges *Inflatella pellicula*, *Poecillastra compressa*, and *Stelletta normani* through PCR screening of the KS domain and also found several sponge-specific KS groups. Also other recent studies have identified other 'sponge-specific' gene clusters related to the biosynthesis of RIPP proteusins, bromotyrosine alkaloids, and ether lipids (Mohanty et al., 2021; Nguyen et al., 2021; Loureiro et al., 2022). These findings imply that the sponge-specific specialised metabolites are prevalent and may play an important role in sponge holobiont defence or communication.

Conclusion

The metagenomes of associated bacteria of three sponges contained a high number of predicted BGCs, ranging from 282 to 463 BGCs per metagenome, with 12 different BGC types. After cf_fatty_acid, PKSs were the most dominant BGCs, followed by terpene BGCs and bacteriocin BGCs. The BGCs were grouped into 363 gene cluster families (GCFs) based on sequence similarity. Interestingly, not a single GCF from the metagenomes was assigned to pathways responsible for the production of known compounds, implying that they might be responsible for production of novel compounds. Based on taxa of the closest reference KS sequences retrieved from the NCBI database, the KS sequences of the identified BGCs from the metagenomes related to 12 bacterial phyla, of which *Actinobacteria*, *Proteobacteria*, and *Firmicutes* were the most predominant. At the genus level, the KS were related to 142 genera, of which *Mycolicibacterium*, *Mycobacterium*, *Burkholderia* and *Streptomyces* were the most predominant. Notably, a large number of KS sequences retrieved from PKS BGCs was present in two known 'sponge-specific' clusters, i.e., *SupA* and *SwfA*, whereas a part of KS sequences retrieved from fatty acid BGCs formed a new sponge-specific KS cluster related to the fatty acid synthesis in *Candidatus* Poribacteria. Our study reinforces that sponge-associated bacteria are a rich source of novel BGCs and as such most likely, bioactive compounds.

Acknowledgements

This research was funded by the Vietnam Ministry of Science and Technology, grant number ĐTĐLCN.17/14; BE-Basic Foundation-FES grant from the Dutch Ministry of Economic Affairs, grant number: F07.003.06; and MC-ITN-BluePharmTrain funded through the

European Union Programme FP7/2007-2013/ under REA grant agreement No. 607786.

Data Availability Statement

Metagenome datasets have been deposited in the NCBI Sequence Read Archive under the BioProject accession number: PRJNA766414.

Supplementary information

Supplementary Table S1. Basic data of the three metagenomes of sponge-associated bacteria

Sample ID	Sponge species	No. reads	Contigs	Contigs sum (bp)	N50	Longest contig (bp)	GC content (%)	No. CDS
CLR	*C. reinwardti*	30,414,932	607,208	475,723,993	56,925	236,946	61	888,255
RHG	*R. globostellata*	49,526,287	466,098	486,959,254	21,342	377,852	62	782,060
SPV	*Spheciospongia* sp.	66,835,559	504,262	598,027,097	23,906	1,109,582	61	903,985

Supplementary Table S2: BGCs identified from the metagenome of *C. reinwardti* by antiSMASH

Supplementary Table S3: BGCs identified from the metagenome of *R. globostellata* by antiSMASH

Supplementary Table S4: BGCs identified from the metagenome of *Spheciospongia* sp. by antiSMASH

Supplementary Table S5: BLAST search of the KS sequences from the metagenome of *C. reinwardti* against the KS sequences of known products from the NaPDos database

Supplementary Table S6: BLAST search of the KS sequences from the metagenome of *R. globostellata* against the KS sequences of known products from the NaPDos database

Supplementary Table S7: BLAST search of the KS sequences from the metagenome of *Spheciospongia* sp. against the KS sequences of known products from the NaPDos database

Supplementary Table S8: BLAST search of the KS sequences from the metagenome of *C. reinwardti* against the *nr* database in NCBI

Supplementary Table S9: BLAST search of the KS sequences from the metagenome of *R. globostellata* against the *nr* database in NCBI

Supplementary Table S10: BLAST search of the KS sequences from the metagenome of *Spheciospongia* sp. against the *nr* database in NCBI

Supplementary Table S11: BLAST search of the KS sequences from the metagenome of *C. reinwardti* against the *refseq_protein* database in NCBI

Supplementary Table S12: BLAST search of the KS sequences from the metagenome of *R. globostellata* against the *refseq_protein* database in NCBI

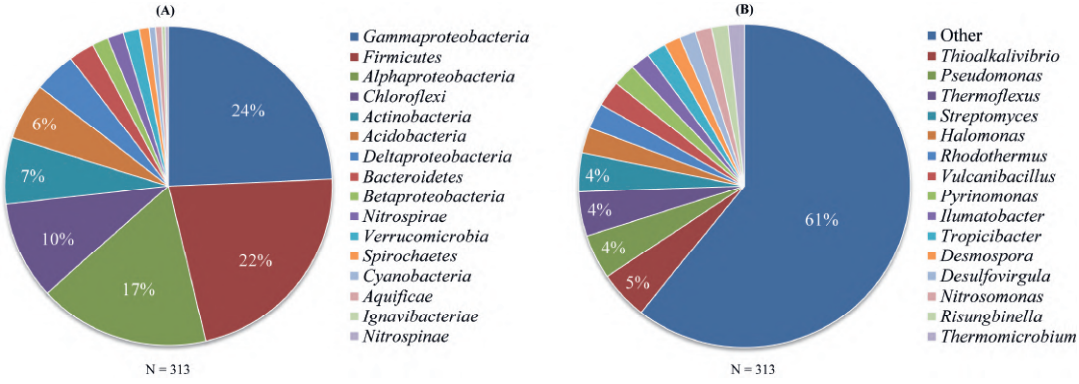
Supplementary Table S13: BLAST search of the KS sequences from the metagenome of *Spheciospongia* sp. against *refseq_protein* database in NCBI

Supplementary Table S14: BLAST search of the BGCs identified from the metagenome of *C. reinwardti* against the MiBIG database

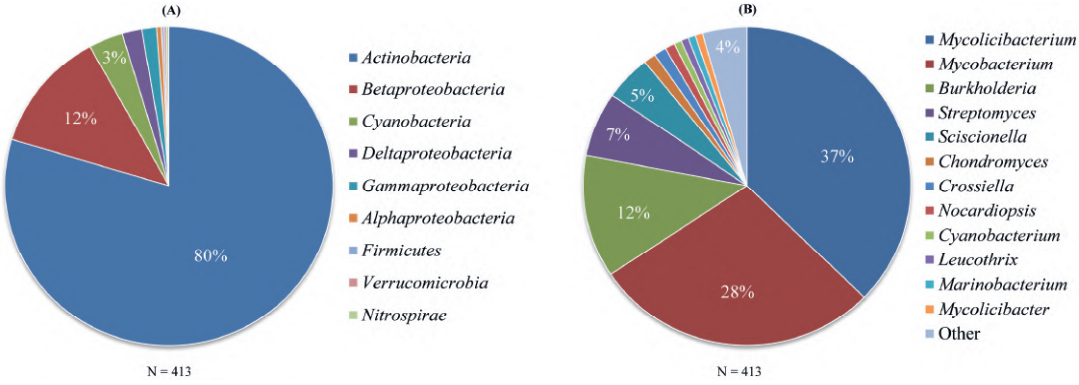
Supplementary Table S15: BLAST search of the BGCs identified from the metagenome of *R. globostellata* against the MiBIG database

Supplementary Table S16: BLAST search of the BGCs identified from the metagenome of *Spheciospongia* sp. against the MiBIG database.

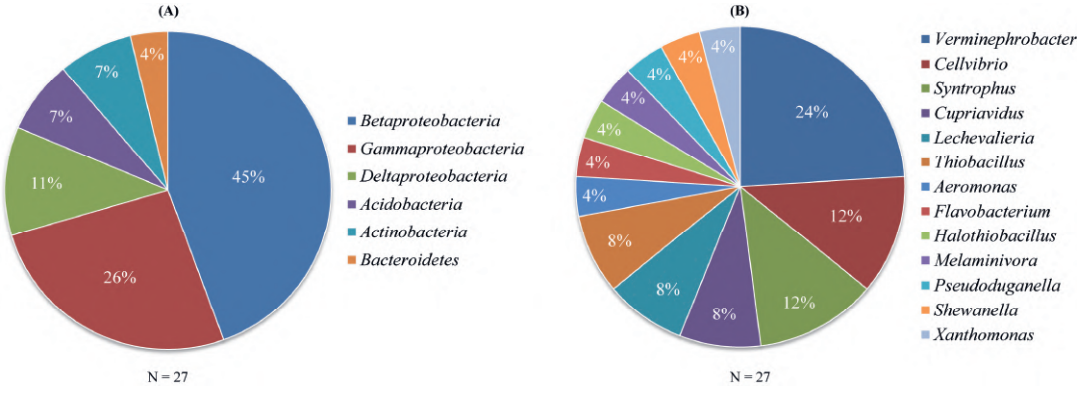
Note: Supplementary Table S2-S16 are available via the following link: https://www.mdpi.com/article/10.3390/md21010029/s1



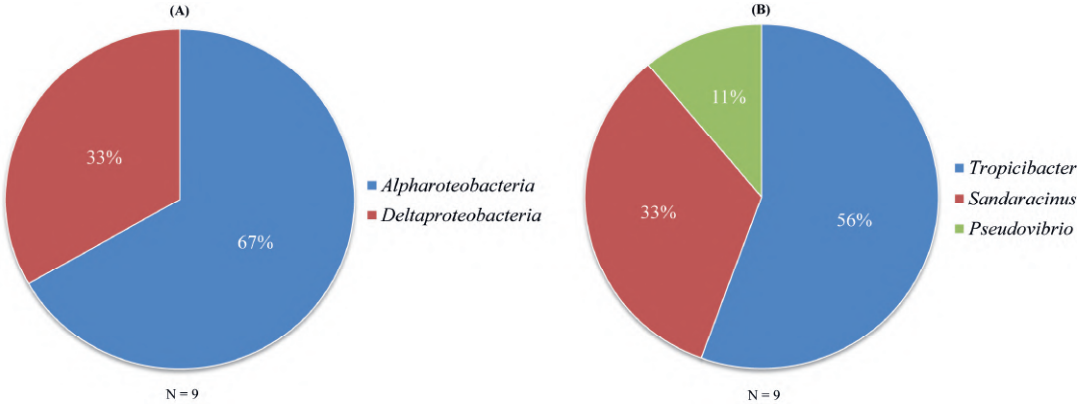
Supplementary Figure S1. Taxonomy of the most homologous KS sequences with the KS sequences of cf_fatty acid BGCs identified from the metagenomes of the sponge-associated bacteria retrieved from NCBI Genbank at phylum/class level (A) and genus level (B)



Supplementary Figure S2. Taxonomy of the most homologous KS sequences with the KS sequences of PKS BGCs identified from the metagenomes of the sponge-associated bacteria retrieved from NCBI Genbank at phylum/class level (A) and genus level (B)



Supplementary Figure S3. Taxonomy of the most homologous KS sequences with the KS sequences of aryl propylene BGCs identified from the metagenomes of the sponge-associated bacteria retrieved from NCBI Genbank at phylum/class level (A) and genus level (B)



Supplementary Figure S4. Taxonomy of the most homologous KS sequences with the KS sequences of ladderane BGCs identified from the metagenomes of the sponge-associated bacteria retrieved from NCBI Genbank at phylum/class level (A) and genus level (B)

Chapter 6

General Discussion

Introduction

Vietnam's marine ecosystem constitutes a high-priority area for marine conservation with distinct biota and is within four of 35 global biodiversity hotspots (Selig et al., 2014). This coastal ecosystem holds high biodiversity conservation values with over 11,000 marine species, including marine sponges (Thuaire et al., 2021). However, research on sponges in Vietnam has received attention in the last few decades. Taxonomic investigations on several shallow sea areas of Vietnam, i.e., Nha Trang Bay, Con Co island, limestone island, and coastal regions of North-Central Vietnam, have revealed a high diversity of marine sponges, with more than 229 identified sponge species belonging to 124 genera, 65 families, 18 orders, and 4 classes (Thai, 2013; Calcinai et al., 2020; Huong et al., 2020; Do Cong, 2022). Subsequent studies on Vietnamese sponge-derived natural products have led to the isolation of more than 250 natural products from about 30 sponge species, including more than 50 new compounds (Kiem et al., 2019), many of which exhibited outstanding anticancer and anti-inflammatory effects (Kiem et al., 2019).

Compared to studies on sponge taxonomy and sponge-derived natural products, Vietnamese sponge-associated microbiota and their natural products have only been studied in the last few years and remain largely unexplored. Cultivation-dependent investigations focused on the isolation of sponge-associated microbes that produce bioactive compounds (Tuan et al., 2017; Cao et al., 2019; Vu et al., 2019; Cao et al., 2020; Quang et al., 2020; Trinh et al., 2020; Quang et al., 2021a; Quang et al., 2021b; Kim et al., 2022; Trinh et al., 2022), resulting in the isolation of nearly 80 compounds with a few new antimicrobial and cytotoxic compounds. Apart from cultivation studies, investigations of Vietnamese sponge microbiota by cultivation-independent methods have yielded several interesting insights into Vietnamese sponge microbiomes such as host-specificity, the influence of environmental quality on their microbiomes, as well as the role of seawater in bacteria recruitment and microbiome stability in sponges (Swierts et al., 2018; Turon et al., 2018; Turon et al., 2019a; Turon et al., 2019b; Cleary et al., 2020; Turon and Uriz, 2020). A summary of results obtained from the previous studies on marine sponges collected in Vietnam is illustrated in Figure 1.

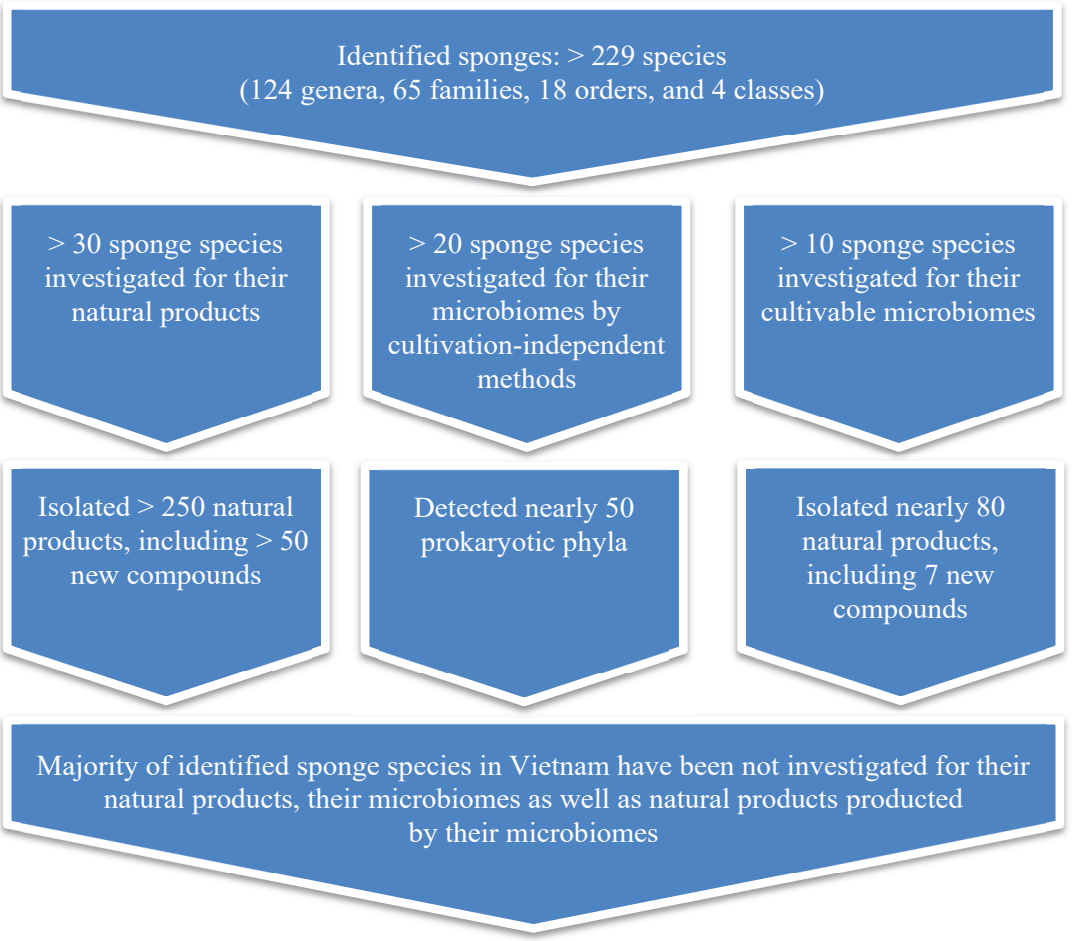


Figure 1. Summary of previous studies on marine sponges collected in Vietnam

Thus, the research described in this thesis set out to provide a better understanding of prokaryotes associated with Vietnamese sponges, their antimicrobial activity as well as and their secondary metabolite gene clusters by applying cultivation-dependent and -independent approaches. A brief overview of results obtained from each chapter is illustrated in Figure 2, which will be further elaborated and discussed in the following sections.

Chapter 2. Diversity of sponge-associated prokaryotes by 16S rRNA gene amplicon Illumina MiSeq

- ❑ 15 prokaryotic phyla detected from Vietnamese sponges
- ❑ Host-specificity of Vietnamese sponge prokaryotic communities
- ❑ Identification of sponge-enriched prokaryotic OTUs

Chapter 3. Cultivation of sponge-associated bacteria and their secondary metabolites

- ❑ Isolated 460 sponge-associated bacteria with 90 strains producing antimicrobials
- ❑ Little overlap between sponge-associated prokaryotes by cultivation-dependent and -independent methods
- ❑ Purification of four antimicrobial compounds

Chapter 4. Literature review of cultivable and not-yet cultivable sponge-associated bacteria

- ❑ Overview of cultivation methods sponge-associated prokaryotes
- ❑ Diversity of cultivable sponge-associated prokaryotes
- ❑ Sponge-specific and sponge-enriched cultivable prokaryotes
- ❑ New methods for cultivation of sponge prokaryotes

Chapter 5. Diversity of biosynthetic gene clusters (BGCs) from sponge-associated prokaryotes by metagenomics

- ❑ Diversity of BGCs detected from sponge microbiome metagenomes of three sponge species
- ❑ High number of BGCs encoding for novel/undescribed compounds
- ❑ Presence of sponge-specific prokaryotic polyketide synthases

Figure 2. A brief overview of results obtained in Chapters 2 – 5

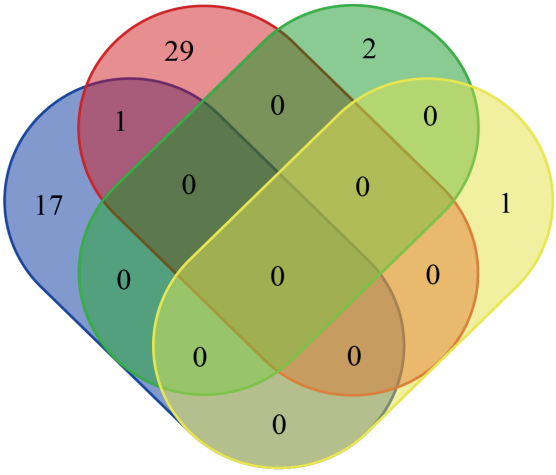
Diversity of Vietnamese sponge-associated prokaryotes

To date, Vietnamese sponge-associated prokaryotes have been largely unexplored with only a few studies on Vietnamese sponge-associated prokaryotes so far. Therefore, we investigated prokaryotic communities associated with 18 Vietnamese sponge species by a cultivation-independent method (**Chapter 2**) and cultivation-dependent method (**Chapter 3**) to expand our understanding of the Vietnamese sponge-associated prokaryotes.

Apart from microbiomes in two sponge species found in Vietnam, *X. testudinaria* and *C. reinwardti*, reported by recent investigations (Swierts et al., 2018; Turon et al., 2018; Turon et al., 2019a), 16 out of 18 Vietnamese sponge species were investigated for their microbiomes for the first time in our study and expanded our knowledge on Vietnamese sponge-associated prokaryotic communities. Generally, *Proteobacteria*, *Acidobacteria, Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes* were the most abundant taxa, followed by *Nitrospirae*,

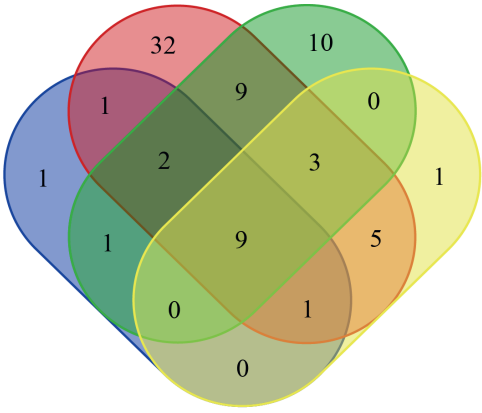
Bacteroidetes, *Cyanobacteria*, *Deferribacteres* and *Spirochaetes* in Vietnamese sponge microbiomes in our study. Studies on bacterial communities associated with Vietnamese sponges in Nha Trang Bay have identified nearly 50 bacterial phyla, with *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Planctomycetes* being the most abundant taxa (Turon et al., 2018; Turon et al., 2019a), whereas bacterial communities associated with Vietnamese sponges *Spheciospongia solida* and *Spongia ceylonensis* in Ha Long Bay harboured 19 and 32 bacterial phyla, respectively, which were dominated by *Proteobacteria*, *Chloroflexi*, *Acidobacteria, Cyanobacteria* and *Bacteroidetes* (Cleary et al., 2020). Furthermore, the analysis of prokaryotic communities associated with Indo-Pacific giant barrel sponges (*Xestospongia* spp.), including two Vietnamese sponges *Xestospongia* spp. in Ha Long Bay, indicated that these sponges harbored 48 prokaryotic phyla, which were predominated by *Proteobacteria*, *Cloroflexi*, *SAR202*, and *Actinobacteria* (Swierts et al., 2018). So far, nearly 50 sponge species in Vietnam have been investigated with respect to their associated prokaryotic communities (Figure 3) with over 50 prokaryotic phyla being identified (Figure 4).

Although the prokaryotic communities associated with Vietnamese sponges harbor relatively similar sets of phyla, their distributions are dissimilar, and different sponge species differ with respect to the most abundant prokaryotic phyla. The majority of Vietnamese sponge species included in our study were characterized by high relative abundances of *Alpha-*, *Beta-* and *Gamma-Proteobacteria* that often characterize LMA sponges (Moitinho-Silva et al., 2017c; Sabrina Pankey et al., 2022). Several Vietnamese sponge species included in our investigation such as *X. testudinaria*, *R. globostellata*, *Halichondria* sp., *Spirastrella* sp., and *Dactylospongia* sp., were found enriched for prokaryotic taxa that usually are almost exclusively found in HMA sponges such *Delta-Proteobacteria*, *Actinobacteria* and *Chloroflexi*. Some of these sponges, including *X. testudinaria* and *R. globostellata* have been reported as HMA sponges by the previous HMA-LMA dichotomy studies (Gloeckner et al., 2014; Moitinho-Silva et al., 2017c; Sabrina Pankey et al., 2022). Investigations of Vietnamese sponge microbiomes by Turon and colleagues observed high abundances of *Alpha-*, *Beta-* and *Gamma-Proteobacteria* in Vietnamese LMA sponges, whereas PAUC34f, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, and *Nitrospinae* are almost exclusively found in HMA sponges including *Aaptos suberitioides*, *Neofibularia hartmani* and *Suberea cf. laboutei* (Turon et al., 2018; Turon et al., 2019a). Cleary et al. (2020) indicated different distributions of abundant bacterial phyla in two Vietnamese sponge species i.e., *S. solida* and *S. ceylonensis*, in which bacterial communities associated with *S. solida* were dominated by the indicator phyla of LMA sponges as *Proteobacteria* (*Alpha- and Gamma-*), *Cyanobacteria* and *Bacteroidetes*; in contrast, the bacterial communities associated with *S. ceylonensis* were dominated by *Proteobacteria* (*Alpha- and Gamma-*), *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* usually enriched in HMA sponges. Also, the Vietnamese sponge species belonging to the genus *Xestospongia* in Ha Long Bay were predominated by *Proteobacteria* (*Alpha- and Gamma-*), *Cloroflexi*, *SAR202*, and *Actinobacteria*, which are usually found in HMA sponges (Swierts et al., 2018).



In this thesis	Turon et al., 2018-2020	Cleary et al., 2019	Swierts et al., 2018
Amphimedon compressa	*Aaptos suberitoides*	*Spheciospongia solida*	*Xestospongia* sp.
Amphimedon sp.	*Amphimedon paraviridis*	*Spongia ceylonensis*	
Axinyssa topsenti	*Amphimedon sulcata*		
Axos cliftoni	*Antho* sp.		
Cinachyrella schulzei	*Callyspongia* sp.		
Clathria reinwardti	*Clathria reinwardti*		
Dactylospongia sp.	*Clathria (Isociella) skia*		
Halichondria sp.	*Clathria* sp.		
Haliclona amboinensis	*Dendroxea* sp.		
Haliclona fascigera	*Dysidea* sp.		
Haplosclerida sp.	*Gellioides cf.gracilis*		
Niphatidae sp.	*Gellioides* sp.		
Rhabdastrella globostellata	*Haliclona (Gellius) toxia*		
Spheciospongia vesparium	*Haliclona (Gellius) toxotes*		
Spirastrella sp.	*Haliclona (Reniera)* sp.		
Tedania sp.	*Monanchora unguiculata*		
Terpios aploos	*Mycale (Arenochalina)* sp.		
Xestospongia testudinaria	*Neofibularia hartmani*		
	Neofibularia sp.		
	Niphates sp.		
	Phorbas sp.		
	Prosuberites proteus		
	Pseudosuberites sp.		
	Suberea cf.laboutei		
	Suberea fusca		
	Terpios cruciatus		
	Terpios sp.		
	Thrinacophora cervicornis		
	Thrinacophora rhaphidophora		

Figure 3. Overview of sponges in Vietnam for which the associated prokaryotic communities were studied in this thesis and/or by others. The Venn diagram indicates how many sponge species overlap between different studies.



In this thesis	Turon et al., 2018 - 2020		Cleary et al., 2019	Swierts et al., 2018
Acidobacteria	*Acetothermia*	*Nanoarchaeaeota*	*Acidobacteria*	*Acidobacteria*
Actinobacteria	*Acidobacteria*	*Nitrospinae*	*Actinobacteria*	*Actinobacteria*
Bacteroidetes	*Actinobacteria*	*Nitrospirae*	*AncK6*	*Bacteroidetes*
Chloroflexi	*Aminicenantes*	*Omnitrophica*	*Bacteroidetes*	*Chloroflexi*
Cyanobacteria	*Armatimonadetes*	*Opisthokonta*	*BHI80.AC0.139*	*Cyanobacteria*
Deferribacteres	*Bacteroidetes*	*Parcubacteria*	*Caldithrix*	*Deinococcus-Thermus*
Deinococcus_Thermus	*BRC1*	*PAUC34f*	*Chlamydiae*	*Gemmatimonadetes*
Firmicutes	*Caldiserica*	*Peregrinibacteria*	*Chlorobi*	*Nitrospinae*
Gemmatimonadetes	*Candidatus Berkelbacteria*	*Planctomycetes*	*Chloroflexi*	*Nitrospirae*
Nitrospirae	*Chlamydiae*	*Poribacteria*	*Cyanobacteria*	*PAUC34f*
Planctomycetes	*Chlorobi*	*Proteobacteria*	*Elusimicrobia*	*Poribacteria*
Proteobacteria	*Chloroflexi*	*RBG-1*	*Fibrobacteres*	*Proteobacteria*
Spirochaetes	*Cloacimonetes*	*Saccharibacteria*	*Firmicutes*	*Saccharibacteria*
Thaumarchaeota	*CPR2*	*SAR*	*Fusobacteria*	*SBR1093*
Verrucomicrobia	*Cyanobacteria*	*SBR1093*	*Gemmatimonadetes*	*Spirochaetae*
	Deferribacteres	*Spirochaetae*	*GN02*	*Tectomicrobia*
	Deinococcus-Thermus	*SR1*	*Lentisphaerae*	*Thaumarchaeota*
	Diapherotrites	*Synergistetes*	*Nitrospirae*	*Verrucomicrobia*
	Elusimicrobia	*Tectomicrobia*	*OD1*	*Woesearchaeota*
	Euryarchaeota	*Tenericutes*	*OP1*	
	FBP	*Thaumarchaeota*	*OP8*	
	Fibrobacteres	*Thermotogae*	*PAUC34f*	
	Firmicutes	*TM6*	*Planctomycetes*	
	Fusobacteria	*Verrucomicrobia*	*Poribacteria*	
	Gemmatimonadetes	*WS1*	*Proteobacteria*	
	Gracilibacteria	*WS2*	*SBR1093*	
	Hydrogenedentes	*WS6*	*Spirochaetes*	
	Ignavibacteriae	*WWE3*	*Tenericutes*	
	KSB3 (Modulibacteria)		*TM6*	
	Latescibacteria		*TM7*	
	LCP-89		*Verrucomicrobia*	
	Lentisphaerae		*WS2*	
	Marinimicrobi		*X.AFs.Thermi.AF0.*	
	Microgenomates		*ZB3*	

Figure 4. Prokaryotic phyla reported to be associated with sponges in Vietnam in this and other studies. The Venn diagram indicates how many prokaryotic phyla overlap between different studies.

Thaumarchaeota was the only archaeal phylum found in Vietnamese sponges in our study, and this phylum was detected in a wide range of Vietnamese sponge species (14 out of 18 species), with the majority of samples exhibiting relative abundances of archaea greater than 10% relative abundance within the prokaryotic communities (**Chapter 1**). *Thaumarchaeota* is the single most widely reported archaea taxon in the Sponge Microbiome Project which includes sponges from all over the world (Moitinho-Silva et al., 2017b). Several high abundant *Thaumarchaeota* OTUs from Vietnamese sponges in our study are enriched in sponges based on comparison with data in the Sponge Microbiome Project (Moitinho-Silva et al., 2017b). In a recent study, Turon et al (2019b) investigated the archaeal communities of 17 Vietnamese sponges in Nha Trang Bay using archaea specific primers and captured higher archaeal diversity than using the universal prokaryotic primers used in our study. More specifically, these authors detected four archaeal phyla, including *Thaumarchaeota*, *Creanoarchaeota*, *Euryarchaeota* and *Diapherotrites*. However, *Thaumarchaeota* was still the predominant phylum and accounted for up 85% of the archaeal community. Among them, *Candidatus* Nitrosopumilus was the dominant genus in the majority of the Vietnamese sponge species analyzed by our study and by Turon and Uriz (2020).

Analysis of archaeal communities associated with Vietnamese sponges by Turon and Uriz (2020) indicated that host identity was the main factor structuring the archaeal assemblages in the majority of the studied Vietnamese sponge species, with only a few exceptions such as *Protosuberites proteus*, *Dendroxea* sp., and *Haliclona (Gellius toxia)*. However, the presence of archaeal indicator phyla for discrimination between HMA and LMA sponges could not be confirmed by the investigated Vietnamese sponges. Although *Thaumarchaeota* and *Euryarchaeota* sequences found in Vietnamese sponges by Turon and Uriz (2020) were more closely related to environmental sequences, the representatives of *Woesarchaeota*, which were major members of the archaeal microbiome of two Vietnamese sponges, i.e., *Mycale* sp. and *Clathria (Isociella) skia*, formed a monophyletic branch in the phylogenetic tree, only distantly related to any known environmental sequence (Turon and Uriz, 2020).

Host-specificity of Vietnamese sponge microbiomes

The structure of sponge microbiomes is often stable and species-specific regardless of geographic, bathymetric, trophic, and temperature ranges as well as irradiance shifts (Hentschel et al., 2002; Erwin et al., 2012; Schmitt et al., 2012; Pita et al., 2013a; Pita et al., 2013b; Luter et al., 2014; Strand et al., 2017; Campana et al., 2021), in contrast to the spatial and temporal variations of seawater microbiomes (Zeglin, 2015; Glasl et al., 2017). The structure of sponge microbiomes has been reported to be stable and specific even at sponge order and family levels (Yang et al., 2019). In line with previous studies, the prokaryotic communities associated with Vietnamese sponge species with replicates (i.e., *Axinyssa* sp.,

C. reinwardti, *Dactylospongia* sp., *H. amboinensis*, *Spirastrella* sp., and *X. testudinaria*) in this thesis show a similar pattern of host-specificity (**Chapter 2**), and specimens sampled from different locations at different time points still clustered together, supporting earlier observations of stable host-specific prokaryotic communities despite geographic and temporal differences (Taylor et al., 2007; Pita et al., 2013b; Hardoim and Costa, 2014; Erwin et al., 2015). Host-specificity of Vietnamese sponge microbiomes was also confirmed for other sponge species collected in Nha Trang Bay and Ha Long Bay (Turon et al., 2018; Turon et al., 2019a; Cleary et al., 2020). In addition, species specificity of Vietnamese sponge microbiomes was found to be still stable when specimens in environmentally-impacted and well-preserved sites were compared (Turon et al., 2019a). These findings support the general perception that the selection of specific bacteria and competition among the selected bacteria (Easson and Thacker, 2014) shape the species-specific patterns of sponge microbiomes and lead to similar communities in geographically distant individuals of the same sponge genus (Montalvo et al., 2014).

Vietnamese sponge-enriched and environment-enriched prokaryotes

Another aspect of sponge-associated prokaryotes is the existence of "sponge-specific" clusters (Hentschel et al., 2002; Taylor et al., 2007; Simister et al., 2012b), which has later been revised to "sponge-enriched" because many prokaryotic members of sponge-specific clusters were also found in seawater and sediment samples, albeit at very low relative abundances (Taylor et al., 2013; Thomas et al., 2016). Examination of the most abundant operational taxonomic units (OTUs) in Vietnamese sponge-associated prokaryotes in our study in comparison with those included in the Sponge Microbiome Project (Moitinho-Silva et al., 2017b) revealed that approximately half of the most abundant OTUs in the studies described in this thesis are significantly enriched in sponges (**Chapter 2**), implying that "sponge-enriched" populations may play crucial ecological roles in Vietnamese sponge holobionts. Apart from several sponge-enriched OTUs in the investigated Vietnamese sponges that belong to taxa known for nitrification and denitrification activities (e.g., *Nitrosomonadaceae*, *Desulfurellaceae*, *Nitrospinaceae*, *Nitrosococcus*, *Nitrospira*), many sponge-enriched OTUs in the investigated Vietnamese sponges represent still uncultured prokaryotes (e.g., *Sva0996 marine group*, *Sh765B-TzT-29*, *PAUC34f*, *Marine Group I*). Hence their ecological functions are still unknown.

