Cultivation of Bacteria from Marine Sponges

by

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Abstract

Sponges (phylum *Porifera*) are among the oldest of the multicellular animals (Metazoans). Sponges have been the focus of much recent interest due to the fact that they harbour a diverse range of symbiotic microorganisms. These symbiotic microorganisms are essential for the host's function and interaction with the environment. Our understanding of sponge-bacteria association is limited due to our inability to cultivate most sponge-associated microorganisms. In recent years many new approaches have been developed for culturing marine bacteria. These include the use of a variety of growth conditions, such as temperature, oxygen levels, different atmospheric pressures and novel culture media. These approaches have largely been neglected when it came to the cultivation of sponge-associated bacteria.

This thesis focuses on the cultivation of sponge-associated bacteria from the marine sponges *Cymbastela concentrica, Tedania* sp. and *Scopalina* sp. using agar plate cultures and floating filter cultures. A variety of low- and high-nutrient media were used, including media amended with sponge extracts.

A total of 202 isolates were identified from the three sponge species. Most of the cultivated bacteria were isolated from agar plate cultures, with highest number of isolates from the sponge *Tedania* sp. Media that were rich in nutrients were more successful and resulted in higher diversity of morphotypes and genetically distinct isolates as compared to low-nutrient media. Media with and without addition of sponge extract showed no difference between number and type of isolated bacteria.

Isolated bacteria were classified into 16 genera, with *Pseudovibrio* being the most dominant genus. Most of the isolated *Pseudovibrio* sp., *Ruegeria* sp., *Aquimarina* sp.,

and *Vibrio* sp., have close matches with microorganisms isolated previously from marine sponges.

The results presented in this thesis highlight the use of multiple cultivation methods to improve cultivability of sponge-associated bacteria.

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List of Abbreviations:

°C	Degree Celsius
µL	Microlitre
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
DNA	Deoxyribonucleic acid
HTC	High-throughput cultivation
L	Litre
mL	Millilitre
NCBI	National center for biotechnology information
NSW	Natural seawater
OTU	Operational taxonomic unit
PC	Polycarbonate
PCR	Polymerase chain reaction
RDP	Ribosomal database project
Rpm	Revolutions per minute
UNSW	University of New South Wales
x g	Times gravity
min	Minutes
sec	Seconds
mm	Millimetre
µm	Micrometre
mg	Milligram

1. Introduction

1.1. Description of Marine Sponges:

Sponges (Phylum Porifera) are ancient metazoans, found in every ocean of the world and also in fresh water, regardless of temperature and depth. They are sessile, filter-feeding animals with a body full of tiny pores allowing water to circulate through them. As filter feeders, sponges efficiently take up nutrients, like organic particles and microorganisms, from the seawater, leaving the expelled water almost sterile (Reiswig, 1971). Sponges are classified by their characteristic skeletal structure comprised of a supporting internal structure, a hard external surface or a mixture of both of these. These 'endo' or 'exo-skeletons' can be comprised of dense or fused spicules, which are made of silica (a glass-like material) or calcareous (calcium or calcium carbonate) materials, and spongin, a protein that supports the spicules. Sponge species may be identified and classified by examining their spicules under a microscope (Bergquist, 2001).

Marine sponges are hosts to various symbiotic microorganisms. These microorganisms include a diverse range of bacteria, archaea and eukaryotes, which can comprise up to 40% of sponge volume and may have a profound impact on host biology (Wilkinson, 1978a, b).

1.2. Diversity of Sponge Microorganisms:

Both culture-dependent and culture-independent approaches have been used to study the diverse microbial communities of sponges. Since the majority of microorganisms seem unable to grow under artificial conditions, the use of molecular tools, such as 16S rRNA gene sequencing, has been extensively used to characterise microbial diversity (Pace et al., 1985; Rappé & Giovannoni, 2003; Amann et al., 1995).

An exceptional diversity of microbial communities associated with sponges has been discovered using 16S rRNA gene sequencing and highlighted the previous failure to identify abundant members. For example, only ~0.1% of the total community in the sponge *Rhopoloides odorabile* and 3-11% of total bacterial population for the sponge *Ceratoporella nicholsoni* was estimated as culturable (Webster and Hill, 2001; Santavy et al., 1990).

Various studies have demonstrated that marine sponges harbour specific, stable microbial communities that are distinct from the surrounding seawater (Wilkinson, 1978c; Santavy et al., 1990; Hentschel et al., 2002; Friedrich et al., 2001), with some of the microorganisms being specific to their respective sponge hosts. A δ-proteobacterial species and the sponge *Theonella swinhoei* show such a specific association (Schmidt et al., 2000). Several sponge species from the Mediterranean and Caribbean also harbor a previously unknown, sponge-associated microbial lineage, defined as the phylum *Poribacteria* (Fieseler et al., 2004). *Poribacteria* were first discovered as highly abundant symbionts of marine sponges (Fieseler et al., 2004). Through amplicon sequencing studied based on 16S rRNA genes they were also detected in seawater though in low abundances (Webster et al., 2010).

The development and use of high-throughput DNA sequencing methods and several molecular biology techniques have given insights into the function and diversity of the sponge microbiome (Webster et al., 2010; Schmitt et al., 2011; Simister et al., 2012). A recent study using 454 amplicon pyrosequencing of 16S rRNA genes, revealed extraordinary species richness in Great Barrier Reef sponges (Webster et al., 2010) and red sea sponges (Lee et al., 2010).

Sponges and symbiotic microorganisms can be beneficial to each other with the sponge providing nourishment and safe habitat to their symbionts. The symbiotic microorganisms in turn help through specific metabolic processes within sponges, such as photosynthesis, sulphate reduction and nitrogen fixation (Wilkinson and Fay, 1979; Hoffmann et al., 2005). A recent metagenomic study showed for example that sponge-associated bacteria from six taxonomically different sponges share functional properties of nitrate respiration (Fan et al., 2012a). Microorganisms may also contribute to host defence via the production of biologically active metabolites (Schmidt et al., 2000; Bultel-Poncé et al., 1998).

1.3. New Approaches in Cultivation:

The culturing of microorganisms has been the basis of microbiology for decades. The development of advanced molecular tools, such as metagenomics, has made it possible to describe the genetic and functional properties of the uncultured bacteria. However, a true understanding of the physiology and the role of these bacteria in ecology, host health, and natural product production often requires their cultivation in the laboratory (Stewart, 2012). Unfortunately, knowledge on how to create natural conditions for all microorganisms is limited and artificial media or culturing conditions are often incapable of mimicking the natural abiotic and biotic situation, chemistry of a microorganism's natural habitat and other environmental factors required for the microbial growth (Alain and Querellou, 2009). Several innovations have however been made in culturing techniques leading to a revival of culturing in the field of microbiology.

1.3.1. Modification of Growth Media and Conditions:

Every bacterium has different growth requirements with the need for specific nutrients, chemicals, pH conditions, incubation times, temperatures and oxygen concentrations (Reichelt & Baumann, 1974; Tripp et al., 2008). Kopke et al. (2005) examined the effect of different substrates and culture conditions on the growth of bacteria from coastal sediments samples and found that the different cultivation approaches resulted in the isolation of different groups of bacteria and were specific to each method used. In other studies on soil, there has been some success in the cultivation of previously uncultured soil bacteria, for example, by using a medium containing soil extract (Hamaki et al., 2005).

Many bacteria are very slow-growing and thus extended incubation times are required for their cultivation. Faster-growing members within the mixed population may initially overgrow slow-growing bacteria, but then may die off over time, reducing the bacterial competition. For example, isolation of the abundant marine strain SAR11 has been successful after long-term incubation for up to 24 weeks (Song et al., 2009). Many studies have shown that longer incubation times (Davis et al., 2005; Stevenson et al., 2004; Stott et al., 2008) and a reduced inoculum size (Davis et al., 2005) can lead to an increased recovery of bacterial diversity.

1.3.2. Coculture or community culture:

Microorganisms always interact with other microorganisms in a community and these interactions can be either to compete for limited resources or cooperate through an exchange of metabolites and signalling molecules. Classic examples can be found in biofilms, where microorganisms communicate with one another (Nadell et al., 2009).

Community culture is an effective way to cultivate organisms. Depending on beneficial bacterial interactions within the source environment, attempts to cultivate certain bacteria under laboratory conditions have been successful only when these bacteria are cocultivated with helper strains (Ohno et al., 1999, 2000; Nichols et al., 2008). Factors released from helper strains into the environment are often growth-stimulatory for bacteria that are otherwise unculturable. Thus, the conditioning of media with culture supernatants or cell-free extracts derived from helper strains has been used for the growth stimulation of several species (Tanaka et al., 2004; Bae et al., 2005; Nichols et al., 2008). Signalling molecules may also be responsible for such growth promotion. Testing of known signal molecules, such as cyclic AMP (cAMP) and acyl homoserine lactones, was shown to significantly increase the cultivation efficiency of marine bacteria (Bruns et al., 2002).

Growth of previously uncultured isolates from marine sediment biofilm was successfully observed in the presence of cultured organisms from the same environment. The growth factors produced by one cultured helper strain were identified as new siderophores. This siderophore-based approach has enabled the culturing of organisms only distantly related to previously cultured microbes (D'Onofrio et al., 2010).

1.3.3. Dilution to Extinction:

Dilution to extinction or extinction culturing is probably the simplest method to obtain single cells from mixed populations. This is done by serially diluting a sample until only single cells remain. Growth is measured after incubating the diluted cultures (Button et al., 1993; Schut et al., 1993). High-throughput cultivation (HTC) method

employs ‘dilution to extinction’ or low-density partitioning of cells in tubes or microwells with low-nutrient media, and exploits the fact that diversity of culturable species observed in microbial isolations apparently increases as inoculum density decreases (Connon and Giovannoni, 2002; Button et al., 1993; Simu & Hagström, 2004; Song et al., 2009; Stingl et al., 2007).

The most successful application of this approach was the isolation and cultivation of the marine strains belonging to the SAR11 clade. This slow-growing, oligotrophic bacterium is ubiquitous and abundant in the ocean (Rappé et al., 2002; Giovannoni and Stingl, 2007). In order to cultivate this organism, Rappe and colleagues used the natural environment in the form of seawater as a growth medium, and by diluting the samples “to extinction”, they were able to separate the bacteria of this clade into microtiter well-plates and grow them in pure culture (Rappé et al., 2002).

1.3.4. Cultivation of Microorganisms using Microcapsules:

To cultivate uncultured bacteria from their environment, Zengler and colleagues described a HTC technique, which combines encapsulating single bacterial cells in gel microdroplets followed by flow cytometry to detect microdroplets containing microcolonies (Zengler et al., 2002). To limit the number of bacteria in each gel microdroplet (GMD) a dilution series was used, and after encapsulation, the GMDs were retained in a flow column bounded by membranes, which prevents free-living cells from contaminating the column and allows them to be washed out. For the marine samples, seawater was used as a growth medium and constantly pumped through the column. After incubation, the GMDs containing a microcolony were sorted with a flow cytometer into 96-well microtiter plate after which phylogenetic analysis and further cultivation were carried out (Figure 1). Interestingly, filtered seawater alone as a

medium yielded a higher diversity than seawater with added nutrients, as shown by 16S rRNA gene sequence analysis, which supports the notion that the natural environment can be important for growing unculturable bacteria in the laboratory (Zengler et al., 2002).

Different environments, such as alkaline lake sediment and soil were also screened using this technique, which resulted in the growth and isolation of a wide range of bacteria, including previously uncultured phylotypes (Zengler et al., 2002, 2005).

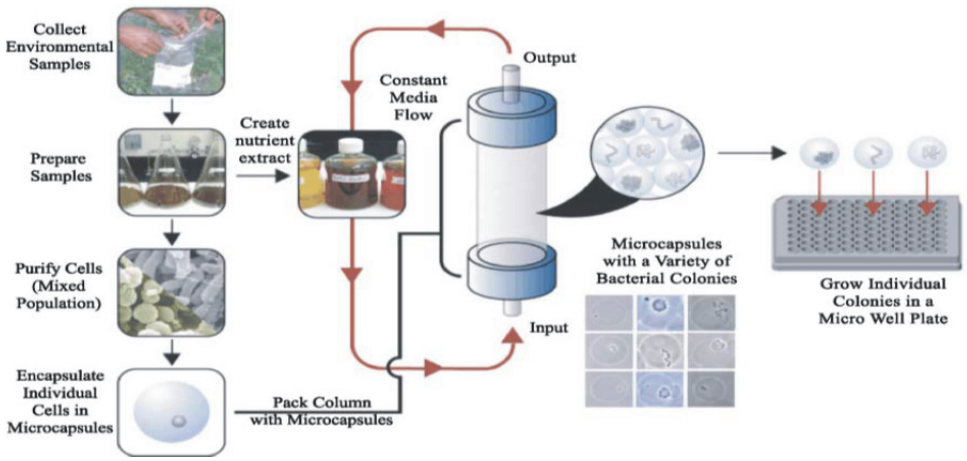

Figure 1: Flow diagram of high-throughput cultivation approach based on the encapsulation of single cells in microcapsules (Zengler et al., 2005).

1.3.5. Diffusion-Chamber based approaches:

A diffusion chamber was designed by Kaeberlein and colleagues (Kaeberlein et al., 2002) and allowed the growth of previously uncultivated microorganisms in a simulated natural environment. This was achieved by enclosing the bacteria inside a semi-permeable chamber so that the nutrients and chemicals from the environment are able to pass through the membrane, but restrict the movement of cells (Kaeberlein et al.,

2002). In one application, a diluted suspension of cells from marine sediment mixed with warm agar made with seawater was poured in these chambers before sealing, and was placed on surface of sediment collected from the tidal flat and incubated in an aquarium of seawater. Microscopic examination of the chambers after incubation revealed microcolonies of bacteria growing within them, the majority of which could be further isolated by reinoculation into fresh chambers. The number of growing microcolonies with microscopic counts of cells in the initial inoculum yielded recovery rates of up to 40%. The same inoculum when examined for colony production on standard petri plates had a recovery rate of 0.05% (Kaeberlein et al., 2002). In a follow-up study, several incubations in multiple diffusion chamber led to an increase in the total number and diversity of environmental isolates capable of growth *in vitro* (Bollmann et al., 2007).

