BACTERIA ASSOCIATED WITH INTERTIDAL SPONGE, *Cinachyra alloclada* AND THEIR FUNCTIONAL DIVERSITY

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By

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2018

Statement

As required under the University ordinance OB-9.9 (v-vi), I state that this thesis entitled "Bacteria associated with intertidal sponge, *Cinachyra alloclada* and their functional diversity" is my original contribution and it has not been submitted on any previous occasion. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

SUBINA N. S.

CSIR-National Institute of Oceanography, Goa.

4th August, 2018

Certificate

This is to certify that the thesis entitled "Bacteria associated with intertidal sponge, *Cinachyra alloclada* and their functional diversity" submitted by Ms. Subina N. S. for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any institution.

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Dedicated to

Shanta ma'am

(Dr. Shanta Achuthankutty)

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CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Marine microbial ecology studies have focused mainly on abundance, diversity and role of free-living microbes in biogeochemical cycling. The associated microbes are still to be well understood. Studies on associated bacteria in marine environment mainly dealt with those associated with particles (Riemann and Winding, 2001; Gonsalves et al., 2009). The interaction of bacteria with marine eukaryotes has been studied in detail on their commensal or mutualistic association with phytoplankton or on their pathogenicity (Riemann and Middelboe, 2002; Oliver, 2005). The complex relationship between microorganisms and eukaryotes range from the host with one single dominant microorganism to hundreds of microorganisms. The animal-associated microorganisms occupying equivalent niches may result in sharing functional aspects. Marine sponge-bacterial association provides an ideal system to study associated bacteria as sponges are sessile filter feeders that pump large volume of water (Hentschel et al., 2002) and bacterioplanktons are able to disperse among individuals of the same host species and adapt to a particular host and thus undergo speciation (Taylor et al., 2005). However, the role of these bacteria in host nutrition and biogeochemical cycling and their mechanism of functional equivalence in this complex microbial community are largely unknown.

1.2. SPONGES: SIMPLEST AND ANCIENT METAZOAN

Sponges originated in Precambrian era more than 600 million years ago and hence form one of the deepest radiations of the Metazoa. Today, more than 8500 living sponge species are found, mostly on tropical reefs and also at increasing latitudes (Dieckmann et al., 2005; Van Soest et al., 2012). Their habitats are from epilittoral to hadal depths and from rocks to mud bottom. They represent a significant component of the deep water as well as shallow water benthic communities especially on coral reefs (Dayton et al., 1974; Dayton, 1989).

Sponges have a simple cellular organisation with no tissue or organ[1]. Sponge body is covered by pinacoderm (Figure 1.1) and made of pinacocyte cells. An outer thick layer of sponge body is known as cortex and inner, gelatinous proteinaceous mass containing cells are known as mesohyl (De Vos et al., 1991). They acquire nutrients by phagocytosis of bacteria that are removed from the surrounding water (Hentschel et al.,

[1]Though sponges do not have tissue level organization, the sponge biomass is commonly called as tissues.

2002). Sponges also can either derive indirect nutritional benefit from their associated microorganisms such as photosynthate from autotrophic organisms or directly use the microorganisms growing within their body as food. Thus they act as key players in the transfer of carbon from the pelagic microbes into the benthos.