It is worth noting that half of the most abundant OTUs among the Vietnamese sponge-associated prokaryotes have also been described to be significantly enriched in non-sponge sources in the Sponge Microbiome Project (Moitinho-Silva et al., 2017b), suggesting that these prokaryotes may be acquired from the environment. In a recent investigation of microbiomes from Vietnamese sponges and seawater, Turon et al. (2018) found a high overlap between the sponge core bacteria and the seawater bacterial core, ranging between

9% and 99% cumulative relative abundance, depending on the sponge species. Half of the sponge species shared more than 50% of their bacterial core with the seawater bacterial core regardless of their status as HMA or LMA sponge, indicating that these bacteria may be acquired from seawater and that seawater acts as a seed bank for sponge microbiomes, as suggested by Webster et al. (2010), Webster and Thomas (2016), and Moitinho-Silva et al. (2014a). Therefore, Turon and colleagues proposed that environmental acquisition plays a major role in the establishment of Vietnamese sponge microbiomes, whereas host-specificity of Vietnamese sponge microbiomes implies that individual sponges have mechanisms to selectively enrich certain environmental bacteria in their tissues (Turon et al. 2018). These mechanisms would allow the maintenance of bacterial communities in a species across geographical ranges. Recent series of investigations and comprehensive reviews support a mixture of both horizontal and vertical transmissions in most sponges (Sipkema et al., 2015; Griffiths et al., 2019; Russell, 2019; de Oliveira et al., 2020; Oliveira et al., 2020; Díez-Vives et al., 2022).

Little overlap of Vietnamese sponge prokaryotes captured by cultivation-dependent and –independent methods

Although marine sponges host a high diversity of associated prokaryotes (Moitinho-Silva et al., 2017b), the majority of sponge-associated prokaryotes have not been cultivated (Olson et al., 2000; Webster and Hill, 2001; Sipkema et al., 2011; Fuerst, 2014; Montalvo et al., 2014). I isolated bacteria associated with sponges from Vietnam using different culture media and recovered isolates of four prokaryotic phyla (**Chapter 3**), which is much lower compared to the 15 prokaryotic phyla that were detected by direct Illumina MiSeq amplicon sequencing analysis of the same sponge samples (**Chapter 2**). The diversity of cultivated sponge-associated prokaryotes was dominated by strains from commonly isolated bacterial genera, e.g. *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces*, which were not detected or were detected at very low relative abundance in the same sponge specimens by direct Illumina MiSeq amplicon sequencing analysis (**Chapter 2**). These results are in line with other efforts in cultivation of Vietnamese sponge-associated microbes for the discovery of novel natural compounds where especially the genera *Bacillus* and *Streptomyces* were frequently recovered (Tuan et al., 2017; Cao et al., 2019; Vu et al., 2019; Cao et al., 2020; Quang et al., 2020; Trinh et al., 2020; Quang et al., 2021a; Quang et al., 2021b; Kim et al., 2022; Trinh et al., 2022). At the OTU level, only six OTUs out of 921 OTUs detected by MiSeq sequencing of 16S rRNA gene amplicons were recovered by cultivation, but the abundance of the shared OTUs was distinct for the two methods (**Chapter 3**). For instance, OTU909 (*Bacillus*), OTU337 (*Pseudovibrio*), OTU541 (*Ruegeria*), and OTU710 (*Pseudoalteromonas*) were cultivated from all sponge species; however, these OTUs were not detected or detected at very low relative abundance from the same sponge species by Illumina MiSeq amplicon sequencing analysis. This discrepancy is in line with previous

investigations of sponge-associated bacteria using both cultivation-dependent and -independent approaches (Li et al., 2011a; Sipkema et al., 2011; Hardoim and Costa, 2014; Montalvo et al., 2014; Versluis et al., 2017), indicating that prokaryotes captured by cultivation are not representative of the diversity found by cultivation-independent methods. A comprehensive review of sponge-associated cultivable prokaryotes (**Chapter 4**) reported representatives of 11 cultivable prokaryotic phyla associated with sponges, with the predominance of the four phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, which were also dominant in cultivable prokaryotes from Vietnamese sponges (**Chapter 3**). In contrast, more than 60 prokaryotic phyla associated with sponges have been detected by cultivation-independent methods so far (Moitinho-Silva et al., 2017b). Looking at the combined efforts of nearly all published studies where bacteria have been isolated from sponges (**Chapter 4**), a few important guidelines can be derived that should be taken into account in new studies focusing on the isolation of the "true sponge symbionts":

1. The use of nutrient-rich media often results in the recovery of numerous colonies of common prokaryotes. Instead, use of nutrient-poor media, which are probably more similar to the nutrient levels in sponge holobionts may recover more diverse sponge-associated prokaryotes.

2. The limited overlap of sponge-associated prokaryotes captured by cultivation-dependent and –independent methods indicates that the majority of sponge-associated prokaryotes recovered by cultivation are probably not from sponges, but other probably free-living microorganisms in the surrounding seawater. Especially, genera such as *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces* are found to be dominant in culture regardless of environmental and culture conditions.

3. In natural environments in general and sponge holobionts in particular, microorganisms often occur in complex networks with other organisms, the majority of which constitutes obligate symbionts that are recalcitrant to cultivation and isolation under laboratory conditions because they have co-evolved with their host, which leads to complex nutritional requirements or other growth conditions that are still unknown or hard to mimic *in vitro*. Therefore, co-culture and culture *in situ* may enhance cultivability of sponge-associated prokaryotes.

4. The failure of capturing 'true' sponge prokaryotes can be partially explained by use of synthetic media, which fail to mimic the native nutrients of sponge-associated prokaryotes. Furthermore, cultivation under laboratory conditions might not be sufficiently similar to the natural growth conditions of sponge-associated bacteria (e.g., host-symbiont interactions, substrates required for growth). Recent developments in reconstructing composite genomes of currently uncultivable

bacteria from metagenomes have provided clues on growth substrates of some sponge-associated bacteria, e.g., predicted consumption of carnitine, spermidine, and sulfated polysaccharides (Slaby et al., 2017; Taylor et al., 2021), compounds that are hardly ever included in bacterial cultivation media. In addition, extensive cultivation efforts by using alternative techniques (e.g., cultivation media and growth condition diversification, liquid culture, floating filters, diffusion chamber cultivation) have improved the cultivability of sponge-associated bacteria (Sipkema et al., 2011; Steinert et al., 2014; Esteves et al., 2016; Versluis et al., 2017). These cultivation approaches should therefore be applied in order to enhance cultivability of Vietnamese sponge-associated microbes, especially not-yet-cultured 'true' sponge prokaryotes.

Re-isolation of secondary metabolites by traditional cultivation-based methods

Marine sponge-associated prokaryotes are among the most prolific producers of secondary metabolites with diverse bioactive properties (Laport et al., 2009; Fuerst, 2014; Juliana et al., 2014; Indraningrat et al., 2016). I investigated the antimicrobial activity of extracts from bacteria isolated from Vietnamese sponges (**Chapter 3**). Nearly 20% of the isolated prokaryotes showed antimicrobial properties, however, diversity of the isolated prokaryotes with antimicrobial properties was low and dominated by several common prokaryotes that are well-known as producers of antimicrobials, such as, e.g., members of the genera *Streptomyces* and *Bacillus* (Zhao and Kuipers, 2016; Quinn et al., 2020; Donald et al., 2022; Xiao et al., 2022). Thus there is a high probability of re-isolating producers of known secondary metabolites. In fact, cultivation and extraction of secondary metabolites from a bacterial isolate from a Vietnamese sponge with strong antimicrobial activity resulted in the isolation of four known antimicrobials, including one cyclic dipeptide and three macrolactins (**Chapter 3**). Only one isolated strain with strong antimicrobial activity was selected because of the time-consuming procedures, limiting the usefulness of this approach for the remaining bioactive strains from this study, but also in general. Indeed, the efforts to discover novel natural compounds from Vietnamese sponge-associated microbes in recent studies have resulted in the isolation of just a few compounds, of which the majority was already known (Tuan et al., 2017; Cao et al., 2019; Vu et al., 2019; Cao et al., 2020; Quang et al., 2020; Trinh et al., 2020; Quang et al., 2021a; Quang et al., 2021b; Kim et al., 2022; Trinh et al., 2022). Therefore, alternative approaches are urgently needed to discover new compounds, including the development of new cultivation methods enhancing the cultivation of novel microbes. In addition, the discovery of novel antimicrobials should be targeted to largely unexplored bacterial taxa with high biosynthetic potential, including *Pseudomonas*, *Clostridium*, *Burkholderia*, *Pseudonocardia*, *Photorhabdus*, *Xenorhabdus*, *Chitinophaga*, *Herpetosiphon*, and *Planctomyces* (Challinor and Bode, 2015; Tracanna et al., 2017; Subramani and Sipkema, 2019).

Striking novelty and diversity of metabolic biosynthesis in Vietnamese sponge prokaryotic communities

Discovery of novel antimicrobials by cultivation-based approaches is time-consuming and hampered due to re-isolation of known prokaryotic taxa and known compounds. Therefore, to discover novel gene clusters responsible for synthesis of secondary metabolites from sponge-associated prokaryotes, particularly not-yet cultured prokaryotes, cultivation-independent approaches, in particular metagenomics, can be used to investigate the diversity of biosynthetic gene clusters (BGCs) in sponge metagenomes. I used this approach to study the biosynthetic potential of three Vietnamese sponges (**Chapter 5**). All three metagenomes contained high numbers of BGCs from various BGCs classes, but especially those encoding the production of polyketides and terpenes, which have diverse structures and biological properties (Gomes et al., 2013; Yamada et al., 2015; Mahizan et al., 2019; Robertsen and Musiol-Kroll, 2019). No BGCs in our dataset could be reliably assigned to a cluster encoding the production of known compounds, implying that BGCs detected in our study are likely involved in the biosynthesis of currently unknown compounds. This finding is in line with a recent comparative metagenomic analysis of the biosynthetic diversity across sponge microbiomes where <0.003% of the BGCs included experimentally characterized reference BGCs from the MIBiG database (Loureiro et al., 2022). Therefore, the gene clusters detected in the metagenomes of Vietnamese sponge microbiomes represent still untapped sources for secondary metabolites with bioactive properties, which can be extrapolated to sponges in general.

Sponge-specific polyketide synthases in sponge microbiomes

Marine sponges are well-known to harbor species-specific microbial communities and sponge-specific or at least sponge-enriched bacteria (Simister et al., 2012b). Recent omics-based studies have also identified 'sponge-specific' gene clusters related to the biosynthesis of ribosomally synthesised and post-translationally modified peptides (RIPPs) (e.g. proteusins), bromotyrosine alkaloids, and ether lipids (Mohanty et al., 2021; Nguyen et al., 2021; Loureiro et al., 2022). In addition, sponges also contain 'sponge-specific' polyketide synthases (PKSs). For instance, a systematic analysis of 150 ketosynthase (KS) sequences from metagenomic DNA from 20 different demosponges through PCR screenings of KS genes by Fieseler et al. (2007) revealed a cluster of sponge-specific PKS sequences, termed "symbiont ubiquitous type I PKS" (*Sup*). Afterwards, Sala et al. (2013) detected another sponge-specific KS group, named "sponge (symbiont) widespread fatty acid synthase" (*Swf*), in several high-microbial-abundance sponges. These sponge-specific *SupA* and *SwfA* are likely involved in the synthesis of methyl-branched fatty acids (Hochmuth et al., 2010; Sala et al., 2013). Interestingly, phylogenetic reconstruction of KS sequences derived from the metagenomes of Vietnamese sponge-associated bacteria in our study showed an additional

monophyletic sponge-specific cluster that is phylogenetically distant from KS sequences from other sources (**Chapter 5**). The third sponge-specific KS group is likely involved in fatty acid biosynthesis in *Candidatus* Poribacteria and includes KS sequences from our study and KS sequences retrieved from *Candidatus* Poribacteria metagenome-assembled genomes (Podell et al., 2019). These findings imply that these sponge-specific specialized metabolites are prevalent and may play an important role in sponge holobiont defense or communication.

Conclusions and outlook

Marine sponges distributed in various regions around the globe have been studied well for diverse aspects, e.g. taxonomy, chemical ecology, sponge-derived natural products, and sponge microbiomes. Particularly sponges in the Caribbean, the Gulf of Mexico, Northeast Atlantic, Mediterranean, and Coral Sea (Van Soest et al., 2012; Moitinho-Silva et al., 2017b) have been well studied, whereas knowledge of Vietnamese sponges is limited. The present thesis provides deeper insights in Vietnamese sponge-associated prokaryotes as well as their natural product potential, contributing to a general picture of Vietnamese sponges and linking them to global sponge microbiomes using cultivation-dependent and -independent methods. Results presented here show that prokaryotes associated with Vietnamese sponges have characteristics that are in line with global sponge microbiomes such as high diversity, host-specificity, and a large fraction of sponge-enriched prokaryotes. Particularly, metagenomic analysis of three Vietnamese sponge-associated prokaryotes revealed the novelty and diversity of BGCs as well as the presence of a newly discovered sponge-specific type of PKS mainly found in the phylum *Candidatus* Poribacteria. However, many aspects of Vietnamese sponge-associated prokaryotes are still not fully understood and need further study also to facilitate the development of biotechnological and pharmaceutical applications:

1. Numerous prokaryotes were isolated from (Vietnamese) sponges, however, these studies failed to isolate novel prokaryotes and the 'true' sponge-associated prokaryotes exemplified by the lack of isolates from clusters of sponge-enriched bacteria. This asks for radically different isolation methods that incorporate knowledge on interactions between the host and microbes and microbes and microbes.

2. The cultivation-independent, metagenomic study of the diversity of BGCs revealed a high diversity of BGCs, and the large majority of the predicted BGCs encode proteins that most likely are responsible for the biosynthesis of undescribed/unknown secondary metabolites. However, linking BGCs to molecules of interest is a long shot for uncultivable bacteria. Therefore, generalized methodologies are urgently needed that can identify links between BGCs and these molecules. Tools to establish these links are currently being developed, hopefully

providing us with improved general knowledge about the structure of an unknown natural product by predicting likely occurring scaffolds or substructures by using chemical compound classification strategies and using that to filter for (more) plausible candidates based on matching chemical compound classes (Louwen et al., 2023). Ultimately, as long as cultivation of these symbionts remains elusive, only heterologous expression of these BGCs can reliably link BGCs to molecules. This is currently a holy grail and although some successes have been reported, they are scarce due to inherent complications due to the large size of many BGCs (Xu et al., 2022). In addition, nearly all BGCs that have been amenable to heterologous expression are derived from culturable bacteria and not from uncultured species, which may indicate that regulation of gene expression of BGCs from currently uncultivable bacteria may be substantially different from their better-studied counterparts.

In summary, the results obtained in this thesis provide additional information on Vietnamese sponge-associated prokaryotes that have not been reported previously and that can serve as a basis for further studies in order to fully unravel ecological and biotechnological insights into Vietnamese sponge microbiomes. Particularly, metagenome mining indicated striking novelty and high diversity of secondary metabolite biosynthesis capacity in the investigated sponge prokaryotic communities, which should be investigated further to deploy this potential bioactive secondary metabolite source.

References

Abadi, A.T.B., Rizvanov, A.A., Haertlé, T., and Blatt, N.L. (2019). World Health Organization Report: Current Crisis of Antibiotic Resistance. *BioNanoScience* 9, 778-788. doi: 10.1007/s12668-019-00658-4.

Abbamondi, G.R., De Rosa, S., Iodice, C., and Tommonaro, G. (2014). Cyclic Dipeptides Produced by Marine Sponge-Associated Bacteria as Quorum Sensing Signals. *Nat. Prod. Commun.* 9, 1934578X1400900225. doi: 10.1177/1934578X1400900225.

Abdelmohsen, U.R., Bayer, K., and Hentschel, U. (2014). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat. Prod. Rep.* 31, 381-399. doi: 10.1039/c3np70111e.

Abdelmohsen, U.R., Pimentel-Elardo, S.M., Hanora, A., Radwan, M., Abou-El-Ela, S.H., Ahmed, S., et al. (2010). Isolation, Phylogenetic Analysis and Anti-infective Activity Screening of Marine Sponge-Associated Actinomycetes. *Mar. Drugs* 8, 399-412. doi: 10.3390/md8030399.

Abe, T., Sahin, F.P., Akiyama, K., Naito, T., Kishigami, M., Miyamoto, K., et al. (2012). Construction of a Metagenomic Library for the Marine Sponge Halichondria okadai. *Biosci., Biotechnol., Biochem.* 76, 633-639. doi: 10.1271/bbb.110533.

Adedeji, W.A. (2016). The treasure called antibiotics. *Ann Ib Postgrad Med* 14, 56-57.

Adnani, N., Chevrette, M.G., Adibhatla, S.N., Zhang, F., Yu, Q., Braun, D.R., et al. (2017). Coculture of Marine Invertebrate-Associated Bacteria and Interdisciplinary Technologies Enable Biosynthesis and Discovery of a New Antibiotic, Keyicin. *ACS Chem. Biol.* 12, 3093-3102. doi: 10.1021/acschembio.7b00688.

Adnani, N., Vazquez-Rivera, E., Adibhatla, S.N., Ellis, G.A., Braun, D.R., and Bugni, T.S. (2015). Investigation of Interspecies Interactions within Marine Micromonosporaceae Using an Improved Co-Culture Approach. *Mar. Drugs* 13, 6082-6098. doi: 10.3390/md13106082.

Aguila-Ramírez, R.N., Hernández-Guerrero, C.J., González-Acosta, B., Id-Daoud, G., Hewitt, S., Pope, J., et al. (2014). Antifouling activity of symbiotic bacteria from sponge *Aplysina gerardogreeni*. *Int. Biodeterior. Biodegrad.* 90, 64-70. doi: 10.1016/j.ibiod.2014.02.003.

Ahmad, M., and Khan, A.U. (2019). Global economic impact of antibiotic resistance: A review. *Journal of Global Antimicrobial Resistance* 19, 313-316. doi: 10.1016/j.jgar.2019.05.024.

Alain, K., and Querellou, J. (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* 13, 583-594. doi: 10.1007/s00792-009-0261-3.

Alanis, A.J. (2005). Resistance to Antibiotics: Are We in the Post-Antibiotic Era? *Arch. Med. Res.* 36, 697-705. doi: 10.1016/j.arcmed.2005.06.009.

Alex, A., and Antunes, A. (2015a). Pyrosequencing Characterization of the Microbiota from Atlantic Intertidal Marine Sponges Reveals High Microbial Diversity and the Lack of Co-Occurrence Patterns. *PLoS One* 10, e0127455. doi: 10.1371/journal.pone.0127455.

Alex, A., and Antunes, A. (2015b). Whole Genome Sequencing of the Symbiont *Pseudovibrio* sp. from the Intertidal Marine Sponge *Polymastia penicillus* Revealed a Gene Repertoire for Host-Switching Permissive Lifestyle. *Genome Biol. Evol.* 7, 3022-3032. doi: 10.1093/gbe/evv199.

Alex, A., and Antunes, A. (2018). Genus-wide comparison of Pseudovibrio bacterial genomes reveal diverse adaptations to different marine invertebrate hosts. *PLoS One* 13, e0194368. doi: 10.1371/journal.pone.0194368.

Aliscioni, N.L., Delbón, N., and Gurvich, D.E. (2021). Spine function in Cactaceae, a review. *Journal of the Professional Association for Cactus Development* 23, 1-11. doi: 10.56890/jpacd.v23i.325.

Alt, S., and Wilkinson, B. (2015). Biosynthesis of the Novel Macrolide Antibiotic Anthracimycin. *ACS Chem. Biol.* 10, 2468-2479. doi: 10.1021/acschembio.5b00525.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *JMBio* 215, 403-410. doi: 10.1016/S0022-2836(05)80360-2.

Aminov, R. (2010). A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology* 1. doi: 10.3389/fmicb.2010.00134.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq-a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)* 31, 166-169. doi: 10.1093/bioinformatics/btu638.

Anjum, K., Abbas, S.Q., Shah, S.a.A., Akhter, N., Batool, S., and Hassan, S.S.U. (2016). Marine Sponges as a Drug Treasure. *Biomol. Ther. (Seoul)* 24, 347-362. doi: 10.4062/biomolther.2016.067.

Antcliffe, J.B., Jessop, W., and Daley, A.C. (2019). Prey fractionation in the Archaeocyatha and its implication for the ecology of the first animal reef systems. *Palb* 45, 652-675. doi: 10.1017/pab.2019.32.

Anteneh, Y.S., Yang, Q., Brown, M.H., and Franco, C.M.M. (2022). Factors affecting the isolation and diversity of marine sponge-associated bacteria. *Appl. Microbiol. Biotechnol.* 106, 1729-1744. doi: 10.1007/s00253-022-11791-8.

Aoi, Y., Kinoshita, T., Hata, T., Ohta, H., Obokata, H., and Tsuneda, S. (2009). Hollow-Fiber Membrane Chamber as a Device for In Situ Environmental Cultivation. *Appl. Environ. Microbiol.* 75, 3826-3833. doi: 10.1128/AEM.02542-08.

Apprill, A., Mcnally, S., Parsons, R., and Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75, 129-137. doi: 10.3354/ame01753.

Arendrup, M.C., Guinea, J., Cuenca-Estrell, M., Meletiadis, J., Mouton, J.W., Lagrou, K., et al. (2015a). *EUCAST definitive document E.DEF 7.3: Method for the determination of broth dilution minimum Inhibitory concentrations of antifungal agents for yeasts.* Växjö, Sweden: EUCAST.

Arendrup, M.C., Guinea, J., Cuenca-Estrell, M., Meletiadis, J., Mouton, J.W., Lagrou, K., et al. (2015b). *EUCAST definitive documnet E.DEF 9.3: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds.* Växjö, Sweden: EUCAST.

Asadzadeh, S.S., Kiørboe, T., Larsen, P.S., Leys, S.P., Yahel, G., and Walther, J.H. (2020). Hydrodynamics of sponge pumps and evolution of the sponge body plan. *eLife* 9, e61012. doi: 10.7554/eLife.61012.

Ashkenazi, S. (2013). Beginning and possibly the end of the antibiotic era. *J. Paediatr. Child Health* 49, E179-E182. doi: 10.1111/jpc.12032.

Astudillo-García, C., Slaby, B.M., Waite, D.W., Bayer, K., Hentschel, U., and Taylor, M.W. (2018). Phylogeny and genomics of SAUL, an enigmatic bacterial lineage frequently associated with marine sponges. *Environ. Microbiol.* 20, 561-576. doi: 10.1111/1462-2920.13965.

Atanasov, A.G., Zotchev, S.B., Dirsch, V.M., Orhan, I.E., Banach, M., Rollinger, J.M., et al. (2021). Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery* 20, 200-216. doi: 10.1038/s41573-020-00114-z.

Bader, J., Mast-Gerlach, E., Popović, M.K., Bajpai, R., and Stahl, U. (2010). Relevance of microbial coculture fermentations in biotechnology. *J. Appl. Microbiol.* 109, 371-387. doi: 10.1111/j.1365-2672.2009.04659.x.

Bai, X., Liu, Y., Wang, H., and Zhang, H. (2021). Natural Products from the Marine Sponge Subgenus *Reniera*. *Molecules* 26, 1097. doi: 10.3390/molecules26041097.

Balskus, E.P. (2014). Sponge symbionts play defense. *Nat. Chem. Biol.* 10, 611-612. doi: 10.1038/nchembio.1588.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology* 19, 455-477. doi: 10.1089/cmb.2012.0021.

Barriere, S.L. (2015). Clinical, economic and societal impact of antibiotic resistance. *Expert Opin. Pharmacother.* 16, 151-153. doi: 10.1517/14656566.2015.983077.

Bayer, K., Jahn Martin, T., Slaby Beate, M., Moitinho-Silva, L., and Hentschel, U. (2018). Marine Sponges as Chloroflexi Hot Spots: Genomic Insights and High-Resolution Visualization of an Abundant and Diverse Symbiotic Clade. *mSystems* 3, e00150-00118. doi: 10.1128/mSystems.00150-18.

Bayer, K., Kamke, J., and Hentschel, U. (2014a). Quantification of bacterial and archaeal symbionts in high and low microbial abundance sponges using real-time PCR. *FEMS Microbiol. Ecol.* 89, 679-690. doi: 10.1111/1574-6941.12369.

Bayer, K., Kamke, J., and Hentschel, U. (2014b). Quantification of bacterial and archaeal symbionts in high and low microbial abundance sponges using real-time PCR. *FEMS microbiology ecology* 89, 679-690. doi: 10.1111/1574-6941.12369.

Bayer, K., Moitinho-Silva, L., Brümmer, F., Cannistraci, C.V., Ravasi, T., and Hentschel, U. (2014c). GeoChip-based insights into the microbial functional gene repertoire of marine sponges (high microbial abundance, low microbial abundance) and seawater. *FEMS Microbiol. Ecol.* 90, 832-843. doi: 10.1111/1574-6941.12441.

Bayer, K., Schmitt, S., and Hentschel, U. (2008a). Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environ Microbiol* 10, 2942-2955. doi: 10.1111/j.1462-2920.2008.01582.x.

Bayer, K., Schmitt, S., and Hentschel, U. (2008b). Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environ. Microbiol.* 10, 2942-2955. doi: 10.1111/j.1462-2920.2008.01582.x.

Beck, J.V. (1971). "[8] Enrichment culture and isolation techniques particularly for anaerobic bacteria," in *Methods Enzymol.* Academic Press, vol. 22, 57-64. doi: 10.1016/0076-6879(71)22010-3.

Beekes, M., Lasch, P., and Naumann, D. (2007). Analytical applications of Fourier transform-infrared (FT-IR) spectroscopy in microbiology and prion research. *Vet. Microbiol.* 123, 305-319. doi: 10.1016/j.vetmic.2007.04.010.

Belbahri, L., Chenari Bouket, A., Rekik, I., Alenezi, F.N., Vallat, A., Luptakova, L., et al. (2017). Comparative Genomics of *Bacillus amyloliquefaciens* Strains Reveals a Core Genome with Traits for Habitat Adaptation and a Secondary Metabolites Rich Accessory Genome. *Frontiers in Microbiology* 8, 1438. doi: 10.3389/fmicb.2017.01438.

Belknap, K.C., Park, C.J., Barth, B.M., and Andam, C.P. (2020). Genome mining of biosynthetic and chemotherapeutic gene clusters in *Streptomyces* bacteria. *Sci. Rep.* 10, 2003. doi: 10.1038/s41598-020-58904-9.

Bell, J.J. (2008). The functional roles of marine sponges. *Estuar. Coast. Shelf Sci.* 79, 341-353. doi: 10.1016/j.ecss.2008.05.002.

Bell, J.J., Mcgrath, E., Biggerstaff, A., Bates, T., Cárdenas, C.A., and Bennett, H. (2015). Global conservation status of sponges. *Conserv. Biol.* 29, 42-53. doi: 10.1111/cobi.12447.

Ben-Dov, E., Kramarsky-Winter, E., and Kushmaro, A. (2009). An in situ method for cultivating microorganisms using a double encapsulation technique. *FEMS Microbiol. Ecol.* 68, 363-371. doi: 10.1111/j.1574-6941.2009.00682.x.

Benítez, X., Gonzalez, E.G., García, J., Zúñiga, P., De La Calle, F., and Cuevas, C. (2021). Detection of a pederin-like compound using a dilution-to-extinction-based platform for the isolation of marine bacteria in drug discovery strategies. *Microbial biotechnology* 14, 241-250. doi: 10.1111/1751-7915.13679.

Bergquist, P.R. (2001). "Porifera (Sponges)," in *ELS*. doi: 10.1038/npg.els.0001582.

Bian, C., Wang, J., Zhou, X., Wu, W., and Guo, R. (2020). Recent Advances on Marine Alkaloids from Sponges. *Chem. Biodivers.* 17, e2000186. doi: 10.1002/cbdv.202000186.

Bin Saidin, J., Abd Wahid, M.E., and Le Pennec, G. (2016). Characterization of the in vitro production of N-acyl homoserine lactones by cultivable bacteria inhabiting the sponge *Suberites domuncula*. *J. Mar. Biol. Assoc. U.K.* 97, 119-127. doi: 10.1017/S0025315416000151.

Björk, J.R., Díez-Vives, C., Astudillo-García, C., Archie, E.A., and Montoya, J.M. (2019). Vertical transmission of sponge microbiota is inconsistent and unfaithful. *Nature Ecology & Evolution* 3, 1172-1183. doi: 10.1038/s41559-019-0935-x.

Blunt, J.W., and Munro, M.H. (2022). "MarinLit: A database of the marine natural products literature". University of Canterbury, New Zealand.

Bollmann, A., Lewis, K., and Epstein, S.S. (2007). Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Appl Environ Microbiol* 73, 6386-6390. doi: 10.1128/AEM.01309-07.

Bolotin, A., Quinquis, B., Sorokin, A., and Ehrlich, S.D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 151, 2551-2561. doi: 10.1099/mic.0.28048-0.

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., and Schulz-Vogt, H.N. (2013). The genus Pseudovibrio contains metabolically versatile bacteria adapted for symbiosis. *Environ. Microbiol.* 15, 2095-2113. doi: 10.1111/1462-2920.12123.

Borchert, E., Jackson, S.A., O'gara, F., and Dobson, A.D.W. (2016). Diversity of Natural Product Biosynthetic Genes in the Microbiome of the Deep Sea Sponges *Inflatella pellicula*, *Poecillastra compressa*, and *Stelletta normani*. *Frontiers in microbiology* 7, 1027-1027. doi: 10.3389/fmicb.2016.01027.

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J., and Le Parco, Y. (2001). Sponge paraphyly and the origin of Metazoa. *J. Evol. Biol.* 14, 171-179. doi: 10.1046/j.1420-9101.2001.00244.x.

Bose, U., Ortori, C.A., Sarmad, S., Barrett, D.A., Hewavitharana, A.K., Hodson, M.P., et al. (2017). Production of N-acyl homoserine lactones by the sponge-associated marine actinobacteria *Salinispora arenicola* and *Salinispora pacifica*. *FEMS Microbiol. Lett.* 364, fnx002. doi: 10.1093/femsle/fnx002.

Bourguet-Kondracki, M.-L., Longeon, A., Debitus, C., and Guyot, M. (2000). New cytotoxic isomalabaricane-type sesterterpenes from the New Caledonian marine sponge *Rhabdastrella globostellata*. *Tetrahedron Lett.* 41, 3087-3090. doi: 10.1016/S0040-4039(00)00345-2.

Boury-Esnault, N., Muricy, G., Gallissian, M.-F., and Vacelet, J. (1995). Sponges without skeleton: A new Mediterranean genus of Homoscleromorpha (Porifera, Demospongiae). *Ophelia* 43, 25-43. doi: 10.1080/00785326.1995.10430575.

Brakhage, A.A. (2013). Regulation of fungal secondary metabolism. *Nature Reviews Microbiology* 11, 21-32. doi: 10.1038/nrmicro2916.

Breitling, R., and Takano, E. (2016). Synthetic Biology of Natural Products. *Cold Spring Harb. Perspect. Biol.* 8, a023994. doi: 10.1101/cshperspect.a023994.

Bringmann, G., Lang, G., Mühlbacher, J., Schaumann, K., Steffens, S., Rytik, P.G., et al. (2003). "Sorbicillactone A: a Structurally Unprecedented Bioactive Novel-Type Alkaloid from a Sponge-Derived Fungus," in *Sponges (Porifera),* ed. W.E.G. Müller. Berlin, Heidelberg: Springer Berlin Heidelberg, 231-253. doi: 10.1007/978-3-642-55519-0_9.

Brinkmann, M.C., Marker, A., and Kurtböke, İ.D. (2017). An Overview on Marine Sponge-Symbiotic Bacteria as Unexhausted Sources for Natural Product Discovery. *Diversity* 9, 40. doi: 10.3390/d9040040.

Brito, P.H., Chevreux, B., Serra, C.R., Schyns, G., Henriques, A.O., and Pereira-Leal, J.B. (2018). Genetic Competence Drives Genome Diversity in *Bacillus subtilis*. *Genome Biol. Evol.* 10, 108-124. doi: 10.1093/gbe/evx270.

Britstein, M., Saurav, K., Teta, R., Sala, G.D., Bar-Shalom, R., Stoppelli, N., et al. (2017). Identification and chemical characterization of N-acyl-homoserine lactone quorum sensing signals across sponge species and time. *FEMS Microbiol. Ecol.* 94, fix182. doi: 10.1093/femsec/fix182.

Brown, E.D., and Wright, G.D. (2016). Antibacterial drug discovery in the resistance era. *Nature* 529, 336-343. doi: 10.1038/nature17042.

Bruns, A., Cypionka, H., and Overmann, J. (2002). Cyclic AMP and Acyl Homoserine Lactones Increase the Cultivation Efficiency of Heterotrophic Bacteria from the Central Baltic Sea. *Appl. Environ. Microbiol.* 68, 3978-3987. doi: 10.1128/AEM.68.8.3978-3987.2002.

Burgsdorf, I., Sizikov, S., Squatrito, V., Britstein, M., Slaby, B.M., Cerrano, C., et al. (2022). Lineage-specific energy and carbon metabolism of sponge symbionts and contributions to the host carbon pool. *The ISME Journal* 16, 1163-1175. doi: 10.1038/s41396-021-01165-9.

Burgsdorf, I., Slaby Beate, M., Handley Kim, M., Haber, M., Blom, J., Marshall Christopher, W., et al. (2015). Lifestyle Evolution in Cyanobacterial Symbionts of Sponges. *mBio* 6, e00391-00315. doi: 10.1128/mBio.00391-15.

Burns, E., Ifrach, I., Carmeli, S., Pawlik, J.R., and Ilan, M. (2003). Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. I. Chemical defense. *Mar. Ecol. Prog. Ser.* 252, 105-114. doi: 10.3354/meps252105.

Burns, E., and Ilan, M. (2003). Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. II. Physical defense. *Mar. Ecol. Prog. Ser.* 252, 115-123. doi: 10.3354/meps252115.

Butler, M.S., Robertson, A.a.B., and Cooper, M.A. (2014). Natural product and natural product derived drugs in clinical trials. *Nat. Prod. Rep.* 31, 1612-1661. doi: 10.1039/C4NP00064A.

Calcabrini, C., Catanzaro, E., Bishayee, A., Turrini, E., and Fimognari, C. (2017). Marine Sponge Natural Products with Anticancer Potential: An Updated Review. *Mar. Drugs* 15, 310. doi: 10.3390/md15100310.