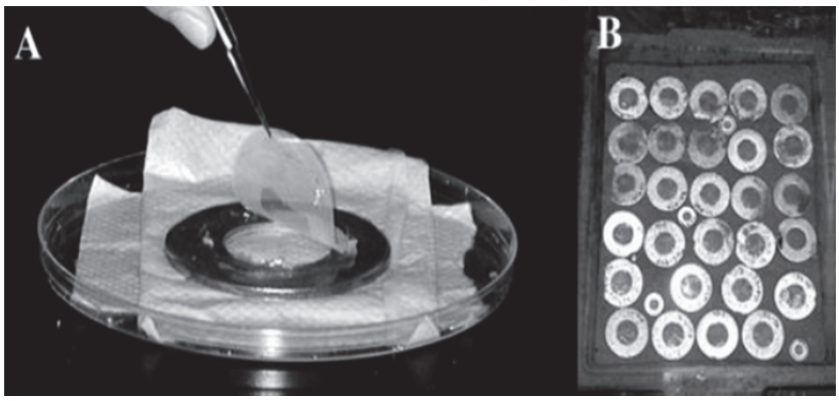

Figure 2: Diffusion growth chamber for cultivation of microorganisms. (A) The chamber formed by a washer sandwiched between two polycarbonate membranes. (B) Growth chambers incubated on the surface of marine sediment (Kaeberlein et al., 2002).

This technique was also later tested with contaminated subsurface soil, which was incubated in the chambers and on petri plates. This chamber-based approach led to the isolation of species that are novel and showed adaptation to environmental conditions (Bollmann et al., 2010). The chamber was further modified, such that one of the enclosing membranes has a pore size just large enough for filamentous bacteria to grow

into the chamber, but not non-filamentous bacteria. This yielded a greater diversity and number of filamentous *Actinobacteria*, including isolates of rare groups (Gavrish et al., 2008).

In a recent study by Steinert and colleagues (2014) used diffusion growth chambers for the cultivation of bacteria from the reef sponge *Rhabdastrella globostellata*. The phylogenetic analyses of the cultivated bacteria revealed their affiliations with the *Alpha-* and *Gammaproteobacteria*, *Bacteroidetes, Actinobacteria*, and *Firmicutes*. In addition, results showed that fifteen sequences represented previously uncultivated bacteria belonging to the *Bacteroidetes* and *Proteobacteria* (Alpha and Gamma classes) (Steinert et al., 2014). The results indicate that the diffusion growth chamber approach can be applied successfully in a living marine environment, such as sponges.

1.3.6. Soil Substrate Membrane System (SSMS):

Ferrari and co-workers (2005) cultured rare soil bacteria as microcolonies by growing them on filters suspended on soil slurry in the absence of added nutrients (Ferrari et al., 2005). A polycarbonate membrane (PC) is used as a solid support for growth and non-sterile soil slurry as the culture medium. The soil slurry is set in an inverted tissue culture insert covered by a fixed membrane. This membrane supplies nutrients for bacteria growing on the PC membrane and also prevents bacterial contaminations (Ferrari et al., 2008). After incubation, diverse microcolonies were observed using total bacterial staining combined with fluorescence in situ hybridization (FISH), and these microcolonies could be micromanipulated for downstream cultivation (Ferrari and Gillings, 2009). This technique has also been used on mercury-

contaminated soil to successfully isolate difficult-to-culture mercury-resistant bacteria (Rasmussen et al., 2008).

A disadvantage of membrane microcultivation is that the bacteria remained part of a mixed community on the membrane and also fluorescence microscopy is needed to identify the cells. The micromanipulation of single microcolonies from the SSMS into artificial media or fluorescence-activated cell sorting might improve recovery of microcolonies, which could increase the isolation of pure cultures from the mixed microcolonies (Ferrari and Gillings, 2009).

1.4. Other new cultivation approaches:

Several other techniques have been used to cultivate microorganisms. A micro-petri dish that is composed of a unique ceramic having millions of compartments, in which cultures can be separately grown, was produced by Ingham et al. (2007). Nutrients are supplied by diffusion through a semi-permeable membrane, resulting in the recovery of exceptional numbers of microorganisms.

Other methods used for cultivation are single-cell isolation techniques using micromanipulators or laser manipulation systems, such as optical tweezers. This method uses a highly focused laser beam to trap and isolate single cells within a cell separation unit from where they are ultimately transferred to growth media for cultivation. Using this method, a bacterial cell can be isolated from a mixture of cells (Frohlich & Konig, 2000; Zhang and Liu, 2008).

Several microfluidic devices have been developed to isolate single cells and to sort cells of interest. A microfluidic device captures, incubates and releases single cells

by controlling the flow rate (Yamaguchi et al., 2009). Liu et al. (2009) also developed a unique plug-based microfluidic device that allows single-cell isolation, incubation, and separation of the clonal population. In this device, single cells from a mixed population are stochastically isolated into plugs and are incubated to grow microcolonies and clonal populations originating from single cells are used for further analyses.

Flow cytometry and cell sorting is a high-throughput method, where individual cells from a large mixed population can be easily analysed in a short time by their similarity in physiological or structural properties. In this technique, the cell sample is mixed with sheath fluid, which is then forced through an opening to generate drops, each containing only a single cell. These droplets pass through a focused beam of light (mainly laser), which scans the cells and emits light of a certain wavelength or scattering. Sorted cells are collected in a tube or, single cells can be obtained in a microtiter plate (Ishii et al., 2010; Link et al., 2007). Isolated or sorted cells can be used for subsequent culture-based (Kalyuzhnaya et al., 2006; Wang et al., 2009) or culture-independent analyses (Fujii and Hiraishi, 2009; Hoefel et al., 2005; Kalyuzhnaya et al., 2006). Main disadvantages of flow cytometry are the instrumentation costs and the time required for sample preparation (Link et al., 2007).

1.5. Cultivation of Sponge Microorganisms:

Marine sponges have been shown by 16S rRNA community analysis to contain remarkably diverse microbial communities that include many novel bacteria found only within sponges (Webster et al., 2001; Hentschel et al., 2002). However, the 16S rRNA – gene based techniques are known to be limited by the short read lengths obtained and sequencing errors. Furthermore, the use of a single marker gene to assess diversity is

challenging, given the prevalence of horizontal gene transfer as well as the limited resolution of the 16S rRNA gene among closely related species (Poretsky et al., 2014; Schloss et al., 2011). Parallel to the development of culture-independent studies, culture-dependent approaches to isolate sponge microorganisms have been extensively used. Having access to pure cultures of microorganisms can be useful as it allows for the physiological and biochemical characterisation as well as potential genetic manipulation.

Different cultivation methods, based on modified traditional approaches, have resulted in the isolation of morphologically diverse bacteria from various marine sponges (Hentschel et al., 2001, Olson et al., 2000, Santavy et al., 1990, Webster and Hill, 2001). Many cultivation studies for sponge-associated bacteria used rich culture media or aerobic conditions, with many studies targeting actinomycetes for drug discovery (Kim et al., 2005; Abdelmohsen et al., 2010). In a recent study, use of aerobic and microaerophilic conditions and diverse culturing media with addition of antibiotics yielded higher diversity of bacteria and led to the isolation of novel sponge-specific bacteria from the Red Sea sponge *Theonella swinhoei* (Lavy et al., 2013).

Olson et al. (2000) also observed an improved recovery of sponge-associated microorganisms by using a variety of low to high-nutrient media in combination with other medium additives. In addition, using oligotrophic media (J agar) with sodium thiosulfate, aqueous sponge extract, and sodium silicate the filamentous symbiotic bacteria from the sponge *Theonella swinhoei* could be cultured (Schmidt et al., 2000). Table 1 summarizes different media and conditions used for the cultivation of sponge-associated microorganisms.

In a study by Sipkema and colleagues, three methods (agar plate cultures, liquid cultures, and floating filter cultures) were tested to cultivate bacteria associated with the marine sponge *Haliclona (gellius)* sp. (Sipkema et al., 2011). A variety of different low-nutrient media and environmental conditions were employed in order to mimic microenvironments that are found in sponges. Floating polycarbonate filters were applied to mimic the inner structures of the filter-feeding sponge. More than 3,900 isolates were analyzed, and 205 operational taxonomic units (OTUs) were identified using these techniques. Out of these 205 OTUs that were obtained from isolates, only 17 matched with OTUs that were also obtained in a clone library from the same sponge specimen (Sipkema et al., 2011). Many other studies have used media amended with sponge extracts, which have led to the isolation of a different set of microorganisms (Webster et al., 2001; Pabel et al., 2003; Wichels et al., 2006; Abdelmohsen et al., 2010).

Table 1: Medium, temperature and conditions used to isolate microorganisms from sponges:

Sponges	Medium	Cultured Bacterial Isolates	Conditions & Additives	Reference
Rhopaloeides odorabile	i) Marine agar 2216 ii) MN+B12 broth	i)StrainNW001 member of α-group *Proteobacteria* ii)Strain NW4194 *Cyanobacteria*	i) 27°C ii) room temperature & natural light condition	Webster and Hill, 2001
Ircinia variabilis	i) Standard medium (MB) & Experimental media (MF, MF+ALI, ALI+Y, MF+Y)	Strain IV-1 genus *Staphylococcus* Strain IV-2 genus *Bacillus*	18.5°C & 37°C Medium containing marine derived proteins	De Rosa et al., 2003
Axinella corrugata, Mycale laxissima, Monanchora unguifera, Niphates digitalis, Didiscus oxeata, Monanchora unguifera, Acanthostronglyophora sp. *and Microciona prolifera*	Marine agar 2216	Isolates belonging to *alphaproteobacteria*, closely related to each other	N/A	Enticknap et al., 2006
Iotrochota sp.	Gause's medium No.1(an isolation medium for Actinomycetes) prepared with 80% seawater &fresh water	*Actinobacteria* isolates belonging to genera *Streptomyces, Cellulosimicrobium*, and *Nocardiopsis*	28°C	Jiang et al., 2008
Aplysina aerophoba	Mineral salt medium prepared with 70% seawater	*Nitrospira*-like bacterium (culture Aa01)	28°C dark, pH 7.8, medium containing 0.5mM nitrite	Off et al., 2010
Amphimedon ochracea	Marine agar and ISP medium 2, both prepared with artificial sea water	Four strains (HA-21, HA-68, HA- MS-105 and HA-MS-119) belonging to *Bacillus* sp. *(Firmicutes)*	28°C for 5 to10 days, Media supplemented with Cycloheximide, Nystatin& Nalidixic	Aboul-Ela et al., 2012
Ircinia muscarum	Marine agar	*Pseudomonas sp.* (IM-1)	19°C pH 7.6 incubation time 72 hr.	M. Mitova et al., 2003
Pseudoceratina clavata, Rhabdastrella globostellata	Marine agar 2216, Modified marine agar, & 1 in 10 dilution Marine Agar 2216 with adjusted salt conc.	Isolates belonging to phylum *Proteobacteria* (class α-*proteobacteria* and *gammaproteobacteria*), the phylum *Bacteroidetes*, the phylum *Firmicutes* and phylum *Actinobacteria*	28°C in dark Aerobic conditions	Lafi et al., 2005

Dendrilla nigra	Marine sponge agar (MSA), modified marine agar (MMA), seawater agar (SA), modified nutrient agar (MNA), halophilic agar (HA), actinomycetes agar (AA), starch casein agar (CSA), raffinose-histidine medium (RH), Fluid-thioglycollate medium (FT), malt agar (MA), Emerson Agar (EA), TCBS Agar (TA), Pseudomonas agar (PA), and anaerobic agar (ANA)	*Micromonospora, Saccharomonospora*, *Streptomyces* was the major culturable *Actinobacteria* in this sponge and the other cultured bacterial associates belongs to genera *Marinobacter, Roseobacter , Alteromonas,* and *Pseudomonas*	25°C dark aerobic conditions different enrichments: (1)Media enriched with sponge extract, (2)enriched with nystatin, (3) enriched with alpha-butyrolactone, and (4) complete enrichment containing sponge extract, nystatin, and alpha-butyrolactone	Selvin et al., 2009
Haliclona simulans	Starch-yeast extract-peptone seawater (SYP_SW) agar, Marine agar, Modified marine agar, Actinomycetes isolation seawater agar	Isolates belonging to four bacterial phyla *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteroidetes*) and 12 bacterial genera	28°C for 8 weeks, Media supplemented with Amphotericin and with and without Nalidixic	Kennedy et al., 2008
Aplysina aerophoba and Aplysina cavernicola	Marine Medium (Difco 2216) and 3/4 strength local seawater- based medium for heterotrophic and Cyanobacteria, a selective medium for gram-positive and Oligotrophic bacteria	Isolates affiliated with the low (Bacillus) and high G+C Gram-positive bacteria (*Arthobacter, Micrococcus*), as well as the α-*Proteobacteria* (unknown isolate) and γ-*Proteobacteria (Vibrio, Pseudoalteromonas*)	(20-25°C) and colonies were picked at 2, 5, 10 and 20 days and plates were retained for additional month at 4³C for the isolation of slow-growing bacteria	Hentschel et al., 2001

1.6. Objective:

A huge diversity of sponge associated bacteria has been identified using molecular methods (Webster et al., 2010; Simister et al., 2012), but the proportion of sponge bacteria that can be isolated has remained low. This is because the majority of microorganisms have proven resistant to standard cultivation techniques (Friedrich et al., 2001). Most cultivated microorganisms from sponges are not dominant in their host microbiomes. In addition, not many of the non-traditional techniques described above in 1.3-1.4 have been used to cultivate sponge-microorganisms. Therefore, the use of innovative and multiple cultivation methods and media can be useful to increase and compare the diversity of the cultivated microorganisms from each sponge species used in this study.

Therefore, the aim of this study is:

- To culture sponge-associated bacteria using floating filters and agar plate culture from three sponges: *Cymbastela concentrica, Tedania* sp. and *Scopalina* sp.

- Use of a variety of low- to high-nutrient media for cultivation. Comparing and assessing the diversity of bacteria obtained by use of different techniques and media from different sponges.

- Assessing cultivation success by comparing the present cultivation-dependent experiments with previous cultivation-independent studies.

2. Methods and materials

2.1. Sponge sample collection and processing:

Samples were collected from the sponges *Cymbastela concentrica, Tedania* sp. and *Scopalina* sp. in triplicate from Bare Island, Sydney, Australia. Each sponge specimen was collected and placed separately in a sterile zip-lock bag with surrounding seawater, transported on ice and processed immediately in the laboratory.

All the work henceforth was carried out in a laminar flow hood under sterile conditions and each sponge specimen was processed separately. Foreign material, like snails or worms, were removed from the sponge sample using sterile scalpels and forceps and the sponge was rinsed twice with sterile natural seawater (NSW).

2.2. Culturing media:

All culturing media were prepared to mimic physiological conditions in the sponge environment. Also some media were designed for targeted cultivation using previous genomic data (Fan et al., 2012a, b). A variety of low- to high-nutrient media were used for the cultivation of sponge-associated bacteria. The low nutrient media used were: Diluted Marine broth 1:20 + sponge extract (MB20 + SE); Diluted Marine broth 1:20 (MB20); Natural seawater + Sponge extract (NSW + SE); Natural seawater (NSW); Artificial sponge medium (Asp); Marine broth 1:20 + SE + nitrate (MB20 + SE + NO_3^-); MB 1:20 + Sponge extract + Ammonia (MB20 + SE + NH_4^+) and Snax media. The high-nutrient media used were: Reasoner´s 2A agar (R2A) and Marine broth half strength (MB 1:2). Table 2 shows the composition of different media used.