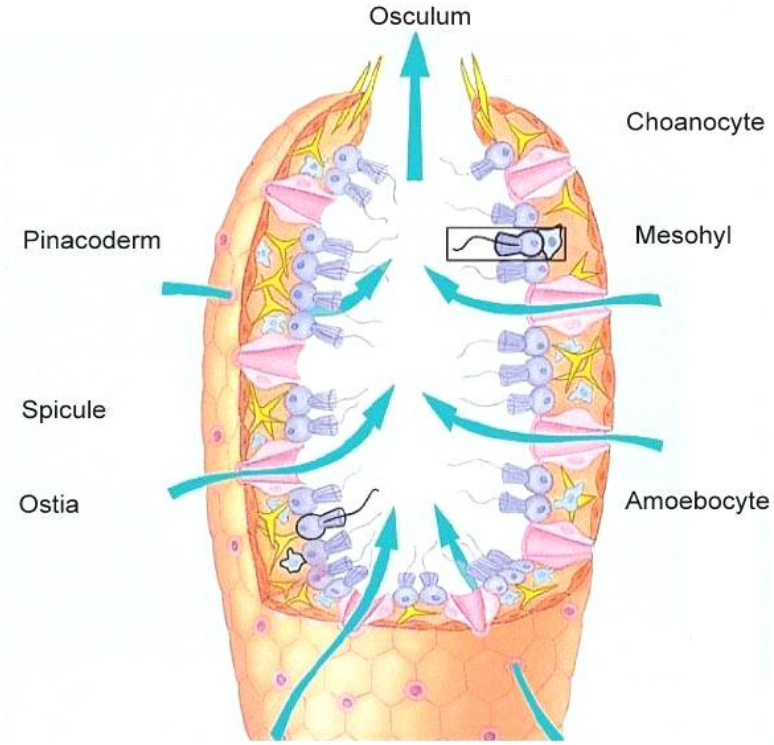


Figure 1.1. Representational image of cellular organisation of sponge

1.3. SPONGES: RESERVOIR OF COMPLEX MICROBIAL COMMUNITIES

One of the main features of sponges is their association with the complex microbial community. Sponge-associated microorganisms include bacteria (Vacelet, 1975; Manz et al., 2000; Gauthier et al., 2016), archaea (Preston et al., 1996; Han et al., 2012), and eukaryotes such as diatoms (Vacelet, 1982; Totti et al., 2005), dinoflagellates (Garson et al., 1998) and fungi (Holler et al., 2000; Bugni and Ireland, 2004; Konig et al., 2006) with bacteria being the predominant domain. A large number of bacteria are present in the mesohyl and are estimated to account for about 40% of the sponge volume (Wilkinson, 1978a, b). Sponges are divided into 2 categories based on the abundance of associated microorganisms. High microbial abundance (HMA) sponges carry up to 10^8 - 10^{10} bacteria per gram of sponge wet weight, whereas low microbial abundance (LMA) sponges carry lower microbial abundance of fewer than 10^6 microorganisms per gram of

sponge wet weight (Vacelet and Donadey, 1977; Hentschel et al., 2003, 2006). The microorganisms which are filtered from seawater grow, divide and retain a balanced state with the sponge and form a symbiotic association (Hooper and van Soest, 2002). The microbial density, location, their morphological and genetic diversity and the type of associations are different in different sponges. Sponge-associated bacteria were divided into different categories based on the abundance, location, and their specificity: 1) Abundant population of sponge-specific mesohyl bacteria, 2) Small population of sponge-specific intracellular bacteria, and 3) Non-specific bacteria in the water canals resembling seawater bacteria (Vacelet, 1975; Wilkinson, 1978a). Based on the local distribution of phylotypes in the host sponge, the bacteria were classified as sponge associate, specialist, and generalist (Meyer and Kuever, 2008). Based on the mode of transfer, Schmitt et al. (2012a) redefined these definitions: core bacterial community (Acquired by horizontal transfer of bacteria from seawater), sponge-specific bacteria (Restricted to sponge or sponge and coral specific organisms, acquired mostly by vertical transmission) and variable bacterial community.

Past decade witnessed great developments in our understanding of the phylogenetic diversity of sponge-associated microorganisms. Various culture-dependent and culture-independent studies reported more than 30 bacterial phyla and two archaeal phyla in the sponge, including sponge-specific phylum Poribacteria (Taylor et al., 2007; Thomas et al., 2010). Sponge-associated bacteria show spatial, temporal and species-specific variations which are different from the surrounding seawater (Thakur et al., 2004; Taylor et al., 2005; Olson and McCarthy, 2005; Turque et al., 2008; Hardoim et al., 2009; Radwan et al., 2010; Cleary et al., 2013; Sipkema et al., 2015). There are also reports that irrespective of the geographic location, species and season, sponges carry uniform bacterial community (Friedrich et al., 1999, 2001; Hentschel et al., 2002; Thoms et al., 2003; Wichels et al., 2006; Thiel et al., 2007; Lee et al., 2003, 2009, 2011), which might be considered as sponge-specific bacteria. In the same way, sponges maintained in artificial conditions may harbour same or different bacterial community from the wild sponges and may or may not show temporal variations (Mohamed et al., 2008b; Isaacs et al., 2009). Increasing evidence is accumulating that highlights the important role of phylogenetically complex yet highly sponge-specific microbial communities, including novel lineages and even candidate phyla in marine sponges.

1.4. SPONGES: MICROBIAL FERMENTERS

Hentschel et al. (2006) termed sponges as microbial fermenters that provide an exciting area in marine microbiology and biotechnology as they harbour a large number of uncultured and elusive microorganisms that may play an important role in the chemistry of these animals. Sponges and their association with bacteria have been extensively studied for the production of bioactive compounds (Schmidt et al., 2000; Kennedy et al., 2007; Siegl and Hentschel, 2010) in the last 3 decades.