Calcinai, B., Cerrano, C., Núñez-Pons, L., Pansini, M., Thung, D.C., and Bertolino, M. (2020). A New Species of Spongilla (Porifera, Demospongiae) from a Karst Lake in Ha Long Bay (Vietnam). *Journal of Marine Science and Engineering* 8, 1008. doi: 10.3390/jmse8121008.

Campana, S., Demey, C., Busch, K., Hentschel, U., Muyzer, G., and De goeij, J.M. (2021). Marine sponges maintain stable bacterial communities between reef sites with different coral to algae cover ratios. *FEMS Microbiol. Ecol.* 97, fiab115. doi: 10.1093/femsec/fiab115.

Cane, D.E., and Ikeda, H. (2012). Exploration and Mining of the Bacterial Terpenome. *Acc. Chem. Res.* 45, 463-472. doi: 10.1021/ar200198d.

Cantón, R., Horcajada, J.P., Oliver, A., Garbajosa, P.R., and Vila, J. (2013). Inappropriate use of antibiotics in hospitals: The complex relationship between antibiotic use and antimicrobial resistance. *Enferm. Infecc. Microbiol. Clin.* 31, 3-11. doi: 10.1016/S0213-005X(13)70126-5.

Cao, D.D., Do, T.Q., Doan Thi Mai, H., Vu Thi, Q., Nguyen, M.A., Le Thi, H.M., et al. (2020). Antimicrobial lavandulylated flavonoids from a sponge-derived actinomycete. *Nat. Prod. Res.* 34, 413-420. doi: 10.1080/14786419.2018.1538219.

Cao, D.D., Trinh, T.T., Doan Thi Mai, H., Vu, V.N., Le, H.M., Vu Thi, Q., et al. (2019). Antimicrobial Lavandulylated Flavonoids from a Sponge-Derived *Streptomyces* sp. G248 in East Vietnam Sea. *Mar. Drugs* 17, 529. doi: 10.3390/md17090529.

Capella-Gutiérrez, S., Silla-Martínez, J.M., and Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25, 1972-1973. doi: 10.1093/bioinformatics/btp348.

Capone, D.G., Bronk, D.A., Mulholland, M.R., and Carpenter, E.J. (2008). *Nitrogen in the marine environment*. Burlington, MA: Academic Press.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335-336. doi: 10.1038/nmeth.f.303.

Cárdenas, P., and Rapp, H.T. (2012). A review of Norwegian streptaster-bearing Astrophorida (Porifera: Demospongiae: Tetractinellida), new records and a new species. *Zootaxa* 3253, 1–52. doi: 10.11646/ZOOTAXA.3253.1.1.

Carrier, T.J., Maldonado, M., Schmittmann, L., Pita, L., Bosch, T.C.G., and Hentschel, U. (2022). Symbiont transmission in marine sponges: reproduction, development, and metamorphosis. *BMC Biol.* 20, 100. doi: 10.1186/s12915-022-01291-6.

Carroll, A.R., Copp, B.R., Davis, R.A., Keyzers, R.A., and Prinsep, M.R. (2019). Marine natural products. *Nat. Prod. Rep.* 36, 122-173. doi: 10.1039/C8NP00092A.

Carroll, A.R., Copp, B.R., Davis, R.A., Keyzers, R.A., and Prinsep, M.R. (2021). Marine natural products. *Nat. Prod. Rep.* 38, 362-413. doi: 10.1039/D0NP00089B.

Cartwright, C.P., Stock, F., and Gill, V.J. (1994). Improved enrichment broth for cultivation of fastidious organisms. *J. Clin. Microbiol.* 32, 1825-1826. doi: 10.1128/jcm.32.7.1825-1826.1994.

Casillas-Vargas, G., Ocasio-Malavé, C., Medina, S., Morales-Guzmán, C., Del Valle, R.G., Carballeira, N.M., et al. (2021). Antibacterial fatty acids: An update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. *Prog. Lipid Res.* 82, 101093. doi: 10.1016/j.plipres.2021.101093.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., and Mahillon, J. (2019). Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Frontiers in Microbiology* 10, 302. doi: 10.3389/fmicb.2019.00302.

Cavalcanti, F.F., and Klautau, M. (2011). Solenoid: a new aquiferous system to Porifera. *Zoomorphology* 130, 255-260. doi: 10.1007/s00435-011-0139-7.

Cerrano, C., Pansini, M., Valisano, L., Calcinai, B., Sarà, M., and Bavestrello, G. (2004). Lagoon sponges from carrie bow cay (Belize): ecological benefits of selective sediment incorporation. *Bulletin of Museums and Biological Institutes of the University of Genoa* 68, 239-252.

Chaib De Mares, M., Jiménez, D.J., Palladino, G., Gutleben, J., Lebrun, L.A., Muller, E.E.L., et al. (2018). Expressed protein profile of a *Tectomicrobium* and other microbial symbionts in the marine sponge *Aplysina aerophoba* as evidenced by metaproteomics. *Sci. Rep.* 8, 11795. doi: 10.1038/s41598-018-30134-0.

Chaib De Mares, M., Sipkema, D., Huang, S., Bunk, B., Overmann, J., and Van Elsas, J.D. (2017). Host Specificity for Bacterial, Archaeal and Fungal Communities Determined for High- and Low-Microbial Abundance Sponge Species in Two Genera. *Frontiers in Microbiology* 8, 2560. doi: 10.3389/fmicb.2017.02560.

Challinor, V.L., and Bode, H.B. (2015). Bioactive natural products from novel microbial sources. *Ann. N. Y. Acad. Sci.* 1354, 82-97. doi: 10.1111/nyas.12954.

Chan, B.C.L., Han, X.Q., Lui, S.L., Wong, C.W., Wang, T.B.Y., Cheung, D.W.S., et al. (2015). Combating against methicillin-resistant *Staphylococcus aureus* – two fatty acids from Purslane (*Portulaca oleracea* L.) exhibit synergistic effects with erythromycin. *J. Pharm. Pharmacol.* 67, 107-116. doi: 10.1111/jphp.12315.

Chanas, B., and Pawlik, J.R. (1995). Defenses of Caribbean sponges against predatory reef fish. II. Spicules, tissue toughness, and nutritional quality. *Mar. Ecol. Prog. Ser.* 127, 195-211. doi: 10.3354/meps127195.

Chanas, B., and Pawlik, J.R. (1996). Does the skeleton of a sponge provide a defense against predatory reef fish? *Oecologia* 107, 225-231. doi: 10.1007/BF00327906.

Chau, K.M., Van, T.T., Quyen, D.V., Le, H.D., Phan, T.H., Ngo, N.D., et al. (2021). Molecular Identification and Characterization of Probiotic *Bacillus* Species with the Ability to Control *Vibrio* spp. in Wild Fish Intestines and Sponges from the Vietnam Sea. *Microorganisms* 9, 1927. doi: 10.3390/microorganisms9091927.

Chen, H., and Du, L. (2016). Iterative polyketide biosynthesis by modular polyketide synthases in bacteria. *Appl. Microbiol. Biotechnol.* 100, 541-557. doi: 10.1007/s00253-015-7093-0.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., et al. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* 25, 1007-1014. doi: 10.1038/nbt1325.

Cheng, M.-M., Tang, X.-L., Sun, Y.-T., Song, D.-Y., Cheng, Y.-J., Liu, H., et al. (2020). Biological and Chemical Diversity of Marine Sponge-Derived Microorganisms over the Last Two Decades from 1998 to 2017. *Molecules* 25, 853. doi: 10.3390/molecules25040853.

Choi, S.-S., Hur, Y.-A., Sherman, D.H., and Kim, E.-S. (2007). Isolation of the biosynthetic gene cluster for tautomycetin, a linear polyketide T cell-specific immunomodulator from *Streptomyces* sp. CK4412. *Microbiology* 153, 1095-1102. doi: 10.1099/mic.0.2006/003194-0.

Choudhary, A., Naughton, L.M., Montánchez, I., Dobson, A.D.W., and Rai, D.K. (2017). Current Status and Future Prospects of Marine Natural Products (MNPs) as Antimicrobials. *Mar. Drugs* 15, 272. doi: 10.3390/md15090272.

Cimmino, A., Puopolo, G., Perazzolli, M., Andolfi, A., Melck, D., Pertot, I., et al. (2014). Cyclo(L-PRO-L-TYR), The Fungicide Isolated From *Lysobacter Capsici* AZ78: A Structure–Activity Relationship Study. *Chemistry of Heterocyclic Compounds* 50, 290-295. doi: 10.1007/s10593-014-1475-6.

Cleary, D.F.R., Becking, L.E., De Voogd, N.J., Pires, A.C.C., Polónia, A.R.M., Egas, C., et al. (2013). Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters. *FEMS Microbiol. Ecol.* 85, 465-482. doi: 10.1111/1574-6941.12135.

Cleary, D.F.R., Ferreira, M.R.S., Bat, N.K., Polónia, A.R.M., Gomes, N.C.M., and De Voogd, N.J. (2020). Bacterial composition of sponges, sediment and seawater in enclosed and open marine lakes in Ha Long Bay Vietnam. *Mar. Biol. Res.* 16, 18-31. doi: 10.1080/17451000.2019.1702214.

Cleary, D.F.R., Swierts, T., Coelho, F.J.R.C., Polónia, A.R.M., Huang, Y.M., Ferreira, M.R.S., et al. (2019). The sponge microbiome within the greater coral reef microbial metacommunity. *Nature Communications* 10, 1644. doi: 10.1038/s41467-019-09537-8.

Clement, J.A., Li, M., Hecht, S.M., and Kingston, D.G.I. (2006). Bioactive Isomalabaricane Triterpenoids from *Rhabdastrella globostellata* that Stabilize the Binding of DNA Polymerase β to DNA. *J. Nat. Prod.* 69, 373-376. doi: 10.1021/np0504107.

Collignon, P., and Beggs, J.J. (2019). Socioeconomic Enablers for Contagion: Factors Impelling the Antimicrobial Resistance Epidemic. *Antibiotics* 8, 86. doi: 10.3390/antibiotics8030086.

Colman, A.S. (2015). Sponge symbionts and the marine P cycle. *Proceedings of the National Academy of Sciences* 112, 4191-4192. doi: 10.1073/pnas.1502763112.

Connon, S.A., and Giovannoni, S.J. (2002). High-Throughput Methods for Culturing Microorganisms in Very-Low-Nutrient Media Yield Diverse New Marine Isolates. *Appl. Environ. Microbiol.* 68, 3878-3885. doi: 10.1128/aem.68.8.3878-3885.2002.

Corredor, J.E., Wilkinson, C.R., Vicente, V.P., Morell, J.M., and Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnol. Oceanogr.* 33, 114-120. doi: 10.4319/lo.1988.33.1.0114.

Costantino, V., Fattorusso, E., Imperatore, C., and Mangoni, A. (2005). Vesparioside from the Marine Sponge *Spheciospongia vesparia*, the First Diglycosylceramide with a Pentose Sugar Residue. *Eur. J. Org. Chem.* 2005, 368-373. doi: 10.1002/ejoc.200400543.

Costantino, V., Fattorusso, E., Imperatore, C., and Mangoni, A. (2008). Glycolipids from Sponges. 20. J-Coupling Analysis for Stereochemical Assignments in Furanosides: Structure Elucidation of Vesparioside B, a Glycosphingolipid from the Marine Sponge *Spheciospongia vesparia*. *The Journal of Organic Chemistry* 73, 6158-6165. doi: 10.1021/jo800837k.

Cox, R.J. (2007). Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. *Org. Biomol. Chem.* 5, 2010-2026. doi: 10.1039/B704420H.

Croué, J., West, N.J., Escande, M.-L., Intertaglia, L., Lebaron, P., and Suzuki, M.T. (2013). A single betaproteobacterium dominates the microbial community of the crambescidine-containing sponge Crambe crambe. *Sci. Rep.* 3, 2583. doi: 10.1038/srep02583.

Cuc, N.T., Anh, H.L.T., Hang, D.T.T., Nhiem, N.X., Dang, N.H., Nam, N.H., et al. (2015). Sesquiterpenes from the Vietnamese Marine Sponge *Dysidea fragilis*. *Nat. Prod. Commun.* 10, 2. doi:

Cuvelier, M.L., Blake, E., Mulheron, R., Mccarthy, P., Blackwelder, P., Vega Thurber, R.L., et al. (2014). Two distinct microbial communities revealed in the sponge *Cinachyrella*. *Frontiers in Microbiology* 5, 518. doi: 10.3389/fmicb.2014.00581.

D'onofrio, A., Crawford, J.M., Stewart, E.J., Witt, K., Gavrish, E., Epstein, S., et al. (2010). Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria. *Chem. Biol.* 17, 254-264. doi: 10.1016/j.chembiol.2010.02.010.

Dahihande, A.S., and Thakur, N.L. (2021). Differences in the Structural Components Influence the Pumping Capacity of Marine Sponges. *Frontiers in Marine Science* 8. doi: 10.3389/fmars.2021.671362.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., et al. (2015). Complete nitrification by Nitrospira bacteria. *Nature* 528, 504-509. doi: 10.1038/nature16461.

Dashti, Y., Grkovic, T., Abdelmohsen, U.R., Hentschel, U., and Quinn, R.J. (2014). Production of Induced Secondary Metabolites by a Co-Culture of Sponge-Associated Actinomycetes, *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163. *Mar. Drugs* 12, 3046-3059. doi: 10.3390/md12053046.

Dat, T.T., Cuc, N.T., Cuong, P.V., Smidt, H., and Sipkema, D. (2021a). Diversity and Antimicrobial Activity of Vietnamese Sponge-Associated Bacteria. *Mar. Drugs* 19, 353. doi: 10.3390/md19070353.

Dat, T.T.H., Steinert, G., Cuc, N.T.K., Smidt, H., and Sipkema, D. (2021b). Bacteria Cultivated From Sponges and Bacteria Not Yet Cultivated From Sponges—A Review. *Frontiers in Microbiology* 12, 3427. doi:

Dat, T.T.H., Steinert, G., Thi Kim Cuc, N., Smidt, H., and Sipkema, D. (2018). Archaeal and bacterial diversity and community composition from 18 phylogenetically divergent sponge species in Vietnam. *PeerJ* 6, e4970. doi: 10.7717/peerj.4970.

Davies, J., and Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* 74, 417-433. doi: 10.1128/MMBR.00016-10.

De Caralt, S., Uriz, M.J., and Wijffels, R.H. (2007). Vertical transmission and successive location of symbiotic bacteria during embryo development and larva formation in *Corticium candelabrum* (Porifera: Demospongiae). *J. Mar. Biol. Assoc. U.K.* 87, 1693-1699. doi: 10.1017/S0025315407056846.

De Goeij, J.M., Van Oevelen, D., Vermeij, M.J.A., Osinga, R., Middelburg, J.J., De Goeij, A.F.P.M., et al. (2013). Surviving in a Marine Desert: The Sponge Loop Retains Resources Within Coral Reefs. *Sci* 342, 108. doi: 10.1126/science.1241981.

De Oliveira, B.F.R., Freitas-Silva, J., Sánchez-Robinet, C., and Laport, M.S. (2020). Transmission of the sponge microbiome: moving towards a unified model. *Environ. Microbiol. Rep.* 12, 619-638. doi: 10.1111/1758-2229.12896.

De Queiroz, J.S., Griswold, D., Nguyen, D.T., and Hall, P. (2013). *Viet Nam tropical forest and biodiversity assessment.* Sun Mountain International and the Cadmus Group, Inc.

De Queiroz, K. (2007). Species Concepts and Species Delimitation. *Syst. Biol.* 56, 879-886. doi: 10.1080/10635150701701083.

De Rosa, S., Mitova, M., and Tommonaro, G. (2003). Marine bacteria associated with sponge as source of cyclic peptides. *Biomol. Eng.* 20, 311-316. doi: 10.1016/S1389-0344(03)00038-8.

De Voogd, N.J., Alvarez, B., Boury-Esnault, N., Carballo, J.L., Cárdenas, P., Díaz, M.-C., et al. (2022). "World Porifera Database".

Degnan, S.M. (2015). The surprisingly complex immune gene repertoire of a simple sponge, exemplified by the NLR genes: A capacity for specificity? *Dev. Comp. Immunol.* 48, 269-274. doi: 10.1016/j.dci.2014.07.012.

Degrassi, G., Aguilar, C., Bosco, M., Zahariev, S., Pongor, S., and Venturi, V. (2002). Plant Growth-Promoting Pseudomonas putida WCS358 Produces and Secretes Four Cyclic Dipeptides: Cross-Talk with Quorum Sensing Bacterial Sensors. *Curr. Microbiol.* 45, 250-254. doi: 10.1007/s00284-002-3704-y.

Desbois, A.P., and Smith, V.J. (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Appl. Microbiol. Biotechnol.* 85, 1629-1642. doi: 10.1007/s00253-009-2355-3.

Dharamshi, J.E., Gaarslev, N., Steffen, K., Martin, T., Sipkema, D., and Ettema, T.J.G. (2022). Genomic diversity and biosynthetic capabilities of sponge-associated chlamydiae. *The ISME Journal* 16, 2725-2740. doi: 10.1038/s41396-022-01305-9.

Dheman, N., Mahoney, N., Cox, E.M., Farley, J.J., Amini, T., and Lanthier, M.L. (2021). An Analysis of Antibacterial Drug Development Trends in the United States, 1980–2019. *Clin. Infect. Dis.* 73, e4444-e4450. doi: 10.1093/cid/ciaa859.

Dhingra, S., Rahman, N.a.A., Peile, E., Rahman, M., Sartelli, M., Hassali, M.A., et al. (2020). Microbial Resistance Movements: An Overview of Global Public Health Threats Posed by Antimicrobial Resistance, and How Best to Counter. *Frontiers in Public Health* 8, 535668. doi: 10.3389/fpubh.2020.535668.

Di, H.J., Cameron, K.C., Shen, J.-P., Winefield, C.S., O'callaghan, M., Bowatte, S., et al. (2010). Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiol. Ecol.* 72, 386-394. doi: 10.1111/j.1574-6941.2010.00861.x.

Diaz, M.C., and Ward, B.B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Mar. Ecol. Prog. Ser.* 156, 97-107. doi: 10.3354/meps156097.

Dickschat, J.S. (2016). Bacterial terpene cyclases. *Nat. Prod. Rep.* 33, 87-110. doi: 10.1039/C5NP00102A.

Dieckmann, R., Graeber, I., Kaesler, I., Szewzyk, U., and Von Döhren, H. (2005). Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by Intact-Cell-MALDI-TOF mass spectrometry (ICM-MS). *Appl. Microbiol. Biotechnol.* 67, 539-548. doi: 10.1007/s00253-004-1812-2.

Dieckmann, R., Helmuth, R., Erhard, M., and Malorny, B. (2008). Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl. Environ. Microbiol.* 74, 7767-7778. doi: 10.1128/AEM.01402-08.

Díez-Vives, C., Esteves, A.I.S., Costa, R., Nielsen, S., and Thomas, T. (2018). Detecting signatures of a sponge-associated lifestyle in bacterial genomes. *Environ. Microbiol. Rep.* 10, 433-443. doi: 10.1111/1758-2229.12655.

Díez-Vives, C., Koutsouveli, V., Conejero, M., and Riesgo, A. (2022). Global patterns in symbiont selection and transmission strategies in sponges. *Frontiers in Ecology and Evolution* 10, 1015592. doi: 10.3389/fevo.2022.1015592.

Do Cong, T. (2022). Research results of biological resources and and biodiversity of Vietnam sea: current status, treats, proposed solutions to use lasting. *Vietnam Journal of Marine Science and Technology* 22, 1-20. doi: 10.15625/1859-3097/17034.

Dobson, A.D.W., Jackson, S.A., Kennedy, J., Margassery, L.M., Flemer, B., O'leary, N., et al. (2015). "Marine Sponges – Molecular Biology and Biotechnology," in *Springer Handbook of Marine Biotechnology,* ed. S.-K. Kim. Berlin, Heidelberg: Springer Berlin Heidelberg, 219-254. doi: 10.1007/978-3-642-53971-8_8.

Doesburg, W., Eekert Miriam, H.A., Middeldorp Peter, J.M., Balk, M., Schraa, G., and Stams Alfons, J.M. (2006). Reductive dechlorination of β-hexachlorocyclohexane (β-HCH) by a Dehalobacter species in coculture with a Sedimentibacter sp. *FEMS Microbiol. Ecol.* 54, 87-95. doi: 10.1016/j.femsec.2005.03.003.

Donadio, S., Maffioli, S., Monciardini, P., Sosio, M., and Jabes, D. (2010). Antibiotic discovery in the twenty-first century: current trends and future perspectives. *The Journal of Antibiotics* 63, 423-430. doi: 10.1038/ja.2010.62.

Donald, L., Pipite, A., Subramani, R., Owen, J., Keyzers, R.A., and Taufa, T. (2022). Streptomyces: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. *Microbiology Research* 13, 418-465. doi: 10.3390/microbiolres13030031.

Dubourg, G., Lagier, J.C., Robert, C., Armougom, F., Hugon, P., Metidji, S., et al. (2014). Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics. *Int. J. Antimicrob. Agents* 44, 117-124. doi: 10.1016/j.ijantimicag.2014.04.020.

Dupont, S., Corre, E., Li, Y., Vacelet, J., and Bourguet-Kondracki, M.-L. (2013). First insights into the microbiome of a carnivorous sponge. *FEMS Microbiol. Ecol.* 86, 520-531. doi: 10.1111/1574-6941.12178.

Duris, Z., Horka, I., Juracka, P.J., Petrusek, A., and Sandford, F. (2011). These squatters are not innocent: the evidence of parasitism in sponge-inhabiting shrimps. *PLoS One* 6, e21987. doi: 10.1371/journal.pone.0021987.

Easson, C.G., and Thacker, R.W. (2014). Phylogenetic signal in the community structure of host-specific microbiomes of tropical marine sponges. *Frontiers in Microbiology* 5, 532. doi: 10.3389/fmicb.2014.00532.

Ebada, S.S., Lin, W., and Proksch, P. (2010). Bioactive Sesterterpenes and Triterpenes from Marine Sponges: Occurrence and Pharmacological Significance. *Mar. Drugs* 8, 313-346. doi: 10.3390/md8020313.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460-2461. doi: 10.1093/bioinformatics/btq461.

El-Demerdash, A., Moriou, C., Toullec, J., Besson, M., Soulet, S., Schmitt, N., et al. (2018). Bioactive Bromotyrosine-Derived Alkaloids from the Polynesian Sponge *Suberea ianthelliformis*. *Mar. Drugs* 16, 146. doi: 10.3390/md16050146.

El-Gendy, M.M.A., and El-Bondkly, A.M.A. (2010). Production and genetic improvement of a novel antimycotic agent, Saadamycin, against Dermatophytes and other clinical fungi from Endophytic *Streptomyces* sp. Hedaya. *J. Ind. Microbiol. Biotechnol.* 37, 831-841. doi: 10.1007/s10295-010-0729-2.

El-Sayed, A.K., Hothersall, J., Cooper, S.M., Stephens, E., Simpson, T.J., and Thomas, C.M. (2003). Characterization of the Mupirocin Biosynthesis Gene Cluster from *Pseudomonas fluorescens* NCIMB 10586. *Chem. Biol.* 10, 419-430. doi: 10.1016/S1074-5521(03)00091-7.

Eltamany, E.E., Ibrahim, A.K., Radwan, M.M., Elsohly, M.A., Hassanean, H.A., and Ahmed, S.A. (2015). Cytotoxic ceramides from the Red Sea sponge *Spheciospongia vagabunda*. *Med. Chem. Res.* 24, 3467-3473. doi: 10.1007/s00044-015-1394-9.

Emmett, J.S., Raj, K.D., Mathews, G., and Laju, R.L. (2021). Opportunistic spongivore fishes in a reef of Gulf of Mannar, India. *Environ. Biol. Fishes* 104, 1251-1262. doi: 10.1007/s10641-021-01150-3.

Engel, S., and Pawlik, J.R. (2000). Allelopathic activities of sponge extracts. *Mar. Ecol. Prog. Ser.* 207, 273-282. doi: 10.3354/meps207273.

Engelberts, J.P., Abdul Wahab, M.A., Maldonado, M., Rix, L., Marangon, E., Robbins, S.J., et al. (2022). Microbes from Mum: symbiont transmission in the tropical reef sponge *Ianthella basta*. *ISME Communications* 2, 90. doi: 10.1038/s43705-022-00173-w.

Engelberts, J.P., Robbins, S.J., De Goeij, J.M., Aranda, M., Bell, S.C., and Webster, N.S. (2020). Characterization of a sponge microbiome using an integrative genome-centric approach. *The ISME Journal* 14, 1100-1110. doi: 10.1038/s41396-020-0591-9.

Enticknap, J.J., Kelly, M., Peraud, O., and Hill, R.T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Appl Environ Microbiol* 72, 3724-3732. doi: 10.1128/AEM.72.5.3724-3732.2006.

Ereskovsky, A.V., and Dondua, A.K. (2006). The problem of germ layers in sponges (Porifera) and some issues concerning early metazoan evolution. *Zoologischer Anzeiger - A Journal of Comparative Zoology* 245, 65-76. doi: 10.1016/j.jcz.2006.04.002.

Ereskovsky, A.V., Gonobobleva, E., and Vishnyakov, A. (2005). Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). *Mar. Biol.* 146, 869-875. doi: 10.1007/s00227-004-1489-1.

Erpenbeck, D., Breeuwer, J.a.J., Parra-Velandia, F.J., and Van Soest, R.W.M. (2006). Speculation with spiculation?—Three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Mol. Phylogen. Evol.* 38, 293-305. doi: 10.1016/j.ympev.2005.11.001.

Erpenbeck, D., Duran, S., Rützler, K., Paul, V., Hooper, J.N.A., and Wörheide, G. (2007). Towards a DNA taxonomy of Caribbean demosponges: a gene tree reconstructed from partial mitochondrial CO1 gene sequences supports previous rDNA phylogenies and provides a new perspective on the systematics of Demospongiae. *J. Mar. Biol. Assoc. U.K.* 87, 1563-1570. doi: 10.1017/S0025315407058195.

Erpenbeck, D., Sutcliffe, P., Cook, S.D.C., Dietzel, A., Maldonado, M., Van Soest, R.W.M., et al. (2012). Horny sponges and their affairs: On the phylogenetic relationships of keratose sponges. *Mol. Phylogen. Evol.* 63, 809-816. doi: 10.1016/j.ympev.2012.02.024.

Erpenbeck, D., and Van Soest, R.W.M. (2007). Status and Perspective of Sponge Chemosystematics. *Mar. Biotechnol.* 9, 2-19. doi: 10.1007/s10126-005-6109-7.

Erwin, P.M., Coma, R., Lopez-Sendino, P., Serrano, E., and Ribes, M. (2015). Stable symbionts across the HMA-LMA dichotomy: low seasonal and interannual variation in sponge-associated bacteria from taxonomically diverse hosts. *FEMS Microbiol. Ecol.* 91, fiv115. doi: 10.1093/femsec/fiv115.

Erwin, P.M., Olson, J.B., and Thacker, R.W. (2011). Phylogenetic Diversity, Host-Specificity and Community Profiling of Sponge-Associated Bacteria in the Northern Gulf of Mexico. *PLoS One* 6, e26806. doi: 10.1371/journal.pone.0026806.

Erwin, P.M., Pita, L., López-Legentil, S., and Turon, X. (2012). Stability of Sponge-Associated Bacteria over Large Seasonal Shifts in Temperature and Irradiance. *Appl. Environ. Microbiol.* 78, 7358-7368. doi: 10.1128/AEM.02035-12.

Erwin, P.M., and Thacker, R.W. (2008). Phototrophic nutrition and symbiont diversity of two Caribbean sponge–cyanobacteria symbioses. *Mar. Ecol. Prog. Ser.* 362, 139-147. doi: 10.3354/meps07464.

Esteves, A.I., Amer, N., Nguyen, M., and Thomas, T. (2016). Sample Processing Impacts the Viability and Cultivability of the Sponge Microbiome. *Front Microbiol* 7, 499. doi: 10.3389/fmicb.2016.00499.

Eucast (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* 9, ix-xv. doi: 10.1046/j.1469-0691.2003.00790.x.

Fan, L., Reynolds, D., Liu, M., Stark, M., Kjelleberg, S., Webster, N.S., et al. (2012). Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proceedings of the National Academy of Sciences* 109, E1878-E1887. doi: 10.1073/pnas.1203287109.

Faulkner, D.J., Harper, M.K., Haygood, M.G., Salomon, C.E., and Schmidt, E.W. (2000). "Symbiotic Bacteria in Sponges: Sources of Bioactive Substances," in *Drugs from the Sea,* ed. N. Fusetani. Basel, Switzerland: Karger Publishers, 107-119. doi: 10.1159/000062486.

Fehmida, B., Muhammad, F., Esam, I.A., Muhammad, Y., Sana, A.A., Mohammad, A.K., et al. (2017). Bacteria From Marine Sponges: A Source of New Drugs. *Curr. Drug Metab.* 18, 11-15. doi: 10.2174/1389200217666161013090610.

Felnagle, E.A., Jackson, E.E., Chan, Y.A., Podevels, A.M., Berti, A.D., Mcmahon, M.D., et al. (2008). Nonribosomal Peptide Synthetases Involved in the Production of Medically Relevant Natural Products. *Mol. Pharm.* 5, 191-211. doi: 10.1021/mp700137g.

Feng, G., Sun, W., Zhang, F., Karthik, L., and Li, Z. (2016). Inhabitancy of active *Nitrosopumilus*-like ammonia-oxidizing archaea and *Nitrospira* nitrite-oxidizing bacteria in the sponge *Theonella swinhoei*. *Sci. Rep.* 6, 24966. doi: 10.1038/srep24966.

Fenical, W. (2006). Marine pharmaceuticals: Past, present, and future. *Oceanography* 19, 110-119. doi: 10.5670/oceanog.2006.74.

Ferrándiz, M.L., Sanz, M.J., Bustos, G., Payá, M., Alcaraz, M.J., and De Rosa, S. (1994). Avarol and avarone, two new anti-inflammatory agents of marine origin. *Eur. J. Pharmacol.* 253, 75-82. doi: 10.1016/0014-2999(94)90759-5.

Ferrari, B.C., and Gillings, M.R. (2009). Cultivation of Fastidious Bacteria by Viability Staining and Micromanipulation in a Soil Substrate Membrane System. *Appl. Environ. Microbiol.* 75, 3352-3354. doi: 10.1128/AEM.02407-08.

Feuda, R., Dohrmann, M., Pett, W., Philippe, H., Rota-Stabelli, O., Lartillot, N., et al. (2017). Improved Modeling of Compositional Heterogeneity Supports Sponges as Sister to All Other Animals. *Curr. Biol.* 27, 3864-3870.e3864. doi: 10.1016/j.cub.2017.11.008.

Fieseler, L., Hentschel, U., Grozdanov, L., Schirmer, A., Wen, G., Platzer, M., et al. (2007). Widespread Occurrence and Genomic Context of Unusually Small Polyketide Synthase Genes in Microbial Consortia Associated with Marine Sponges. *Appl. Environ. Microbiol.* 73, 2144-2155. doi: 10.1128/aem.02260-06.

Fieseler, L., Horn, M., Wagner, M., and Hentschel, U. (2004). Discovery of the Novel Candidate Phylum "Poribacteria" in Marine Sponges. *Appl. Environ. Microbiol.* 70, 3724-3732. doi: 10.1128/AEM.70.6.3724-3732.2004.

Fieth, R.A., Gauthier, M.-E.A., Bayes, J., Green, K.M., and Degnan, S.M. (2016). Ontogenetic Changes in the Bacterial Symbiont Community of the Tropical Demosponge Amphimedon queenslandica: Metamorphosis Is a New Beginning. *Frontiers in Marine Science* 3, 228. doi: 10.3389/fmars.2016.00228.

Finkelstein, R.A., and Lankford, C.E. (1957). A bacteriotoxic substance in autoclaved culture media containing glucose and phosphate. *ApMic* 5, 74-79. doi: 10.1128/am.5.2.74-79.1957.

Fiore, C.L., Labrie, M., Jarett, J.K., and Lesser, M.P. (2015). Transcriptional activity of the giant barrel sponge, Xestospongia muta Holobiont: molecular evidence for metabolic interchange. *Frontiers in Microbiology* 6, 364. doi: 10.3389/fmicb.2015.00364.

Fisch, K.M., Gurgui, C., Heycke, N., Van Der Sar, S.A., Anderson, S.A., Webb, V.L., et al. (2009). Polyketide assembly lines of uncultivated sponge symbionts from structure-based gene targeting. *Nat. Chem. Biol.* 5, 494-501. doi: 10.1038/nchembio.176.

Fjærvik, E., and Zotchev, S.B. (2005). Biosynthesis of the polyene macrolide antibiotic nystatin in *Streptomyces noursei*. *Appl. Microbiol. Biotechnol.* 67, 436-443. doi: 10.1007/s00253-004-1802-4.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *Br. J. Exp. Pathol.* 10, 226-236. doi:

Flowers, A.E., Garson, M.J., Webb, R.I., Dumdei, E.J., and Charan, R.D. (1998). Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). *Cell Tissue Res.* 292, 597-607. doi: 10.1007/s004410051089.

Fouad, M., Edrada, R.A., Ebel, R., Wray, V., Müller, W.E.G., Lin, W.H., et al. (2006). Cytotoxic Isomalabaricane Triterpenes from the Marine Sponge *Rhabdastrella globostellata*. *J. Nat. Prod.* 69, 211-218. doi: 10.1021/np050346t.

Freeman, C.J., Thacker, R.W., Baker, D.M., and Fogel, M.L. (2013). Quality or quantity: is nutrient transfer driven more by symbiont identity and productivity than by symbiont abundance? *The ISME Journal* 7, 1116-1125. doi: 10.1038/ismej.2013.7.

Freeman, M.F., Vagstad, A.L., and Piel, J. (2016). Polytheonamide biosynthesis showcasing the metabolic potential of sponge-associated uncultivated '*Entotheonella*' bacteria. *Curr. Opin. Chem. Biol.* 31, 8-14. doi: 10.1016/j.cbpa.2015.11.002.

Fuerst, J.A. (2014). Diversity and biotechnological potential of microorganisms associated with marine sponges. *Appl. Microbiol. Biotechnol.* 98, 7331-7347. doi: 10.1007/s00253-014-5861-x.

Fuqua, C., Parsek, M.R., and Greenberg, E.P. (2001). Regulation of Gene Expression by Cell-to-Cell Communication: Acyl-Homoserine Lactone Quorum Sensing. *Annu. Rev. Genet.* 35, 439-468. doi: 10.1146/annurev.genet.35.102401.090913.