MB20 + SE + NH_4^+ and MB20 + SE + NO_3^- were designed to target microorganism involved in sponge nitrogen cycle. Modified version of R2A agar was used to target *Gammaproteobacteria*. The liquid medium Snax was used in order to target *Cyanobacteria.*

All media were prepared using natural sea water (NSW), except medium Asp. Antibiotics were added to all media after autoclaving at final concentrations of 20 mg/L nalidixic acid (Sigma Aldrich, Sydney, Australia), 20 mg/L cycloheximide (Sigma Aldrich, Sydney, Australia) and 25 mg/L nystatin (Sigma Aldrich, Sydney, Australia). Cycloheximide and nystatin were added to inhibit fungal growth. Nalidixic acid inhibits many rapidly growing bacteria (Webster et al., 2001).

Aqueous sponge extract (SE) was also added at final concentration of 10% to Asp and NSW + SE and 2% to MB20 + SE; MB20 + SE + NH_4^+; MB20 + SE + NO_3^-. Sponge extract was prepared separately for all three sponge species. For preparing SE, a sponge sample was cut into small cubes and homogenized in NSW (10 mL NSW/ g of sponge) for two min or until complete tissue disruption. The sample was incubated on ice for 30 min with agitation at 130 rpm, then filtered through a 125 µm metal sieve into a sterile centrifuge tube and centrifuged for 30 min at 15000 x g at 4°C. The resultant supernatant was filtered through a series of 12µm, 3µm and 0.2µm sterile filter papers (Whatman cellulose nitrate membrane filters) using a vacuum filtration unit. This constituted the SE that was added to the autoclaved media. In addition to SE, silicon dioxide (SiO_2) was also added to five different growth media as shown in Table 2.

Table 2: Growth media composition

Growth medium	Composition	Targeted bacteria	Reference
MB20 + SE	MB 1.87 g/L (Marine broth 2216 Difco), Noble agar (Difco) 15 g/L, NSW 1000 mL, SiO_2 25 g/L♦ (Sigma Aldrich, 0.5-10µm), SE 20 mL	Slow growing bacteria	This study
MB20	MB 1.87 g/L, Noble agar 15 g/L, NSW 1000 mL, SiO_2 25 g/L♦	Slow growing bacteria	This study
NSW + SE	Noble agar 15 g/L, NSW 1000 mL, SiO_2 25 g/L♦, SE 100 mL	Slow growing bacteria	This study
NSW	Noble agar 15 g/L, NSW 1000 mL, SiO_2 25 g/L♦	Slow growing bacteria	This study
MB 1:2	MB 18.7 g/L, Noble agar 15 g/L, NSW 500 mL, H_2O 500 mL	Heterotrophic bacteria	The prokaryotes-volume 5
R2A	R2A agar 18.4 g/L (Difco), NSW 750 mL, H_2O 250 mL	*Gammaproteobacteria*	The prokaryotes-volume 5
MB20 + SE + NH_4^+	Noble agar 15 g/L, H_2O 1000 mL, SE 20 mL, 10 mg/L $(NH_4)_2SO_4$ (McCaig et al., 1994)	To target bacteria involve in nitrogen cycle	The prokaryotes-volume 2
Asp	SE 100 mL, SiO_2 25 g/L♦, Gellan gum 8 g/L (Sigma Aldrich), CMFSW* 1L	Heterotrophic bacteria	This study
MB20 + SE + NO_3^-	MB 1.87 g/L, Noble agar 15 g/L, NSW 1000 mL, SE 20 mL, KNO_3 2 g/L, pH 7.5	To target bacteria involve in nitrogen cycle	The prokaryotes-volume 2
Snax (liquid media)	NSW 750 mL, H_2O 250 mL, 13 mg $(NH_4)2SO_4$, 1.5 mg K_2HPO_4, 0.5 mg Na_2 EDTA.$2H_2O$, Micronutrients† 0.1 mL, 1 mg Na_2CO_3, 90 mg KNO_3	*Cyanobacteria*	The prokaryotes-volume 4

*CMFSW (Calcium-magnesium free sea water): 25 g NaCl, 0.8 g KCl, 1 g Na_2SO_4, 0.04 g $NaHCO_3$, per litre of MilliQ water.
†Micronutrients composition: 10 mL H_2O, 60 mg/mL Iron citrate, 14 mg/mL $MnCl_2.4H_2O$, 0.2 mg/mL $CoCl_2.6H_2O$, 2.2 mg/mL $ZnSO_4.7H_2O$.
♦The amount of SiO_2 in media was optimized according to the amount of silicate in each sponge species (results provided by Ana I.S. Esteves, post-doctoral researcher at UNSW).
Names for media and some components have been abbreviated here; the full names of the media can be found in the text.

2.2.1. Media for floating filters:

Four different low-nutrient liquid media were used for cultivation with floating filters: 1) MB20 (MB 1.87 g/L, NSW 1000 mL, SiO_2 25 g/L); 2) MB20 + SE (MB 1.87 g/L, SE 20 mL, SiO_2 25 g/L, NSW 1000 mL); 3) NSW (NSW 1000 mL, SiO_2 25 g/L); 4) NSW + SE (NSW 1000 mL, SE 100 mL, SiO_2 25 g/L).

2.3. Microbial cell isolation:

For microbial cell isolation, each sponge specimen was processed separately; 2.5 g of sponge (comprise of both endosome and ectosome of sponge) was cut and washed four times with sterile CMFSW at 200 rpm for 10 min. The washed sponge was then homogenized for 10-15 sec in CMFSW using a homogenizer (IKA ultra-turrax homogenizer). The sample was incubated on ice for 30 min on a rocking platform at 150 rpm and then filtered through a 125 µm metal sieve. The filtrate was then centrifuged for 15 min at 100 x g at 4°C. The collected supernatant was centrifuged twice at 300 x g for 15 min at 4°C. The resultant supernatant was filtered through a 12 µm filter and subsequently filtered through a 3 µm filter (Whatman cellulose nitrate membrane filters) using a vacuum filtration unit. The final filtrate was centrifuged at 15,000 x g for 20 min at 4°C and washed twice with CMFSW. The pellets were then finally resuspended in CMFSW. This cell inoculum was used for cultivation, which will be explained in the following section.

2.4. Cultivation:

2.4.1. Agar plate cultivation:

For inoculation in agar media, the prepared cell suspension was serially diluted in sterile CMFSW and dilutions 10^{-3} to 10^{-5} were plated on all agar media plates in triplicates. For cultivation in Asp medium, a natural bath sponge was used as source of spongin fibre, it was cut into thin slices of ~ 2 mm and autoclaved. These autoclaved spongin pieces were layered on a petri dish and overlayed with the gellan gum based

medium. One hundred µL of cell suspension dilution 10^{-3} was plated in triplicates and plates were incubated at 18 °C in the dark.

2.4.2. Floating filter cultivation:

For floating filters, PVP-Nuclepore Polycarbonate filters (Whatman) with a diameter of 47 mm and a pore size of 0.2 µm were autoclaved and mounted on a sterile glass filter holder. Each filter was first rinsed with 10 mL CMFSW and then 5 mL diluted cell suspension was filtered through it (dilutions 10^{-3} and 10^{-4}). The filters were placed in a petri dish with liquid medium by using sterile tweezers in triplicates (figure 4). The petri dishes were sealed and incubated in the dark at 18 °C.

2.4.3. Direct inoculation:

Small cubes from each sponge sample were also cut and washed four times at 200 rpm for 10 min in CMFSW and directly placed on all solid media using sterile tweezers in triplicates (figure 3). Plates were incubated at 18 °C.

2.4.4. Liquid medium cultivation:

For inoculation in Snax liquid medium, a small cube ~ $1cm^3$ from each sponge specimen was placed in 100 mL of Snax media in a sterile conical flask and incubated at room temperature under cool white light with intensity of 20 µE m^{-2} s^{-1} on a rocking platform. For isolation on solid medium, 100 µL of liquid culture was plated onto SNAX + 15 g/L agar and colonies were then restreaked in MB1:2 Agar.

All the agar media and floating filter plates were incubated for a period of 6 months, and growth was monitored regularly. Colonies with different morphologies were picked time to time along the experiment and purified by repeated streak plating on new MB 1:2 agar plates until pure. Purity was assessed based on colony morphology. Pure isolated colonies were transferred to 3 mL sterile liquid medium (MB 1:2) and allowed to grow for 3 days at 18 °C with shaking (200 rpm) in the dark. An aliquot of each liquid culture was taken and stored in 20% glycerol at -80 °C until further use. Two millilitres of the remaining culture was centrifuged at 17 000 x g for 5 min, the supernatant was discarded, and the bacterial pellet was stored at -20 °C for DNA extraction.

2.5. Live/Dead staining:

For Live/Dead staining Live/Dead BacLight Bacterial Viability Kit L7007 was used according to the manufacturer's instructions. This rapid epifluorescence staining method was applied to estimate viability of bacteria in cell suspension. It utilizes mixtures of green-fluorescent nucleic acid stain SYTO 9, and the red-fluorescent nucleic acid stain propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. Total (red+green) and viable (green) cells can hence be counted simultaneously.

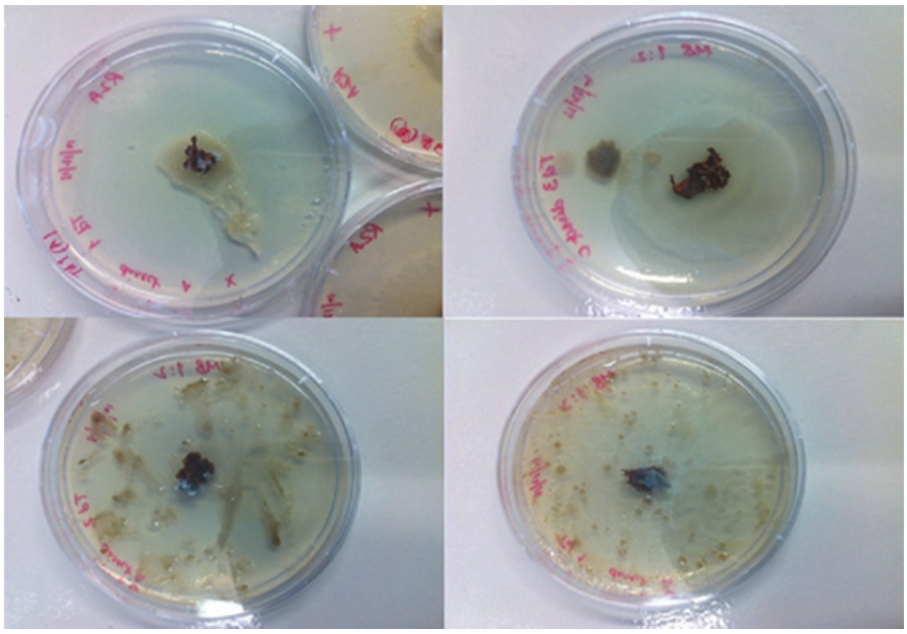

Figure 3: Direct inoculation plates: Bacterial growth on different medium inoculated with sponge pieces.

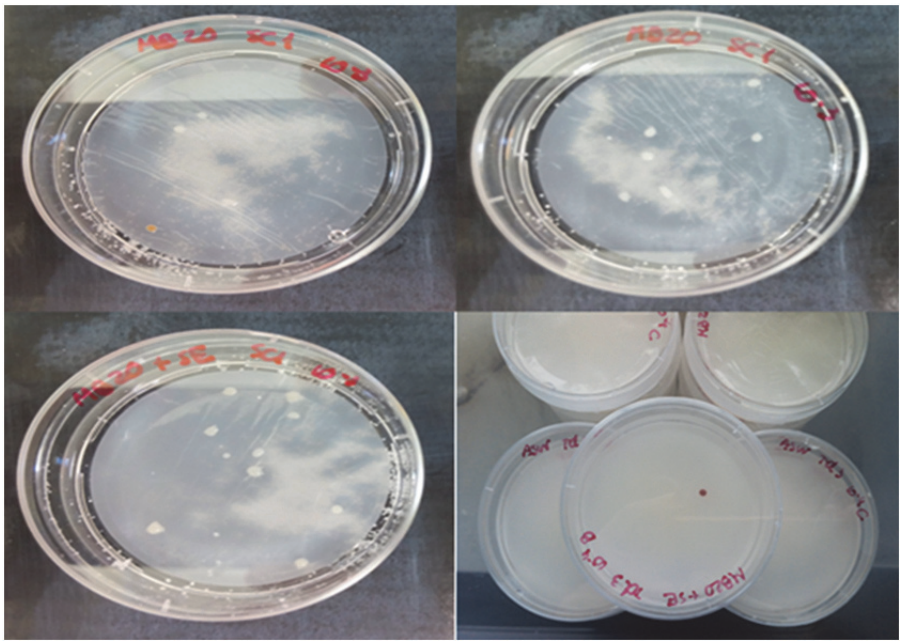

Figure 4: Floating filters plates: Visible bacterial colonies on top of floating filters on different medium.

2.6. DNA extraction and identification:

DNA was extracted from frozen bacterial pellets with the Qiagen DNeasy Blood & Tissue Kit according to manufacturer's instructions. Identification of isolates was determined by 16S rRNA gene sequencing. 16S rRNA gene fragments of approximately

1400 bp in length were amplified using the universal bacterial primers F27 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and R1492 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Weisburg et al., 1991). Reaction mixtures (25 µL) were prepared as follows: 10.5 µL of H_2O, 12.5 µL of EconoTaq PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, WI, USA), 0.5 µL of each primer 10 µM and 1 µL of template DNA. Thermal cycling started with an initial denaturation step of 94 °C for 5 minutes, 25 cycles of 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 45 sec and a final extension step at 72 °C for 10 min. The PCR products were identified on 1% agarose gel stained with GelRed (Biotium) to ensure they were of the correct size and quantified with the Image Lab Software (Bio-Rad). Products from successful PCRs were purified using Sephadex G50 (BioReagent, for molecular biology, DNA grade, fine, Sigma Aldrich) columns and subjected to sequencing with the chain termination method in an Applied Biosystems 3730 Capillary Sequencer at the Ramaciotti centre for genomics at the University of New South Wales (Sydney, Australia) using the 27F primer. Sequences were manually trimmed using Sequence Scanner v1.0 software (Applied Bio systems), based on the quality value attributed to each base (cut-off = 20) and the resolution of peaks in electropherogram. Related sequences were identified using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) database. Taxonomic assignment of bacterial isolates was obtained using the classifier tool of the Ribosomal Database Project (RDP) (Wang et al., 2007) at 80% confidence threshold. Closest type strains to all sequences were determined using the RDP sequence match tool (RDP version 11).