Garland, J.L., Cook, K.L., Adams, J.L., and Kerkhof, L. (2001). Culturability as an Indicator of Succession in Microbial Communities. *MicEc* 42, 150-158. doi: 10.1007/s00248-001-0002-3.

Gauthier, M.-E.A., Watson, J.R., and Degnan, S.M. (2016). Draft Genomes Shed Light on the Dual Bacterial Symbiosis that Dominates the Microbiome of the Coral Reef Sponge *Amphimedon queenslandica*. *Frontiers in Marine Science* 3, 196. doi: 10.3389/fmars.2016.00196.

Gavriilidou, A., Gutleben, J., Versluis, D., Forgiarini, F., Van Passel, M.W.J., Ingham, C.J., et al. (2020). Comparative genomic analysis of Flavobacteriaceae: insights into carbohydrate metabolism, gliding motility and secondary metabolite biosynthesis. *BMC Genomics* 21, 569. doi: 10.1186/s12864-020-06971-7.

Gavriilidou, A., Mackenzie, T.A., Sánchez, P., Tormo, J.R., Ingham, C., Smidt, H., et al. (2021). Bioactivity Screening and Gene-Trait Matching across Marine Sponge-Associated Bacteria. *Mar. Drugs* 19, 75. doi: 10.3390/md19020075.

Gavrish, E., Bollmann, A., Epstein, S., and Lewis, K. (2008). A trap for in situ cultivation of filamentous actinobacteria. *J. Microbiol. Methods* 72, 257-262. doi: 10.1016/j.mimet.2007.12.009.

Gazave, E., Lapébie, P., Ereskovsky, A.V., Vacelet, J., Renard, E., Cárdenas, P., et al. (2012). No longer Demospongiae: Homoscleromorpha formal nomination as a fourth class of Porifera. *Hydrobiologia* 687, 3-10. doi: 10.1007/s10750-011-0842-x.

Geller, J., Meyer, C., Parker, M., and Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Mol. Ecol. Resour.* 13, 851-861. doi: 10.1111/1755-0998.12138.

Gerardo Toledo, Wayne Green, Ricardo A. Gonzalez, Leif Christoffersen, Mircea Podar, Hwai W. Chang, et al. (2012). Case Study High Throughput Cultivation for Isolation of Novel Marine Microorganisms. *Oceanography* 19, 120-125. doi: 10.5670/oceanog.2006.75.

Ghareeb, M.A., Tammam, M.A., El-Demerdash, A., and Atanasov, A.G. (2020). Insights about clinically approved and Preclinically investigated marine natural products. *Current Research in Biotechnology* 2, 88-102. doi: 10.1016/j.crbiot.2020.09.001.

Ghobrial, O., Derendorf, H., and Hillman, J.D. (2010). Pharmacokinetic and pharmacodynamic evaluation of the lantibiotic MU1140. *J. Pharm. Sci.* 99, 2521-2528. doi: 10.1002/jps.22015.

Ghurye, J.S., Cepeda-Espinoza, V., and Pop, M. (2016). Metagenomic Assembly: Overview, Challenges and Applications. *Yale J Biol Med* 89, 353-362. doi:

Giles, E.C., Kamke, J., Moitinho-Silva, L., Taylor, M.W., Hentschel, U., Ravasi, T., et al. (2013). Bacterial community profiles in low microbial abundance sponges. *FEMS Microbiol. Ecol.* 83, 232-241. doi: 10.1111/j.1574-6941.2012.01467.x.

Glasl, B., Webster, N.S., and Bourne, D.G. (2017). Microbial indicators as a diagnostic tool for assessing water quality and climate stress in coral reef ecosystems. *Mar. Biol.* 164, 91. doi: 10.1007/s00227-017-3097-x.

Gloeckner, V., Wehrl, M., Moitinho-Silva, L., Gernert, C., Schupp, P., Pawlik, J.R., et al. (2014). The HMA-LMA Dichotomy Revisited: an Electron Microscopical Survey of 56 Sponge Species *Biol. Bull.* 227, 78–88. doi: 10.1086/BBLv227n1p78.

Godefroy, N., Le Goff, E., Martinand-Mari, C., Belkhir, K., Vacelet, J., and Baghdiguian, S. (2019). Sponge digestive system diversity and evolution: filter feeding to carnivory. *Cell Tissue Res.* 377, 341-351. doi: 10.1007/s00441-019-03032-8.

Gold, D.A., Grabenstatter, J., De Mendoza, A., Riesgo, A., Ruiz-Trillo, I., and Summons, R.E. (2016). Sterol and genomic analyses validate the sponge biomarker hypothesis. *Proceedings of the National Academy of Sciences* 113, 2684-2689. doi: 10.1073/pnas.1512614113.

Gomes, E.S., Schuch, V., and De Macedo Lemos, E.G. (2013). Biotechnology of polyketides: new breath of life for the novel antibiotic genetic pathways discovery through metagenomics. *Braz J Microbiol* 44, 1007-1034. doi: 10.1590/s1517-83822013000400002.

González, J.E., and Keshavan, N.D. (2006). Messing with Bacterial Quorum Sensing. *Microbiol. Mol. Biol. Rev.* 70, 859. doi: 10.1128/MMBR.00002-06.

Goud, T.V., Krishnaiah, P., Reddy, S.M., Srinivasulu, M., Rao, M.R., and Venkateswarlu, Y. (2005). Chemical investigation of the marine sponges *Clathria reinwardti* and *Haliclona cribricutis*. *Indian J. Chem.*, 607-610.

Grasemann, H., Stehling, F., Brunar, H., Widmann, R., Laliberte, T.W., Molina, L., et al. (2007). Inhalation of Moli1901 in Patients With Cystic Fibrosis. *Chest* 131, 1461-1466. doi: 10.1378/chest.06-2085.

Griffiths, S.M., Antwis, R.E., Lenzi, L., Lucaci, A., Behringer, D.C., Butler Iv, M.J., et al. (2019). Host genetics and geography influence microbiome composition in the sponge *Ircinia campana*. *J. Anim. Ecol.* 88, 1684-1695. doi: 10.1111/1365-2656.13065.

Gross, H., and König, G.M. (2006). Terpenoids from Marine Organisms: Unique Structures and their Pharmacological Potential. *Phytochem. Rev.* 5, 115-141. doi: 10.1007/s11101-005-5464-3.

Grubbs, K.J., Bleich, R.M., Santa Maria, K.C., Allen, S.E., Farag, S., Agbiome, T., et al. (2017). Large-Scale Bioinformatics Analysis of *Bacillus* Genomes Uncovers Conserved Roles of Natural Products in Bacterial Physiology. *mSystems* 2, e00040-00017. doi: 10.1128/mSystems.00040-17.

Guerrero-Garzón, J.F., Zehl, M., Schneider, O., Rückert, C., Busche, T., Kalinowski, J., et al. (2020). *Streptomyces* spp. From the Marine Sponge Antho dichotoma: Analyses of Secondary Metabolite Biosynthesis Gene Clusters and Some of Their Products. *Frontiers in Microbiology* 11, 437. doi: 10.3389/fmicb.2020.00437.

Gunasekera, S., Abraham, S., Stegger, M., Pang, S., Wang, P., Sahibzada, S., et al. (2021). Evaluating coverage bias in next-generation sequencing of *Escherichia coli*. *PLoS One* 16, e0253440. doi: 10.1371/journal.pone.0253440.

Gutleben, J. (2019). *Ecological and biotechnological aspects of Aplysina-associated microorganisms*. PhD, Wageningen University, Wageningen.

Gutleben, J., Chaib De Mares, M., Van Elsas, J.D., Smidt, H., Overmann, J., and Sipkema, D. (2018). The multi-omics promise in context: from sequence to microbial isolate. *Crit Rev Microbiol* 44, 212-229. doi: 10.1080/1040841X.2017.1332003.

Gutleben, J., Loureiro, C., Ramírez Romero, L.A., Shetty, S., Wijffels, R.H., Smidt, H., et al. (2020). Cultivation of Bacteria From *Aplysina aerophoba*: Effects of Oxygen and Nutrient Gradients. *Frontiers in microbiology* 11, 175-175. doi: 10.3389/fmicb.2020.00175.

Haft, D.H., Selengut, J., Mongodin, E.F., and Nelson, K.E. (2005). A Guild of 45 CRISPR-Associated (Cas) Protein Families and Multiple CRISPR/Cas Subtypes Exist in Prokaryotic Genomes. *PLoS Comp. Biol.* 1, e60. doi: 10.1371/journal.pcbi.0010060.

Halanych, K.M. (2004). The New View of Animal Phylogeny. *Annu. Rev. Ecol. Evol. Syst.* 35, 229-256. doi: 10.1146/annurev.ecolsys.35.112202.130124.

Hallam, S.J., Konstantinidis, K.T., Putnam, N., Schleper, C., Watanabe, Y.-I., Sugahara, J., et al. (2006). Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. *Proc. Natl. Acad. Sci. U. S. A.* 103, 18296-18301. doi: 10.1073/pnas.0608549103.

Han, B.-N., Hong, L.-L., Gu, B.-B., Sun, Y.-T., Wang, J., Liu, J.-T., et al. (2019). "Natural Products from Sponges," in *Symbiotic Microbiomes of Coral Reefs Sponges and Corals,* ed. Z. Li. Dordrecht: Springer Netherlands), 329-463. doi: 10.1007/978-94-024-1612-1_15.

Hardoim, C.C.P., Cardinale, M., Cúcio, A.C.B., Esteves, A.I.S., Berg, G., Xavier, J.R., et al. (2014). Effects of sample handling and cultivation bias on the specificity of bacterial communities in keratose marine sponges. *Frontiers in microbiology* 5, 611-611. doi: 10.3389/fmicb.2014.00611.

Hardoim, C.C.P., and Costa, R. (2014). Microbial Communities and Bioactive Compounds in Marine Sponges of the Family Irciniidae—A Review. *Mar. Drugs* 12, 5089-5122. doi: 10.3390/md12105089.

Harjes, J., Ryu, T., Abdelmohsen Usama, R., Moitinho-Silva, L., Horn, H., Ravasi, T., et al. (2014). Draft Genome Sequence of the Antitrypanosomally Active Sponge-Associated Bacterium *Actinokineospora* sp. Strain EG49. *Genome Announcements* 2, e00160-00114. doi: 10.1128/genomeA.00160-14.

Harris, J.E. (1985). GELRITE as an Agar Substitute for the Cultivation of Mesophilic *Methanobacterium* and *Methanobrevibacter* Species. *Appl. Environ. Microbiol.* 50, 1107-1109. doi: 10.1128/aem.50.4.1107-1109.1985.

Harwood, C.R., Mouillon, J.-M., Pohl, S., and Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiol. Rev.* 42, 721-738. doi: 10.1093/femsre/fuy028.

Hatzenpichler, R. (2012). Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Appl Environ Microbiol* 78, 7501-7510. doi: 10.1128/AEM.01960-12.

Hayashi, K., Hamada, Y., and Shioiri, T. (1992). Synthesis of nazumamide A, a thrombin-inhibitory linear tetrapeptide, from a marine sponge, *Theonella* sp. *Tetrahedron Lett.* 33, 5075-5076. doi: 10.1016/S0040-4039(00)61193-0.

Haygood, M.G., Schmidt Ew Fau - Davidson, S.K., Davidson Sk Fau - Faulkner, D.J., and Faulkner, D.J. (1999). Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. *J Mol Microbiol Biotechnol* 1, 33-43.

Helber, S.B., Hoeijmakers, D.J.J., Muhando, C.A., Rohde, S., and Schupp, P.J. (2018). Sponge chemical defenses are a possible mechanism for increasing sponge abundance on reefs in Zanzibar. *PLoS One* 13, e0197617. doi: 10.1371/journal.pone.0197617.

Helfrich, E.J.N., and Piel, J. (2016). Biosynthesis of polyketides by trans-AT polyketide synthases. *Nat. Prod. Rep.* 33, 231-316. doi: 10.1039/C5NP00125K.

Helfrich, E.J.N., Ueoka, R., Chevrette, M.G., Hemmerling, F., Lu, X., Leopold-Messer, S., et al. (2021). Evolution of combinatorial diversity in trans-acyltransferase polyketide synthase assembly lines across bacteria. *Nature Communications* 12, 1422. doi: 10.1038/s41467-021-21163-x.

Hentschel, U., Hopke, J., Horn, M., Friedrich, A.B., Wagner, M., Hacker, J., et al. (2002). Molecular Evidence for a Uniform Microbial Community in Sponges from Different Oceans. *Appl. Environ. Microbiol.* 68, 4431-4440. doi: 10.1128/AEM.68.9.4431-4440.2002.

Hentschel, U., Piel, J., Degnan, S.M., and Taylor, M.W. (2012). Genomic insights into the marine sponge microbiome. *Nat. Rev. Microbiol.* 10, 641-654. doi: 10.1038/nrmicro283.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., and Hacker, J. (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol. Ecol.* 35, 305-312. doi: 10.1111/j.1574-6941.2001.tb00816.x.

Hentschel, U., Usher, K.M., and Taylor, M.W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiol. Ecol.* 55, 167-177. doi: 10.1111/j.1574-6941.2005.00046.x.

Herfort, L., Schouten, S., Abbas, B., Veldhuis, M.J.W., Coolen, M.J.L., Wuchter, C., et al. (2007). Variations in spatial and temporal distribution of Archaea in the North Sea in relation to environmental variables. *FEMS Microbiol. Ecol.* 62, 242-257. doi: 10.1111/j.1574-6941.2007.00397.x.

Hernández-González, I.L., Moreno-Hagelsieb, G., and Olmedo-Álvarez, G. (2018). Environmentally-driven gene content convergence and the *Bacillus* phylogeny. *BMC Evol. Biol.* 18, 148. doi: 10.1186/s12862-018-1261-7.

Hertweck, C. (2009). The Biosynthetic Logic of Polyketide Diversity. *Angew. Chem. Int. Ed.* 48, 4688-4716. doi: 10.1002/anie.200806121.

Hertweck, C., Luzhetskyy, A., Rebets, Y., and Bechthold, A. (2007). Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. *Nat. Prod. Rep.* 24, 162-190. doi: 10.1039/B507395M.

Hess Donavon, J., Henry-Stanley Michelle, J., and Wells Carol, L. (2014). Antibacterial Synergy of Glycerol Monolaurate and Aminoglycosides in *Staphylococcus aureus* Biofilms. *Antimicrob. Agents Chemother.* 58, 6970-6973. doi: 10.1128/AAC.03672-14.

Hestetun, J.T., Tompkins-Macdonald, G., and Rapp, H.T. (2017). A review of carnivorous sponges (Porifera: Cladorhizidae) from the Boreal North Atlantic and Arctic. *Zool. J. Linn. Soc.* 181, 1-69. doi: 10.1093/zoolinnean/zlw022.

Hifnawy, M.S., Hassan, H.M., Mohammed, R., Fouda, M.M., Sayed, A.M., Hamed, A.A., et al. (2020). Induction of Antibacterial Metabolites by Co-Cultivation of Two Red-Sea-Sponge-Associated Actinomycetes *Micromonospora* sp. UR56 and *Actinokinespora* sp. EG49. *Mar. Drugs* 18, 243. doi: 10.3390/md18050243.

Hildebrand, M., Waggoner, L.E., Lim, G.E., Sharp, K.H., Ridley, C.P., and Haygood, M.G. (2004). Approaches to identify, clone, and express symbiont bioactive metabolite genes. *Nat. Prod. Rep.* 21, 122-142. doi: 10.1039/B302336M.

Hill, M.S., and Hill, A.L. (2002). Morphological Plasticity in the Tropical Sponge *Anthosigmella varians*: Responses to Predators and Wave Energy. *The Biological Bulletin* 202, 86-95. doi: 10.2307/1543225.

Hirashima, M., Tsuda, K., Hamada, T., Okamura, H., Furukawa, T., Akiyama, S.-I., et al. (2010). Cytotoxic Isomalabaricane Derivatives and a Monocyclic Triterpene Glycoside from the Sponge *Rhabdastrella globostellata*. *J. Nat. Prod.* 73, 1512-1518. doi: 10.1021/np100302a.

Hirata, Y., and Uemura, D. (1986). Halichondrins - antitumor polyether macrolides from a marine sponge. 58, 701-710. doi: doi:10.1351/pac198658050701.

Hochmuth, T., Niederkrüger, H., Gernert, C., Siegl, A., Taudien, S., Platzer, M., et al. (2010). Linking Chemical and Microbial Diversity in Marine Sponges: Possible Role for Poribacteria as Producers of Methyl-Branched Fatty Acids. *ChemBioChem* 11, 2572-2578. doi: 10.1002/cbic.201000510.

Hoffmann, F., Larsen, O., Thiel, V., Rapp, H.T., Pape, T., Michaelis, W., et al. (2005). An Anaerobic World in Sponges. *GeomJ* 22, 1-10. doi: 10.1080/01490450590922505.

Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H.T., et al. (2009). Complex nitrogen cycling in the sponge *Geodia barretti*. *Environ. Microbiol.* 11, 2228-2243. doi: 10.1111/j.1462-2920.2009.01944.x.

Hoffmann, F., Røy, H., Bayer, K., Hentschel, U., Pfannkuchen, M., Brümmer, F., et al. (2008). Oxygen dynamics and transport in the Mediterranean sponge *Aplysina aerophoba*. *Mar. Biol.* 153, 1257-1264. doi: 10.1007/s00227-008-0905-3.

Holden, M.T.G., Ram Chhabra, S., De Nys, R., Stead, P., Bainton, N.J., Hill, P.J., et al. (1999). Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Mol. Microbiol.* 33, 1254-1266. doi: 10.1046/j.1365-2958.1999.01577.x.

Holmes, K.K., Bertozzi, S., Bloom, B.R., Jha, P., Gelband, H., Demaria, L.M., et al. (2017). "Major Infectious Diseases: Key Messages from Disease Control Priorities, Third Edition," in *Disease Control Priorities, Third Edition (Volume 6): Major Infectious Diseases*. The World Bank, 1-27. doi: 10.1596/978-1-4648-0524-0_ch1.

Hong, J.-K., and Cho, J.-C. (2015). Environmental Variables Shaping the Ecological Niche of *Thaumarchaeota* in Soil: Direct and Indirect Causal Effects. *PLoS One* 10, e0133763. doi: 10.1371/journal.pone.0133763.

Hong, L.-L., Ding, Y.-F., Zhang, W., and Lin, H.-W. (2022). Chemical and biological diversity of new natural products from marine sponges: a review (2009–2018). *Marine Life Science & Technology* 4, 356-372. doi: 10.1007/s42995-022-00132-3.

Hooper, J.N.A., and Van Soest, R.W.M. (2002). "Systema Porifera. A Guide to the Classification of Sponges," in *Systema Porifera: A Guide to the Classification of Sponges,* eds. J.N.A. Hooper, R.W.M. Van Soest & P. Willenz. Boston, MA: Springer US, 1-7. doi: 10.1007/978-1-4615-0747-5_1.

Hooper, J.N.A., Van Soest, R.W.M., and Debrenne, F. (2002). "Phylum Porifera Grant, 1836," in *Systema Porifera: A Guide to the Classification of Sponges,* eds. J.N.A. Hooper, R.W.M. Van Soest & P. Willenz. Boston, MA: Springer US, 9-13. doi: 10.1007/978-1-4615-0747-5_2.

Horn, H., Slaby, B.M., Jahn, M.T., Bayer, K., Moitinho-Silva, L., Förster, F., et al. (2016). An Enrichment of CRISPR and Other Defense-Related Features in Marine Sponge-Associated Microbial Metagenomes. *Frontiers in Microbiology* 7, 1751. doi: 10.3389/fmicb.2016.01751.

Hu, Y., Chen, J., Hu, G., Yu, J., Zhu, X., Lin, Y., et al. (2015). Statistical Research on the Bioactivity of New Marine Natural Products Discovered during the 28 Years from 1985 to 2012. *Mar. Drugs* 13, 202-221. doi: 10.3390/md13010202.

Huang, J., Xin, Y., Cao, X., and Zhang, W. (2011). Phylogenetic diversity and characterization of 2-haloacid degrading bacteria from the marine sponge *Hymeniacidon perlevis*. *World J. Microbiol. Biotechnol.* 27, 1787-1794. doi: 10.1007/s11274-010-0636-8.

Huayue, L., Byung Cheol, L., Tae Sung, K., Kyung Sook, B., Jongki, H., Sang Ho, C., et al. (2008). Bioactive Cyclic Dipeptides from a Marine Sponge-Associated Bacterium, *Psychrobacter* sp. *Biomol. Ther. (Seoul)* 16, 356-363. doi: 10.4062/biomolther.2008.16.4.356.

Hughes, C.C., and Fenical, W. (2010). Antibacterials from the sea. *Chemistry* 16, 12512-12525. doi: 10.1002/chem.201001279.

Huong, T.V., Bat, N.K., Hieu, N.V., Thien, N.H., and Dat, D.T. (2020). Porifera composition, distribution in costal regions in Con Co island, Quang Tri province. *Can Tho University Journal of Science* 56, 75-85. doi: 10.22144/ ctu.jvn.200.008.

Hutchings, M.I., Truman, A.W., and Wilkinson, B. (2019). Antibiotics: past, present and future. *Curr. Opin. Microbiol.* 51, 72-80. doi: 10.1016/j.mib.2019.10.008.

Hyatt, D., Chen, G.-L., Locascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11, 119. doi: 10.1186/1471-2105-11-119.

Ian, E., Malko, D.B., Sekurova, O.N., Bredholt, H., Rückert, C., Borisova, M.E., et al. (2014). Genomics of Sponge-Associated *Streptomyces* spp. Closely Related to *Streptomyces albus* J1074: Insights into Marine Adaptation and Secondary Metabolite Biosynthesis Potential. *PLoS One* 9, e96719. doi: 10.1371/journal.pone.0096719.

Indraningrat, A.A., Smidt, H., and Sipkema, D. (2016). Bioprospecting Sponge-Associated Microbes for Antimicrobial Compounds. *Mar. Drugs* 14, 87. doi: 10.3390/md14050087.

Indraningrat, A.a.G., Micheller, S., Runderkamp, M., Sauerland, I., Becking, L.E., Smidt, H., et al. (2019). Cultivation of Sponge-Associated Bacteria from *Agelas sventres* and *Xestospongia muta* Collected from Different Depths. *Mar. Drugs* 17, 578. doi: 10.3390/md17100578.

Ishii, S., Tago, K., and Senoo, K. (2010). Single-cell analysis and isolation for microbiology and biotechnology: methods and applications. *Appl. Microbiol. Biotechnol.* 86, 1281-1292. doi: 10.1007/s00253-010-2524-4.

Ishikawa, M., Nakajima, K., Yanagi, M., Yamamoto, Y., and Yamasato, K. (2003). *Marinilactibacillus psychrotolerans gen. nov., sp. nov.*, a halophilic and alkaliphilic marine lactic acid bacterium isolated

from marine organisms in temperate and subtropical areas of Japan. *Int. J. Syst. Evol. Microbiol.* 53, 711-720. doi: doi:10.1099/ijs.0.02446-0.

Isobe, K., Koba, K., Suwa, Y., Ikutani, J., Fang, Y., Yoh, M., et al. (2012). High abundance of ammonia-oxidizing archaea in acidified subtropical forest soils in southern China after long-term N deposition. *FEMS Microbiol. Ecol.* 80, 193-203. doi: 10.1111/j.1574-6941.2011.01294.x.

Izumi, H., Sagulenko, E., Webb, R.I., and Fuerst, J.A. (2013). Isolation and diversity of planctomycetes from the sponge *Niphates* sp., seawater, and sediment of Moreton Bay, Australia. *Antonie Van Leeuwenhoek* 104, 533-546. doi: 10.1007/s10482-013-0003-5.

Jabés, D., Brunati, C., Candiani, G., Riva, S., Romanó, G., and Donadio, S. (2011). Efficacy of the New Lantibiotic NAI-107 in Experimental Infections Induced by Multidrug-Resistant Gram-Positive Pathogens. *Antimicrob. Agents Chemother.* 55, 1671-1676. doi: 10.1128/AAC.01288-10.

Jackson, S.A., Crossman, L., Almeida, E.L., Margassery, L.M., Kennedy, J., and Dobson, A.D.W. (2018). Diverse and Abundant Secondary Metabolism Biosynthetic Gene Clusters in the Genomes of Marine Sponge Derived Streptomyces spp. Isolates. *Mar. Drugs* 16, 67. doi: 10.3390/md16020067.

Jackson, S.A., Flemer, B., Mccann, A., Kennedy, J., Morrissey, J.P., O'gara, F., et al. (2013). Archaea appear to dominate the microbiome of *Inflatella pellicula* deep sea sponges. *PLoS One* 8, e84438-e84438. doi: 10.1371/journal.pone.0084438.

Jahn, M.T., Markert, S.M., Ryu, T., Ravasi, T., Stigloher, C., Hentschel, U., et al. (2016). Shedding light on cell compartmentation in the candidate phylum Poribacteria by high resolution visualisation and transcriptional profiling. *Sci. Rep.* 6, 35860. doi: 10.1038/srep35860.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Appl. Environ. Microbiol.* 68, 2391-2396. doi: 10.1128/aem.68.5.2391-2396.2002.

Jasmin, C., Anas, A., and Nair, S. (2015). Bacterial Diversity Associated with *Cinachyra cavernosa* and *Haliclona pigmentifera*, Cohabiting Sponges in the Coral Reef Ecosystem of Gulf of Mannar, Southeast Coast of India. *PLoS One* 10, e0123222. doi: 10.1371/journal.pone.0123222.

Jaspars, M., De Pascale, D., Andersen, J.H., Reyes, F., Crawford, A.D., and Ianora, A. (2016). The marine biodiscovery pipeline and ocean medicines of tomorrow. *J. Mar. Biol. Assoc. U.K.* 96, 151-158. doi: 10.1017/S0025315415002106.

Jayatilake, G.S., Thornton, M.P., Leonard, A.C., Grimwade, J.E., and Baker, B.J. (1996). Metabolites from an Antarctic Sponge-Associated Bacterium, Pseudomonas aeruginosa. *J. Nat. Prod.* 59, 293-296. doi: 10.1021/np960095b.

Jenner, R.A. (2004). When molecules and morphology clash: reconciling conflicting phylogenies of the Metazoa by considering secondary character loss. *Evol Dev* 6, 372-378. doi: 10.1111/j.1525-142X.2004.04045.x.

Jensen, S., Fortunato, S.a.V., Hoffmann, F., Hoem, S., Rapp, H.T., Øvreås, L., et al. (2017). The Relative Abundance and Transcriptional Activity of Marine Sponge-Associated Microorganisms Emphasizing Groups Involved in Sulfur Cycle. *MicEc* 73, 668-676. doi: 10.1007/s00248-016-0836-3.

Jiang, C.-Y., Dong, L., Zhao, J.-K., Hu, X., Shen, C., Qiao, Y., et al. (2016). High-Throughput Single-Cell Cultivation on Microfluidic Streak Plates. *Appl. Environ. Microbiol.* 82, 2210. doi: 10.1128/AEM.03588-15.

Jiménez, E., and Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnol. Oceanogr.* 52, 948-958. doi: 10.4319/lo.2007.52.3.0948.

Joachimiak, M.P., Weisman, J.L., and May, B.C.H. (2006). JColorGrid: software for the visualization of biological measurements. *BMC Bioinformatics* 7, 225. doi: 10.1186/1471-2105-7-225.

Jones, A.C., Blum, J.E., and Pawlik, J.R. (2005). Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *J. Exp. Mar. Biol. Ecol.* 322, 67-81. doi: 10.1016/j.jembe.2005.02.009.

Juliana, F.S.-G., Marcia, G.-D., Walter, M.R.O., and Marinella, S.L. (2014). Biotechnological Potential of Sponge-Associated Bacteria. *Curr. Pharm. Biotechnol.* 15, 143-155. doi: 10.2174/1389201015666140711115033.

Jung, D., Machida, K., Nakao, Y., Kindaichi, T., Ohashi, A., and Aoi, Y. (2021). Triggering Growth via Growth Initiation Factors in Nature: A Putative Mechanism for in situ Cultivation of Previously Uncultivated Microorganisms. *Frontiers in Microbiology* 12, 537194. doi: 10.3389/fmicb.2021.537194.

Jung, D., Machida, K., Nakao, Y., Owen, J.S., He, S., Kindaichi, T., et al. (2022). Cultivation of previously uncultured sponge-associated bacteria using advanced cultivation techniques: A perspective on possible key mechanisms. *Frontiers in Marine Science* 9, 963277. doi: 10.3389/fmars.2022.963277.

Jung, D., Seo, E.Y., Epstein Slava, S., Joung, Y., Han, J., Parfenova Valentina, V., et al. (2014). Application of a new cultivation technology, I-tip, for studying microbial diversity in freshwater sponges of Lake Baikal, Russia. *FEMS Microbiol. Ecol.* 90, 417-423. doi: 10.1111/1574-6941.12399.

Kaboré, O.D., Godreuil, S., and Drancourt, M. (2019). Improved culture of fastidious *Gemmata* spp. bacteria using marine sponge skeletons. *Sci. Rep.* 9, 11707. doi: 10.1038/s41598-019-48293-z.

Kamke, J., Sczyrba, A., Ivanova, N., Schwientek, P., Rinke, C., Mavromatis, K., et al. (2013). Single-cell genomics reveals complex carbohydrate degradation patterns in poribacterial symbionts of marine sponges. *The ISME Journal* 7, 2287-2300. doi: 10.1038/ismej.2013.111.

Kanoh, S., and Rubin, B.K. (2010). Mechanisms of action and clinical application of macrolides as immunomodulatory medications. *Clin. Microbiol. Rev.* 23, 590-615. doi: 10.1128/CMR.00078-09.

Karimi, E., Keller-Costa, T., Slaby, B.M., Cox, C.J., Da Rocha, U.N., Hentschel, U., et al. (2019). Genomic blueprints of sponge-prokaryote symbiosis are shared by low abundant and cultivatable *Alphaproteobacteria*. *Sci. Rep.* 9, 1999. doi: 10.1038/s41598-019-38737-x.

Karimi, E., Ramos, M., Gonçalves, J.M.S., Xavier, J.R., Reis, M.P., and Costa, R. (2017). Comparative Metagenomics Reveals the Distinctive Adaptive Features of the *Spongia officinalis* Endosymbiotic Consortium. *Frontiers in Microbiology* 8, 2499. doi: 10.3389/fmicb.2017.02499.

Karimi, E., Slaby, B.M., Soares, A.R., Blom, J., Hentschel, U., and Costa, R. (2018). Metagenomic binning reveals versatile nutrient cycling and distinct adaptive features in alphaproteobacterial symbionts of marine sponges. *FEMS Microbiol. Ecol.* 94, fiy074. doi: 10.1093/femsec/fiy074.

Karpiński, T.M. (2019). Marine Macrolides with Antibacterial and/or Antifungal Activity. *Mar. Drugs* 17, 241. doi: 10.3390/md17040241.

Kashman, Y., Groweiss, A., and Shmueli, U. (1980). Latrunculin, a new 2-thiazolidinone macrolide from the marine sponge *Latrunculia magnifica*. *Tetrahedron Lett.* 21, 3629-3632. doi: 10.1016/0040-4039(80)80255-3.

Kataoka, N., Tokiwa, Y., Tanaka, Y., Takeda, K., and Suzuki, T. (1996). Enrichment culture and isolation of slow-growing bacteria. *Appl. Microbiol. Biotechnol.* 45, 771-777. doi: 10.1007/s002530050761.

Katoh, K., and Standley, D.M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* 30, 772-780. doi: 10.1093/molbev/mst010.

Katz, L., and Baltz, R.H. (2016). Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* 43, 155-176. doi: 10.1007/s10295-015-1723-5.

Kawasaki, K., and Kamagata, Y. (2017). Phosphate-Catalyzed Hydrogen Peroxide Formation from Agar, Gellan, and κ-Carrageenan and Recovery of Microbial Cultivability via Catalase and Pyruvate. *Appl. Environ. Microbiol.* 83, e01366-01317. doi: 10.1128/AEM.01366-17.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647-1649. doi: 10.1093/bioinformatics/bts199.

Kennedy, J., Flemer, B., Jackson, S.A., Morrissey, J.P., O'gara, F., and Dobson, A.D.W. (2014). Evidence of a Putative Deep Sea Specific Microbiome in Marine Sponges. *PLoS One* 9, e91092. doi: 10.1371/journal.pone.0091092.

Keren, R., Lavy, A., Mayzel, B., and Ilan, M. (2015). Culturable associated-bacteria of the sponge *Theonella swinhoei* show tolerance to high arsenic concentrations. *Front Microbiol* 6, 154. doi: 10.3389/fmicb.2015.00154.

Khalifa, S.a.M., Elias, N., Farag, M.A., Chen, L., Saeed, A., Hegazy, M.-E.F., et al. (2019). Marine Natural Products: A Source of Novel Anticancer Drugs. *Mar. Drugs* 17, 419. doi: 10.3390/md17090491.

Khan, S.T., Musarrat, J., Alkhedhairy, A.A., and Kazuo, S. (2013). Diversity of bacteria and polyketide synthase associated with marine sponge *Haliclona* sp. *Annals of Microbiology* 64, 199-207. doi: 10.1007/s13213-013-0652-7.

Kiem, P.V., Minh, C.V., Nhiem, N.X., Cuc, N.T., Quang, N.V., Tuan Anh, H.L., et al. (2014). Muurolane-type sesquiterpenes from marine sponge *Dysidea cinerea*. *Magn. Reson. Chem.* 52, 51-56. doi: 10.1002/mrc.4030.

Kiem, P.V., Nhiem, N.X., Tai, B.H., Anh, H.L.T., Hang, D.T.T., Cuc, N.T., et al. (2016). Bis-sesquiterpene from the Marine Sponge *Dysidea fragilis*. *Nat. Prod. Commun.* 11, 3. doi: 10.1177/1934578X1601100403.

Kiem, P.V., Van Minh, C., Nhiem, N.X., Tai, B.H., Yen, P.H., Hang, D.T.T., et al. (2019). The chemical constituents and biological activity of some sponges in Northern Vietnam: A review. *Vietnam Journal of Chemistry* 57, 261-271. doi: 10.1002/vjch.201900041.

Kikuchi, H., Ito, I., Takahashi, K., Ishigaki, H., Iizumi, K., Kubohara, Y., et al. (2017). Isolation, Synthesis, and Biological Activity of Chlorinated Alkylresorcinols from Dictyostelium Cellular Slime Molds. *J. Nat. Prod.* 80, 2716-2722. doi: 10.1021/acs.jnatprod.7b00456.

Kim, D.-C., Quang, T.H., Tien, N.T., Kim, K.-W., Kim, Y.-C., Ngan, N.T.T., et al. (2022). Anti-neuroinflammatory effect of oxaline, isorhodoptilometrin, and 5-hydroxy-7-(2′-hydroxypropyl)-2-methyl-chromone obtained from the marine fungal strain *Penicillium oxalicum* CLC-MF05. *Arch. Pharmacal Res.* 45, 90-104. doi: 10.1007/s12272-022-01370-w.

Kim, D.H., Kim, H.K., Kim, K.M., Kim, C.K., Jeong, M.H., Ko, C.Y., et al. (2011). Antibacterial activities of macrolactin a and 7-O-succinyl macrolactin a from *Bacillus polyfermenticus* KJS-2 against vancomycin-resistant *Enterococci* and methicillin-resistant *Staphylococcus aureus*. *Arch. Pharmacal Res.* 34, 147-152. doi: 10.1007/s12272-011-0117-0.

Kim, Y.-G., Lee, J.-H., and Lee, J. (2021). Antibiofilm activities of fatty acids including myristoleic acid against *Cutibacterium acnes* via reduced cell hydrophobicity. *Phytomedicine* 91, 153710. doi: 10.1016/j.phymed.2021.153710.

Kiran, G.S., Sekar, S., Ramasamy, P., Thinesh, T., Hassan, S., Lipton, A.N., et al. (2018). Marine sponge microbial association: Towards disclosing unique symbiotic interactions. *Mar. Environ. Res.* 140, 169-179. doi: 10.1016/j.marenvres.2018.04.017.

Kirchman, D.L., Elifantz, H., Dittel, A.I., Malmstrom, R.R., and Cottrell, M.T. (2007). Standing stocks and activity of Archaea and Bacteria in the western Arctic Ocean. *Limnol. Oceanogr.* 52, 495-507. doi: 10.4319/lo.2007.52.2.0495.

Kiriiri, G.K., Njogu, P.M., and Mwangi, A.N. (2020). Exploring different approaches to improve the success of drug discovery and development projects: a review. *Future Journal of Pharmaceutical Sciences* 6, 27. doi: 10.1186/s43094-020-00047-9.

Kirschner, C., Maquelin, K., Pina, P., Ngo Thi, N.A., Choo-Smith, L.P., Sockalingum, G.D., et al. (2001). Classification and identification of enterococci: a comparative phenotypic, genotypic, and vibrational spectroscopic study. *J. Clin. Microbiol.* 39, 1763-1770. doi: 10.1128/JCM.39.5.1763-1770.2001.

Klautau, M., and Russo, C.a.M. (2016). "Metazoans, Origins of," in *Encyclopedia of Evolutionary Biology,* ed. R.M. Kliman. Oxford: Academic Press, 1-6. doi: 10.1016/B978-0-12-800049-6.00270-5.

Knobloch, S., Jóhannsson, R., and Marteinsson, V. (2019). Co-cultivation of the marine sponge *Halichondria panicea* and its associated microorganisms. *Sci. Rep.* 9, 10403. doi: 10.1038/s41598-019-46904-3.

Komaki, H., Ichikawa, N., Hosoyama, A., Takahashi-Nakaguchi, A., Matsuzawa, T., Suzuki, K.-I., et al. (2014). Genome based analysis of type-I polyketide synthase and nonribosomal peptide synthetase gene clusters in seven strains of five representative *Nocardia* species. *BMC Genomics* 15, 323. doi: 10.1186/1471-2164-15-323.

Komaki, H., Sakurai, K., Hosoyama, A., Kimura, A., Igarashi, Y., and Tamura, T. (2018). Diversity of nonribosomal peptide synthetase and polyketide synthase gene clusters among taxonomically close *Streptomyces* strains. *Sci. Rep.* 8, 6888. doi: 10.1038/s41598-018-24921-y.

Konstantinou, D., Gerovasileiou, V., Voultsiadou, E., and Gkelis, S. (2018). Sponges-Cyanobacteria associations: Global diversity overview and new data from the Eastern Mediterranean. *PLoS One* 13, e0195001. doi: 10.1371/journal.pone.0195001.

Konstantinou, D., Popin, R.V., Fewer, D.P., Sivonen, K., and Gkelis, S. (2021). Genome Reduction and Secondary Metabolism of the Marine Sponge-Associated Cyanobacterium *Leptothoe*. *Mar. Drugs* 19, 298. doi: 10.3390/md19060298.

Korotkova, G.P., and Aizenshtadt, T.B. (1976). A study of oogenesis of the marine sponge *Haliscarca dujardini*. I. The origin of the oogonia and early stages of oocyte development. *Tsitologiya* 18, 549-555. doi:

Kowalchuk, G.A., and Stephen, J.R. (2001). Ammonia-Oxidizing Bacteria: A Model for Molecular Microbial Ecology. *Annu. Rev. Microbiol.* 55, 485-529. doi: 10.1146/annurev.micro.55.1.485.

Kubanek, J., Whalen, K.E., Engel, S., Kelly, S.R., Henkel, T.P., Fenical, W., et al. (2002). Multiple defensive roles for triterpene glycosides from two Caribbean sponges. *Oecologia* 131, 125-136. doi: 10.1007/s00442-001-0853-9.

Kumar, P., Lee, J.-H., Beyenal, H., and Lee, J. (2020). Fatty Acids as Antibiofilm and Antivirulence Agents. *Trends Microbiol.* 28, 753-768. doi: 10.1016/j.tim.2020.03.014.

Kumar, S.N., Lankalapalli, R.S., and Kumar, B.S.D. (2014). In Vitro Antibacterial Screening of Six Proline-Based Cyclic Dipeptides in Combination with β-Lactam Antibiotics Against Medically Important Bacteria. *Appl. Biochem. Biotechnol.* 173, 116-128. doi: 10.1007/s12010-014-0808-3.

Kunakom, S., and Eustáquio, A.S. (2019). Natural Products and Synthetic Biology: Where We Are and Where We Need To Go. *mSystems* 4, e00113-00119. doi: 10.1128/mSystems.00113-19.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., et al. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390, 249-256. doi: 10.1038/36786.

Kuster, E., and William, S.T. (1964). Selection of media for isolation of *Streptomyces*. *Nature* 202, 928-929. doi:

Lackner, G., Peters, E.E., Helfrich, E.J.N., and Piel, J. (2017a). Insights into the lifestyle of uncultured bacterial natural product factories associated with marine sponges. *Proceedings of the National Academy of Sciences* 114, E347. doi: 10.1073/pnas.1616234114.

Lackner, G., Peters, E.E., Helfrich, E.J.N., and Piel, J. (2017b). Insights into the lifestyle of uncultured bacterial natural product factories associated with marine sponges. *Proceedings of the National Academy of Sciences* 114, E347-E356. doi: 10.1073/pnas.1616234114.

Lafi, F.F., Garson, M.J., and Fuerst, J.A. (2005). Culturable Bacterial Symbionts Isolated from Two Distinct Sponge Species (*Pseudoceratina clavata* and *Rhabdastrella globostellata*) from the Great Barrier Reef Display Similar Phylogenetic Diversity. *MicEc* 50, 213-220. doi: 10.1007/s00248-004-0202-8.

Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., et al. (2012). Microbial culturomics: paradigm shift in the human gut microbiome study. *Clin. Microbiol. Infect.* 18, 1185-1193. doi: 10.1111/1469-0691.12023.

Lane, D.J. (1991). "16S/23S rRNA sequencing," in *Nucleic acid techniques in bacterial systematics,* eds. E. Stackebrandt & M. Goodfellow. New York, NY, USA: John Wiley & Sons.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences* 82, 6955. doi: 10.1073/pnas.82.20.6955.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357-359. doi: 10.1038/nmeth.1923.

Lapidus, A.L., and Korobeynikov, A.I. (2021). Metagenomic Data Assembly – The Way of Decoding Unknown Microorganisms. *Frontiers in Microbiology* 12, 613791. doi: 10.3389/fmicb.2021.613791.

Laport, M.S., Santos, O.C.S., and Muricy, G. (2009). Marine Sponges: Potential Sources of New Antimicrobial Drugs. *Curr. Pharm. Biotechnol.* 10, 86-105. doi: 10.2174/138920109787048625.

Lattig, P., and Martín, D. (2011). Sponge-associated Haplosyllis (Polychaeta: Syllidae: Syllinae) from the Caribbean Sea, with the description of four new species. *Sci Mar* 75, 733-758. doi: 10.3989/scimar.2011.75n4733.

Lavy, A., Keren, R., Haber, M., Schwartz, I., and Ilan, M. (2014). Implementing sponge physiological and genomic information to enhance the diversity of its culturable associated bacteria. *FEMS Microbiol. Ecol.* 87, 486-502. doi: 10.1111/1574-6941.12240.

Lee, O.O., Chui, P.Y., Wong, Y.H., Pawlik, J.R., and Qian, P.-Y. (2009). Evidence for Vertical Transmission of Bacterial Symbionts from Adult to Embryo in the Caribbean Sponge *Svenzea zeai*. *Appl. Environ. Microbiol.* 75, 6147-6156. doi: 10.1128/AEM.00023-09.

Lee, O.O., Wang, Y., Yang, J., Lafi, F.F., Al-Suwailem, A., and Qian, P.-Y. (2011). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *ISME J* 5, 650-664. doi: 10.1038/ismej.2010.165.

Lee, S.-J., Cho, J.-Y., Cho, J.-I., Moon, J.-H., Park, K.D., Lee, Y.J., et al. (2004). Isolation and Characterization of Antimicrobial Substance Macrolactin A Produced from *Bacillus amyloliquefaciens* CHO104 Isolated from Soil. *J. Microbiol. Biotechnol.* 14, 525-531. doi:

Lepore, E., Sciscioli, M., Gherardi, M., and Laici, S. (1995). The ultrastructure of the mature oocyte and the nurse cells of the ceractinomorpha *Petrosia ficiformis*. *Cahiers Biol. Mar* 36, 15-20.

Lesser, M.P. (2006). Benthic–pelagic coupling on coral reefs: Feeding and growth of Caribbean sponges. *J. Exp. Mar. Biol. Ecol.* 328, 277-288. doi: 10.1016/j.jembe.2005.07.010.

Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *NAR* 49, W293-W296. doi: 10.1093/nar/gkab301.

Levy, S.B. (2002). "From tragedy the antibiotic era is born," in *The Antibiotic Paradox: How the misuse of antibiotics destroys their curative powers,* ed. S.B. Levy. 2nd ed., Cambridge, MA: Perseus Publishing, 1-14.

Lewis, W.H., Tahon, G., Geesink, P., Sousa, D.Z., and Ettema, T.J.G. (2021). Innovations to culturing the uncultured microbial majority. *Nature Reviews Microbiology* 19, 225-240. doi: 10.1038/s41579-020-00458-8.

Li, C.Q., Liu, W.C., Zhu, P., Yang, J.L., and Cheng, K.D. (2011a). Phylogenetic diversity of bacteria associated with the marine sponge *Gelliodes carnosa* collected from the Hainan Island coastal waters of the South China Sea. *Microb Ecol* 62, 800-812. doi: 10.1007/s00248-011-9896-6.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078-2079. doi: 10.1093/bioinformatics/btp352.

Li, J., Wang, W., Xu, S.X., Magarvey, N.A., and Mccormick, J.K. (2011b). *Lactobacillus reuteri*-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in *Staphylococci*. *Proceedings of the National Academy of Sciences* 108, 3360. doi: 10.1073/pnas.1017431108.

Li, J., Xu, B., Cui, J., Deng, Z., De Voogd, N.J., Proksch, P., et al. (2010). Globostelletins A–I, cytotoxic isomalabaricane derivatives from the marine sponge *Rhabdastrella globostellata*. *Biorg. Med. Chem.* 18, 4639-4647. doi: 10.1016/j.bmc.2010.05.029.

Li, W., Tang, X.-X., Yan, X., Wu, Z., Yi, Z.-W., Fang, M.-J., et al. (2016a). A new macrolactin antibiotic from deep sea-derived bacteria *Bacillus subtilis* B5. *Nat. Prod. Res.* 30, 2777-2782. doi: 10.1080/14786419.2016.1155576.

Li, Z., He, L., and Miao, X. (2007a). Cultivable bacterial community from South China Sea sponge as revealed by DGGE fingerprinting and 16S rDNA phylogenetic analysis. *Curr. Microbiol.* 55, 465-472. doi: 10.1007/s00284-007-9035-2.

Li, Z., Hu, Y., Liu, Y., Huang, Y., He, L., and Miao, X. (2007b). 16S rDNA clone library-based bacterial phylogenetic diversity associated with three South China Sea sponges. *World J. Microbiol. Biotechnol.* 23, 1265-1272. doi: 10.1007/s11274-007-9359-x.

Li, Z., Wang, Y., Li, J., Liu, F., He, L., He, Y., et al. (2016b). Metagenomic Analysis of Genes Encoding Nutrient Cycling Pathways in the Microbiota of Deep-Sea and Shallow-Water Sponges. *Mar. Biotechnol.* 18, 659-671. doi: 10.1007/s10126-016-9725-5.

Li, Z.Y., and Liu, Y. (2006). Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis. *Lett. Appl. Microbiol.* 43, 410-416. doi: 10.1111/j.1472-765X.2006.01976.x.

Lin, C.C., and Casida, L.E. (1984). GELRITE as a Gelling Agent in Media for the Growth of Thermophilic Microorganisms. *Appl. Environ. Microbiol.* 47, 427-429. doi: 10.1128/AEM.47.2.427-429.1984.

Lin, H., Yu, M., Wang, X., and Zhang, X.-H. (2018). Comparative genomic analysis reveals the evolution and environmental adaptation strategies of vibrios. *BMC Genomics* 19, 135-135. doi: 10.1186/s12864-018-4531-2.

Lin, Z., Nielsen, J., and Liu, Z. (2020). Bioprospecting Through Cloning of Whole Natural Product Biosynthetic Gene Clusters. *Frontiers in Bioengineering and Biotechnology* 8, 526. doi: 10.3389/fbioe.2020.00526.

Lindequist, U. (2016). Marine-Derived Pharmaceuticals ? Challenges and Opportunities. *Biomol. Ther. (Seoul)* 24, 561-571. doi: 10.4062/biomolther.2016.181.

Linh, V.T.T., Kiem, D.T., Ngoc, P.H., Phu, L.H., Tam, P.H., and Vinh, L.T. (2015). Coastal sea water quality of Nha Trang bay, Khanh Hoa, Viet Nam. *Journal of Shipping and Ocean Engineering* 5, 123-130. doi: 10.17265/2159-5879/2015.03.003.

Liu, M., Fan, L., Zhong, L., Kjelleberg, S., and Thomas, T. (2012). Metaproteogenomic analysis of a community of sponge symbionts. *The ISME Journal* 6, 1515-1525. doi: 10.1038/ismej.2012.1.

Liu, M.Y., Kjelleberg, S., and Thomas, T. (2011). Functional genomic analysis of an uncultured δ-proteobacterium in the sponge *Cymbastela concentrica*. *The ISME journal* 5, 427-435. doi: 10.1038/ismej.2010.139.

Loh, T.-L., and Pawlik, J.R. (2014). Chemical defenses and resource trade-offs structure sponge communities on Caribbean coral reefs. *Proceedings of the National Academy of Sciences* 111, 4151-4156. doi: 10.1073/pnas.1321626111.

Lok, C. (2015). Mining the microbial dark matter. *Nature* 522, 270-273. doi: 10.1038/522270a.

Lopanik, N.B. (2014). Chemical defensive symbioses in the marine environment. *Funct. Ecol.* 28, 328-340. doi: 10.1111/1365-2435.12160.

Loureiro, C., Galani, A., Gavriilidou, A., Chaib De Mares, M., Van Der Oost, J., Medema Marnix, H., et al. (2022). Comparative Metagenomic Analysis of Biosynthetic Diversity across Sponge Microbiomes Highlights Metabolic Novelty, Conservation, and Diversification. *mSystems* 7, e00357-00322. doi: 10.1128/msystems.00357-22.

Loureiro, C., Medema, M.H., Van Der Oost, J., and Sipkema, D. (2018). Exploration and exploitation of the environment for novel specialized metabolites. *Curr. Opin. Biotechnol.* 50, 206-213. doi: 10.1016/j.copbio.2018.01.017.

Louwen, J.J.R., Medema, M.H., and Van Der Hooft, J.J.J. (2023). Enhanced correlation-based linking of biosynthetic gene clusters to their metabolic products through chemical class matching. *Microbiome* 11, 13. doi: 10.1186/s40168-022-01444-3.

Love, G.D., Grosjean, E., Stalvies, C., Fike, D.A., Grotzinger, J.P., Bradley, A.S., et al. (2009). Fossil steroids record the appearance of Demospongiae during the Cryogenian period. *Nature* 457, 718-721. doi: 10.1038/nature07673.

Love, G.D., and Summons, R.E. (2015). The molecular record of Cryogenian sponges – a response to Antcliffe (2013). *Palaeontology* 58, 1131-1136. doi: 10.1111/pala.12196.

Lu, X.L., Xu, Q.Z., Shen, Y.H., Liu, X.Y., Jiao, B.H., Zhang, W.D., et al. (2008). Macrolactin S, a novel macrolactin antibiotic from marine *Bacillus* sp. *Nat. Prod. Res.* 22, 342-347. doi: 10.1080/14786410701768162.

Łukowiak, M., Pisera, A., and O'dea, A. (2013). Do spicules in sediments reflect the living sponge community? A test in a Caribbean shallow water lagoon. *Palaios* 28, 373-385. doi: 10.2110/palo.2012.p12-082r.

Lutaenko, K.A., Choi, K.-S., and Thai, N.C. (2011). *Coastal Marine Biodiversity of Vietnam : Regional and Local Challenges and Coastal Zone Management for Sustainable Development.* Asia-Pacific Network for Global Change Research.

Luter, H.M., Gibb, K., and Webster, N.S. (2014). Eutrophication has no short-term effect on the *Cymbastela stipitata* holobiont. *Frontiers in Microbiology* 5, 216. doi: 10.3389/fmicb.2014.00216.

Ma, L., Kim, J., Hatzenpichler, R., Karymov, M.A., Hubert, N., Hanan, I.M., et al. (2014). Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in Human Microbiome Project's Most Wanted taxa. *Proc. Natl. Acad. Sci. U. S. A.* 111, 9768-9773. doi: 10.1073/pnas.1404753111.

Mahajan, G.B., and Balachandran, L. (2017). Sources of antibiotics: Hot springs. *Biochem. Pharmacol.* 134, 35-41. doi: 10.1016/j.bcp.2016.11.021.

Mahizan, N.A., Yang, S.-K., Moo, C.-L., Song, A.A., Chong, C.-M., Chong, C.-W., et al. (2019). Terpene Derivatives as a Potential Agent against Antimicrobial Resistance (AMR) Pathogens. *Molecules* 24, 2631. doi: 10.3390/molecules24142631.

Malcolm, S.H., Nora, A.L., and Kimberly, A.Y. (2005). Anti-predator defenses in western North Atlantic sponges with evidence of enhanced defense through interactions between spicules and chemicals. *Mar. Ecol. Prog. Ser.* 291, 93-102. doi: 10.3354/meps291093.

Maldonado, M. (2004). Choanoflagellates, choanocytes, and animal multicellularity. *Invertebr. Biol.* 123, 1-22. doi: 10.1111/j.1744-7410.2004.tb00138.x.

Maldonado, M. (2016). Sponge waste that fuels marine oligotrophic food webs: a re-assessment of its origin and nature. *Mar. Ecol.* 37, 477-491. doi: 10.1111/maec.12256.

Maldonado, M., Carmona, M.C., Velásquez, Z., Puig, A., Cruzado, A., López, A., et al. (2005). Siliceous sponges as a silicon sink: An overlooked aspect of benthopelagic coupling in the marine silicon cycle. *Limnol. Oceanogr.* 50, 799-809. doi: 10.4319/lo.2005.50.3.0799.

Maldonado, M., Navarro, L., Grasa, A., Gonzalez, A., and Vaquerizo, I. (2011). Silicon uptake by sponges: a twist to understanding nutrient cycling on continental margins. *Sci. Rep.* 1, 30. doi: 10.1038/srep00030.

Maldonado, M., Ribes, M., and Van Duyl, F.C. (2012). Nutrient fluxes through sponges: biology, budgets, and ecological implications. *Adv Mar Biol* 62, 113-182. doi: 10.1016/B978-0-12-394283-8.00003-5.

Maldonado, M., and Riesgo, A. (2009). Gametogenesis, embryogenesis, and larval features of the oviparous sponge *Petrosia ficiformis* (Haplosclerida, Demospongiae). *Mar. Biol.* 156, 2181-2197. doi: 10.1007/s00227-009-1248-4.

Malik, B., and Bhattacharyya, S. (2019). Antibiotic drug-resistance as a complex system driven by socio-economic growth and antibiotic misuse. *Sci. Rep.* 9, 9788. doi: 10.1038/s41598-019-46078-y.

Maloof, A.C., Rose, C.V., Beach, R., Samuels, B.M., Calmet, C.C., Erwin, D.H., et al. (2010). Possible animal-body fossils in pre-Marinoan limestones from South Australia. *Nature Geosci* 3, 653-659. doi: 10.1038/ngeo934.

Manconi, R., and Pronzato, R. (2008). Global diversity of sponges (Porifera: Spongillina) in freshwater. *Hydrobiologia* 595, 27-33. doi: 10.1007/s10750-007-9000-x.

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. *Molecules* 23, 795. doi: 10.3390/molecules23040795.

Margot, H., Acebal, C., Toril, E., Amils, R., and Fernandez Puentes, J. (2002). Consistent association of crenarchaeal Archaea with sponges of the genus *Axinella*. *Mar. Biol.* 140, 739-745. doi: 10.1007/s00227-001-0740-2.

Martins, T.P., Rouger, C., Glasser, N.R., Freitas, S., De Fraissinette, N.B., Balskus, E.P., et al. (2019). Chemistry, bioactivity and biosynthesis of cyanobacterial alkylresorcinols. *Nat. Prod. Rep.* 36, 1437-1461. doi: 10.1039/C8NP00080H.

Masschelein, J., Mattheus, W., Gao, L.-J., Moons, P., Van Houdt, R., Uytterhoeven, B., et al. (2013). A PKS/NRPS/FAS Hybrid Gene Cluster from Serratia plymuthica RVH1 Encoding the Biosynthesis of Three Broad Spectrum, Zeamine-Related Antibiotics. *PLoS One* 8, e54143. doi: 10.1371/journal.pone.0054143.

Mast, Y., Weber, T., Gölz, M., Ort-Winklbauer, R., Gondran, A., Wohlleben, W., et al. (2011). Characterization of the 'pristinamycin supercluster' of *Streptomyces pristinaespiralis*. *Microbial Biotechnology* 4, 192-206. doi: 10.1111/j.1751-7915.2010.00213.x.

Mathur, H., O'connor Paula, M., Hill, C., Cotter Paul, D., and Ross, R.P. (2013). Analysis of Anti-Clostridium difficile Activity of Thuricin CD, Vancomycin, Metronidazole, Ramoplanin, and Actagardine, both Singly and in Paired Combinations. *Antimicrob. Agents Chemother.* 57, 2882-2886. doi: 10.1128/AAC.00261-13.

Matobole, R.M., Van Zyl, L.J., Parker-Nance, S., Davies-Coleman, M.T., and Trindade, M. (2017). Antibacterial Activities of Bacteria Isolated from the Marine Sponges *Isodictya compressa* and Higgins ia bidentifera Collected from Algoa Bay, South Africa. *Mar. Drugs* 15, 47. doi: 10.3390/md15020047.

Matroodi, S., Siitonen, V., Baral, B., Yamada, K., Akhgari, A., and Metsä-Ketelä, M. (2020). Genotyping-Guided Discovery of Persiamycin A From Sponge-Associated Halophilic *Streptomonospora* sp. PA3. *Frontiers in Microbiology* 11, 1237. doi: 10.3389/fmicb.2020.01237.

Mau, L.D., Lam, T.H., and Cuong, N.M. (2015). Present State of Ocean Observations and Service in Vietnam. *Vietnam Journal of Marine Science and Technology* 15, 309-319. doi: 10.15625/1859-3097/7376.

Mclain, J.E., Cytryn, E., Durso, L.M., and Young, S. (2016). Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge. *J. Environ. Qual.* 45, 432-440. doi: 10.2134/jeq2015.06.0317.

Medema, M.H., Kottmann, R., Yilmaz, P., Cummings, M., Biggins, J.B., Blin, K., et al. (2015). Minimum Information about a Biosynthetic Gene cluster. *Nat. Chem. Biol.* 11, 625-631. doi: 10.1038/nchembio.1890.

Medlin, L., Elwood, H.L., S., S., and Sogin, M.L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71, 491-499. doi: 10.1016/0378-1119(88)90066-2.

Mehbub, F.M., Lei, J., Franco, C., and Zhang, W. (2014). Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives. *Mar. Drugs* 12, 4539-4577. doi: 10.3390/md12084539.

Mehbub, M.F., Perkins, M.V., Zhang, W., and Franco, C.M.M. (2016). New marine natural products from sponges (Porifera) of the order Dictyoceratida (2001 to 2012): a promising source for drug discovery, exploration and future prospects. *Biotechnol. Adv.* 34, 473-491. doi: 10.1016/j.biotechadv.2015.12.008.

Mendola, D. (2003). Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: process developments and economics. *Biomol. Eng.* 20, 441-458. doi: 10.1016/S1389-0344(03)00075-3.

Miller, B.R., and Gulick, A.M. (2016a). Structural Biology of Nonribosomal Peptide Synthetases. *Methods in molecular biology (Clifton, N.J.)* 1401, 3-29. doi: 10.1007/978-1-4939-3375-4_1.

Miller, B.R., and Gulick, A.M. (2016b). "Structural Biology of Nonribosomal Peptide Synthetases," in *Nonribosomal Peptide and Polyketide Biosynthesis: Methods and Protocols,* ed. B.S. Evans. New York, NY, USA: Humana Press, 3-29. doi: 10.1007/978-1-4939-3375-4_1.

Mincer, T.J., Jensen, P.R., Kauffman, C.A., and Fenical, W. (2002). Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Appl. Environ. Microbiol.* 68, 5005-5011. doi: 10.1128/aem.68.10.5005-5011.2002.

Mithöfer, A., and Maffei, M.E. (2017). "General Mechanisms of Plant Defense and Plant Toxins," in *Plant Toxins,* eds. C.R. Carlini, R. Ligabue-Braun & P. Gopalakrishnakone. Dordrecht: Springer Netherlands, 3-24. doi: 10.1007/978-94-007-6464-4_21.

Mitova, M., Tommonaro, G., Hentschel, U., Müller, W.E.G., and De Rosa, S. (2004). Exocellular Cyclic Dipeptides from a *Ruegeria* Strain Associated with Cell Cultures of *Suberites domuncula*. *Mar. Biotechnol.* 6, 95-103. doi: 10.1007/s10126-003-0018-4.

Mizuno, C.M., Kimes, N.E., López-Pérez, M., Ausó, E., Rodriguez-Valera, F., and Ghai, R. (2013). A Hybrid NRPS-PKS Gene Cluster Related to the Bleomycin Family of Antitumor Antibiotics in *Alteromonas macleodii* Strains. *PLoS One* 8, e76021. doi: 10.1371/journal.pone.0076021.

Moeller, F.U., Webster, N.S., Herbold, C.W., Behnam, F., Domman, D., Albertsen, M., et al. (2019). Characterization of a thaumarchaeal symbiont that drives incomplete nitrification in the tropical sponge *Ianthella basta*. *Environ. Microbiol.* 21, 3831-3854. doi: 10.1111/1462-2920.14732.

Mohanty, I., Tapadar, S., Moore Samuel, G., Biggs Jason, S., Freeman Christopher, J., Gaul David, A., et al. (2021). Presence of Bromotyrosine Alkaloids in Marine Sponges Is Independent of Metabolomic and Microbiome Architectures. *mSystems* 6, e01387-01320. doi: 10.1128/mSystems.01387-20.

Moitinho-Silva, L., Bayer, K., Cannistraci, C.V., Giles, E.C., Ryu, T., Seridi, L., et al. (2014a). Specificity and transcriptional activity of microbiota associated with low and high microbial abundance sponges from the Red Sea. *Mol. Ecol.* 23, 1348-1363. doi: 10.1111/mec.12365.

Moitinho-Silva, L., Díez-Vives, C., Batani, G., Esteves, A.I.S., Jahn, M.T., and Thomas, T. (2017a). Integrated metabolism in sponge–microbe symbiosis revealed by genome-centered metatranscriptomics. *The ISME Journal* 11, 1651-1666. doi: 10.1038/ismej.2017.25.

Moitinho-Silva, L., Nielsen, S., Amir, A., Gonzalez, A., Ackermann, G.L., Cerrano, C., et al. (2017b). The sponge microbiome project. *GigaScience* 6, 1-7. doi: 10.1093/gigascience/gix077.

Moitinho-Silva, L., Seridi, L., Ryu, T., Voolstra, C.R., Ravasi, T., and Hentschel, U. (2014b). Revealing microbial functional activities in the Red Sea sponge *Stylissa carteri* by metatranscriptomics. *Environ. Microbiol.* 16, 3683-3698. doi: 10.1111/1462-2920.12533.

Moitinho-Silva, L., Steinert, G., Nielsen, S., Hardoim, C.C.P., Wu, Y.-C., Mccormack, G.P., et al. (2017c). Predicting the HMA-LMA Status in Marine Sponges by Machine Learning. *Frontiers in Microbiology* 8, 752. doi: 10.3389/fmicb.2017.00752.

Molinski, T.F., Dalisay, D.S., Lievens, S.L., and Saludes, J.P. (2009). Drug development from marine natural products. *Nature Reviews Drug Discovery* 8, 69-85. doi: 10.1038/nrd2487.

Mondol, M.a.M., Shin, H.J., and Islam, M.T. (2013). Diversity of secondary metabolites from marine *Bacillus* species: chemistry and biological activity. *Mar. Drugs* 11, 2846-2872. doi: 10.3390/md11082846.

Mondol, M.a.M., Tareq, F.S., Kim, J.H., Lee, M.A., Lee, H.-S., Lee, Y.-J., et al. (2011). Cyclic Ether-Containing Macrolactins, Antimicrobial 24-Membered Isomeric Macrolactones from a Marine *Bacillus* sp. *J. Nat. Prod.* 74, 2582-2587. doi: 10.1021/np200487k.

Monre (2014). *Vietnam's Fifth National Report To The United Nations Convention On Biological Diversity: Reporting period 2009-2013*. Ha noi, Vietnam: Ministry of Natural Resources and Environment (MONRE).

Monre (2016). *National environmental status in the period from 2011 to 2015*. Ha Noi, Vietnam: Ministry of Natural Resources and Environment (MONRE), Vietnam Publishing House of Natural Resources, Environment and Cartography.

Montalvo, N.F., Davis, J., Vicente, J., Pittiglio, R., Ravel, J., and Hill, R.T. (2014). Integration of culture-based and molecular analysis of a complex sponge-associated bacterial community. *PLoS One* 9, e90517. doi: 10.1371/journal.pone.0090517.

Montalvo, N.F., Mohamed, N.M., Enticknap, J.J., and Hill, R.T. (2005). Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek* 87, 29-36. doi: 10.1007/s10482-004-6536-x.

Montaser, R., and Luesch, H. (2011). Marine natural products: a new wave of drugs? *Future Med. Chem.* 3, 1475-1489. doi: 10.4155/fmc.11.118.

Moore, B.S., and Hopke, J.N. (2001). Discovery of a New Bacterial Polyketide Biosynthetic Pathway. *ChemBioChem* 2, 35-38. doi: 10.1002/1439-7633(20010105)2:1<35::AID-CBIC35>3.0.CO;2-1.

Mori, T., Cahn, J.K.B., Wilson, M.C., Meoded, R.A., Wiebach, V., Martinez, A.F.C., et al. (2018). Single-bacterial genomics validates rich and varied specialized metabolism of uncultivated *Entotheonella* sponge symbionts. *Proceedings of the National Academy of Sciences* 115, 1718-1723. doi: 10.1073/pnas.1715496115.

Morrow, C., and Cárdenas, P. (2015). Proposal for a revised classification of the Demospongiae (Porifera). *Front Zool* 12, 7. doi: 10.1186/s12983-015-0099-8.

Muller, E.E.L., Glaab, E., May, P., Vlassis, N., and Wilmes, P. (2013). Condensing the omics fog of microbial communities. *Trends Microbiol.* 21, 325-333. doi: 10.1016/j.tim.2013.04.009.

Mullis, M.M., Rambo, I.M., Baker, B.J., and Reese, B.K. (2019). Diversity, Ecology, and Prevalence of Antimicrobials in Nature. *Frontiers in Microbiology* 10, 2518. doi: 10.3389/fmicb.2019.02518.

Mumby, P.J., and Steneck, R.S. (2018). Paradigm Lost: Dynamic Nutrients and Missing Detritus on Coral Reefs. *BioSc* 68, 487-495. doi: 10.1093/biosci/biy055.

Murray, C.J.L., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* 399, 629-655. doi: 10.1016/S0140-6736(21)02724-0.

Muscholl-Silberhorn, A., Thiel, V., and Imhoff, J.F. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean sea. *MicEc* 55, 94-106. doi: 10.1007/s00248-007-9255-9.

Nagao, T., Adachi, K., Sakai, M., Nishijima, M., and Sano, H. (2001). Novel Macrolactins as antibiotic lactones from a marine bacterium. *The Journal of antibiotics* 54, 333-339. doi: 10.7164/antibiotics.54.333.

Naim, M.A., Morillo, J.A., Sorensen, S.J., Waleed, A.A., Smidt, H., and Sipkema, D. (2014). Host-specific microbial communities in three sympatric North Sea sponges. *FEMS Microbiol. Ecol.* 90, 390-403. doi: 10.1111/1574-6941.12400.

Nakashima, T., Seki, T., Matsumoto, A., Miura, H., Sato, E., Niwano, Y., et al. (2010). Generation of reactive oxygen species from conventional laboratory media. *J. Biosci. Bioeng.* 110, 304-307. doi: 10.1016/j.jbiosc.2010.03.003.

Nakashima, Y., Egami, Y., Kimura, M., Wakimoto, T., and Abe, I. (2016). Metagenomic Analysis of the Sponge *Discodermia* Reveals the Production of the Cyanobacterial Natural Product Kasumigamide by '*Entotheonella*'. *PLoS One* 11, e0164468. doi: 10.1371/journal.pone.0164468.