2.7. Phylogenetic analysis:

For phylogenetic analysis, all 16S rRNA gene sequences were aligned using the SINA web aligner tool version 1.2.11 (Pruesse et al., 2012) before importing into the ARB software package (Ludwig et al., 2004). Alignments were manually refined using the ARB alignment tool. The 16S rRNA gene sequences of closest matches found in the BLAST analysis above were included in the alignment procedure. Maximum likelihood phylogenetic analyses were performed by RAxML using the GTRMIX model with 100 replicates, each starting from a random tree. Bootstrap analyses for phylogenetic trees were determined by RAxML using the rapid bootstrap analysis algorithm implemented in ARB (Stamatakis et al., 2008).

3. Results

3.1. Live/ Dead staining of inoculum:

To assess the viability of bacteria in the cell suspension of each sponge species, the rapid epifluorescence staining method was applied. By visual inspection of each sample treated with live/dead stain, the cell viability was observed to be above 50 %.

3.2. Colony number with different cultivation media and methods:

A total of 202 isolates (each isolate is representative of a different morphotype) were retrieved from the three sponge species *C. concentrica, Tedania* sp. and *Scopalina* sp., using a broad range of culture media that differed in nutrient composition and concentration (see Appendix for details of each isolate). Most of the isolates were cultivated from agar-based media and around 15 from floating filters, with the highest number of isolates obtained from *Tedania* sp. (Table 3). For *C. concentri*ca and *Tedania* sp., no differences were found regarding the number of isolates from medium MB20, with and without the addition of sponge extract. In general, fewer number of colony types were obtained from the low-nutrient media, such as MB 1:20+SE, MB20, NSW+SE and NSW agar, compared to high-nutrient media, such as MB 1:2 and R2A (figure 5). Several isolates were obtained from two very low-nutrient media i.e. SNAX (25 isolates) and Asp (16 isolates) media for *Tedania* sp. and *Scopalina* sp., respectively (figure 5).

For direct inoculation cultivation used in this study, no differences based on morphology were found between isolates from agar plate cultivation and isolates from direct cultivation; hence the results obtained from these two cultivation techniques were combined.

For floating filters, most of colony formation occurred on MB1:20 and MB1:20+SE and only few on NSW+SE, whereas on NSW medium no growth was observed. The colonies obtained from agar based media were comparable morphologically to the colonies observed in floating filters; and colonies picked from different sponge species were morphologically different (for details see appendix table 1).

Table 3: Number of isolates from all sponge species

Sponge species	No. of isolates	Isolation method
Cymbastela concentrica	59	Agar-based media
	1	Floating filters
Tedania sp.	97	Agar based media
	14	Floating filters
Scopalina sp.	24	Agar based media

Total number of morphotype from each sponge species with different isolation techniques.

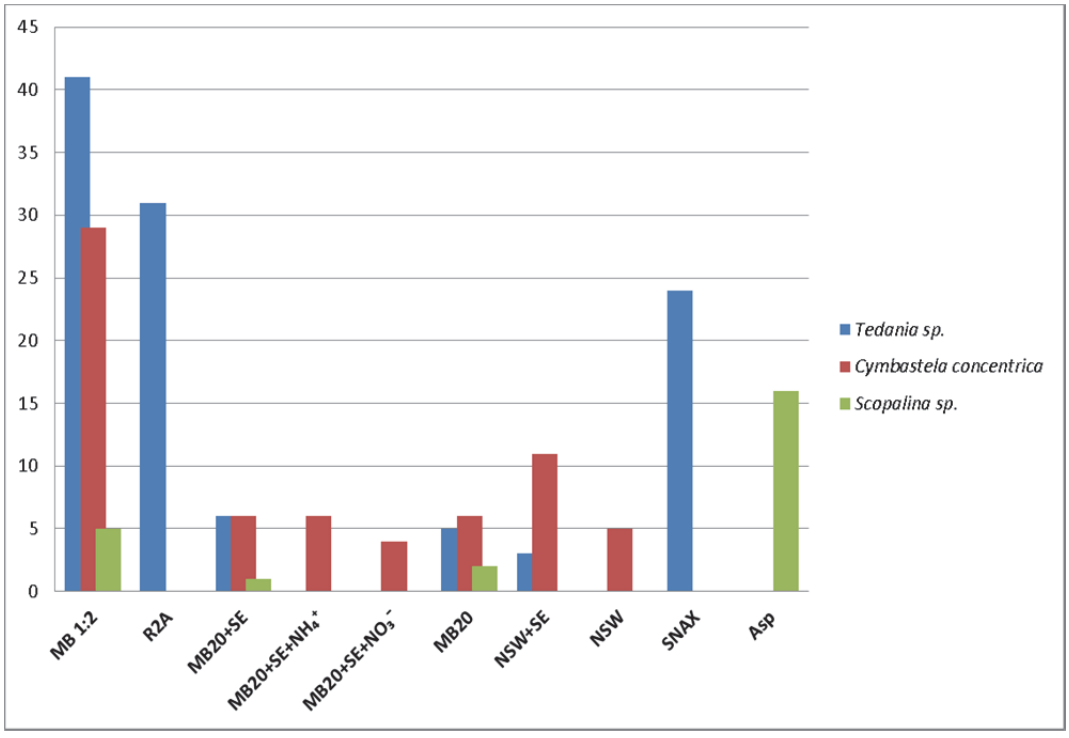

Figure 5: Total number of isolates from each sponge species. Graph shows the total number of isolated bacteria from this study on different media used. Each isolate is representative of a different morphotype from each medium.

3.3. Identification of cultivated bacteria:

The 16S rRNA gene amplification and subsequent partial gene sequencing (≥ 600 bp) were successful for 202 isolates. Isolated bacteria were distributed among four bacterial phyla: *Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes,* according to the RDP classification. The phylum *Proteobacteria* was the most dominant among all phyla. Within the phylum *Proteobacteria*, the class *Alphaproteobacteria* was the most abundant group in all three sponges. The isolated bacteria fell into 16 different genera. The most frequently isolated genus was *Pseudovibrio* (111 isolates), followed by *Ruegeria* (18 isolates), *Labrenzia* (14 isolates), *Cellulophaga* (14 isolates) and *Vibrio* (12 isolates). The other genera isolated in smaller numbers included: *Enterovibrio* (6 isolates), *Bacillus* (5 isolates), *Aquimarina* (4 isolates), *Streptomyces* (4 isolates), *Erythrobacter* (2 isolates) and *Pseudoalteromonas, Neptuniibacter, Colwellia, Tenacibaculum, Micrococcus* and *Sphingopyxis*, represented each by one isolate.

Pseudovibrio spp. were isolated from all three sponges used in this study, with the highest number of isolates retrieved from the high-nutrient media MB 1:2 and R2A. *Ruegeria* spp. were cultivated from the sponge *Tedania* sp. on media MB1:2, R2A and MB20+SE, with highest number of isolates from medium MB1:2 (see Methods and materials for media used for cultivation experiments) (figure 6). *Labrenzia* and *Cellulophaga* were also cultivated from *Tedania* sp. on SNAX medium. Figure 6 also shows a clear difference between the diversity of bacteria cultivated from each sponge species on different media used. A higher diversity of bacteria was cultivated from high-nutrient media (i.e. MB 1:2 and R2A) as compared to the bacterial diversity from low-nutrient media used in this study (figure 6). Few of the isolated bacteria that showed a confidence threshold of less than 80 % according to RDP classification were considered unclassified, these included unclassified *Vibrionaceae*, unclassified *Rhodobacteraceae*

and unclassified *Flavobacteriaceae*. *Vibrio* and *Enterovibrio* spp. were cultivated mostly from *C. concentrica* on different media as shown in figure 6.

When considering the total diversity of bacteria per sponge species the most genera were isolated from *Tedania* sp. (12 genera), followed by *C. concentrica* (10 genera) and *Scopalina* sp. (3 genera) (figure 7).

The bacterial diversity isolated from traditional cultivation technique was high, when compared with diversity from floating filters. The most dominant genera isolated from floating filters were *Pseudovibrio*; other genera isolated were *Ruegeria, Aquimarina* and *Streptomyces* (figure 8).

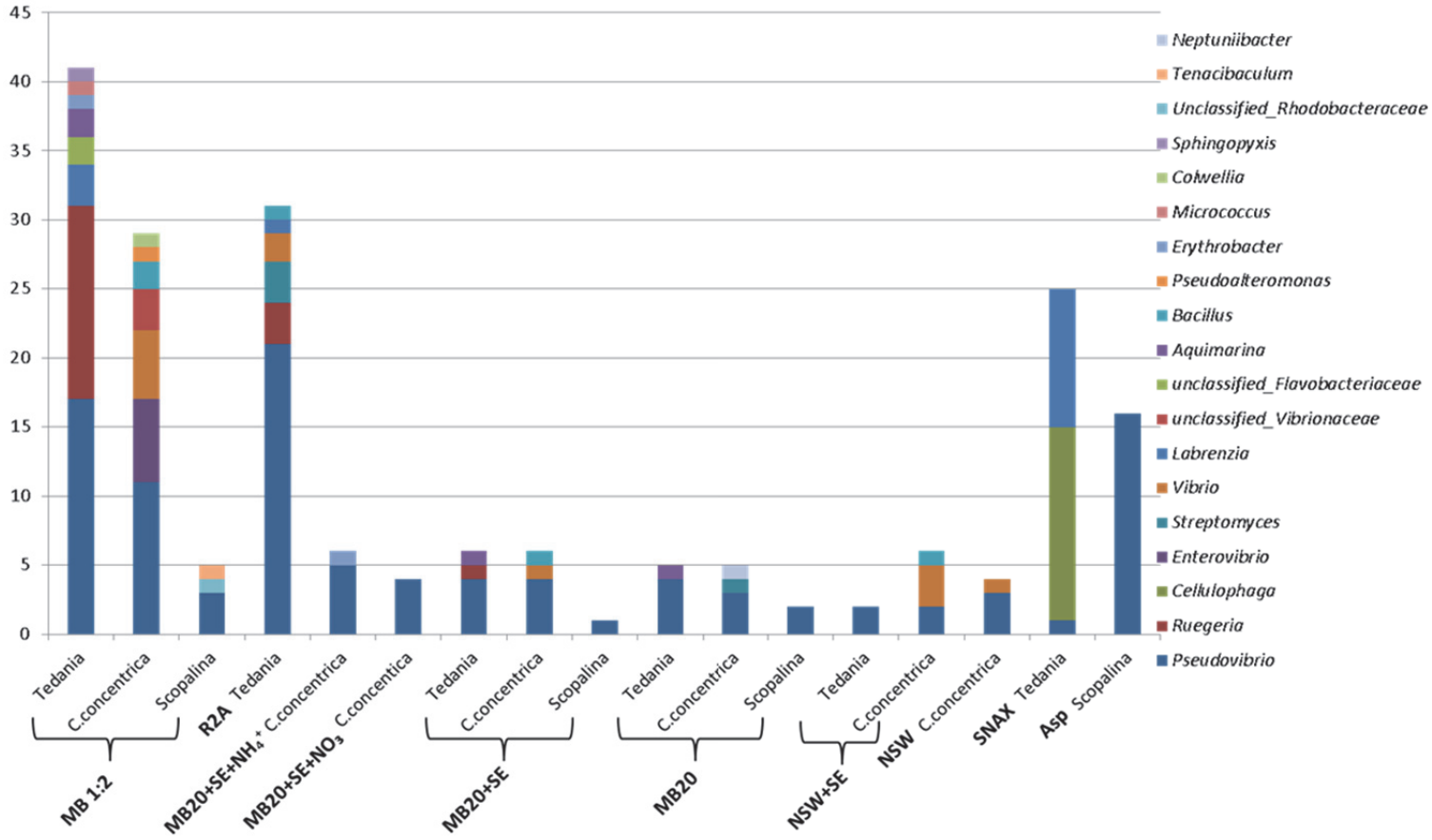

Figure 6: The diversity of bacteria per medium. Diversity in *Tedania* sp., *Cymbastela concentrica* and *Scopalina* sp. The graph shows the difference in diversity observed in each medium and each sponge species.

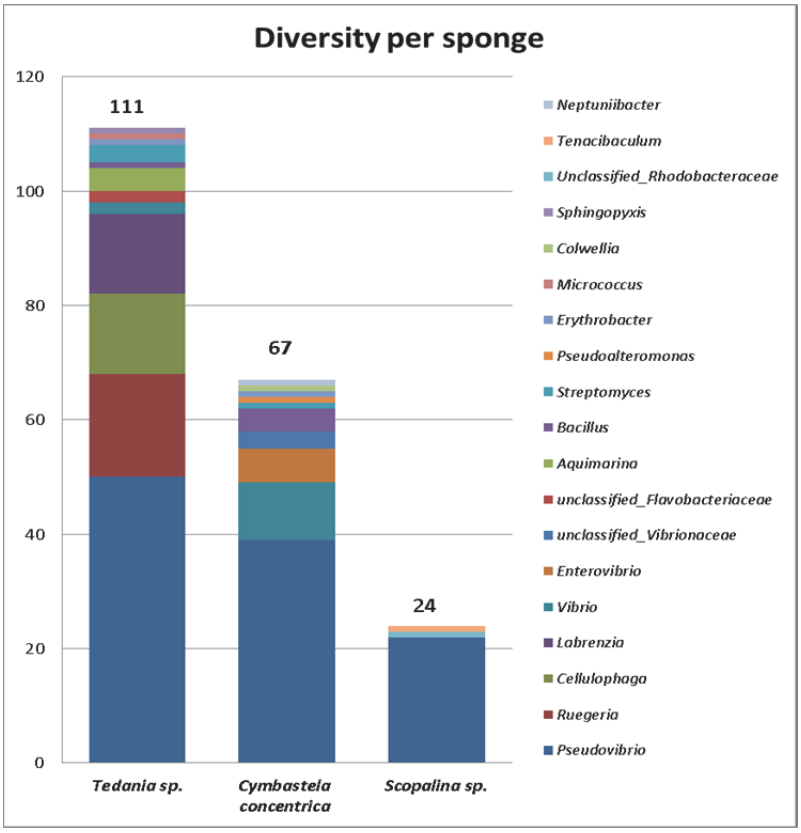

Figure 7: Total bacterial diversity per sponge species. The number on top of each bar represents the total number of isolates from each sponge species

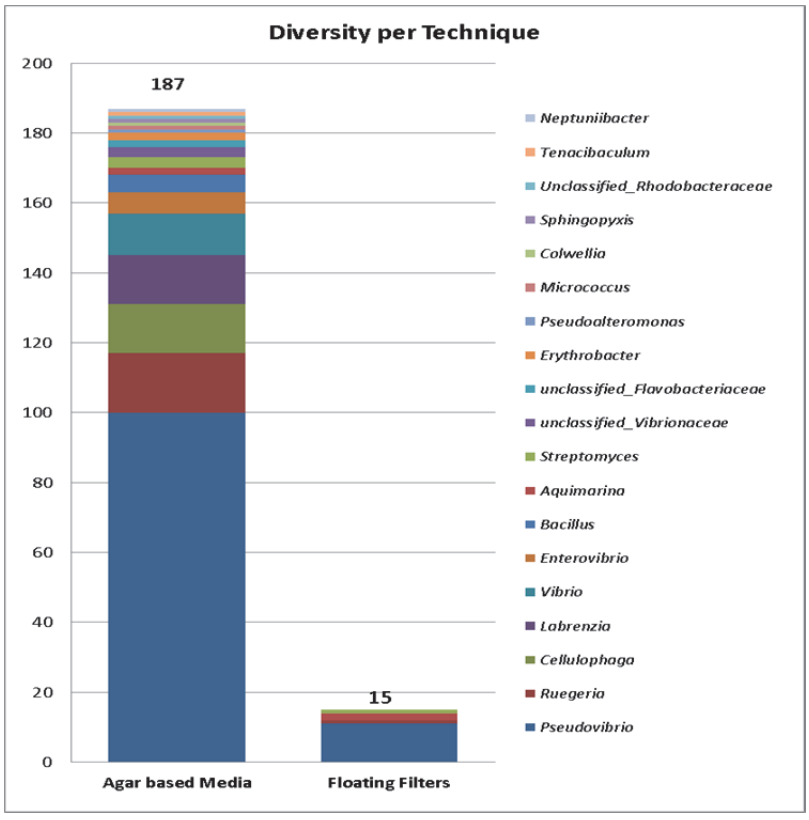

Figure 8: Diversity per technique. Graph shows diversity of bacteria per isolation technique. Number on top of each bar represents the total isolated bacteria from each technique.