Ňancucheo, I., Rowe, O.F., Hedrich, S., and Johnson, D.B. (2016). Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. *FEMS Microbiol. Lett.* 363, fnw083-fnw083. doi: 10.1093/femsle/fnw083.

Naughton, L.M., Romano, S., O'gara, F., and Dobson, A.D.W. (2017). Identification of Secondary Metabolite Gene Clusters in the *Pseudovibrio* Genus Reveals Encouraging Biosynthetic Potential toward the Production of Novel Bioactive Compounds. *Frontiers in Microbiology* 8, 1494. doi: 10.3389/fmicb.2017.01494.

Naumann, D. (2006). "Infrared Spectroscopy in Microbiology," in *Encyclopedia of Analytical Chemistry,* eds. R.A. Meyers & H.H. Mantsch. Hoboken, NJ, USA: John Wiley & Sons. doi: 10.1002/9780470027318.a0117.

Newman, D.J., and Cragg, G.M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79, 629-661. doi: 10.1021/acs.jnatprod.5b01055.

Newman, D.J., and Hill, R.T. (2006). New drugs from marine microbes: the tide is turning. *J. Ind. Microbiol. Biotechnol.* 33, 539-544. doi: 10.1007/s10295-006-0115-2.

Ngo-Thi, N.A., Kirschner, C., and Naumann, D. (2003). Characterization and identification of microorganisms by FT-IR microspectrometry. *JMoSt* 661-662, 371-380. doi: 10.1016/j.molstruc.2003.08.012.

Nguyen, H.T., Chau, V.M., Tran, T.H., Phan, V.K., Hoang, T.H., Nguyen, T.D., et al. (2009). C29 sterols with a cyclopropane ring at C-25 and 26 from the Vietnamese marine sponge *Ianthella* sp. and their anticancer properties. *Bioorg. Med. Chem. Lett.* 19, 4584-4588. doi: 10.1016/j.bmcl.2009.06.097.

Nguyen, M.T.H.D., Liu, M., and Thomas, T. (2014). Ankyrin-repeat proteins from sponge symbionts modulate amoebal phagocytosis. *Mol. Ecol.* 23, 1635-1645. doi: 10.1111/mec.12384.

Nguyen, N.A., Lin, Z., Mohanty, I., Garg, N., Schmidt, E.W., and Agarwal, V. (2021). An Obligate Peptidyl Brominase Underlies the Discovery of Highly Distributed Biosynthetic Gene Clusters in Marine Sponge Microbiomes. *J. Am. Chem. Soc.* 143, 10221-10231. doi: 10.1021/jacs.1c03474.

Nguyen, T., Ishida, K., Jenke-Kodama, H., Dittmann, E., Gurgui, C., Hochmuth, T., et al. (2008). Exploiting the mosaic structure of trans-acyltransferase polyketide synthases for natural product discovery and pathway dissection. *Nat. Biotechnol.* 26, 225-233. doi: 10.1038/nbt1379.

Nguyen, X.C., Longeon, A., Pham, V.C., Urvois, F., Bressy, C., Trinh, T.T., et al. (2013). Antifouling 26,27-cyclosterols from the Vietnamese marine sponge *Xestospongia testudinaria*. *J Nat Prod* 76, 1313-1318. doi: 10.1021/np400288j.

Nguyen, X.N., Nguyen, T.C., Dan, T.T., Do, T.T., Nguyen, H.N., Pham, H.Y., et al. (2015). (1)H and (13)C NMR assignments of sesquiterpenes from *Dysidea fragilis*. *Magn. Reson. Chem.* 53, 1057-1060. doi: 10.1002/mrc.4288.

Nicacio, K.J., Ióca, L.P., Fróes, A.M., Leomil, L., Appolinario, L.R., Thompson, C.C., et al. (2017). Cultures of the Marine Bacterium *Pseudovibrio denitrificans* Ab134 Produce Bromotyrosine-Derived Alkaloids Previously Only Isolated from Marine Sponges. *J. Nat. Prod.* 80, 235-240. doi: 10.1021/acs.jnatprod.6b00838.

Nichols, D. (2007). Cultivation gives context to the microbial ecologist. *FEMS Microbiol. Ecol.* 60, 351-357. doi: 10.1111/j.1574-6941.2007.00332.x.

Nichols, D., Cahoon, N., Trakhtenberg, E.M., Pham, L., Mehta, A., Belanger, A., et al. (2010). Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. *Appl. Environ. Microbiol.* 76, 2445-2450. doi: 10.1128/AEM.01754-09.

Nickel, M., Scheer, C., Hammel, J.U., Herzen, J., and Beckmann, F. (2011). The contractile sponge epithelium sensu lato – body contraction of the demosponge *Tethya wilhelma* is mediated by the pinacoderm. *J. Exp. Biol.* 214, 1692-1698. doi: 10.1242/jeb.049148.

Noyer, C., Hamilton, A., Sacristan-Soriano, O., and Becerro, M.A. (2010). Quantitative comparison of bacterial communities in two Mediterranean sponges. *Symbiosis* 51, 239-243. doi: 10.1007/s13199-010-0082-2.

O'brien, R.V., Davis, R.W., Khosla, C., and Hillenmeyer, M.E. (2014). Computational identification and analysis of orphan assembly-line polyketide synthases. *The Journal of Antibiotics* 67, 89-97. doi: 10.1038/ja.2013.125.

O'neill, J. (2014). Antimicrobial Resistance: tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance: Chaired by Jim O'Neill, 2014*.

O' Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O' Gara, F., and A, D.W.D. (2011). Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *J. Appl. Microbiol.* 110, 1495-1508. doi: 10.1111/j.1365-2672.2011.05008.x.

Oberreuter, H., Seiler, H., and Scherer, S. (2002). Identification of coryneform bacteria and related taxa by Fourier-transform infrared (FT-IR) spectroscopy. *Int. J. Syst. Evol. Microbiol.* 52, 91-100. doi: doi:10.1099/00207713-52-1-91.

Ogle, D.H. (2017). FSA: Fisheries Stock Analysis. R package version 0.8.17.

Oksanen, J., Blanchett, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R.B., et al. (2016). Vegan: community Ecology Package. R Package 2.4.0.

Oliveira, B.F.R., Lopes, I.R., Canellas, A.L.B., Muricy, G., Dobson, A.D.W., and Laport, M.S. (2020). Not That Close to Mommy: Horizontal Transmission Seeds the Microbiome Associated with the Marine Sponge *Plakina cyanorosea*. *Microorganisms* 8, 1978. doi: 10.3390/microorganisms8121978.

Olson, J.B., Lord, C.C., and Mccarthy, P.J. (2000). Improved Recoverability of Microbial Colonies from Marine Sponge Samples. *Microb Ecol* 40, 139-147. doi: 10.1007/s002480000058.

Ortiz-Castro, R., Díaz-Pérez, C., Martínez-Trujillo, M., Del Río, R.E., Campos-García, J., and López-Bucio, J. (2011). Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proceedings of the National Academy of Sciences* 108, 7253. doi: 10.1073/pnas.1006740108.

Osbourn, A. (2010). Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends Genet.* 26, 449-457. doi: 10.1016/j.tig.2010.07.001.

Oton, E.V., Quince, C., Nicol, G.W., Prosser, J.I., and Gubry-Rangin, C. (2015). Phylogenetic congruence and ecological coherence in terrestrial *Thaumarchaeota*. *The Isme Journal* 10, 85-96. doi: 10.1038/ismej.2015.101.

Overmann, J., Abt, B., and Sikorski, J. (2017). Present and Future of Culturing Bacteria. *Annu. Rev. Microbiol.* 71, 711-730. doi: 10.1146/annurev-micro-090816-093449.

Öztürk, B., De Jaeger, L., Smidt, H., and Sipkema, D. (2013). Culture-dependent and independent approaches for identifying novel halogenases encoded by *Crambe crambe* (marine sponge) microbiota. *Sci. Rep.* 3, 2780. doi: 10.1038/srep02780.

Page, M.G.P. (2012). "The Role of the Outer Membrane of Gram-negative Bacteria in Antibiotic Resistance: Ajax' Shield or Achilles' Heel?," in *Antibiotic Resistance. Handbook of Experimental Pharmacology,* ed. C. A. Berlin, Heidelberg: Springer, vol. 211, 67-86. doi: 10.1007/978-3-642-28951-4_5.

Pagliara, P., and Caroppo, C. (2011). Cytotoxic and antimitotic activities in aqueous extracts of eight cyanobacterial strains isolated from the marine sponge *Petrosia ficiformis*. *Toxicon* 57, 889-896. doi: 10.1016/j.toxicon.2011.03.006.

Park, J., Kerner, A., Burns, M.A., and Lin, X.N. (2011). Microdroplet-Enabled Highly Parallel Co-Cultivation of Microbial Communities. *PLoS One* 6, e17019. doi: 10.1371/journal.pone.0017019.

Park, S., Lee, J.-H., Kim, Y.-G., Hu, L., and Lee, J. (2022). Fatty Acids as Aminoglycoside Antibiotic Adjuvants Against *Staphylococcus aureus*. *Frontiers in Microbiology* 13, 876932. doi: 10.3389/fmicb.2022.876932.

Pawlik, J.R. (2011). The Chemical Ecology of Sponges on Caribbean Reefs: Natural Products Shape Natural Systems. *BioSc* 61, 888-898. doi: 10.1525/bio.2011.61.11.8.

Pawlik, J.R., Burkepile, D.E., and Thurber, R.V. (2016). A Vicious Circle? Altered Carbon and Nutrient Cycling May Explain the Low Resilience of Caribbean Coral Reefs. *BioSc* 66, 470-476. doi: 10.1093/biosci/biw047.

Pawlik, J.R., Chanas, B., Toonen, R.J., and Fenical, W. (1995). Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127, 183-194. doi: 10.3354/meps127183.

Pawlik, J.R., and Mcmurray, S.E. (2020). The Emerging Ecological and Biogeochemical Importance of Sponges on Coral Reefs. *Ann. Rev. Mar. Sci.* 12, 315-337. doi: 10.1146/annurev-marine-010419-010807.

Pfister, C.A., Gilbert, J.A., and Gibbons, S.M. (2014). The role of macrobiota in structuring microbial communities along rocky shores. *PeerJ* 2, e631. doi: 10.7717/peerj.631.

Pfleiderer, A., Lagier, J.C., Armougom, F., Robert, C., Vialettes, B., and Raoult, D. (2013). Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. *Eur. J. Clin. Microbiol. Infect. Dis.* 32, 1471-1481. doi: 10.1007/s10096-013-1900-2.

Pham, V.H.T., and Kim, J. (2012). Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30, 475-484. doi: 10.1016/j.tibtech.2012.05.007.

Phan, T.H.T., Yurchenko, E.A., Yurchenko, A.N., Ngo, T.D.N., Vo, T.D.T., Cao, T.T.H., et al. (2022). Evaluation of cytotoxic activity of marine fungi isolated from sponges in Nha Trang bay. *Vietnam Journal of Marine Science and Technology* 22, 51-57. doi: 10.15625/1859-3097/16631.

Phelan, R.W., Barret, M., Cotter, P.D., O'connor, P.M., Chen, R., Morrissey, J.P., et al. (2013). Subtilomycin: a new lantibiotic from *Bacillus subtilis* strain MMA7 isolated from the marine sponge Haliclona simulans. *Mar. Drugs* 11, 1878-1898. doi: 10.3390/md11061878.

Piel, J., Hui, D., Wen, G., Butzke, D., Platzer, M., Fusetani, N., et al. (2004). Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16222-16227. doi: 10.1073/pnas.0405976101.

Pinto, A.J., Marcus, D.N., Ijaz, U.Z., Bautista-De Lose Santos, Q.M., Dick, G.J., and Raskin, L. (2016). Metagenomic Evidence for the Presence of Comammox Nitrospira-Like Bacteria in a Drinking Water System. *mSphere* 1, e00054-00015. doi: 10.1128/mSphere.00054-15.

Piper, C., Casey, P.G., Hill, C., Cotter, P.D., and Ross, R.P. (2012). The Lantibiotic Lacticin 3147 Prevents Systemic Spread of *Staphylococcus aureus* in a Murine Infection Model. *International journal of microbiology* 2012, 806230-806230. doi: 10.1155/2012/806230.

Pita, L., Erwin, P.M., Turon, X., and López-Legentil, S. (2013a). Till Death Do Us Part: Stable Sponge-Bacteria Associations under Thermal and Food Shortage Stresses. *PLoS One* 8, e80307. doi: 10.1371/journal.pone.0080307.

Pita, L., Rix, L., Slaby, B.M., Franke, A., and Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome* 6, 46. doi: 10.1186/s40168-018-0428-1.

Pita, L., Turon, X., López-Legentil, S., and Erwin, P.M. (2013b). Host rules: spatial stability of bacterial communities associated with marine sponges (*Ircinia* spp.) in the Western Mediterranean Sea. *FEMS Microbiol. Ecol.* 86, 268-276. doi: 10.1111/1574-6941.12159.

Pivetal, J., Toru, S., Frenea-Robin, M., Haddour, N., Cecillon, S., Dempsey, N.M., et al. (2014). Selective isolation of bacterial cells within a microfluidic device using magnetic probe-based cell fishing. *Sensors Actuators B: Chem.* 195, 581-589. doi: 10.1016/j.snb.2014.01.004.

Podell, S., Blanton, J.M., Neu, A., Agarwal, V., Biggs, J.S., Moore, B.S., et al. (2019). Pangenomic comparison of globally distributed *Poribacteria* associated with sponge hosts and marine particles. *The ISME Journal* 13, 468-481. doi: 10.1038/s41396-018-0292-9.

Podell, S., Blanton, J.M., Oliver, A., Schorn, M.A., Agarwal, V., Biggs, J.S., et al. (2020). A genomic view of trophic and metabolic diversity in clade-specific *Lamellodysidea* sponge microbiomes. *Microbiome* 8, 97. doi: 10.1186/s40168-020-00877-y.

Polónia, A.R.M., Cleary, D.F.R., Duarte, L.N., De Voogd, N.J., and Gomes, N.C.M. (2014). Composition of Archaea in Seawater, Sediment, and Sponges in the Kepulauan Seribu Reef System, Indonesia. *MicEc* 67, 553-567. doi: 10.1007/s00248-013-0365-2.

Projan, S.J. (2003). Why is big Pharma getting out of antibacterial drug discovery? *Curr. Opin. Microbiol.* 6, 427-430. doi: 10.1016/j.mib.2003.08.003.

Proksch, P., Edrada-Ebel, R., and Ebel, R. (2003). Drugs from the Sea - Opportunities and Obstacles. *Mar. Drugs* 1, 5-17. doi: 10.3390/md101005.

Prosser, J.I., and Nicol, G.W. (2008). Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. *Environ. Microbiol.* 10, 2931-2941. doi: 10.1111/j.1462-2920.2008.01775.x.

Prozanto, R., Pisera, A., and Manconi, R. (2017). Fossil freshwater sponges: taxonomy, geographic distribution, and critical review. *Acta Palaeontol. Pol.* 62, 467–495. doi: 10.4202/app.00354.2017.

Puopolo, G., Cimmino, A., Palmieri, M.C., Giovannini, O., Evidente, A., and Pertot, I. (2014). *Lysobacter capsici* AZ78 produces cyclo(l-Pro-l-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of

Phytophthora infestans and *Plasmopara viticola*. *J. Appl. Microbiol.* 117, 1168-1180. doi: 10.1111/jam.12611.

Qceem (2015). *Monitoring report of environmental quality of Quang Tri province in 2015*. Quang Tri, Vietnam: Quang Tri Center for Environmental Engineering and Monitoring (QCEEM).

Quail, M.A., Smith, M., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., et al. (2012). A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 13, 341. doi: 10.1186/1471-2164-13-341.

Quang, T.H., Huong, P.T.M., Ngan, N.T.T., Hanh, T.T.H., Cuong, N.X., Nam, N.H., et al. (2020). Secondary metabolites from a marine sponge-associated fungus *Xenomyrothecium* sp. IMBC-FP2.11. *Vietnam Journal of Chemistry* 58, 752-758. doi: 10.1002/vjch.202000067.

Quang, T.H., Phong, N.V., Hanh, T.T.H., Cuong, N.X., Ngan, N.T.T., Oh, H., et al. (2021a). Cytotoxic and immunomodulatory phenol derivatives from a marine sponge-derived fungus *Ascomycota* sp. VK12. *Nat. Prod. Res.* 35, 5153-5159. doi: 10.1080/14786419.2020.1786829.

Quang, T.H., Vien, L.T., Anh, L.N., Ngan, N.T.T., Hanh, T.T.H., Cuong, N.X., et al. (2021b). Anti-inflammatory metabolites from a marine sponge-associated fungus *Aspergillus* sp. IMBC-FP2.05. *Vietnam Journal of Chemistry* 59, 52-56. doi: 10.1002/vjch.202000099.

Quang, T.M. (2013). "A review of the diversity of sponges (Porifera) in Vietnam", in: *The Proceedings of the 2nd international workshop on marine bioresources of Vietnam*, 109-115.

Quinn, G.A., Banat, A.M., Abdelhameed, A.M., and Banat, I.M. (2020). *Streptomyces* from traditional medicine: sources of new innovations in antibiotic discovery. *J Med Microbiol* 69, 1040-1048. doi: 10.1099/jmm.0.001232.

Quyen, T.V., Ngan, T.B., Huong, D.T.M., Kondracki, M.L.B., Longeon, A., Murphy, B., et al. (2015). Secondary metabolites from *Micromonospora ectrinospora* G017. *Vietnam Journal of Chemistry* 53, 146-149. doi: 10.15625/0866-7144.2015-2e-034.

R Core Team (2016). "R: A language and environment for statistical computing". R Foundation for Statistical Computing. Vienna, Austria. Available at: http://www.R-project.org/.

Radax, R., Hoffmann, F., Rapp, H.T., Leininger, S., and Schleper, C. (2012). Ammonia-oxidizing archaea as main drivers of nitrification in cold-water sponges. *Environ Microbiol* 14, 909-923. doi: 10.1111/j.1462-2920.2011.02661.x.

Ramiro-Garcia, J., Hermes, G.D.A., Giatsis, C., Sipkema, D., Zoetendal, E.G., Schaap, P.J., et al. (2016). NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000Research* 5, 1791. doi: 10.12688/f1000research.9227.1.

Rawlings, B.J. (2001). Type I polyketide biosynthesis in bacteria (Part A—erythromycin biosynthesis). *Nat. Prod. Rep.* 18, 190-227. doi: 10.1039/B009329G.

Ray, K., Adi, L., and Micha, I. (2016). Increasing the Richness of Culturable Arsenic-Tolerant Bacteria from *Theonella swinhoei* by Addition of Sponge Skeleton to the Growth Medium. *Microb Ecol* 71, 873-876. doi: 10.1007/s00248-015-0726-0.

Reasoner, D.J., and Geldreich, A.E.E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Appl Environ Microbiol.* 49, 1-7. doi: 10.1128/aem.49.1.1-7.1985.

Rebuffo, C.A., Schmitt, J., Wenning, M., Von Stetten, F., and Scherer, S. (2006). Reliable and rapid identification of *Listeria monocytogenes* and *Listeria* species by artificial neural network-based Fourier transform infrared spectroscopy. *Appl. Environ. Microbiol.* 72, 994-1000. doi: 10.1128/AEM.72.2.994-1000.2006.

Redmond, N.E., Raleigh, J., Van Soest, R.W.M., Kelly, M., Travers, S.a.A., Bradshaw, B., et al. (2011). Phylogenetic Relationships of the Marine Haplosclerida (Phylum Porifera) Employing Ribosomal (28S rRNA) and Mitochondrial (cox1, nad1) Gene Sequence Data. *PLoS One* 6, e24344. doi: 10.1371/journal.pone.0024344.

Rego, A., Fernandez-Guerra, A., Duarte, P., Assmy, P., Leão, P.N., and Magalhães, C. (2021). Secondary metabolite biosynthetic diversity in Arctic Ocean metagenomes. *Microbial genomics* 7, 000731. doi: 10.1099/mgen.0.000731.

Reinhold, C.E., Nickolai Dj Fau - Piccinini, T.E., Piccinini Te Fau - Byford, B.A., Byford Ba Fau - York, M.K., York Mk Fau - Brooks, G.F., and Brooks, G.F. (1988). Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am J Clin Pathol* 89, 671-674. doi: 10.1093/ajcp/89.5.671.

Reiswig, H.M. (1981). Partial Carbon and Energy Budgets of the Bacteriosponge *Verohgia fistularis* (Porifera: Demospongiae) in Barbados. *Mar. Ecol.* 2, 273-293. doi: 10.1111/j.1439-0485.1981.tb00271.x.

Renard, E., Gazave, E., Fierro-Constain, L., Schenkelaars, Q., Ereskovsky, A., Vacelet, J., et al. (2013). "Porifera (Sponges): Recent Knowledge and New Perspectives," in *ELS*., doi: 10.1002/9780470015902.a0001582.pub2.

Rettedal, E.A., Gumpert, H., and Sommer, M.O.A. (2014). Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nature Communications* 5, 4714. doi: 10.1038/ncomms5714.

Reveillaud, J., Maignien, L., Eren, A.M., Huber, J.A., Apprill, A., Sogin, M.L., et al. (2014). Host-specificity among abundant and rare taxa in the sponge microbiome. *ISME J* 8, 1198-1209. doi: 10.1038/ismej.2013.227.

Reynolds, D., and Thomas, T. (2016). Evolution and function of eukaryotic-like proteins from sponge symbionts. *Mol. Ecol.* 25, 5242-5253. doi: 10.1111/mec.13812.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., et al. (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* 499, 431-437. doi: 10.1038/nature12352.

Rischer, M., Raguž, L., Guo, H., Keiff, F., Diekert, G., Goris, T., et al. (2018). Biosynthesis, Synthesis, and Activities of Barnesin A, a NRPS-PKS Hybrid Produced by an Anaerobic *Epsilonproteobacterium*. *ACS Chem. Biol.* 13, 1990-1995. doi: 10.1021/acschembio.8b00445.

Rix, L., Goeij, J., Oevelen, D., Struck, U., Al-Horani, F.A., Wild, C., et al. (2018). Reef sponges facilitate the transfer of coral-derived organic matter to their associated fauna via the sponge loop. *Mar. Ecol. Prog. Ser.* 589, 85-96. doi: 10.3354/meps12443.

Rix, L., Ribes, M., Coma, R., Jahn, M.T., De Goeij, J.M., Van Oevelen, D., et al. (2020). Heterotrophy in the earliest gut: a single-cell view of heterotrophic carbon and nitrogen assimilation in sponge-microbe symbioses. *The ISME Journal* 14, 2554-2567. doi: 10.1038/s41396-020-0706-3.

Robbins, S.J., Song, W., Engelberts, J.P., Glasl, B., Slaby, B.M., Boyd, J., et al. (2021). A genomic view of the microbiome of coral reef demosponges. *The ISME Journal* 15, 1641-1654. doi: 10.1038/s41396-020-00876-9.

Robbins, T., Liu, Y.-C., Cane, D.E., and Khosla, C. (2016). Structure and mechanism of assembly line polyketide synthases. *Curr. Opin. Struct. Biol.* 41, 10-18. doi: 10.1016/j.sbi.2016.05.009.

Robertsen, H.L., and Musiol-Kroll, E.M. (2019). Actinomycete-Derived Polyketides as a Source of Antibiotics and Lead Structures for the Development of New Antimicrobial Drugs. *Antibiotics* 8, 157. doi: 10.3390/antibiotics8040157.

Rodríguez-Marconi, S., De La Iglesia, R., Díez, B., Fonseca, C.A., Hajdu, E., and Trefault, N. (2015). Characterization of Bacterial, Archaeal and Eukaryote Symbionts from Antarctic Sponges Reveals a High Diversity at a Three-Domain Level and a Particular Signature for This Ecosystem. *PLoS One* 10, e0138837. doi:

Rohde, S., Nietzer, S., and Schupp, P.J. (2015). Prevalence and Mechanisms of Dynamic Chemical Defenses in Tropical Sponges. *PLoS One* 10, e0132236-e0132236. doi: 10.1371/journal.pone.0132236.

Rohde, S., and Schupp, P.J. (2011). Allocation of chemical and structural defenses in the sponge *Melophlus sarasinorum*. *J. Exp. Mar. Biol. Ecol.* 399, 76-83. doi: 10.1016/j.jembe.2011.01.012.

Romano, S. (2018). Ecology and Biotechnological Potential of Bacteria Belonging to the Genus *Pseudovibrio*. *Appl. Environ. Microbiol.* 84, e02516-02517. doi: 10.1128/AEM.02516-17.

Romano, S., Jackson, S.A., Patry, S., and Dobson, A.D.W. (2018). Extending the "One Strain Many Compounds" (OSMAC) Principle to Marine Microorganisms. *Mar. Drugs* 16, 244. doi: 10.3390/md16070244.

Romero-Tabarez, M., Jansen, R., Sylla, M., Lünsdorf, H., Häussler, S., Santosa, D.A., et al. (2006). 7-O-malonyl macrolactin A, a new macrolactin antibiotic from *Bacillus subtilis* active against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *enterococci*, and a small-colony variant of *Burkholderia cepacia*. *Antimicrob. Agents Chemother.* 50, 1701-1709. doi: 10.1128/AAC.50.5.1701-1709.2006.

Rua, C.P.J., Trindade-Silva, A.E., Appolinario, L.R., Venas, T.M., Garcia, G.D., Carvalho, L.S., et al. (2014). Diversity and antimicrobial potential of culturable heterotrophic bacteria associated with the endemic marine sponge Arenosclera brasiliensis. *PeerJ* 2, e419. doi: 10.7717/peerj.419.

Ruetzler, K. (2003). Sponges on coral reefs: a community shaped by competitive cooperation. *Bulletin of Museums and Biological Institutes of the University of Genoa* 68, 85-148.

Rule, P.L., and Alexander, A.D. (1986). Gellan gum as a substitute for agar in leptospiral media. *J. Clin. Microbiol.* 23, 500-504. doi: 10.1128/jcm.23.3.500-504.1986.

Russell, S.L. (2019). Transmission mode is associated with environment type and taxa across bacteria-eukaryote symbioses: a systematic review and meta-analysis. *FEMS Microbiol. Lett.* 366, fnz013. doi: 10.1093/femsle/fnz013.

Ryan, R.P., and Dow, J.M. (2008). Diffusible signals and interspecies communication in bacteria. *Microbiology* 154, 1845-1858. doi: 10.1099/mic.0.2008/017871-0.

Rygaard, A.M., Thøgersen, M.S., Nielsen, K.F., Gram, L., and Bentzon-Tilia, M. (2017). Effects of Gelling Agent and Extracellular Signaling Molecules on the Culturability of Marine Bacteria. *Appl. Environ. Microbiol.* 83, e00243-00217. doi: 10.1128/AEM.00243-17.

Sabrina Pankey, M., Plachetzki, D.C., Macartney, K.J., Gastaldi, M., Slattery, M., Gochfeld, D.J., et al. (2022). Cophylogeny and convergence shape holobiont evolution in sponge–microbe symbioses. *Nature Ecology & Evolution* 6, 750-762. doi: 10.1038/s41559-022-01712-3.

Sacristán-Soriano, O., Banaigs, B., Casamayor Emilio, O., and Becerro Mikel, A. (2011). Exploring the Links between Natural Products and Bacterial Assemblages in the Sponge *Aplysina aerophoba*. *Appl. Environ. Microbiol.* 77, 862-870. doi: 10.1128/AEM.00100-10.

Sagar, S., Kaur, M., and Minneman, K.P. (2010). Antiviral Lead Compounds from Marine Sponges. *Mar. Drugs* 8, 2619-2638. doi: 10.3390/md8102619.

Sala, G.D., Hochmuth, T., Costantino, V., Teta, R., Gerwick, W., Gerwick, L., et al. (2013). Polyketide genes in the marine sponge *Plakortis simplex*: a new group of mono-modular type I polyketide synthases from sponge symbionts. *Environ. Microbiol. Rep.* 5, 809-818. doi: 10.1111/1758-2229.12081.

Saleem, Z., Saeed, H., Hassali, M.A., Godman, B., Asif, U., Yousaf, M., et al. (2019). Pattern of inappropriate antibiotic use among hospitalized patients in Pakistan: a longitudinal surveillance and implications. *Antimicrobial Resistance & Infection Control* 8, 188. doi: 10.1186/s13756-019-0649-5.

Sánchez, C., Méndez, C., and Salas, J.A. (2006). Indolocarbazole natural products: occurrence, biosynthesis, and biological activity. *Nat. Prod. Rep.* 23, 1007-1045. doi: 10.1039/B601930G.

Sansinenea, E., Salazar, F., Jiménez, J., Mendoza, Á., and Ortiz, A. (2016). Diketopiperazines derivatives isolated from *Bacillus thuringiensis* and *Bacillus endophyticus*, establishment of their configuration by X-ray and their synthesis. *Tetrahedron Lett.* 57, 2604-2607. doi: 10.1016/j.tetlet.2016.04.117.

Santavy, D.L. (1985). "The symbiontic relationship between a blue-pigmented bacterium and the coral reef sponge, *Terpios granulosa*," in *In: Harmelin Vivien M, Salvat B (eds) Proc Fifth Int Coral Reef Congress, Tahiti*. Antenne Museum Ephe, Moorea, Tahiti, vol. 5, 135-140.

Santavy, D.L., and Colwell, R.R. (1990). Comparison of bacterial communities associated with the Caribbean selerosponge *Ceratoporella nicholsoni* and ambient seawater. *Mar. Ecol. Prog. Ser.* 67, 73-82. doi: 10.3354/meps067073.

Santos-Gandelman, J., Giambiagi-Demarval, M., Oelemann, W., and Laport, M. (2014). Biotechnological Potential of Sponge-Associated Bacteria. *Curr. Pharm. Biotechnol.* 15, 143-155. doi: 10.2174/1389201015666140711115033.

Santos, O.C., Pontes, P.V., Santos, J.F., Muricy, G., Giambiagi-Demarval, M., and Laport, M.S. (2010). Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Res Microbiol* 161, 604-612. doi: 10.1016/j.resmic.2010.05.013.

Sarà, M. (1971). Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). *Mar. Biol.* 11, 214-221. doi: 10.1007/BF00401270.

Sata, N.U., Matsunaga, S., Fusetani, N., and Van Soest, R.W.M. (1999). Aurantosides D, E, and F: New Antifungal Tetramic Acid Glycosides from the Marine Sponge *Siliquariaspongia japonica*. *J. Nat. Prod.* 62, 969-971. doi: 10.1021/np9900021.

Sato, M.P., Ogura, Y., Nakamura, K., Nishida, R., Gotoh, Y., Hayashi, M., et al. (2019). Comparison of the sequencing bias of currently available library preparation kits for Illumina sequencing of bacterial genomes and metagenomes. *DNA Res.* 26, 391-398. doi: 10.1093/dnares/dsz017.

Sawangwong, P., Wattanadilok, R., Kijjoa, A., Silva, A.M.S., Eaton, G., and Herz, W. (2008). Secondary metabolites from a marine sponge *Cliona patera*. *Biochem. Syst. Ecol.* 36, 493-496. doi: 10.1016/j.bse.2008.01.002.

Sayed, A.M., Hassan, M.H.A., Alhadrami, H.A., Hassan, H.M., Goodfellow, M., and Rateb, M.E. (2020). Extreme environments: microbiology leading to specialized metabolites. *J. Appl. Microbiol.* 128, 630-657. doi: 10.1111/jam.14386.

Schirmer, A., Gadkari, R., Reeves, C.D., Ibrahim, F., Delong, E.F., and Hutchinson, C.R. (2005). Metagenomic Analysis Reveals Diverse Polyketide Synthase Gene Clusters in Microorganisms Associated with the Marine Sponge Discodermia dissoluta. *Appl. Environ. Microbiol.* 71, 4840-4849. doi: 10.1128/AEM.71.8.4840-4849.2005.

Schläppy, M.-L., Schöttner, S.I., Lavik, G., Kuypers, M.M.M., De Beer, D., and Hoffmann, F. (2010). Evidence of nitrification and denitrification in high and low microbial abundance sponges. *Mar. Biol.* 157, 593-602. doi: 10.1007/s00227-009-1344-5.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl. Environ. Microbiol.* 75, 7537-7541. doi: 10.1128/AEM.01541-09.

Schmidt, E.W., Bewley, C.A., and Faulkner, D.J. (1998). Theopalauamide, a Bicyclic Glycopeptide from Filamentous Bacterial Symbionts of the Lithistid Sponge *Theonella swinhoei* from Palau and Mozambique. *The Journal of Organic Chemistry* 63, 1254-1258. doi: 10.1021/jo9718455.

Schmitt, S., Angermeier, H., Schiller, R., Lindquist, N., and Hentschel, U. (2008). Molecular Microbial Diversity Survey of Sponge Reproductive Stages and Mechanistic Insights into Vertical Transmission of Microbial Symbionts. *Appl. Environ. Microbiol.* 74, 7694-7708. doi: 10.1128/AEM.00878-08.

Schmitt, S., Deines, P., Behnam, F., Wagner, M., and Taylor, M.W. (2011). *Chloroflexi* bacteria are more diverse, abundant, and similar in high than in low microbial abundance sponges. *FEMS Microbiol. Ecol.* 78, 497-510. doi: 10.1111/j.1574-6941.2011.01179.x.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., et al. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J* 6, 564-576. doi: 10.1038/ismej.2011.116.

Schmitt, S., Weisz, J.B., Lindquist, N., and Hentschel, U. (2007). Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. *Appl Environ Microbiol* 73, 2067-2078. doi: 10.1128/AEM.01944-06.

Schorn, M.A., Alanjary, M.M., Aguinaldo, K., Korobeynikov, A., Podell, S., Patin, N., et al. (2016). Sequencing rare marine actinomycete genomes reveals high density of unique natural product biosynthetic gene clusters. *Microbiology* 162, 2075-2086. doi: 10.1099/mic.0.000386.

Schweizer, E., and Hofmann, J. (2004). Microbial Type I Fatty Acid Synthases (FAS): Major Players in a Network of Cellular FAS Systems. *Microbiol. Mol. Biol. Rev.* 68, 501. doi: 10.1128/MMBR.68.3.501-517.2004.

Selig, E.R., Turner, W.R., Troëng, S., Wallace, B.P., Halpern, B.S., Kaschner, K., et al. (2014). Global Priorities for Marine Biodiversity Conservation. *PLoS One* 9, e82898. doi: 10.1371/journal.pone.0082898.

Selvin, J., Gandhimathi, R., Kiran, G.S., Priya, S.S., Ravji, T.R., and Hema, T.A. (2009). Culturable heterotrophic bacteria from the marine sponge *Dendrilla nigra*: isolation and phylogenetic diversity of actinobacteria. *HMR* 63, 239-247. doi: 10.1007/s10152-009-0153-z.

Sfanos, K., Harmody, D., Dang, P., Ledger, A., Pomponi, S., Mccarthy, P., et al. (2005). A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. *Syst. Appl. Microbiol.* 28, 242-264. doi: 10.1016/j.syapm.2004.12.002.

Shady, N.H., El-Hossary, E.M., Fouad, M.A., Gulder, T.a.M., Kamel, M.S., and Abdelmohsen, U.R. 2017. Bioactive Natural Products of Marine Sponges from the Genus *Hyrtios*. *Molecules* 22. doi: 10.3390/molecules22050781.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* 13, 2498-2504. doi: 10.1101/gr.1239303.

Sharp, K.H., Eam, B., Faulkner, D.J., and Haygood, M.G. (2007). Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. *Appl Environ Microbiol* 73, 622-629. doi: 10.1128/AEM.01493-06.

Shungu, D., Valiant, M., Tutlane, V., Weinberg, E., Weissberger, B., Koupal, L., et al. (1983). GELRITE as an Agar Substitute in Bacteriological Media. *Appl. Environ. Microbiol.* 46, 840-845. doi: 10.1128/aem.46.4.840-845.1983.

Siegl, A., and Hentschel, U. (2010). PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. *Environ. Microbiol. Rep.* 2, 507-513. doi: 10.1111/j.1758-2229.2009.00057.x.

Simion, P., Philippe, H., Baurain, D., Jager, M., Richter, D.J., Di Franco, A., et al. (2017). A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals. *Curr. Biol.* 27, 958-967. doi: 10.1016/j.cub.2017.02.031.

Simister, R., Taylor, M.W., Tsai, P., Fan, L., Bruxner, T.J., Crowe, M.L., et al. (2012a). Thermal stress responses in the bacterial biosphere of the Great Barrier Reef sponge, *Rhopaloeides odorabile*. *Environ. Microbiol.* 14, 3232-3246. doi: 10.1111/1462-2920.12010.

Simister, R.L., Deines, P., Botté, E.S., Webster, N.S., and Taylor, M.W. (2012b). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environ. Microbiol.* 14, 517-524. doi: 10.1111/j.1462-2920.2011.02664.x.

Simons, A., Alhanout, K., and Duval, R.E. (2020). Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. *Microorganisms* 8, 639. doi: 10.3390/microorganisms8050639.

Simu, K., and Hagström, A. (2004). Oligotrophic bacterioplankton with a novel single-cell life strategy. *Appl. Environ. Microbiol.* 70, 2445-2451. doi: 10.1128/AEM.70.4.2445-2451.2004.

Singh, A., and Thakur, N.L. (2016). Significance of investigating allelopathic interactions of marine organisms in the discovery and development of cytotoxic compounds. *Chem.-Biol. Interact.* 243, 135-147. doi: 10.1016/j.cbi.2015.09.009.

Singh, R., Kumar, M., Mittal, A., and Mehta, P.K. (2017). Microbial metabolites in nutrition, healthcare and agriculture. *3 Biotech* 7, 15-15. doi: 10.1007/s13205-016-0586-4.

Sipkema, D., De Caralt, S., Morillo, J.A., Al-Soud, W.A., Sørensen, S.J., Smidt, H., et al. (2015). Similar sponge-associated bacteria can be acquired via both vertical and horizontal transmission. *Environ. Microbiol.* 17, 3807-3821. doi: 10.1111/1462-2920.12827.

Sipkema, D., Franssen, M.C.R., Osinga, R., Tramper, J., and Wijffels, R.H. (2005). Marine Sponges as Pharmacy. *Mar. Biotechnol.* 7, 142-162. doi: 10.1007/s10126-004-0405-5.

Sipkema, D., Holmes, B., Nichols, S.A., and Blanch, H.W. (2009). Biological Characterisation of *Haliclona* (?*gellius*) sp.: Sponge and Associated Microorganisms. *MicEc* 58, 903-920. doi: 10.1007/s00248-009-9534-8.

Sipkema, D., Schippers, K., Maalcke, W.J., Yang, Y., Salim, S., and Blanch, H.W. (2011). Multiple Approaches To Enhance the Cultivability of Bacteria Associated with the Marine Sponge *Haliclona* (*gellius*) sp. *Appl. Environ. Microbiol.* 77, 2130-2140. doi: 10.1128/AEM.01203-10.

Slaby, B.M., Hackl, T., Horn, H., Bayer, K., and Hentschel, U. (2017). Metagenomic binning of a marine sponge microbiome reveals unity in defense but metabolic specialization. *The ISME Journal* 11, 2465-2478. doi: 10.1038/ismej.2017.101.

Slattery, M., Gochfeld, D.J., Diaz, M.C., Thacker, R.W., and Lesser, M.P. (2016). Variability in chemical defense across a shallow to mesophotic depth gradient in the Caribbean sponge *Plakortis angulospiculatus*. *Coral Reefs* 35, 11-22. doi: 10.1007/s00338-015-1324-9.

Smanski, M.J., Zhou, H., Claesen, J., Shen, B., Fischbach, M.A., and Voigt, C.A. (2016). Synthetic biology to access and expand nature's chemical diversity. *Nature reviews. Microbiology* 14, 135-149. doi: 10.1038/nrmicro.2015.24.

Smaoui, S., Mathieu, F., Elleuch, L., Coppel, Y., Merlina, G., Karray-Rebai, I., et al. (2012). Taxonomy, purification and chemical characterization of four bioactive compounds from new *Streptomyces* sp. TN256 strain. *World J. Microbiol. Biotechnol.* 28, 793-804. doi: 10.1007/s11274-011-0872-6.

Smith, D., Wang, J.-H., Swatton, J.E., Davenport, P., Price, B., Mikkelsen, H., et al. (2006). Variations on a Theme: Diverse N-Acyl Homoserine Lactone-Mediated Quorum Sensing Mechanisms in Gram-Negative Bacteria. *ScPr* 89, 167-211. doi: 10.3184/003685006783238335.

Smith, P.W., Watkins, K., and Hewlett, A. (2012). Infection control through the ages. *Am. J. Infect. Control* 40, 35-42. doi: 10.1016/j.ajic.2011.02.019.

Song, H., Hewitt, O.H., and Degnan, S.M. (2021). Arginine Biosynthesis by a Bacterial Symbiont Enables Nitric Oxide Production and Facilitates Larval Settlement in the Marine-Sponge Host. *Curr. Biol.* 31, 433-437.e433. doi: 10.1016/j.cub.2020.10.051.

Song, J., Oh, H.-M., and Cho, J.-C. (2009). Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. *FEMS Microbiol. Lett.* 295, 141-147. doi: 10.1111/j.1574-6968.2009.01623.x.

Sperling, E.A., Robinson, J.M., Pisani, D., and Peterson, K.J. (2010). Where's the glass? Biomarkers, molecular clocks, and microRNAs suggest a 200-Myr missing Precambrian fossil record of siliceous sponge spicules. *Geobiology* 8, 24-36. doi: 10.1111/j.1472-4669.2009.00225.x.

Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.* 39, 321-346. doi: 10.1146/annurev.mi.39.100185.001541.

Stamatakis, A. (2014). RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* 30, 1312-1313. doi: 10.1093/bioinformatics/btu033.

Stasiuk, M., and Kozubek, A. (2010). Biological activity of phenolic lipids. *Cell. Mol. Life Sci.* 67, 841-860. doi: 10.1007/s00018-009-0193-1.

Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. *Nat. Prod. Rep.* 18, 380-416. doi: 10.1039/A909079G.

Steger, D., Ettinger-Epstein, P., Whalan, S., Hentschel, U., De Nys, R., Wagner, M., et al. (2008). Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ. Microbiol.* 10, 1087-1094. doi: 10.1111/j.1462-2920.2007.01515.x.

Steil, L., Hoffmann, T., Budde, I., Völker, U., and Bremer, E. (2003). Genome-Wide Transcriptional Profiling Analysis of Adaptation of *Bacillus subtilis* to High Salinity. *J. Bacteriol.* 185, 6358. doi: 10.1128/JB.185.21.6358-6370.2003.

Stein, T. (2005). Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* 56, 845-857. doi: 10.1111/j.1365-2958.2005.04587.x.

Steinert, G., Rohde, S., Janussen, D., Blaurock, C., and Schupp, P.J. (2017). Host-specific assembly of sponge-associated prokaryotes at high taxonomic ranks. *Sci. Rep.* 7, 2542. doi: 10.1038/s41598-017-02656-6.

Steinert, G., Stauffer, C.H., Aas-Valleriani, N., Borchert, E., Bhushan, A., Campbell, A., et al. (2018). "BluePharmTrain: Biology and Biotechnology of Marine Sponges" in *Grand Challenges in Marine Biotechnology,* eds. P.H. Rampelotto & A. Trincone. Cham: Springer International Publishing, 505-553. doi: 10.1007/978-3-319-69075-9_13.

Steinert, G., Taylor, M.W., Deines, P., Simister, R.L., De Voogd, N.J., Hoggard, M., et al. (2016). In four shallow and mesophotic tropical reef sponges from Guam the microbial community largely depends on host identity. *PeerJ* 4, e1936. doi: 10.7717/peerj.1936.

Steinert, G., Whitfield, S., Taylor, M.W., Thoms, C., and Schupp, P.J. (2014). Application of Diffusion Growth Chambers for the Cultivation of Marine Sponge-Associated Bacteria. *Mar. Biotechnol.* 16, 594-603. doi: 10.1007/s10126-014-9575-y.

Stingl, U., Tripp, H.J., and Giovannoni, S.J. (2007). Improvements of high-throughput culturing yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast and the Bermuda Atlantic Time Series study site. *The Isme Journal* 1, 361. doi: 10.1038/ismej.2007.49.

Storey, M.A., Andreassend, S.K., Bracegirdle, J., Brown, A., Keyzers, R.A., Ackerley, D.F., et al. (2020). Metagenomic Exploration of the Marine Sponge *Mycale hentscheli* Uncovers Multiple Polyketide-Producing Bacterial Symbionts. *mBio* 11, e02997-02919. doi: 10.1128/mBio.02997-19.

Strand, R., Whalan, S., Webster, N.S., Kutti, T., Fang, J.K.H., Luter, H.M., et al. (2017). The response of a boreal deep-sea sponge holobiont to acute thermal stress. *Sci. Rep.* 7, 1660. doi: 10.1038/s41598-017-01091-x.

Strejcek, M., Smrhova, T., Junkova, P., and Uhlik, O. (2018). Whole-Cell MALDI-TOF MS Versus 16S rRNA Gene Analysis for Identification and Dereplication of Recurrent Bacterial Isolates. *Frontiers in microbiology* 9, 1294-1294. doi: 10.3389/fmicb.2018.01294.

Subramani, R., and Sipkema, D. (2019). Marine Rare Actinomycetes: A Promising Source of Structurally Diverse and Unique Novel Natural Products. *Mar. Drugs* 17, 249. doi: 10.3390/md17050249.

Sullivan, B., Faulkner, D.J., and Webb, L. (1983). Siphonodictidine, a Metabolite of the Burrowing Sponge *Siphonodictyon* sp. That Inhibits Coral Growth. *Sci* 221, 1175-1176. doi: 10.1126/science.221.4616.1175.

Sumi, C.D., Yang, B.W., Yeo, I.-C., and Hahm, Y.T. (2014). Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Can. J. Microbiol.* 61, 93-103. doi: 10.1139/cjm-2014-0613.

Sun, W., Dai, S., Jiang, S., Wang, G., Liu, G., Wu, H., et al. (2010). Culture-dependent and culture-independent diversity of Actinobacteria associated with the marine sponge *Hymeniacidon perleve* from the South China Sea. *Antonie Van Leeuwenhoek* 98, 65-75. doi: 10.1007/s10482-010-9430-8.

Svenning, M.M., Wartiainen, I., Hestnes, A.G., and Binnerup, S.J. (2003). Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. *FEMS Microbiol. Ecol.* 44, 347-354. doi: 10.1016/S0168-6496(03)00073-4.

Swierts, T., Cleary, D.F.R., and De voogd, N.J. (2018). Prokaryotic communities of Indo-Pacific giant barrel sponges are more strongly influenced by geography than host phylogeny. *FEMS Microbiol. Ecol.* 94, fiy194. doi: 10.1093/femsec/fiy194.

Tai, B.H., Hang, D.T., Trang, D.T., Yen, P.H., Huong, P.T.T., Nhiem, N.X., et al. (2021). Conjugated Polyene Ketones From the Marine Sponge *Clathria* (*Thalysias*) *reinwardti* (Vosmaer, 1880) and Their Cytotoxic Activity. *Nat. Prod. Commun.* 16. doi: 10.1177/1934578X21104373.

Takenaka, S., Nomura, R., Minegishi, A., and Yoshida, K.-I. (2013). Enrichment and characterization of a bacterial culture that can degrade 4-aminopyridine. *BMC Microbiol.* 13, 62-62. doi: 10.1186/1471-2180-13-62.

Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y., and Kamagata, Y. (2009). Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environ. Microbiol.* 11, 1827-1834. doi: 10.1111/j.1462-2920.2009.01907.x.

Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M., et al. (2005). Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Appl. Environ. Microbiol.* 71, 2162-2169. doi: 10.1128/AEM.71.4.2162-2169.2005.

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38, 3022-3027. doi: 10.1093/molbev/msab120.

Tanaka, T., Kawasaki, K., Daimon, S., Kitagawa, W., Yamamoto, K., Tamaki, H., et al. (2014). A Hidden Pitfall in the Preparation of Agar Media Undermines Microorganism Cultivability. *Appl. Environ. Microbiol.* 80, 7659-7666. doi: 10.1128/AEM.02741-14.

Tanaka, Y., and Benno, Y. (2014). Application of a single-colony coculture technique to the isolation of hitherto unculturable gut bacteria. *Microbiol. Immunol.* 59, 63-70. doi: 10.1111/1348-0421.12220.

Tang, S.L., Hong, M.J., Liao, M.H., Jane, W.N., Chiang, P.W., Chen, C.B., et al. (2011). Bacteria associated with an encrusting sponge (*Terpios hoshinota*) and the corals partially covered by the sponge. *Environ Microbiol* 13, 1179-1191. doi: 10.1111/j.1462-2920.2010.02418.x.

Tareq, F.S., Kim, J.H., Lee, M.A., Lee, H.-S., Lee, J.-S., Lee, Y.-J., et al. (2013). Antimicrobial Gageomacrolactins Characterized from the Fermentation of the Marine-Derived Bacterium *Bacillus subtilis* under Optimum Growth Conditions. *J. Agric. Food Chem.* 61, 3428-3434. doi: 10.1021/jf4009229.

Tasdemir, D., Mangalindan, G.C., Concepción, G.P., Verbitski, S.M., Rabindran, S., Miranda, M., et al. (2002). Bioactive Isomalabaricane Triterpenes from the Marine Sponge *Rhabdastrella globostellata*. *J. Nat. Prod.* 65, 210-214. doi: 10.1021/np0104020.

Tawfike, A., Attia, E.Z., Desoukey, S.Y., Hajjar, D., Makki, A.A., Schupp, P.J., et al. (2019). New bioactive metabolites from the elicited marine sponge-derived bacterium *Actinokineospora spheciospongiae* sp. nov. *AMB Express* 9, 12. doi: 10.1186/s13568-018-0730-0.

Taylor, J.A., Palladino, G., Wemheuer, B., Steinert, G., Sipkema, D., Williams, T.J., et al. (2021). Phylogeny resolved, metabolism revealed: functional radiation within a widespread and divergent clade of sponge symbionts. *The ISME Journal* 15, 503-519. doi: 10.1038/s41396-020-00791-z.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiol. Mol. Biol. Rev.* 71, 295-347. doi: 10.1128/MMBR.00040-06.

Taylor, M.W., Schupp, P.J., Baillie, H.J., Charlton, T.S., De Nys, R., Kjelleberg, S., et al. (2004a). Evidence for acyl homoserine lactone signal production in bacteria associated with marine sponges. *Appl. Environ. Microbiol.* 70, 4387-4389. doi: 10.1128/AEM.70.7.4387-4389.2004.

Taylor, M.W., Schupp, P.J., Dahllöf, I., Kjelleberg, S., and Steinberg, P.D. (2004b). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environ. Microbiol.* 6, 121-130. doi: 10.1046/j.1462-2920.2003.00545.x.

Taylor, M.W., Tsai, P., Simister, R.L., Deines, P., Botte, E., Ericson, G., et al. (2013). 'Sponge-specific' bacteria are widespread (but rare) in diverse marine environments. *ISME J* 7, 438-443. doi: 10.1038/ismej.2012.111.

Taylor, P.L., and Wright, G.D. (2008). Novel approaches to discovery of antibacterial agents. *Anim. Health Res. Rev.* 9, 237-246. doi: 10.1017/S1466252308001527.

Teasdale, M.E., Donovan, K.A., Forschner-Dancause, S.R., and Rowley, D.C. (2011). Gram-Positive Marine Bacteria as a Potential Resource for the Discovery of Quorum Sensing Inhibitors. *Mar. Biotechnol.* 13, 722-732. doi: 10.1007/s10126-010-9334-7.

Thacker, R.W., Becerro, M.A., Lumbang, W.A., and Paul, V.J. (1998). Allelopathic Interactions between Sponges on a Tropical Reef. *Ecology* 79, 1740-1750. doi: 10.2307/176792.

Thacker, R.W., and Freeman, C.J. (2012). Sponge-microbe symbioses: recent advances and new directions. *Adv Mar Biol* 62, 57-111. doi: 10.1016/B978-0-12-394283-8.00002-3.

Thai, M.Q. (2013). "A review of the diversity of sponges (Porifera) in Vietnam", in: *The 2nd international workshop on marine bioresources of Vietnam.* Ha noi, Vietnam.

Thakur, N.L., and Singh, A. (2016). "Chemical Ecology of Marine Sponges," in *Marine Sponges: Chemicobiological and Biomedical Applications,* eds. R. Pallela & H. Ehrlich. New Delhi: Springer India, 37-52. doi: 10.1007/978-81-322-2794-6_3.

Thiel, V., Neulinger, S.C., Staufenberger, T., Schmaljohann, R., and Imhoff, J.F. (2007). Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. *FEMS Microbiol. Ecol.* 59, 47-63. doi: 10.1111/j.1574-6941.2006.00217.x.

Thomas, T., Moitinho-Silva, L., Lurgi, M., Bjork, J.R., Easson, C., Astudillo-Garcia, C., et al. (2016). Diversity, structure and convergent evolution of the global sponge microbiome. *Nat Commun* 7, 11870. doi: 10.1038/ncomms11870.

Thomas, T., Rusch, D., Demaere, M.Z., Yung, P.Y., Lewis, M., Halpern, A., et al. (2010a). Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *ISME J* 4, 1557-1567. doi: 10.1038/ismej.2010.74.

Thomas, T.R.A., Kavlekar, D.P., and Lokabharathi, P.A. (2010b). Marine Drugs from Sponge-Microbe Association—A Review. *Mar. Drugs* 8, 1417-1468. doi: 10.3390/md8041417.

Thompson, J.E., Walker, R.P., and Faulkner, D.J. (1985). Screening and bioassays for biologically-active substances from forty marine sponge species from San Diego, California, USA. *Mar. Biol.* 88, 11-21. doi: 10.1007/BF00393038.

Thoms, C., and Schupp, P. (2005). Biotechnological Potential of Marine Sponges and their Associated Bacteria as Producers of New Pharmaceuticals (Part II). *Journal of International Biotechnology Law* 2, 257. doi: 10.1515/jibl.2005.2.6.257.

Thoms, C., and Schupp, P.J. (2007). "Chemical defense strategies in sponges: a review," in *Porifera research: biodiversity, innovation and sustainability,* eds. M.R. Custodia, E. Lobo-Hadju & G. Muricy. Série Livros 28, Museu Nacional, Rio de Janeiro), 627–637.

Thornburg, C.C., Zabriskie, T.M., and Mcphail, K.L. (2010). Deep-Sea Hydrothermal Vents: Potential Hot Spots for Natural Products Discovery? *J. Nat. Prod.* 73, 489-499. doi: 10.1021/np900662k.

Thuaire, B., Allanic, Y., Hoang, V.A., Le, K.Q., Luu, H.T., Nguyen, T.C., et al. (2021). *Assessing the biodiversity in Viet Nam – Analysis of the impacts from the economic sectors.* Ha Noi, Viet Nam: WWF-Viet Nam.

Tian, R.-M., Zhang, W., Cai, L., Wong, Y.-H., Ding, W., and Qian, P.-Y. (2017). Genome Reduction and Microbe-Host Interactions Drive Adaptation of a Sulfur-Oxidizing Bacterium Associated with a Cold Seep Sponge. *mSystems* 2, e00184-00116. doi: 10.1128/mSystems.00184-16.

Tian, X., Zhang, Z., Yang, T., Chen, M., Li, J., Chen, F., et al. (2016). Comparative Genomics Analysis of Streptomyces Species Reveals Their Adaptation to the Marine Environment and Their Diversity at the Genomic Level. *Frontiers in microbiology* 7, 998-998. doi: 10.3389/fmicb.2016.00998.

Tianero, M.D., Balaich, J.N., and Donia, M.S. (2019). Localized production of defence chemicals by intracellular symbionts of *Haliclona* sponges. *Nature Microbiology* 4, 1149-1159. doi: 10.1038/s41564-019-0415-8.

Tillich, U.M., Wolter, N., Schulze, K., Kramer, D., Brödel, O., and Frohme, M. (2014). High-throughput cultivation and screening platform for unicellular phototrophs. *BMC Microbiol.* 14, 239. doi: 10.1186/s12866-014-0239-x.

Tolar, B., King, G., and Hollibaugh, J. (2013). An Analysis of *Thaumarchaeota* Populations from the Northern Gulf of Mexico. *Frontiers in Microbiology* 4, 72. doi: 10.3389/fmicb.2013.00072.

Tracanna, V., De Jong, A., Medema, M.H., and Kuipers, O.P. (2017). Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiol. Rev.* 41, 417-429. doi: 10.1093/femsre/fux014.

Trang, D.T., Dung, D.T., Hang, D.T.T., Dung, N.V., Linh, T.M., Mai, N.C., et al. (2022a). Diketopiperazines from *Rhabdastrella globostellata*. *Vietnam Journal of Chemistry* 60, 27-31. doi: 10.1002/vjch.202200054.

Trang, D.T., Hang, D.T.T., Dung, D.T., Bang, N.A., Huong, P.T.T., Yen, P.H., et al. (2022b). Chemical constituents of *Clathria reinwardti*. *Vietnam Journal of Chemistry* 60, 21-26. doi: 10.1002/vjch.202200053.

Tréguer, P.J., and De La Rocha, C.L. (2013). The World Ocean Silica Cycle. *Ann. Rev. Mar. Sci.* 5, 477-501. doi: 10.1146/annurev-marine-121211-172346.

Trinh, P.T., Yurchenko, A.N., Khmel, O.O., Dieu, T.V., Ngoc, N.T., Girich, E.V., et al. (2022). Cytoprotective Polyketides from Sponge-Derived Fungus *Lopadostoma pouzarii*. *Molecules* 27. doi: 10.3390/molecules27217650.

Trinh, P.T.H., Ngoc, N.T.D., Ly, B.M., Hung, L.D., Hang, C.T.T., Trang, V.T.D., et al. (2016). Antibacterial activity of marine bacteria isolated from marine sponges at Phu Quoc island, Vietnam. *Academia Journal of Biology* 38, 109-114. doi: 10.15625/0866-7160/v38n1.7074.

Trinh, P.T.H., Thanh Van, T.T., Minh Ly, B., Kyu Choi, B., Jae Shin, H., Seok Lee, J., et al. (2020). Antimicrobial activity of natural compounds from sponge – derived fungus *Aspergillus flocculosus* 01NT.1.1.5. *Vietnam Journal of Biotechnology* 16, 729-735. doi: 10.15625/1811-4989/16/4/8866.

Tuan, C.D., Ngan, T.B., Huong, D.T.M., Quyen, V.T., Minh, L.T.H., Murphy, B., et al. (2017). Secondary metabolites from *Micromonospora* sp. (G044). *Vietnam Journal of Science and Technology* 55, 251-257. doi: 10.15625/2525-2518/55/3/8641.

Tully, B.J., Graham, E.D., and Heidelberg, J.F. (2018). The reconstruction of 2,631 draft metagenome-assembled genomes from the global oceans. *Scientific Data* 5, 170203. doi: 10.1038/sdata.2017.203.

Turner, E.C. (2021). Possible poriferan body fossils in early Neoproterozoic microbial reefs. *Nature* 596, 87-91. doi: 10.1038/s41586-021-03773-z.

Turon, M., Cáliz, J., Garate, L., Casamayor, E.O., and Uriz, M.J. (2018). Showcasing the role of seawater in bacteria recruitment and microbiome stability in sponges. *Sci. Rep.* 8, 15201. doi: 10.1038/s41598-018-33545-1.

Turon, M., Cáliz, J., Triadó-Margarit, X., Casamayor, E.O., and Uriz, M.J. (2019a). Sponges and Their Microbiomes Show Similar Community Metrics Across Impacted and Well-Preserved Reefs. *Frontiers in Microbiology* 10, 1961. doi: 10.3389/fmicb.2019.01961.

Turon, M., Uriz Maria, J., and Martin, D. (2019b). Multipartner Symbiosis across Biological Domains: Looking at the Eukaryotic Associations from a Microbial Perspective. *mSystems* 4, e00148-00119. doi: 10.1128/mSystems.00148-19.

Turon, M., and Uriz, M.J. (2020). New Insights Into the Archaeal Consortium of Tropical Sponges. *Frontiers in Marine Science* 6. doi: 10.3389/fmars.2019.00789.

Turque, A.S., Batista, D., Silveira, C.B., Cardoso, A.M., Vieira, R.P., Moraes, F.C., et al. (2010). Environmental shaping of sponge associated archaeal communities. *PLoS One* 5, e15774. doi: 10.1371/journal.pone.0015774.

Ueoka, R., Uria, A.R., Reiter, S., Mori, T., Karbaum, P., Peters, E.E., et al. (2015). Metabolic and evolutionary origin of actin-binding polyketides from diverse organisms. *Nat. Chem. Biol.* 11, 705-712. doi: 10.1038/nchembio.1870.

Unson, M.D., and Faulkner, D.J. (1993). Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera). *Experientia* 49, 349-353. doi: 10.1007/BF01923420.

Unson, M.D., Holland, N.D., and Faulkner, D.J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar. Biol.* 119, 1-11. doi: 10.1007/BF00350100.

Uriz, M.-J., Turon, X., Becerro, M.A., and Agell, G. (2003). Siliceous spicules and skeleton frameworks in sponges: Origin, diversity, ultrastructural patterns, and biological functions. *Microsc. Res. Tech.* 62, 279-299. doi: 10.1002/jemt.10395.

Uriz, M.J., Agell, G., Blanquer, A., Turon, X., and Casamayor, E.O. (2012). Endosymbiotic calcifying bacteria: a new cue to the origin of calcification in metazoa? *Evolution* 66, 2993-2999. doi: 10.1111/j.1558-5646.2012.01676.x.

Uriz, M.J., Turon, X., and Becerro, M.A. (2001). Morphology and ultrastructure of the swimming larvae of *Crambe crambe* (Demospongiae, Poecilosclerida). *Invertebr. Biol.* 120, 295-307. doi: 10.1111/j.1744-7410.2001.tb00039.x.

Usher, K.M. (2008). The ecology and phylogeny of cyanobacterial symbionts in sponges. *Mar. Ecol.* 29, 178-192. doi: 10.1111/j.1439-0485.2008.00245.x.

Usher, K.M., Sutton, D.C., Toze, S., Kuo, J., and Fromont, J. (2005). Inter-generational transmission of microbial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Mar Freshw Res* 56, 125-131. doi: 10.1071/MF04304.

Vacelet, J. (1975). Etude en mircrosopie électronique de l'association entre bactéries et spongiaires du genre Verongia (Dictyoceratida). *J. Microsc. Biol. Cell.* 23, 271-288.

Vacelet, J., and Donadey, C. (1977). Electron microscope study of the association between some sponges and bacteria. *J. Exp. Mar. Biol. Ecol.* 30, 301-314. doi: 10.1016/0022-0981(77)90038-7.

Valentine, J.W. (2004). *On the Origin of Phyla.* Chicago, IL: The University of Chicago Press.

Van Goethem, M.W., Osborn, A.R., Bowen, B.P., Andeer, P.F., Swenson, T.L., Clum, A., et al. (2021). Long-read metagenomics of soil communities reveals phylum-specific secondary metabolite dynamics. *Communications Biology* 4, 1302. doi: 10.1038/s42003-021-02809-4.

Van Kessel, M.a.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op Den Camp, H.J.M., Kartal, B., et al. (2015). Complete nitrification by a single microorganism. *Nature* 528, 555-559. doi: 10.1038/nature16459.

Van Niel, C.B. (1971). "[1] Techniques for the enrichment, isolation, and maintenance of the photosynthetic bacteria," in *Methods Enzymol.* Academic Press, vol. 23, 3-28. doi: 10.1016/S0076-6879(71)23077-9.

Van Soest, R.W.M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., De Voogd, N.J., et al. (2012). Global Diversity of Sponges (Porifera). *PLoS One* 7, e35105. doi: 10.1371/journal.pone.0035105.

Vanwonterghem, I., and Webster, N.S. (2020). Coral Reef Microorganisms in a Changing Climate. *iScience* 23. doi: 10.1016/j.isci.2020.100972.

Vekeman, B., Dumolin, C., De Vos, P., and Heylen, K. (2017). Improved enrichment culture technique for methane-oxidizing bacteria from marine ecosystems: the effect of adhesion material and gas composition. *Antonie Van Leeuwenhoek* 110, 281-289. doi: 10.1007/s10482-016-0787-1.

Ventola, C.L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics* 40, 277-283.

Verhamme, D.T., Prosser, J.I., and Nicol, G.W. (2011). Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *The ISME journal* 5, 1067-1071. doi: 10.1038/ismej.2010.191.

Verhoeven, J.T.P., Kavanagh, A.N., and Dufour, S.C. (2017). Microbiome analysis shows enrichment for specific bacteria in separate anatomical regions of the deep-sea carnivorous sponge *Chondrocladia grandis*. *FEMS Microbiol. Ecol.* 93, fiw214-fiw214. doi: 10.1093/femsec/fiw214.

Versluis, D., Mcpherson, K., Van Passel, M.W.J., Smidt, H., and Sipkema, D. (2017). Recovery of Previously Uncultured Bacterial Genera from Three Mediterranean Sponges. *Mar. Biotechnol.* 19, 454-468. doi: 10.1007/s10126-017-9766-4.

Versluis, D., Nijsse, B., Naim, M.A., Koehorst, J.J., Wiese, J., Imhoff, J.F., et al. (2018). Comparative Genomics Highlights Symbiotic Capacities and High Metabolic Flexibility of the Marine Genus *Pseudovibrio*. *Genome Biol. Evol.* 10, 125-142. doi: 10.1093/gbe/evx271.

Vicente, J., Stewart, A., Song, B., Hill, R.T., and Wright, J.L. (2013). Biodiversity of Actinomycetes associated with Caribbean sponges and their potential for natural product discovery. *Mar. Biotechnol. (N. Y.)* 15, 413-424. doi: 10.1007/s10126-013-9493-4.

Vogel, S. (1977). Current-induced flow through living sponges in nature. *Proceedings of the National Academy of Sciences* 74, 2069-2071. doi: 10.1073/pnas.74.5.2069.

Vu, V.N., Huong, D.T.M., Dao, P.T., Anh, N.M., Minh, L.T.H., and Van Cuong, P. (2019). Secondary metabolites produced by marine actinomycete *Micromonospora* sp. A258. *Vietnam Journal of Chemistry* 57, 707-711. doi: 10.1002/vjch.201900098.

Waddell, B., and Pawlik, J.R. (2000). Defenses of Caribbean sponges against invertebrate predators. I. Assays with hermit crabs. *Mar. Ecol. Prog. Ser.* 195, 125-132. doi: 10.3354/meps195125.

Wagner-Döbler, I., Beil, W., Lang, S., Meiners, M., and Laatsch, H. (2002). "Integrated Approach To Explore the Potential of Marine Microorganisms for the Production of Bioactive Metabolites," in *Tools and Applications of Biochemical Engineering Science,* eds. K. Schügerl, A.P. Zeng, J.G. Aunins, A. Bader, W. Bell, H. Biebl, M. Biselli, M.J.T. Carrondo, L.R. Castilho, H.N. Chang, P.E. Cruz, C. Fuchs, S.J. Han, M.R. Han, E. Heinzle, B. Hitzmann, D. Köster, I. Jasmund, N. Jelinek, S. Lang, H. Laatsch, J. Lee, H. Miirkl, L. Maranga, R.A. Medronho, M. Meiners, S. Nath, T. Noll, T. Scheper, S. Schmidt, K. Schüigerl, E. Stäirk, A. Tholey, I. Wagner-Döbler, C. Wandrey, C. Wittmann, S.C. Yim & A.P. Zeng. Berlin, Heidelberg, Germany: Springer Berlin Heidelberg, 207-238. doi: 10.1007/3-540-45736-4_10.

Wakimoto, T., Egami, Y., Nakashima, Y., Wakimoto, Y., Mori, T., Awakawa, T., et al. (2014). Calyculin biogenesis from a pyrophosphate protoxin produced by a sponge symbiont. *Nat. Chem. Biol.* 10, 648-655. doi: 10.1038/nchembio.1573.

Wang, J.-H., Quan, C.-S., Qi, X.-H., Li, X., and Fan, S.-D. (2010). Determination of diketopiperazines of Burkholderia cepacia CF-66 by gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 396, 1773-1779. doi: 10.1007/s00216-009-3379-3.

Ward, B.B. (2011). "Nitrification in the ocean," in *Nitrification,* eds. B.B. Ward, D.J. Arp & M.G. Klotz. Washington, D.C.: ASM Press, 325-345. doi: 10.1128/9781555817145.ch13.

Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T., et al. (2016). "gplots: various R programming tools for plotting data. R package 3.0.1".

Waters, A.L., Peraud, O., Kasanah, N., Sims, J.W., Kothalawala, N., Anderson, M.A., et al. (2014). An analysis of the sponge *Acanthostrongylophora igens*' microbiome yields an actinomycete that produces the natural product manzamine A. *Frontiers in Marine Science* 1, 54. doi: 10.3389/fmars.2014.00054.