3.4. Phylogenetic analysis of isolates

Phylogenetic analysis based on the 16S rRNA gene sequence showed that *Pseudovibrio* spp. isolates from all three sponges are closely related to bacteria previously isolated from a wide range of marine sponges (*Pseudovibrio* sp. P1MA4, Dupont et al., 2013; *Pseudovibrio* sp. Pv266, Esteves et al., 2013) and some isolates closely related to bacteria isolated from seaweed (*Pseudovibrio* sp. D323, Penesyan et al., 2011) (figure 9). Details on the phylogeny of individual isolates are given below in figure 14.

Closest matches to *Ruegeria* isolates cultivated in this study included bacteria isolated from irciniid sponge species (Ruegeria sp. Rg351 and 290, Esteves et al., 2013), from the sponge *Halichondria panicea* (sponge bacterium Ex11, Wichels et al., 2006) and from the sponge *Leucosolenia* sp. (Bacterium W15M27b, Flemer et al., 2011). *Ruegeria atlantica* AB255399 represents the type strains of the genus *Ruegeria*, the closest 16S rRNA gene relative to the *Ruegeria* sequences found in the three sponges of this study (Fig. 10).

Aquimarina sp. cultivated from this study is closely related with bacteria isolated from seawater (*Aquimarina* sp. SW2 and *Aquimarina macrocephali)* and also closely related with bacteria isolated from marine sponges (sponge bacterium Zo26, Wichels et al., 2006; Bacterium W15C32, Flemer et al., 2011). The closest type strain to *Aquimarina* isolates cultivated here is *Aquimarina muelleri* AY608406. *Cellulophaga* isolates cultivated in this study have no near neighbours that were previously isolated from marine sponges (figure 11).

A maximum likelihood tree for *Labrenzia* sp. isolated from this study was also constructed (figure 12). Most of the *Labrenzia* sp. isolates have no close neighbours that

were derived from marine sponges, except for isolate 104, which is closely related to sponge derived sequences (figure 12).

The isolated *Vibrio* sp. isolates (isolates 2, 5, 7, 39 and 15) were closely related to bacteria previously found in marine sponges according to the phylogenetic analysis (Su et al., 2014; Dupont et al., 2013; Esteves et al., 2013), and also related to sequences found in other marine organisms, such as oysters and seaweeds (figure 13). For isolates 23 and 16A, B no sponge-derived relative was found.

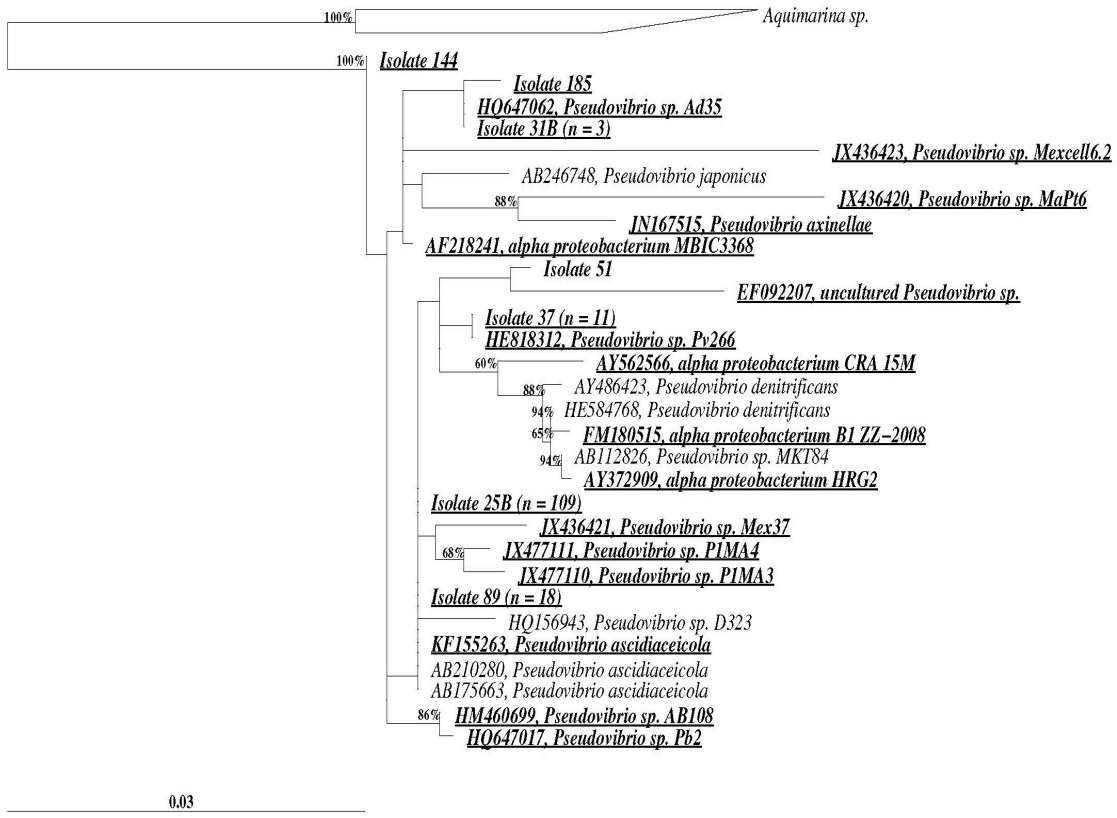

Figure 9: Maximum likelihood tree of *Pseudovibrio* spp. based on 16S rRNA gene sequences. Isolates retrieved in this study and sponge-associated bacteria are underline and highlighted in bold. In brackets is the number of isolates from this study, which had 100% identity to the isolate listed. ML bootstrap values (> 50%) and are shown above branches. Genus *Aquimarina (Flavobacteria, Flavobacteriaceae)* was used as an outgroup. The scale bar indicates the number of substitutions per nucleotide position.

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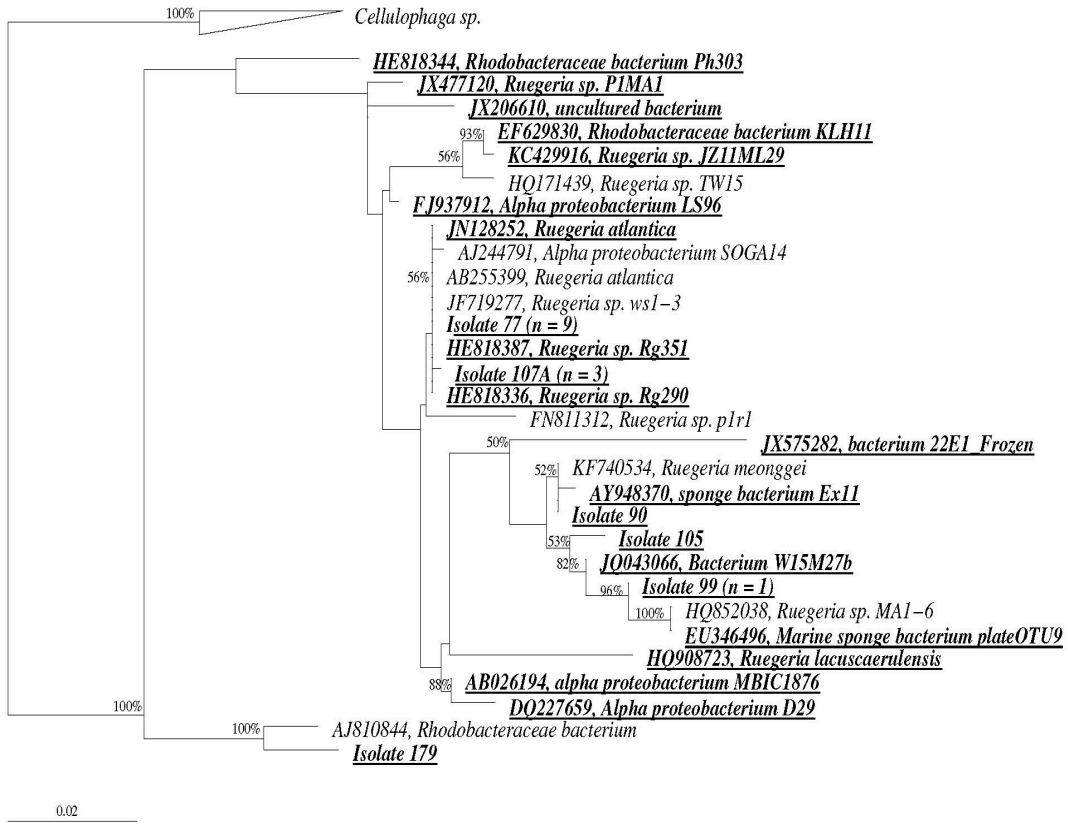

Figure 10: Maximum-likelihood (ML) tree for *Ruegeria* spp. Details are as provided for Fig. 9. The tree is rooted with the *Cellulophaga* sp. *(Flavobacteria, Flavobacteriaceae).*

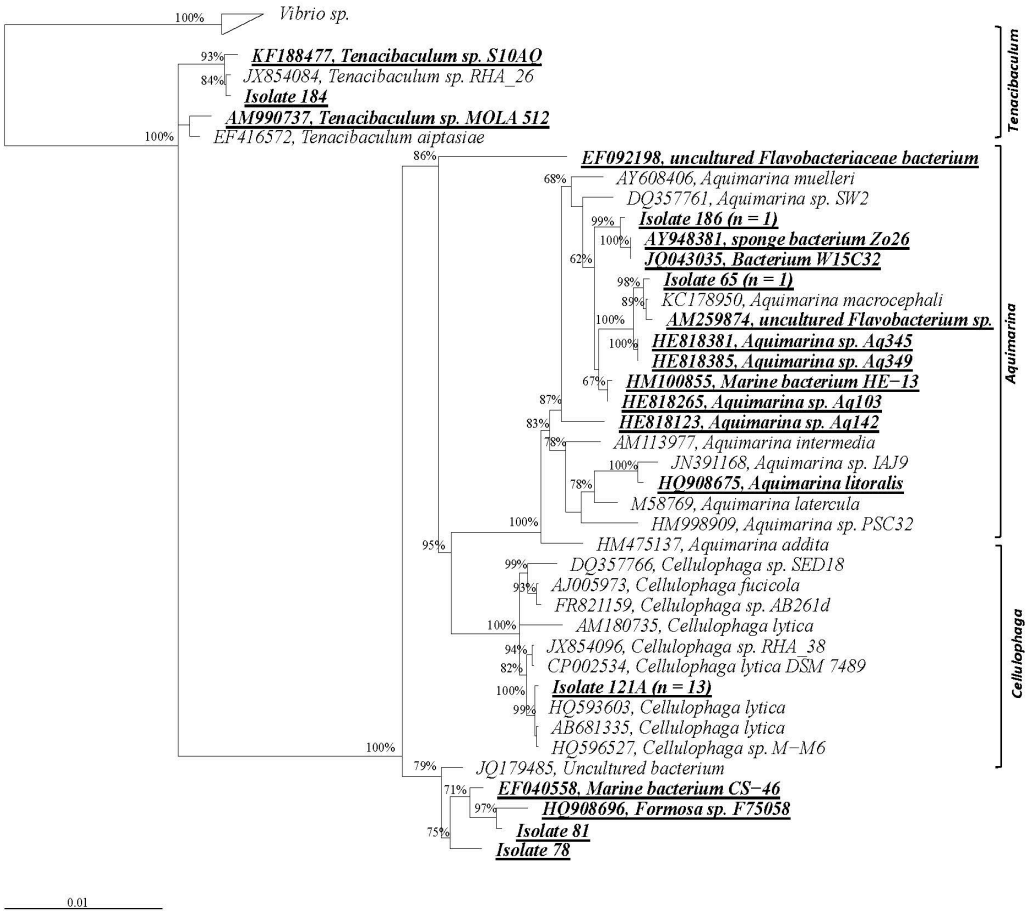

Figure 11: Maximum-likelihood (ML) tree for *Aquimarina spp., Tenacibaculum* spp. *and Cellulophaga spp.* Details are as provided for Fig. 9. The tree is rooted with the *Vibrio* sp. *(Gammaproteobacteria, Vibrionaceae).*

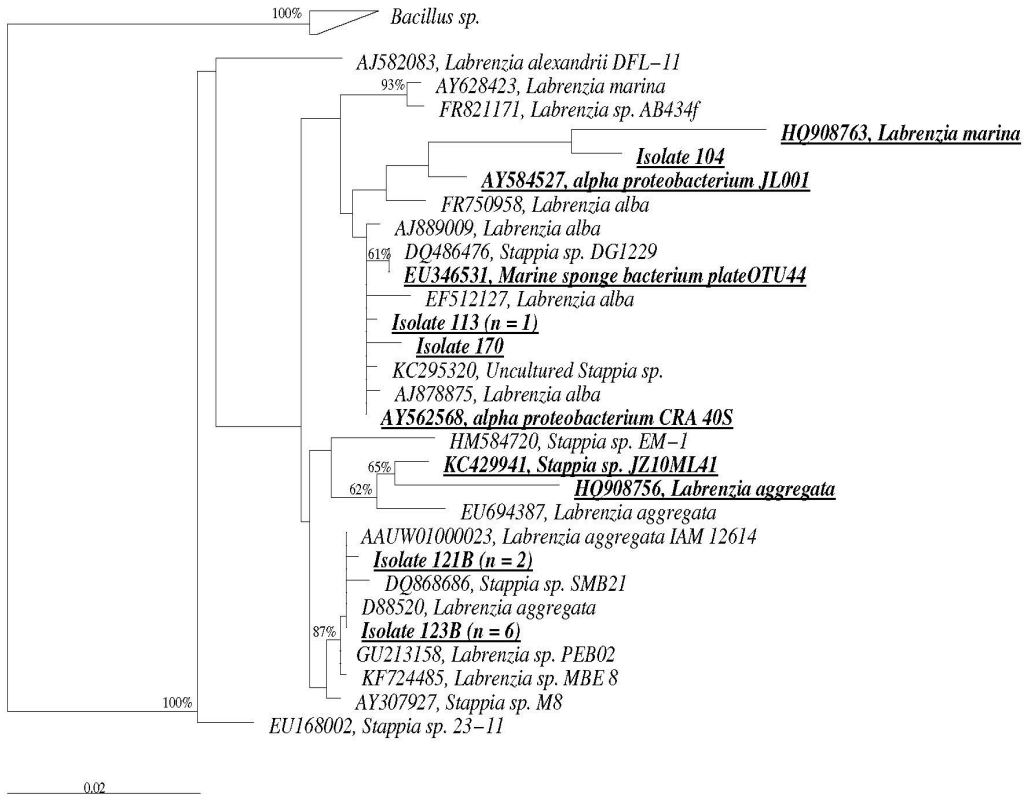

Figure 12: Maximum-likelihood (ML) tree for *Labrenzia* spp. Details are as provided for Fig. 9. The out group was *Bacillus* sp. *(Bacilli, Bacillaceae)*.

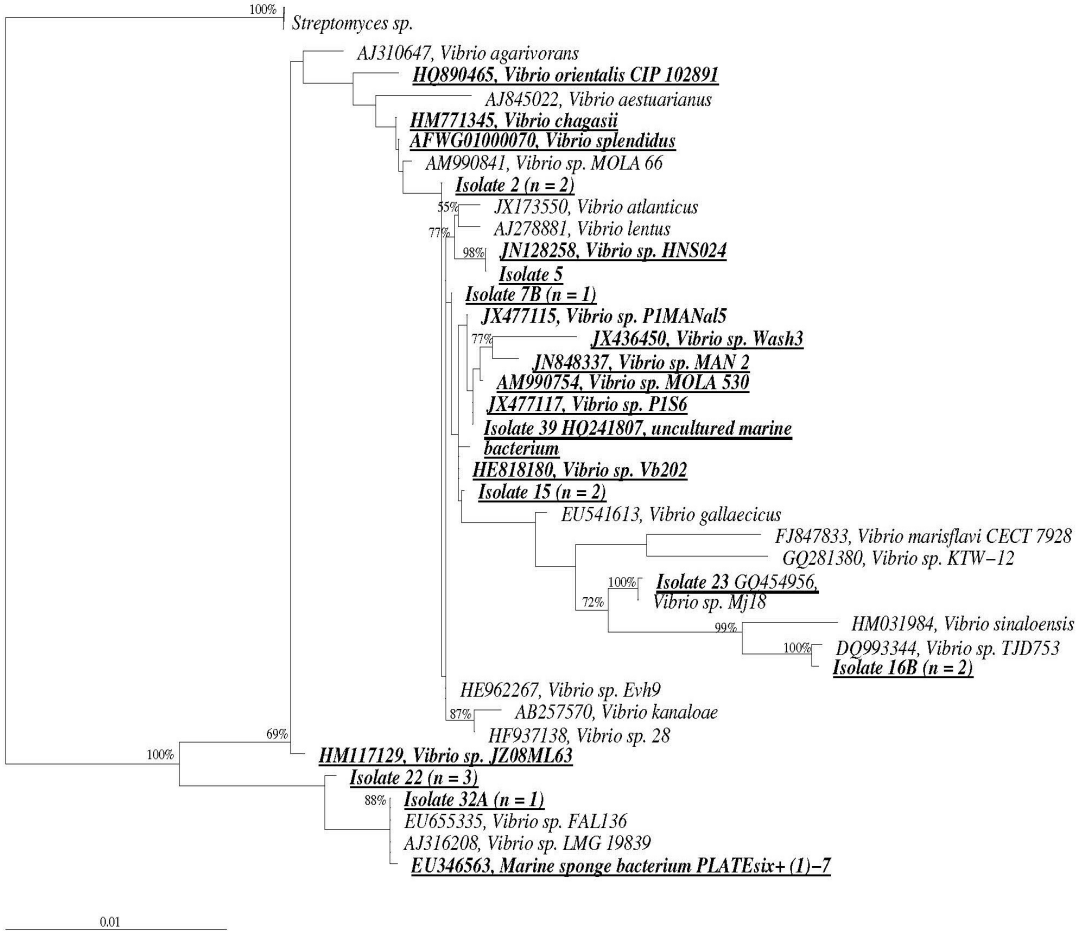

Figure 13: Maximum-likelihood (ML) tree for *Vibrio* spp. Details are as provided for Fig. 9. *Streptomyces* sp. *(Actinobacteria, Streptomycetaceae*) is used as an out group.

3.5. Genus diversity between different sponges and cultivation approaches

A maximum likelihood tree of all individual *Pseudovibrio* isolates cultivated from this study was also determined. Figure 14 shows the clustering of isolated *Pseudovibrio* from different sponge species and media used in this study. Most of isolates from *Scopalina* sp. are clustered together (isolates highlighted in yellow in figure 14), along with isolates from *Tedania* sp. and *C. concentrica*. Isolates belonging to *Tedania* sp. and *C. concentrica* are clustered mostly with each other (isolates highlighted in grey and blue, respectively, in figure 14). However, only a few of the *Pseudovibrio* isolates from *Tedania* sp. and *C. concentrica* form sequence clusters that are specific to certain sponge species as shown in figure 14. In terms of media used in this study, the *Pseudovibrio* isolates cultivated did not form clusters that were specific to any media used (figure 14).

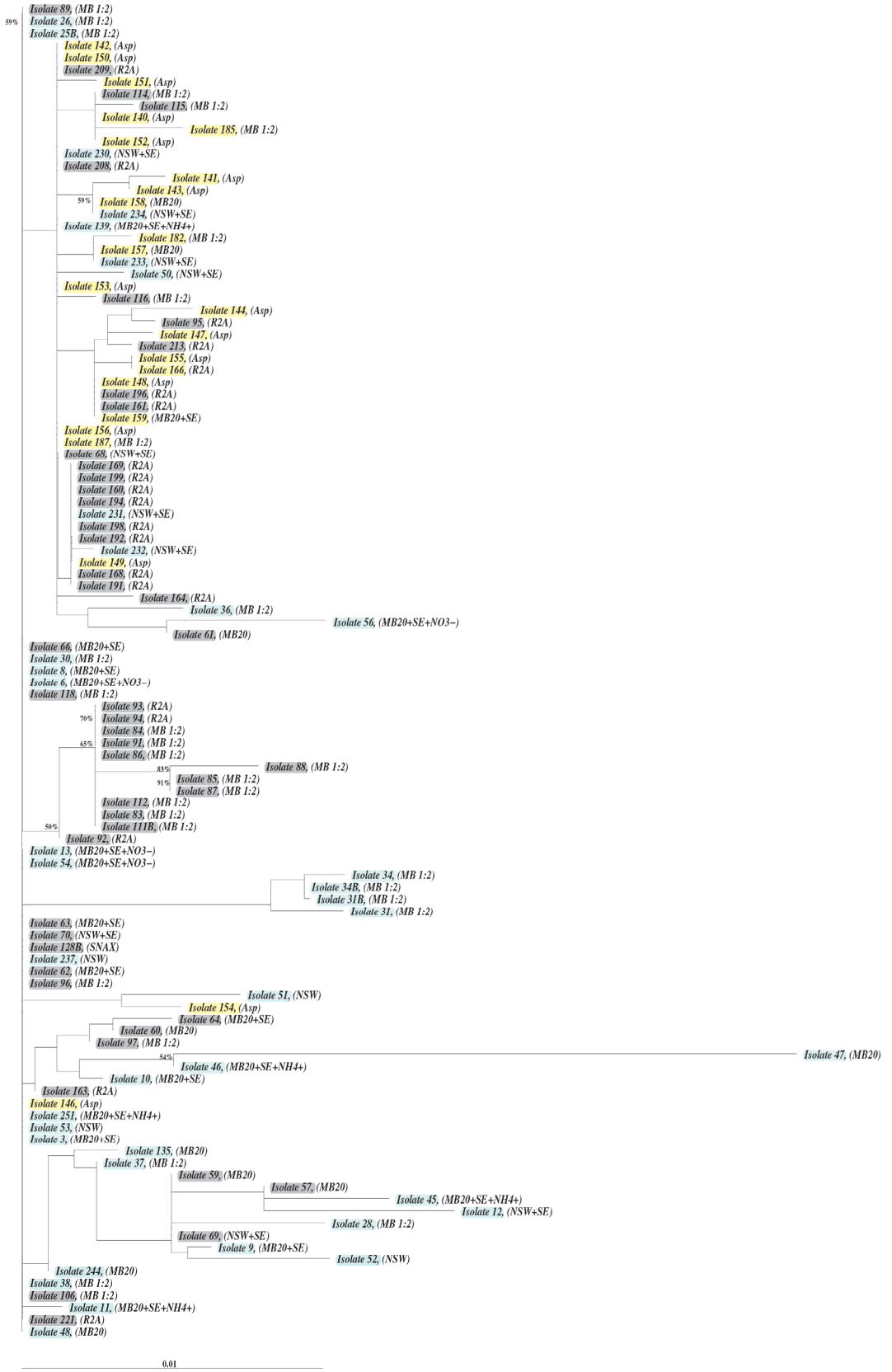

Figure 14: Maximum likelihood tree of the 16S rRNA gene sequence for all individual *Pseudovibrio* isolates cultivated in this study. Isolates highlighted in grey colour, represents isolates cultivated from *Tedania* sp., in blue are the isolates from *Cymbastela concentrica* and in yellow are the isolates from *Scopalina* sp.. Names of cultivation media for each isolate are represented in brackets. Names for media have been abbreviated here; the full names of the media can be found in the text.

3.6. Overlap with cultivation independent studies:

In order to assess the cultivability of sponge-associated bacteria, 16S rRNA gene sequences of the isolates retrieved in this study were compared to previously generated 16S rRNA gene database from six different sponge species (*Cymbastela coralliophila, C. concentrica, Scopalina* sp., *Tedania anhelans, Rhopaloeides odorabile* and *Stylissa* sp. 445) (Fan et al., 2012a). The analysis was done by BLAST searches (http://www.ncbi.nlm.nih.gov/books/NBK52637/) against databases for the six sponges (Fan et al., 2012a). The results show an overall overlap of 20 isolates with the culture-independent study (table 4). Most of the isolates from this study shows closest matches with sequences obtained from sponge *C. coralliophila* and some isolates show closest match with sequences from *Tedania anhelans* (table 4).

Table 4: Overlap between cultivation-dependent and cultivation-independent studies

Isolate number and isolation source from current study	Closest match with sequences from culture-independent studies	Similarity %	RDP classification
Isolates: 67, 77, 79, 80, 82, 90, 99, 101B, 102, 103, 105, 111A, 165, 178 (*Tedania* sp.)	BBAY31_F5ZGUVP02HK0UO (from sponge *Cymbastela coralliophila*)	97.5-98.97%	*Ruegeria* 94-100%
Isolates: 46, 53 (*Cymbastela concentrica*)	BBAY63_GR3VXG201DB8ED (from sponge *Tedania anhelans*)	100%	*Pseudovibrio* 100%
Isolates: 177, 100B, 101A, 107A (*Tedania* sp.)	BBAY31_F5ZGUVP01DM6QX (from sponge *Cymbastela coralliophila*)	98.59-98.79%	*Ruegeria* 94-100%

Table shows the closest match of isolates from this study with previous culture-independent study.

4. Discussion

The culturing experiments and the phylogenetic analyses carried out in this thesis have demonstrated that the use of multiple culturing techniques and conditions results in a diverse range of cultivated bacteria from three different sponge species, which share the same geographical location.

4.1. Bacterial diversity from different sponge species:

The culturable bacterial community of the sponges *Tedania* sp., *C. concentrica* and *Scopalina* sp. consists of bacteria belonging to four phyla: *Proteobacteria, Bacteroidetes, Firmicutes* and *Actinobacteria.* These four bacterial phyla represent the most frequently isolated bacteria from marine sponges (Taylor et al., 2007).

4.1.1. Phylum *Proteobacteria*:

The class *Alphaproteobacteria* with its genus *Pseudovibrio* was most dominant among the culturable community of all three investigated sponge species. Many other cultivation-dependent studies also reported a similar dominance of *Pseudovibrio* for different sponge species and with different culture condition (Webster and Hill, 2001; Thiel & Imhoff, 2003; Sipkema et al., 2011; O'Halloran et al., 2011).

Pseudovibrio sp. has been found associated with sponge larvae and may thus represent a group of vertically transmitted sponge symbionts (Enticknap et al., 2006). The consistent occurrence of *Pseudovibrio*-related bacteria in marine sponges suggests that this genus contains indeed true sponge symbionts (Webster and Hill, 2001; Enticknap et al., 2006). The frequent identification and isolation of *Pseudovibrio* strains

suggests an important role for this genus in marine habitats. Bacteria belonging to the genus *Pseudovibrio* are also found in other marine habitats, mainly in association with corals, seawater, ascidian and tunicates (Shieh et al., 2004; Menezes et al., 2010; Riesenfeld et al., 2008). However, *Pseudovibrio* were not present in one of the largest culture surveys to date (Sfanos et al., 2005).

The phylogenetic clustering of *Pseudovibrio* isolates retrieved in this study (figure 14) shows that most of isolates from *Scopalina* sp. are clustered closely together along with isolates from *Tedania* sp. and *C. concentrica*,. This could potentially be due to the fact that some *Pseudovibrio* strains are specific to a particular sponge host. In a recent study, comparison of bacterial communities using different OTU definitions (Core community: OTUs present in at least 70% of the analyzed sponges; variable community: OTUs present in <70% of sponges but in at least two species; and species-specific community: OTUs present in only a single sponge species) revealed a minimal core and large host species-specific bacterial community (Schmitt et al., 2011). This indicates that each sponge contains a large set of unique bacteria and shares only few bacteria with other sponges (Schmitt et al., 2011).