Waterworth, S.C., Jiwaji, M., Kalinski, J.-C.J., Parker-Nance, S., and Dorrington, R.A. (2017). A Place to Call Home: An Analysis of the Bacterial Communities in Two *Tethya rubra* Samaai and Gibbons 2005 Populations in Algoa Bay, South Africa. *Mar. Drugs* 15, 95. doi: 10.3390/md15040095.

Waterworth, S.C., Parker-Nance, S., Kwan, J.C., and Dorrington, R.A. (2021). Comparative Genomics Provides Insight into the Function of Broad-Host Range Sponge Symbionts. *mBio* 12, e01577-01521. doi: 10.1128/mBio.01577-21.

Wattana-Amorn, P., Charoenwongsa, W., Williams, C., Crump, M.P., and Apichaisataienchote, B. (2016). Antibacterial activity of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) from *Streptomyces* sp. strain 22-4 against phytopathogenic bacteria. *Nat. Prod. Res.* 30, 1980-1983. doi: 10.1080/14786419.2015.1095747.

Webster, N.S., and Hill, R.T. (2001). The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-protebacterium. *Mar. Biol.* 138, 843-851. doi: 10.1007/s002270000503.

Webster, N.S., and Taylor, M.W. (2012). Marine sponges and their microbial symbionts: love and other relationships. *Environ. Microbiol.* 14, 335-346. doi: 10.1111/j.1462-2920.2011.02460.x.

Webster, N.S., Taylor, M.W., Behnam, F., Lucker, S., Rattei, T., Whalan, S., et al. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ Microbiol* 12, 2070-2082. doi: 10.1111/j.1462-2920.2009.02065.x.

Webster, N.S., and Thomas, T. (2016). The Sponge Hologenome. *MBio* 7. doi: 10.1128/mBio.00135-16.

Webster, N.S., Wilson, K.J., Blackall, L.L., and Hill, R.T. (2001a). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl. Environ. Microbiol.* 67, 434-444. doi: 10.1128/AEM.67.1.434-444.2001.

Webster, S.N., Watts, E.M.J., and Hill, T.R. (2001b). Detection and Phylogenetic Analysis of Novel Crenarchaeote and Euryarchaeote 16S Ribosomal RNA Gene Sequences from a Great Barrier Reef Sponge. *Mar. Biotechnol.* 3, 600-608. doi: 10.1007/s10126-001-0065-7.

Wehrl, M., Steinert, M., and Hentschel, U. (2007). Bacterial Uptake by the Marine Sponge *Aplysina aerophoba*. *MicEc* 53, 355-365. doi: 10.1007/s00248-006-9090-4.

Weigel, B.L., and Erwin, P.M. (2015). Intraspecific Variation in Microbial Symbiont Communities of the Sun Sponge, *Hymeniacidon heliophila*, from Intertidal and Subtidal Habitats. *Appl Environ Microbiol* 82, 650-658. doi: 10.1128/AEM.02980-15.

Weiner, R.M., Segall, A.M., and Colwell, A.R.R. (1985). Characterization of a Marine Bacterium Associated with *Crassostrea virginica* (the Eastern Oyster) *Appl Environ Microbiol.* 49, 83-90.

Weissman, K.J. (2015). The structural biology of biosynthetic megaenzymes. *Nat. Chem. Biol.* 11, 660-670. doi: 10.1038/nchembio.1883.

White, J.R., Patel, J., Ottesen, A., Arce, G., Blackwelder, P., and Lopez, J.V. (2012). Pyrosequencing of Bacterial Symbionts within *Axinella corrugata* Sponges: Diversity and Seasonal Variability. *PLoS One* 7, e38204. doi: 10.1371/journal.pone.0038204.

White, R.J. (2012). "The Early History of Antibiotic Discovery: Empiricism Ruled," in *Antibiotic Discovery and Development,* eds. T.J. Dougherty & M.J. Pucci. Boston, MA: Springer US, 3-31. doi: 10.1007/978-1-4614-1400-1_1.

Whitson, E.L., Bugni, T.S., Chockalingam, P.S., Concepcion, G.P., Harper, M.K., He, M., et al. (2008). Sphecioserol Sulfates, PKCζ Inhibitors from a Philippine Sponge *Spheciospongia* sp. *J. Nat. Prod.* 71, 1213-1217. doi: 10.1021/np8001628.

Wilkinson, C.R. (1978a). Microbial associations in sponges. I. Ecology, physiology and microbial populations of coral reef sponges. *Mar. Biol.* 49, 161-167. doi: 10.1007/BF00387115.

Wilkinson, C.R. (1978b). Microbial associations in sponges. II. Numerical analysis of sponge and water bacterial populations. *Mar. Biol.* 49, 169-176. doi: 10.1007/BF00387116.

Wilkinson, C.R. (1978c). Microbial associations in sponges. III. Ultrastructure of the in situ associations in coral reef sponges. *Mar. Biol.* 49, 177-185. doi: 10.1007/BF00387117.

Wilkinson, C.R. (1978d). Significance of microbial symbionts in sponge evolution and ecology. *Symbiosis* 4, 135-146. doi:

Wilkinson, C.R., Nowak, M., Austin, B., and Colwell, R.R. (1981). Specificity of bacterial symbionts in Mediterranean and Great Barrier Reef sponges. *MicEc* 7, 13-21. doi: 10.1007/BF02010474.

Wilkinson, C.R., and Smith, D.C. (1984). Immunological evidence for the Precambrian origin of bacterial symbioses in marine sponges. *Proceedings of the Royal Society of London. Series B. Biological Sciences* 220, 509-518. doi: 10.1098/rspb.1984.0017.

Wilson, D.M., Puyana, M., Fenical, W., and Pawlik, J.R. (1999). Chemical Defense of the Caribbean Reef Sponge *Axinella corrugata* Against Predatory Fishes. *J. Chem. Ecol.* 25, 2811-2823. doi: 10.1023/A:1020811810223.

Wilson, M.C., Mori, T., Rückert, C., Uria, A.R., Helf, M.J., Takada, K., et al. (2014). An environmental bacterial taxon with a large and distinct metabolic repertoire. *Nature* 506, 58-62. doi: 10.1038/nature12959.

Wohlleben, W., Mast, Y., Stegmann, E., and Ziemert, N. (2016). Antibiotic drug discovery. *Microbial Biotechnology* 9, 541-548. doi: 10.1111/1751-7915.12388.

Wood, R., Andrey Yu, Z., and Debrenne, F. (1992). Functional Biology and Ecology of Archaeocyatha. *Palaios* 7, 131-156. doi: 10.2307/3514925.

Wright, G.D. (2019). Unlocking the potential of natural products in drug discovery. *Microbial Biotechnology* 12, 55-57. doi: 10.1111/1751-7915.13351.

Wright, P.M., Seiple, I.B., and Myers, A.G. (2014). The Evolving Role of Chemical Synthesis in Antibacterial Drug Discovery. *Angew. Chem. Int. Ed.* 53, 8840-8869. doi: 10.1002/anie.201310843.

Wu, S., Ou, H., Liu, T., Wang, D., and Zhao, J. (2018). Structure and dynamics of microbiomes associated with the marine sponge *Tedania* sp. during its life cycle. *FEMS Microbiol. Ecol.* 94, fiy055. doi: 10.1093/femsec/fiy055.

Wuchter, C., Abbas, B., Coolen, M.J.L., Herfort, L., Van Bleijswijk, J., Timmers, P., et al. (2006). Archaeal nitrification in the ocean. *Proceedings of the National Academy of Sciences* 103, 12317-12322.

Wulff, J. (2016). "Sponge Contributions to the Geology and Biology of Reefs: Past, Present, and Future," in *Coral Reefs at the Crossroads,* eds. D.K. Hubbard, C.S. Rogers, J.H. Lipps & J.G.D. Stanley. Dordrecht: Springer Netherlands, 103-126. doi: 10.1007/978-94-017-7567-0_5.

Wulff, J.L. (2006). Ecological interactions of marine sponges. *Can. J. Zool.* 84, 146-166. doi: 10.1139/z06-019.

Xiao, S., Chen, N., Chai, Z., Zhou, M., Xiao, C., Zhao, S., et al. (2022). Secondary Metabolites from Marine-Derived Bacillus: A Comprehensive Review of Origins, Structures, and Bioactivities. *Mar. Drugs* 20, 567. doi: 10.3390/md20090567.

Xu, L., Ye, K.-X., Dai, W.-H., Sun, C., Xu, L.-H., and Han, B.-N. (2019). Comparative Genomic Insights into Secondary Metabolism Biosynthetic Gene Cluster Distributions of Marine *Streptomyces*. *Marine drugs* 17. doi: 10.3390/md17090498.

Xu, Y., Du, X., Yu, X., Jiang, Q., Zheng, K., Xu, J., et al. (2022). Recent Advances in the Heterologous Expression of Biosynthetic Gene Clusters for Marine Natural Products. *Mar. Drugs* 20, 341. doi: 10.3390/md20060341.

Yamada, Y., Kuzuyama, T., Komatsu, M., Shin-Ya, K., Omura, S., Cane, D.E., et al. (2015). Terpene synthases are widely distributed in bacteria. *Proceedings of the National Academy of Sciences* 112, 857. doi: 10.1073/pnas.1422108112.

Yang, Q., Franco, C.M.M., Lin, H.-W., and Zhang, W. (2019). Untapped sponge microbiomes: structure specificity at host order and family levels. *FEMS Microbiol. Ecol.* 95, fiz136. doi: 10.1093/femsec/fiz136.

Yang, Q., Franco, C.M.M., Sorokin, S.J., and Zhang, W. (2017). Development of a multilocus-based approach for sponge (phylum Porifera) identification: refinement and limitations. *Sci. Rep.* 7, 41422. doi: 10.1038/srep41422.

Yang, S.-C., Lin, C.-H., Sung, C.T., and Fang, J.-Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology* 5, 241. doi: 10.3389/fmicb.2014.00241.

Yao, H., Campbell, C.D., Chapman, S.J., Freitag, T.E., Nicol, G.W., and Singh, B.K. (2013). Multi-factorial drivers of ammonia oxidizer communities: evidence from a national soil survey. *Environ. Microbiol.* 15, 2545-2556. doi: 10.1111/1462-2920.12141.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res* 42, D643-648. doi: 10.1093/nar/gkt1209.

Yin, Z., Zhu, M., Davidson, E.H., Bottjer, D.J., Zhao, F., and Tafforeau, P. (2015a). Sponge grade body fossil with cellular resolution dating 60 Myr before the Cambrian. *Proc Natl Acad Sci USA* 112, E1453-1460. doi: 10.1073/pnas.1414577112.

Yin, Z., Zhu, M., Davidson, E.H., Bottjer, D.J., Zhao, F., and Tafforeau, P. (2015b). Sponge grade body fossil with cellular resolution dating 60 Myr before the Cambrian. *Proceedings of the National Academy of Sciences* 112, E1453-E1460. doi: 10.1073/pnas.1414577112.

Yonezawa, K., Yamada, K., and Kouno, I. (2011). New Diketopiperazine Derivatives Isolated from Sea Urchin-Derived *Bacillus* sp. *Chem. Pharm. Bull.* 59, 106-108. doi: 10.1248/cpb.59.106.

Yoon, B.K., Jackman, J.A., Valle-González, E.R., and Cho, N.-J. (2018). Antibacterial Free Fatty Acids and Monoglycerides: Biological Activities, Experimental Testing, and Therapeutic Applications. *International journal of molecular sciences* 19, 1114. doi: 10.3390/ijms19041114.

Yu, Y., Zhang, Q., and Van Der Donk, W.A. (2013). Insights into the evolution of lanthipeptide biosynthesis. *Protein Sci.* 22, 1478-1489. doi: 10.1002/pro.2358.

Yung, P.Y., Burke, C., Lewis, M., Kjelleberg, S., and Thomas, T. (2011). Novel Antibacterial Proteins from the Microbial Communities Associated with the Sponge *Cymbastela concentrica* and the Green Alga *Ulva australis*. *Appl. Environ. Microbiol.* 77, 1512-1515. doi: 10.1128/AEM.02038-10.

Zeglin, L.H. (2015). Stream microbial diversity in response to environmental changes: review and synthesis of existing research. *Frontiers in Microbiology* 6, 454. doi: 10.3389/fmicb.2015.00454.

Zeng, Z., Zhao, J., Ke, C., and Wang, D. (2013). Antimicrobial activities of novel cultivable bacteria isolated from marine sponge *Tedania anhelans*. *Chin. J. Oceanol. Limnol.* 31, 581-590. doi: 10.1007/s00343-013-2223-y.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E.J., Short, J.M., et al. (2002). Cultivating the uncultured. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15681-15686. doi: 10.1073/pnas.252630999.

Zengler, K., Walcher, M., Clark, G., Haller, I., Toledo, G., Holland, T., et al. (2005). "High-Throughput Cultivation of Microorganisms Using Microcapsules," in *Methods Enzymol.,* ed. J.R. Leadbetter. Cambridge, MA: Academic Press, vol. 397, 124-130. doi: 10.1016/s0076-6879(05)97007-9.

Zhang, F., Blasiak, L.C., Karolin, J.O., Powell, R.J., Geddes, C.D., and Hill, R.T. (2015). Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. *Proceedings of the National Academy of Sciences* 112, 4381-4386. doi: 10.1073/pnas.1423768112.

Zhang, F., Jonas, L., Lin, H., and Hill, R.T. (2019). Microbially mediated nutrient cycles in marine sponges. *FEMS Microbiol. Ecol.* 95, fiz155. doi: 10.1093/femsec/fiz155.

Zhang, H., Dong, M., Wang, H., and Crews, P. (2017a). Secondary Metabolites from the Marine Sponge Genus *Phyllospongia*. *Mar. Drugs* 15, 12. doi: 10.3390/md15010012.

Zhang, H., Lee, Y.K., Zhang, W., and Lee, H.K. (2006). Culturable Actinobacteria from the Marine Sponge *Hymeniacidon perleve*: Isolation and Phylogenetic Diversity by 16S rRNA gene-RFLP Analysis. *Antonie Van Leeuwenhoek* 90, 159-169. doi: 10.1007/s10482-006-9070-1.

Zhang, H., Zhao, Z., and Wang, H. (2017b). Cytotoxic Natural Products from Marine Sponge-Derived Microorganisms. *Mar. Drugs* 15, 68. doi: 10.3390/md15030068.

Zhao, X., and Kuipers, O.P. (2016). Identification and classification of known and putative antimicrobial compounds produced by a wide variety of Bacillales species. *BMC Genomics* 17, 882. doi: 10.1186/s12864-016-3224-y.

Zhou, K., Zhang, R., Sun, J., Zhang, W., Tian, R.-M., Chen, C., et al. (2019). Potential Interactions between Clade SUP05 Sulfur-Oxidizing Bacteria and Phages in Hydrothermal Vent Sponges. *Appl. Environ. Microbiol.* 85, e00992-00919. doi: 10.1128/AEM.00992-19.

Zhou, N., Sun, Y.-T., Chen, D.-W., Du, W., Yang, H., and Liu, S.-J. (2018). Harnessing microfluidic streak plate technique to investigate the gut microbiome of *Reticulitermes chinensis*. *MicrobiologyOpen* 0, e00654. doi: 10.1002/mbo3.654.

Zhu, J., Liu, Y., Liu, Z., Wang, H., and Zhang, H. (2019). Bioactive Nitrogenous Secondary Metabolites from the Marine Sponge Genus *Haliclona*. *Mar. Drugs* 17, 682. doi: 10.3390/md17120682.

Ziemert, N., Podell, S., Penn, K., Badger, J.H., Allen, E., and Jensen, P.R. (2012). The Natural Product Domain Seeker NaPDoS: A Phylogeny Based Bioinformatic Tool to Classify Secondary Metabolite Gene Diversity. *PLoS One* 7, e34064. doi: 10.1371/journal.pone.0034064.

Zotchev, S.B., Stepanchikova, A.V., Sergeyko, A.P., Sobolev, B.N., Filimonov, D.A., and Poroikov, V.V. (2006). Rational Design of Macrolides by Virtual Screening of Combinatorial Libraries Generated through in Silico Manipulation of Polyketide Synthases. *J. Med. Chem.* 49, 2077-2087. doi: 10.1021/jm051035i.

Zumberge, J.A., Love, G.D., Cárdenas, P., Sperling, E.A., Gunasekera, S., Rohrssen, M., et al. (2018). Demosponge steroid biomarker 26-methylstigmastane provides evidence for Neoproterozoic animals. *Nature Ecology & Evolution* 2, 1709-1714. doi: 10.1038/s41559-018-0676-2.

Summary

Vietnam is one of the sixteen greatest biologically diverse countries in the world and Vietnam's coastal region is also among the global biodiversity hotspots with unique biota and is a high-priority area for marine conservation. This coastal region holds high biodiversity conservation values with over 11,000 marine species, including 128 identified sponge species. However, the diversity of prokaryotes associated with sponges as well as their biotechnological potential has been largely unexplored. The research described in this thesis aimed to investigate the prokaryotic communities associated with sponges in Vietnam and their antimicrobial potential by applying cultivation-dependent and -independent approaches.

Chapter 1 provided an overview of marine sponges and their associated microbial communities within their evolutionary and ecological contexts, as well as their biotechnological potential. This general information serves as a starting point for our current understanding of the sponge-microbe association in general and the Vietnam sponge-microbe association in particular.

Chapter 2 investigated the prokaryotic communities associated with 18 sponge species in Vietnam using 16S rRNA gene Illumina MiSeq sequencing. Insights with respect to prokaryotic communities associated with sponges, including prokaryotic diversity, the influence of host identity, and sponge-enriched OTUs were discussed. Overall, 14 bacterial phyla and one archaeal phylum were identified among the investigated sponge species. The phylum *Proteobacteria* was present in all sponges and represented the most prevalent phylum in the majority of sponge species, albeit with pronounced differences at the class level. *Chloroflexi* was found to be the most abundant phylum in the sponge *Halichondria* sp., whereas the sponges *Spirastrella* sp. and *Dactylospongia* sp. were dominated by *Actinobacteria*. The phylum *Thaumarchaeota*, which is known to comprise nitrifying archaea, was highly abundant among the majority of the investigated sponge species. This chapter also highlighted the pattern of host-specificity among sponge species. The presence of OTUs significantly enriched in the sponge microbiome database found in this chapter supported the general consensus that sponges host certain prokaryotic taxa that are not found or found only in a few samples of other environments. In addition, the presence of prokaryotic taxa particularly known for nitrification in sponge species in Vietnam indicated nitrification might be an important microbial process in Vietnam sponge hosts.

In **Chapter 3**, the diversity and antimicrobial properties of cultivable bacteria associated with sponge species in Vietnam were investigated. This cultivation-based study recovered four bacterial phyla with 58.3% of all isolates belonging to *Proteobacteria*, 16.5% to *Actinobacteria*, 18.0% to *Firmicutes*, and 7.2% to *Bacteroidetes*. At the genus level,

cultivable strains represented 55 genera with the most predominant members of the genera *Bacillus*, *Pseudovibrio*, *Ruegeria*, *Vibrio*, and *Streptomyces*. Little overlap was observed of bacterial composition associated with the investigated sponge species in Vietnam detected by the cultivation-dependent and cultivation-independent approaches. Subsequent antimicrobial assays showed that nearly 20% of the cultivable bacteria exhibited antimicrobial activity against at least one of the tested indicator microorganisms, which lead to the isolation of four known secondary metabolites, including cyclo(L-Pro-L-Tyr) (**1**), macrolactin A (**2**), macrolactin H (**3**), and 15,17-epoxy-16-hydroxy macrolactin A (**4**) from the culture broth of *Bacillus* sp. M1_CRV_171. These compounds exhibited antimicrobial activity against a broad spectrum of the indicator microorganisms. Although these results confirmed the antimicrobial potential of sponge-associated cultivable bacteria, they also stressed the risks of rediscovery of known compounds based on isolates obtained through traditional cultivation techniques. Therefore, the uncultivated bacterial majority should be targeted to get access to the full arsenal of bioactive molecules from sponge-associated bacteria.

Although **Chapter 2** showed a high diversity of the prokaryotes associated with the investigated sponges in Vietnam, the cultivation-based investigation in **Chapter 3** indicated that the majority of them remained recalcitrant to cultivation. Therefore, **Chapter 4** provided a comprehensive review of the cultivability of sponge-associated bacteria, including discussions on cultivation approaches used to cultivate sponge-associated bacteria, evaluation and analysis of the cultivable bacterial diversity from sponges and their associated culture conditions, and suggestion of promising cultivation strategies for cultivating bacteria associated with sponges that are currently uncultivable. Furthermore, sponge-associated cultivable bacterial taxa that were previously labelled as “sponge-specific” and “sponge-enriched” were discussed in this chapter. The cumulative cultivation efforts of sponge-associated bacteria have resulted in the recovery of eleven cultivable bacterial phyla, which were dominated by the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* and the genera *Pseudovibrio*, *Bacillus*, *Streptomyces*, *Vibrio*, and *Ruegeria*. The cultivable bacteria included representatives of 21 sponge-enriched bacterial clusters as defined by the sponge EMP and 37 SC(C)s, whereas 168 SC(C)s had no representative isolate. Meta-analysis of culture conditions and growth media used suggested a substantial impact of these on the cultivable bacterial taxa, with temperature and carbon concentration present in the culture media being the most obvious factors affecting cultivation. This review indicated that the cultivation of most abundant sponge-associated bacteria is still a long way off due to our inadequate understanding of the intricate sponge-symbiont interactions and translating them to pseudo-natural growing conditions for these symbionts in the laboratory. A higher level of cultivation strategies would be required to recover those uncultivable bacteria. This would also be a significant step forward in the exploitation and use of bioactive compounds found in sponges.

The overall diversity of secondary metabolites present in sponge-associated bacteria is difficult to access due to the fact that the majority of bacteria are not readily cultured in the laboratory. Thus, **Chapter 5** used a cultivation-independent approach to investigate the diversity of secondary metabolite biosynthetic gene clusters (BGCs) in metagenomes of bacterial communities associated with three sponge species: *C. reinwardti*, *R. globostellata*, and *Spheciospongia* sp. The metagenomes of sponge-associated bacteria contained a high number of predicted BGCs, ranging from 282 to 463 BGCs per metagenome and represented 12 different cluster types. Among them, clusters predicted to encode fatty acid synthases and polyketide synthases (PKSs) were the most dominant BGC types, followed by clusters encoding the synthesis of terpenes and bacteriocins. Although 363 gene cluster families (GCFs) were identified, no GCFs were assigned to pathways responsible for the production of known compounds, implying that the clusters might be responsible for the production of several novel compounds. Ketosynthase (KS) sequences were predicted to be related to 12 bacterial phyla with *Actinobacteria*, *Proteobacteria*, and *Firmicutes* as the most predominant. At the genus level, the KSs were most related to those found in the genera *Mycolicibacterium*, *Mycobacterium*, *Burkholderia*, and *Streptomyces*. Notably, a large number of KS sequences was present in two known 'sponge-specific' clusters, i.e., *SupA* and *SwfA*, whereas a part of KS sequences formed a new sponge-specific KS cluster related to the fatty acid synthesis in *Candidatus* Poribacteria. The results obtained in this chapter reinforced that sponge-associated bacteria are a rich source of novel BGCs and as such most likely, bioactive compounds.

Finally, **Chapter 6** elaborated and integrated the findings obtained in the previous chapters, and portrayed them in the light of recent studies related to aspects of sponge prokaryotic symbionts and their biosynthesis of bioactive secondary metabolites. The prokaryotes associated with sponges in Vietnam share characteristics of global sponge microbiomes such as high diversity, host-specificity, and a large fraction of sponge-enriched prokaryotes. This chapter furthermore summarized the challenges and failures of isolating novel prokaryotes, the 'true' sponge-associated prokaryotes, and their novel secondary metabolites, and highlighted the next avenues for cultivating these fastidious bacteria as well as mining novel secondary metabolite BGCs detected through metagenomics.

Acknowledgements

The PhD journey is a great, exceptional, yet challenging experience. It is impossible to complete without the assistance and encouragement of supervisors, colleagues, and relatives. Therefore, I would like to express my heartfelt appreciation to all of you who have been a part of my life during my PhD journey.

First, I would like to thank the financial sponsors who provided me with grants to pursue my PhD at Wageningen University: Vietnam Ministry of Science and Technology, grant number ĐTĐLCN.17/14; BE-Basic Foundation-FES grant from the Dutch Ministry of Economic Affairs, grant number: F07.003.06; and MC-ITN-BluePharmTrain funded through the European Union Programme FP7/2007-2013/ under REA grant agreement n° 607786.

I would like to express my profound gratitude to Prof. **Abraham Brouwer**, CEO and Founder of BioDetection Systems B.V. and Prof. **Chu Hoang Ha**, Vice-President of Vietnam Academy of Science and Technology for giving me the opportunity to start my PhD at Wageningen University, which allowed me to pursue my academic career.

I would like to express my sincere gratitude to my promotors, Assoc. Prof. **Detmer Sipkema**, Prof. **Hauke Smidt**, and Dr. **Nguyen Thi Kim Cuc** for their advice, encouragement, and inspiration in helping me achieve my PhD. You always come with insightful comments and suggestions on my manuscripts as well as my PhD thesis. **Detmer**, thank you for choosing me to work within your Marine group. I feel so really lucky and grateful that I had the opportunity to work with you. **Hauke**, thank you for all your patience, kindness and support. Being a member of your MolEco group was a true honor and pleasure.

I am very grateful that I could be part of the Laboratory of Microbiology, under the guidance of Prof. **Willem de Vos**. I always felt comfortable and welcomed for who I am among the warm-hearted and charming group of people from all over the world who work at the Laboratory of Microbiology.

I was lucky to be part of **MolEco**, one of the most diverse groups with members from many nationalities. As part of the multinational MolEco family, I enjoyed the cakes, Christmas dinners and Lab Trips, which offered so many insights into different cultures and philosophies.

I would like to thank my office mates **Johanna Gutleben**, **Jie Lian**, **Sudarshan Shetty**, **Dennis Versluis**, **Loo Wee, and Asimenia Gavriilidou** for making the PhD experience more enjoyable with funny stories, jokes, and office lunches. I would also like to thank the **Marine group** members: **Akhirta Atikana**, **Johanna Gutleben**, **Jie Lian**, **Asimenia**

Gavriilidou, **Georg Steinert**, and **Catarina Loureiro** for their discussion and support. Thank you for sharing all of the ups and downs during the PhD experience. I wish you all the best in the future and hope to see you around.

I would like to thank Dr. **Georg Steinert** for his helpful advice, valuable comments, and feedback on my research and for always being so supportive of my work. You assisted me a lot with numerous critical experiments and metagenomic analyses in my PhD project.

I would like to thank Prof. **Pham Viet Cuong**, Director of the Mientrung Institute for Scientific Research for his support and motivation in pursuing my PhD. I would sincerely thank **Tran Thi Kim Dung** for her assistance in the marine sponge collection. I would sincerely thank Dr. **Le Canh Viet Cuong** and Dr. **Nguyen Tien Dat** for their highly appreciated assistance regarding the structure elucidation of secondary metabolites from sponge-associated bacteria. I would like to thank Dr. **Dang Do Hung Viet**, Institute of Marine Environment and Resources, for providing wonderful photos for the thesis and chapter cover design.

I would like to thank our lab technicians: **Ineke Heikamp-de Jong**, **Steven Aalvink**, **Tom Schonewille**, and **Ton van Gelder** for your assistance whenever I need it. I would also like to thank **Anja van Holland** and **Heidi van den Top** for your administrative assistance, **Philippe Puylaert** for your lab arrangement, **Wim Roelofsen** for your assistance whenever I had problems with my PC and laptop, **Bart Nijsse** for your assistance whenever I needed help on bioinformatics.

I am very thankful to Mr **Binh** and Mrs **Vi** for our warm and delicious dinners in Wageningen. Thank you to all of the Wageningen Vietnamese students who have been studying MSc and PhD at Wageningen University for all of the laughing and enjoyment that has made my life in Wageningen so much more joyful. I am grateful for the time I spent with all of you for the memorable dinners, barbecue on the campus and travels to various destinations in the Netherlands and Europe. Hope to see you in Vietnam soon. I would like to thank my paranymphs **Nguyen Thi Huyen** and **Le Thi Dung**, for your assistance throughout the final stages of this journey. Best wishes and heartfelt gratitude to my peers who have shared a PhD journey in the Netherlands **Nguyen Thi Lan Anh**, **Hoang Ha**, **Le Ngoc Giang**, **Dao Thi Ngoc Anh**, **Pham Ngoc Long**.

Finally, I would like to express my heartfelt thank you to my wife, my parents, and my parents-in-law for always believing in me and encouraging me to follow my dreams. They always give me mental support during my PhD journey. I sincerely thank all my close relatives as well. My PhD journey would not be achieved without the support of my family and friends.

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From 2011 until 2013 he continued his master's program in Environmental Science at Hue University, Vietnam. He completed his master's thesis at the Laboratory of Environmental Engineering under the supervision of Dr. Pham Khac Lieu on the treatment of landfill leachate using an H_2O_2-assisted submerged aerated biofilter. In this period, he also participated in an internship program at the Department of Applied Chemistry and Biochemical Engineering, Shizuoka University, Japan. In this internship program, he conducted research on the isolation of dibenzofuran and dibenzo-p-dioxin degrading bacteria from soil samples under the supervision of Prof. Hiroyuki Futamata and Prof. Kazuhide Kimbara. After completing the master's program, he returned to work at the Mientrung Institute for Scientific Research, Vietnam Academy of Science and Technology.

In October 2015, Johanna started in the Molecular Ecology group of the Laboratory of Microbiology at Wageningen University as a PhD fellow funded by the Vietnam Ministry of Science and Technology, grant number ĐTĐLCN.17/14; BE-Basic Foundation-FES grant from the Dutch Ministry of Economic Affairs, grant number: F07.003.06; and MC-ITN-BluePharmTrain funded through the European Union Programme FP7/2007-2013/ under REA grant agreement n° 607786. His PhD project focussed on the discovery of prokaryotic communities associated with sponges in Vietnam and their secondary metabolite biosynthetic potential. His research was supervised by Assoc. Prof. Detmer Sipkema, Prof. Hauke Smidt and Assoc. Prof. Nguyen Thi Kim Cuc, most of this work is described in this thesis.

At the moment, he continues his academic career at Vietnam National Museum of Nature, Vietnam Academy of Science and Technology as a researcher.

List of publications

Dat TTH, Steinert G[1], Cuc NTK[2], Smidt H[1], Sipkema D[1] (2018). Archaeal and bacterial diversity and community composition from 18 phylogenetically divergent sponge species in Vietnam. PeerJ 6, e4970.

Dat TTH, Cuc NTK[2], Cuong PV[3], Smidt H[1], Sipkema D[1] (2021). Diversity and antimicrobial activity of Vietnamese sponge-associated bacteria. Marine Drugs 19 (7), 353.

Dat TTH, Steinert G[1], Cuc NTK[2], Smidt H[1], Sipkema D[1] (2021). Bacteria cultivated from sponges and bacteria not yet cultivated from sponges–A review. Frontiers in Microbiology 12, 737925.

Dat TTH, Steinert G[1], Cuc NTK[2], Cuong PV[3], Smidt H[1], Sipkema D[1] (2023). Diversity of Bacterial Secondary Metabolite Biosynthetic Gene Clusters in Three Vietnamese Sponges. Marine Drugs 21 (1), 29.

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2. Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi 10072, Vietnam

3. Mientrung Institute for Scientific Research, Vietnam Academy of Science and Technology, 321 Huynh Thuc Khang, Hue City, Thua Thien Hue 531600, Vietnam

Overview of Completed Training Activities

Discipline specific activities	Organizing and Institute(s) & Place	Year
Courses		
Principles of Ecological and Evolutionary Genomics	SENSE/EE/PE&RC, Wageningen	2018
Spring School in Microbial Bioinformatics	VLAG/University of Turku, Wageningen	2018
Workshops, symposia, conferences		
ECO-BIO Conference 2016	BE Basic Foundation/Elsevier, Rotterdam	2016
3rd Wageningen PhD Symposium	Wageningen PhD Council, Wageningen	2016
1st Annual AcroporaNet Symposium	AcroporaNet, Wageningen	2016
VAST-BE Basic Annual Workshop	VAST/BE Basic Foundation, Hanoi, Vietnam	2017
Microbiology Centennial Symposium	WUR (MIB), Wageningen	2017
KNVM Microbial Ecology Fall Symposium	KNVM, Wageningen	2017
Microbial Population Biology series	WUR (MPB), Wageningen	2016, 2018
Science week "What is life"	WUR, Wageningen	2018
Scientific Spring Meeting KNVM & NVMM	KNVM/NVMM, Arnhem	2018
VAST-BE Basic Annual Workshop	VAST/BE Basic Foundation, Hanoi, Vietnam	2018
5rd Wageningen PhD Symposium	Wageningen PhD Council, Wageningen	2018
BE-Basic Annual Meeting	BE Basic Foundation, Utrecht	2018
General courses	**Organizing and Institute(s) & Place**	**Year**
Project and time management	WGS, Wageningen	2015
VLAG PhD Week	VLAG, Baarlo	2016
Introduction to R for statistical analysis	PE&RC, Wageningen	2016
The essential scientific writing and presenting	WGS, Wageningen	2016
Presenting with impact	WGS, Wageningen	2016
Techniques for writing and presenting a scientific paper	WGS, Wageningen	2016
Reviewing scientific paper	WGS, Wageningen	2016
PhD Workshop Carousel	WGS, Wageningen	2016
R course	BluePharmTrain, Wageningen	2016
Big data in life sciences	VLAG, Wageningen	2017
Scientific Artwork - Vector graphics and images	WUR, Wageningen	2018
Optionals	**Organizing and Institute(s) & Place**	**Year**
Preparation of PhD research proposal	WUR (MIB), Wageningen	2016
Monthly PhD meeting	WUR (MIB), Wageningen	2016, 2018
Weekly group meeting	WUR (MIB), Wageningen	2016, 2018
Teaching activities	**Organizing and Institute(s) & Place**	**Year**
MIB-31306 Microbial Ecology	WUR (MIB), Wageningen	2016
MIB-03033 Research Method Microbiology	WUR (MIB), Wageningen	2016, 2018

The research described in this thesis was financially supported by Vietnam Ministry of Science and Technology, grant number ĐTĐLCN.17/14; BE-Basic Foundation-FES grant from the Dutch Ministry of Economic Affairs, grant number: F07.003.06; and MC-ITN-BluePharmTrain funded through the European Union Programme FP7/2007-2013/ under REA grant agreement n° 607786.

Financial support from Laboratory of Microbiology, Wageningen University for printing this thesis is gratefully acknowledged.

Colophon

Cover photo by: Dang Do Hung Viet
Cover design and layout by: Ton That Huu Dat
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