The other dominant genera isolated from the sponges examined in this study include *Ruegeria, Labrenzia* and *Vibrio*. Interestingly, *Ruegeria* and *Labrenzia* were only isolated from the *Tedania* sp.. Some past studies have shown that many of the known sponge-associated microbes are unique to sponges (Hentschel et al., 2002, Taylor et al., 2007). Among these sponge-specific microorganisms, some are common among different types of sponges from different geographical locations, while some are only found in certain sponges (Hentschel et al., 2002, Hill et al., 2006, Taylor et al., 2005). Most *Ruegeria* isolates from this study are closely related to *Ruegeria* sp.

previously isolated from marine sponge *Sarcotragus spinosulus* and *Ircinia variabilis* in the north-east Atlantic (Esteves et al., 2013) (Fig. 10) and therefore could be considered to be consistently associated with sponges. Bacteria related to genus *Ruegeria* require NaCl or sea salts for growth and have been isolated from seawater and marine macroorganisms, including other sponge species, ascidian and algae (Muscholl-Silberhorn et al., 2008; Menezes et al., 2010). The prevalence of the genus *Ruegeria* associated to marine organisms could be related with quorum sensing systems commonly found in *Proteobacteria*, which allows cell-to-cell communication and may be involved in the symbiotic interactions between bacteria and their hosts (Mohamed et al., 2008).

Vibrio spp. were mostly isolated from the sponge *C. concentrica* (figure 7). Previous investigations using both community fingerprinting analysis and metagenomics analysis revealed that the sponge *C. concentrica* harbors a stable bacterial community over time and space and that the microbial community in sponges is significantly different from that of the surrounding seawater (Taylor et al., 2004, Thomas et al., 2010; Fan et al., 2012a). Bacterial groups that have been identified from *C. concentric*a through cultivation-independent studies, include *Proteobacteria* (Alpha, Beta and Gamma), *Actinobacteria* and *Nitrospira* (Fan et al., 2012 a, b). Several cultivation-dependent and cultivation-independent studies have shown that vibrios live in and/or on marine organisms, such as corals, fish, molluscs, seagrasses, sponges, shrimps and zooplankton (Thompson et al., 2004; Hoffmann et al., 2012).

4.1.2. Other less dominant phyla:

Aquimarina and *Cellulophaga* (phylum *Bacteroidetes*, class *Flavobacteria*) were also only isolated from the sponge *Tedania* sp. in this study. *Flavobacteria* are common in epipelagic oceanic and coastal waters as well as in benthic habitats, accounting for 10-30% and sometimes up to 70% of bacterial populations (Hahnke and Harder, 2013). *Aquimarina* species have been previously isolated from marine environments, including seawater, alga and marine sponges (Zhang et al., 2014; Bokun lin et al., 2012; Kennedy et al., 2013; Yoon et al., 2011). *Cellulophaga* sp. has been previously isolated from seawater, but not from sponges (Kientz et al., 2013; Hahnke and Harder, 2013).

Bacillus spp. (phylum *Firmicutes*) and *Streptomyces* spp. (phylum *Actinobacteria*) were also isolated from sponge *C. concentrica* and *Tedania* sp.. Bacteria belonging to the genus *Bacillus* and the phylum *Actinobacteria* are common producers of bioactive metabolites (Kennedy et al., 2009; Su et al., 2014; Phelan et al., 2012). Many studies focused on isolation of genera known to be producers of secondary metabolites from marine sponges, in particular, *Actinobacteria* (Jiang et al., 2007; Kim et al., 2005; Zhang et al., 2006). Both culture-independent and culture-dependent studies have shown the abundance of *Actinobacteria* in marine sponges (Webster et al., 2001; Montalvo et al., 2005), however some culture-dependent approaches have also shown that their abundance varies significantly among sponge species (Zhang et al., 2008; Muscholl-Silberhorn et al., 2008).

4.2. Bacterial diversity from different media:

In the present work, the cultivability of bacteria associated with three sponges was assessed using a variety of media. The general approach for cultivation is to provide an

environment and nutrients that are similar to the natural environment of the target species. Many culture-independent methods have uncovered the diversity and function of bacterial communities associated with sponges (Taylor et al., 2007; Webster et al., 2010; Schmitt et al., 2011; Fan et al., 2012a). Genomic-based methods provide valuable data on metabolic traits, such as primary metabolism, adaptation to anaerobic conditions, resistance to antibiotics, and even potential interaction with the host through eukaryotic-like proteins (Fan et al., 2012a). Using that information, culturing conditions can be created to cultivate bacteria from marine sponges.

Most of the isolated bacteria from this study were cultivated on high nutrient media, with most genera retrieved from MB 1:2 as compared with media low in carbon sources. *Pseudovibrio* spp. were isolated from all media used in this study. The genus *Pseudovibrio* contains metabolically versatile bacteria that are capable of importing and oxidizing a wide range of organic and inorganic compounds to meet their carbon, nitrogen, phosphorous and energy requirements under both oxic and anoxic conditions (Bondarev et al., 2013).

Aqueous sponge extract was also used as medium additive in this study (see methods and materials), however the diversity of isolated bacteria from media with sponge extract was almost similar to media without sponge extract (figure 6). In another study, use of aqueous sponge extract agar and sponge spicule extract as medium additive resulted in higher numbers of OTUs as compared to organic sponge extract, which resulted in low number of observed OTUs (Sipkema et al., 2011). Webster et al. (2001) also reported that the addition of sponge extracts to the media decreased the total number of morphotypes isolated. Antibiotics were also used to inhibit growth of fast growing bacteria and fungi in this study. Slow-growing bacteria are often outcompeted

by fast-growing ones; therefore the addition of antibiotics can be useful. The addition of antibiotics to growth media can also affect the type of bacteria cultured, as observed elsewhere (Sipkema et al., 2011; Lavy et al 2013).

Many sponges have been found to host *Cyanobacteria* that are producers of numerous bioactive compounds and require light for growth (Usher et al., 2008; Pagliara and Caroppo, 2011). Symbiotic *Cyanobacteria* are situated both inter- and intracellularly and have been reported in a large variety of marine sponges (Usher et al., 2004; Usher et al., 2008; Steindler et al., 2005). In another culture-independent study, an uncultured δ-proteobacterium associated with the sponge *C. concentrica* was identified and was proposed to live in association with a cyanobacterium (Liu et al., 2010). Tylor et al (2005) also reported presence of *Cyanobacteria* in sponge *C. concentrica*. *Scopalina* and *Tedania* sp. also show presence of *Cyanobacteria* based on metagenomic information (Fan et al., 2012a). Snax medium was used to target *Cyanobacteria* in this study. However, the characteristics of *Cyanobacteria* make it more difficult to culture; for example, the genera *Lyngbya, Phormidium* and *Oscillatoria* are characterized by multicellular filaments and are therefore difficult to separate during isolation from other bacteria. *Cyanobacteria* are also easily contaminated with diatoms and heterotrophic bacteria and traditional culturing methods are often unsuccessful in the case of cyanobacterial symbionts, because of the complex unknown microenvironmental conditions in the host (Li, Zhiyong, 2009; Steindler et al., 2005). This might explain the lack of cyanobacterial isolates on Snax medium during the cultivation of this study.

In addition to different media, two different isolation techniques were used in this study: agar plate culture method and floating filters techniques. Most of the observed

genera were isolated from agar plate culture as compared to floating filters (figure 8). This could be due to the fact that media used for floating filter cultivation here had very low amount of nutrients available for bacterial growth. In a previous study, the floating filter technique resulted in the isolation of the moderately thermophilic bacterium *Thiobacillus ferrooxidans*, while use of conventional solid media was unsuccessful for growing this species (Bruyn et al., 1990). In another study, using multiple cultivation techniques (agar plate, liquid cultures and floating filters) to cultivate sponge-associated bacteria, a total of 60 OTUs were obtained from floating filters. When compared with the cultivation on agar plates and in liquid media, 23 floating filter-specific OTUs were obtained (Sipkema et al., 2011).

4.3. Cultivation-dependent and cultivation-independent approaches:

To compare between cultivation-dependent and cultivation-independent approach, each 16S rRNA gene sequence obtained from the isolates in this study was compared to previously prepared 16S rRNA gene library (Fan et al., 2012a, b). The bacterial groups that were present in the sponge *C. concentrica* according to previous metagenomics analysis include *Robiginitomaculum, Phyllobacteriaceae, Oceanospirillaceae, Vibrionaceae, Nitrosomonadaceae, Nitrospira*, *Actinobacteria* and the bacterial clade OCS116 (Fan et al., 2012a, b). The bacterial groups present in sponge *Tedania anhelans* and *Scopalina* sp. were dominated by two very similar phylotypes belonging to the *Nitrosomonadaceae (Betaproteobacteria)* (Fan et al., 2012a).

Some media used in this study were prepared using previous metagenomics information (Fan et al., 2012a, b). R2A agar was used to target *Gammaproteobacteria.* Most of the *Gammaproteobacteria* cultivated in this study were isolated from sponge *C.*

concentrica and belong to Family *Vibrionaceae.* Presence of *Vibrionaceae* has been reported in *C. concentrica* previously by a culture-independent study (Fan et al., 2012b), but the *Vibrio* isolates obtained here did not match the *Vibrionaceae* sequences found in the metagenomic dataset (Fan et al., 2012b). Most of these *Gammaproteobacteria* were isolated from media MB 1:2, NSW + SE and only a few were isolated from R2A (see methods and materials for Media used). Another *Gammaproteobacterium* that was cultivated from *C. concentrica* belongs to family *Oceanospirillaceae*, which was also present in *C. concentrica* according to metagenomics analysis. However the *Neptuniibacter* (*Oceanospirillaceae*) isolated here did not match the *Oceanospirillaceae* sequence from previous metagenomic study (Fan et al., 2012b).

Media containing nitrate and ammonia were used in order to target bacteria that are involved in nitrogen cycling, in particular focusing on nitrate reduction and ammonium oxidation which have been previously described in marine sponges (Fan et al., 2012a). In *Scopalina* sp. and *Tedania anhelans*, two closely related, uncultured phylotypes of the family *Nitrosomonadaceae (Betaproteobacteria)* dominated the microbial communities, as determined by culture-independent studies. These phylotypes were related to *Nitrosomonas* spp. and *Nitrosospira* spp., both of which are ammonia-oxidizing bacteria (Fan et al., 2012a). In *C. concentrica,* a phylotype belonging to the family *Phyllobacteriacea* was also dominant. Members of the *Mesorhizobium* and *Nitratireductor* in this family are capable of fixing nitrogen and reducing nitrate to nitrite, respectively (Fan et al., 2012a). However, none of these bacteria likely involved in nitrogen cycle were cultivated in this study. This could be due to the fact that sponge symbionts are dependent on specific concentration of N-compounds being present inside sponge, which were not given during the *in vitro* culturing. For example, the

widespread presence of nitrite oxidizers belonging to *Nitrospira* genus in sponges may indicate low nitrite availability in these hosts, as members of the *Nitrospira* typically favour low-nitrite habitats (Tylor et al., 2007; Off et al., 2010).

Around 18 isolates cultivated from *Tedania* sp. in this study, classified as *Ruegeria* sp. showed overlap with the 16S rRNA sequences previously found in the sponge *Cymbastela coralliophila* (table 4). *Ruegeria* spp. have previously been reported in *C. coralliophila*, however it was not present in any of the three sponges used in this study according to a culture-independent study (Fan et al., 2012a, b). Two *Pseudovibrio* isolates cultivated from the sponge *C. concentrica* in this study showed overlap with a 16S rRNA sequence of previously prepared gene library from *Tedania anhelans* (table 4). The culture independent study does have some *Pseudovibrio* sequences (hence the overlap), but they were not abundant (Fan et al., 2012a). .

Different studies have shown that sponge-associated bacterial phylotypes retrieved in cultivation-independent studies show little or no phylogenetic overlap with cultivation-dependent studies (Montalvo et al., 2014; Esteves et al., 2013). A possible explanation for this could be that the cultured bacteria are only very minor constituents of the community and may not be detected in a 16S rRNA gene clone library. Another reason could be that the isolated bacteria were not truly associated with sponges but were from seawater, and although before preparing the inoculum the sponges were rinsed four times with sterile CMFSW, this rinsing does not remove bacteria that are present in the canal system and choanocyte chambers. These bacterial species are likely to be present at low concentrations, but they might be easily cultivable.

4.4. Conclusion:

In conclusion, this thesis provides data on the cultivation of sponge-associated bacteria from the three sponge species *Cymbastela concentrica, Tedania* sp. and *Scopalina* sp. using different techniques and media.

The work showed a large diversity of bacteria cultivated from the examined sponge species using multiple cultivation methods. It also showed that some of the cultivated bacteria were specific to only one sponge species and some cultured bacteria were found in all examined sponge species.

The use of a variety of low- and high-nutrients media also yields high diversity of cultivated bacteria. This finding indicates that use of wide variety of media in culturing experiments can be useful to assess culturable bacterial diversity. Comparison between culture-dependent and culture-independent results shows that the bacteria that appear to be dominant by culture-independent techniques are still escaping culturing.

The results presented in this thesis underline the importance of using multiple cultivation methods to improve the cultivability of bacteria associated with marine sponges.

4.5. Future work regarding cultivation:

To further improve cultivability of bacteria from sponges, improved culture media formulations that more closely mimic natural conditions present inside the sponge mesohyl will be needed. Target cultivation using previous metagenomic data can also be useful to cultivate sponge specific bacteria. Metagenomic-based methods provide valuable information on functions, metabolic traits and adaptation to different

conditions present inside host which can be useful for creating culturing conditions for growth of bacteria from marine sponges. Fluorescence-activated cell sorting FACS approaches for separation of microbes of interest from natural samples also represent a promising tool for the characterization of as-yet-uncultivated microorganisms. Isolated or sorted cells using FACS can be used for subsequent culture-based (Kalyuzhnaya et al. 2006) or culture-independent analyses (Fujii and Hiraishi, 2009; Kalyuzhnaya et al., 2006). Other new approaches which rely on extinction culturing or simulated environments can also be used to cultivate sponge-associated microorganisms.

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Appendix:

Appendix 1: Details on isolated bacteria from each sponge specie with colony morphology

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
1	*Vibrio 100%*	NSW	*C. concentrica*	Round, big , transparent colony
2	*Vibrio 100%*	NSW+SE	*C. concentrica*	white, round colony
3	*Pseudovibrio 100%*	MB20+SE	*C. concentrica*	White colony with irregular edges
5	*Vibrio 100%*	MB20+SE	*C. concentrica*	Small, round, shiny colony
6	*Pseudovibrio 100%*	MB20+SE+NO3-	*C. concentrica*	round, white colony
7A	*Vibrio 100%*	NSW+SE	*C. concentrica*	Big, round, slightly transparent colony
7B	*Vibrio 100%*	NSW+SE	*C. concentrica*	White Round colony
8	*Pseudovibrio 100%*	MB20+SE	*C. concentrica*	Small, round, white colony
9	*Pseudovibrio 100%*	MB20+SE	*C. concentrica*	White, big colony
10	*Pseudovibrio 100%*	MB20+SE	*C. concentrica*	White, agarolytic, round colony
11	*Pseudovibrio 100%*	MB20+SE+NH4+	*C. concentrica*	White colony with round edges
12	*Pseudovibrio 100%*	NSW+SE	*C. concentrica*	white, mucus colony
13	*Pseudovibrio 100%*	MB20+SE+NO3-	*C. concentrica*	white, big colony with irregular edges
14	*Vibrio 100%*	MB1:2	*C. concentrica*	Very agarolytic, forming a big hole in the agar; white big colony
15	*Vibrio 100%*	MB1:2	*C. concentrica*	Agarolytic, pinkish colony
16A	*Unclassified_ Gammaproteobacteria 100%*	MB1:2	*C. concentrica*	Tiny transparent colony
16B	*Unclassified_ Gammaproteobacteria 100%*	MB1:2	*C. concentrica*	White colour tiny colony
17	*Unclassified_ Gammaproteobacteria 100%*	MB1:2	*C. concentrica*	White round big colony
18	*Colwellia 97%*	MB1:2	*C. concentrica*	Yellowish round colony, very filamentous in liquid culture
21	*Enterovibrio 100%*	MB1:2	*C. concentrica*	Tiny round colony
22	*Enterovibrio 100%*	MB1:2	*C. concentrica*	Agarolytic, pinkish

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
				colony
23	*Vibrio 100%*	MB1:2	*C. concentrica*	Agarolytic colony
24	*Enterovibrio 100%*	MB1:2	*C. concentrica*	Agarolytic pinkish colony
25A	*Pseudoalteromonas 100%*	MB1:2	*C. concentrica*	Yellow, colony
25B	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	Big white colony, mucus
26	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	Pinkish, mucus, big colony
27	*Bacillus 100%*	MB1:2	*C. concentrica*	White colony with round edges
28	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White, irregular edges colony
29	*Bacillus 99%*	MB1:2	*C. concentrica*	white, irregular edges colony
30	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White, round colony
31	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	Pink/red colony that turn to white according to temperature
31B	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White round colony
32A	*Enterovibrio 100%*	MB1:2	*C. concentrica*	Creamy white big colony
32B	*Enterovibrio 100%*	MB1:2	*C. concentrica*	White small round colony
33	*Vibrio 100%*	MB1:2	*C. concentrica*	White, mucus, round edges colony
34	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	Similar as 31
34B	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White, round colony
35	*Enterovibrio 100%*	MB1:2	*C. concentrica*	Small round colony of white colour
36	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White, mucus colony
37	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	White, mucus colony
38	*Pseudovibrio 100%*	MB1:2	*C. concentrica*	
39	*Vibrio 100%*	MB1:2	*C. concentrica*	Pinkish, round edges colony
40	*Bacillus 100%*	MB20+SE	*C. concentrica*	Orange colour colony
45	*Pseudovibrio 100%*	MB20+SE+NH4+	*C. concentrica*	White, round colony
46	*Pseudovibrio 100%*	MB20+SE+NH4+	*C. concentrica*	White, round, big colony, agarolytic
47	*Pseudovibrio 100%*	MB20	*C. concentrica*	White, round
48	*Pseudovibrio 100%*	MB20	*C. concentrica*	Very big colony, irregular edge, off white
49	*Neptuniibacter 100%*	MB20	*C. concentrica*	Very big, round, off white colony

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
50	*Pseudovibrio 100%*	NSW+SE	*C. concentrica*	Small, white, round colony
51	*Pseudovibrio 100%*	NSW	*C. concentrica*	Small, round, transparent colony
52	*Pseudovibrio 100%*	NSW	*C. concentrica*	Round, transparent colony
53	*Pseudovibrio 100%*	NSW	*C. concentrica*	Round, small, transparent colony
54	*Pseudovibrio 100%*	MB20+SE+NO3-	*C. concentrica*	Small, round white colony , agarolytic
56	*Pseudovibrio 100%*	MB20+SE+NO3-	*C. concentrica*	White round small colony
57	*Pseudovibrio 100%*	MB20	*Tedania sp.*	White, irregular shape colony
58	*Aquimarina 100%*	MB20	*Tedania sp.*	Orange, irregular shape colony
59	*Pseudovibrio 100%*	MB20	*Tedania sp.*	White, round, medium size colony
60	*Pseudovibrio 100%*	MB20	*Tedania sp.*	White, round, medium size
61	*Pseudovibrio 100%*	MB20	*Tedania sp.*	Orange/brown colour colony
62	*Pseudovibrio 100%*	MB20+SE	*Tedania sp.*	White, irregular shape, mucus
63	*Pseudovibrio 100%*	MB20+SE	*Tedania sp.*	White, round, big colony
64	*Pseudovibrio 100%*	MB20+SE	*Tedania sp.*	White, round, big colony
65	*Aquimarina 100%*	MB20+SE	*Tedania sp.*	Orange, irregular shape, similar to 58
66	*Pseudovibrio 100%*	MB20+SE	*Tedania sp.*	White, round, big, similar to 63
67	*Ruegeria 99%*	MB20+SE	*Tedania sp.*	White, round, small
68	*Pseudovibrio 100%*	NSW+SE	*Tedania sp.*	White, round, medium size
69	*Pseudovibrio 100%*	NSW+SE	*Tedania sp.*	Similar to 62
70	*Pseudovibrio 100%*	NSW+SE	*Tedania sp.*	white, round, big colony
71	*Streptomyces 100%*	MB20	*C. concentrica*	White, grainy, irregular shape colony
77	*Ruegeria 97%*	MB1:2	*Tedania sp.*	Yellow translucid colony
78	*Unclassified-Flavobacteriaceae 100%*	MB1:2	*Tedania sp.*	Yellow, opaque, round colony
79	*Ruegeria 98%*	MB1:2	*Tedania sp.*	Yellow/green/br-own translucid colony
80	*Ruegeria 96%*	MB1:2	*Tedania sp.*	pink/beige colony
81	*Unclassified-Flavobacteriaceae 100%*	MB1:2	*Tedania sp.*	Similar to 78
82	*Ruegeria 96%*	MB1:2	*Tedania sp.*	Similar to 80

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
83	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Small colony with brown dot in the centre
84	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Similar to 80; small
85	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Beige colony with translucid edges
86	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Brown, small, round colony
87	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Similar to 80
88	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Brown colony with concentric darker rings
89	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Yellow, small colonies
90	*Ruegeria 98%*	MB1:2	*Tedania sp.*	Beige, small colony with irregular edges
91	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Beige colour colony with darker concentric rings
92	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Large, irregular edges, beige colour colony
93	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Small, round, white colony
94	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Very big, concentric, off white colony with darker edges
95	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Small, round, orange colour colony
96	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Dark brown, irregular edges colony
97	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Small, white colony with irregular edges
99	*Ruegeria 98%*	MB1:2	*Tedania sp.*	Big, irregular edges, dark orange colony
100A	*Ruegeria 96%*	MB1:2	*Tedania sp.*	Round off white big colony
100B	*Sphingopyxis 100%*	MB1:2	*Tedania sp.*	Small, round, dark yellow colony
101A	*Ruegeria 99%*	MB1:2	*Tedania sp.*	Big white colonies with irregular edges
101B	*Ruegeria 95%*	MB1:2	*Tedania sp.*	Big, creamy white, round colony
102	*Ruegeria 92%*	MB1:2	*Tedania sp.*	Big colony of white colour
103	*Ruegeria 97%*	MB1:2	*Tedania sp.*	Big, irregular edges, off white colony

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
104	*Labrenzia 98%*	MB1:2	*Tedania sp.*	Very small, white, round colony
105	*Ruegeria 99%*	MB1:2	*Tedania sp.*	Big, round, beige colour colony
106	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Big, irregular edges colony of beige colour
107A	*Ruegeria 100%*	MB1:2	*Tedania sp.*	Small, round, brown colour colony
107B	*Micrococcus 100%*	MB1:2	*Tedania sp.*	Very big, irregular edges, white creamy colony
111A	*Ruegeria 97%*	MB1:2	*Tedania sp.*	Small, irregular edges, beige/light pink colony
111B	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Large colony with irregular edges of light brown colour
112	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Orange colour , small round colony
113	*Labrenzia 100%*	MB1:2	*Tedania sp.*	Small, round, dark orange colony
114	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Round, beige colour colony
115	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Very big, concentric, dark brown colony
116	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Small, round, off white colony
117	*Erythrobacter 86%*	MB1:2	*Tedania sp.*	Irregular edges, yellow colour colony
118	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Round, orange colour colony
119	*Aquimarina 100%*	MB1:2	*Tedania sp.*	Big, irregular edges, light orange colony
120A	*Cellulophaga 100%*	MB1:2	*Tedania sp.*	Yellow, agarolytic colony
120B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Yellow/green colour, round, slightly agarolytic; colony
121A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	White colour, round colony
121B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Off white/light yellow, irregular edges colony
122A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
123A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
123B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Similar to 120 but smaller and lighter in colour
124	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Yellow agarolytic round colony

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
125A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
125B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Large, green/yellow, irregular shape colony drawing lines in the agar
125C	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
126A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Yellow/green medium size colony, slightly irregular
126B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Yellow/green, medium size, slightly irregular colony with dot in the middle
127A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
127B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
128A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 125
128B	*Pseudovibrio 100%*	SNAX Agar	*Tedania sp.*	Similar to 125 but bigger and lighter
129A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Green big colony
129B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Green, big colony drawing lines in the agar
131A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 129
131B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Off white/light, big yellow, dispersed colony
132A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
132B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Similar to 120
133A	*Cellulophaga 100%*	SNAX Agar	*Tedania sp.*	White colour, round colony
133B	*Labrenzia 100%*	SNAX Agar	*Tedania sp.*	Green, "sprayed" colony
135	*Pseudovibrio 100%*	MB20	*C. concentrica*	Swarming; forming white round colony when isolated
139	*Pseudovibrio 100%*	MB1:2	*Scopalina sp.*	White, irregular edges colony
140	*Pseudovibrio 100%*	MB20+SE+NH4+	*C. concentrica*	White, round colony
141	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Brownish colour round spreading colony
142	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	white, mucus
143	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Spreading colony mucus type
144	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	white colony with brown centre
146	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Beige, round colony transparent edges

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
147	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Similar to 143 but bigger
148	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Similar to 145 but bigger
149	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Golden brown colony, spreading (encrusted)
150	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Transparent, irregular shape colony
151	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Off-white/beige round small colony, dominant in the plate
152	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	White colony with transparent irregular edges
153	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	"Semi" agarolytic, golden brown colony
154	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Golden/orange small colony
155	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Round white/beige colony
156	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Similar to 151
157	*Pseudovibrio 100%*	Asp	*Scopalina sp.*	Similar to 150
158	*Pseudovibrio 100%*	MB20	*Scopalina sp.*	White round colony
159	*Pseudovibrio 100%*	MB20	*Scopalina sp.*	White round colony
160	*Pseudovibrio 100%*	MB20+SE	*Scopalina sp.*	White round colony
161	*Pseudovibrio 100%*	R2A	*Tedania sp.*	White round colony
163	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Cloudy spread of white colour
164	*Pseudovibrio 100%*	R2A	*Tedania sp.*	White, Dominant in plate; looks like lysis plaques
165	*Ruegeria 96%*	R2A	*Tedania sp.*	White round shiny medium size
166	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Big colony with central nucleus and concentric ring
167	*Vibrio 100%*	R2A	*Tedania sp.*	Similar to 165
168	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Similar to 162
169	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Grainy colony spreading around 166
170	*Labrenzia 100%*	R2A	*Tedania sp.*	Transparent spread like colony
173	*Bacillus 100%*	R2A	*Tedania sp.*	Dominant white cloudy spread around edges of plate

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
177	*Ruegeria 96%*	R2A	*Tedania sp.*	Pinkish/off white big colony
178	*Ruegeria 98%*	R2A	*Tedania sp.*	Grainy colony growing around 176
179	*Unclassified_ Rhodobacteraceae 100%*	R2A	*Tedania sp.*	White grainy colony
181	*Erythrobacter 97%*	MB1:2	*Scopalina sp.*	Yellow, round colony
182	*Pseudovibrio 100%*	MB20+SE+NH4+	*C. concentrica*	Bright yellow, big, round colony
183	*Labrenzia 100%*	MB1:2	*Scopalina sp.*	Brown, concentric colony
184	*Tenacibaculum 100%*	MB1:2	*Tedania sp.*	Off white, round colony
185	*Pseudovibrio 100%*	MB1:2	*Scopalina sp.*	Bright yellow spread (fluorescent)
186	*Aquimarina 100%*	MB1:2	*Scopalina sp.*	Red big colony, spreading
187	*Pseudovibrio 100%*	MB1:2	*Tedania sp.*	Dark yellow, agarolytic colony
189	*Vibrio 100%*	MB1:2	*Scopalina sp.*	Brown, big, concentric colony
191	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Off white, round colony
192	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Yellow round colony
194	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Yellow big colony (dark)
196	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Yellow round colony
198	*Pseudovibrio 100%*	R2A	*Tedania sp.*	White colony
199	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Yellow round colony
204	*Streptomyces 100%*	R2A	*Tedania sp.*	Off white round colony
205	*Streptomyces 100%*	R2A	*Tedania sp.*	Pinkish colour colony
208	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Bright yellow round colony
209	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Pinkish, round colony
211	*Streptomyces 100%*	R2A	*Tedania sp.*	grey/green colony
213	*Pseudovibrio 100%*	R2A	*Tedania sp.*	yellow, mixed with 205
221	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Yellow colour colony
230	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Orange/ brown round colony
231	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Pinkish, medium size colony
232	*Pseudovibrio 100%*	R2A	*Tedania sp.*	White, big, spreading with halo
233	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Bright yellow,

Isolate #	RDP Classification	Isolation Medium	Sponge species	Colony Morphology
				colony
234	*Pseudovibrio 100%*	R2A	*Tedania sp.*	Orange big colony
235	*Bacillus 100%*	R2A	*Tedania sp.*	Yellow colony
237	*Pseudovibrio 100%*	R2A	*Tedania sp.*	White, big colony with irregular edge
244	*Pseudovibrio 100%*	NSW+SE	*C. concentrica*	Yellow colour colony
251	*Pseudovibrio 100%*	NSW	*C. concentrica*	Orange. colony