The functions of sponge-associated microorganisms in the host metabolism and biogeochemical cycling were overlooked while understanding their diversity (Taylor et al., 2007). It is equally important to analyse the nature of the relationship and biological functions of sponge-associated bacteria. They may help in nutrition, structural rigidity, and chemical defence of sponge (Wilkinson and Garrone, 1979; Rutzler, 1985; Schmidt et al., 2000; Kennedy et al., 2007; Siegl and Hentschel, 2010). Metabolites produced by the sponges may be utilised by sponge-associated bacteria for energy generation and also for their protection. It was believed that sponge nutrition is mainly heterotrophic by intake of microorganisms from the surrounding water via filtration or by the intake of dissolved organic matter from the symbiotic heterotrophic bacteria (Yahel et al., 2003, 2007). The particles filtered by sponge might be degraded by the extracellular enzymes produced by the associated heterotrophic bacteria. Deep-sea sponges without aquiferous system harboured methanotrophic bacteria and were suggested to provide nutrients to host sponge (Vacelet et al., 1995; Vacelet and Boury-Esnault, 2002). Few studies showed the dominance of phototrophic bacteria, Chloroflexi, in the microbiome of some sponge species. It is not clear whether these bacteria form a simple transient association with the sponge or whether they are contributing to the host sponge's nutrition. So the understanding of bacterial abundance, extracellular enzyme production, the metabolic potential to utilise different substrates by heterotrophic bacteria and inorganic carbon fixation by autotrophic bacteria are all important to decipher the role of these bacteria in sponge nutrition. However, symbiont function in nitrogen metabolism (Nitrification) in sponges has become a major focus of recent studies. In nutrient-limited environments, nitrogen-fixing bacteria associated with the sponge might provide fixed nitrogen to the host (Mohamed et al., 2008a). Brusca and Brusca (1990) discovered that sponges ingest nitrogen and excrete ammonia as a metabolic end-product which can be utilised by

nitrifiers (Bayer et al., 2008b) and convert it into nitrate which can be used as a nutrient for sponges. Also, 16S rRNA gene sequences of ammonia-oxidising and nitrite-oxidising bacteria were recovered from sponge tissues, making microbial nitrification a likely scenario inside sponge (Hentschel et al., 2002; Diaz et al., 2004). Nitrification process in sponges has been reported from Mediterranean, and cold-water sponges (Hoffmann et al., 2009; Schlappy et al., 2010; Radax et al., 2012a).

Until recently, sponge metabolism was viewed based on aerobic respiration as sponges pump large amounts of water saturated with oxygen through their body. However, sponges reduce or stop filtration at irregular intervals (Reiswig, 1971; Vogel, 1977; Gerodette and Flechsig, 1979; Pile et al., 1997) and become anoxic which leads to unstable oxygen concentrations in sponges (Hoffmann et al., 2005a; 2009). Hence, both the presence and activity of aerobic and anaerobic bacteria can be expected in sponges. Some sponges from temperate and artic waters harboured the genes which carry out denitrification and anammox (Hoffmann et al., 2009). In order to evaluate the impact of sponges as nitrogen sink or source in the ocean, it will be important to measure such processes in more sponge species from other marine environments such as tropical and intertidal regions. Another group of anaerobic bacteria, the sulfate-reducing bacteria and their activity have been detected in the sponges (SchumannKindel et al., 1997; Hoffmann et al., 2005b, 2006) from cold water. However, these bacteria were not cultivated from sponges and their physiology is unknown.

As sponges are sessile organisms and do not have evasive or behavioural defence (Braekman and Daloze, 1996), they depend largely on chemical defence to prevent predation and biofilm formation on their surface (Davis et al., 1989; Wahl, 1989; Kelly et al., 2003; Braekman and Daloze, 2004), although they exhibit some sort of mechanical prevention by spicules (Reiswig, 2010). These compounds are synthesised wholly or partially by symbiotic microorganisms, as evidenced by the structural similarity of these compounds which were isolated from sponges and their symbionts (Piel et al., 2004; Fisch et al., 2009; Thomas et al., 2010; Hentschel et al., 2012). Antagonistic activity of sponge symbiotic bacteria against other bacteria from the same environment might be an effective mechanism of defence against pathogenic bacteria. It also helps in establishing a stable association with the host and forming a dominant community in the sponge. This type of response has been overlooked while assessing antibacterial activity of sponge-associated

bacteria against known pathogens of human and other animals. Sponge-associated bacteria were reported to be the source of several bioactive compounds and studies are still being carried out to explore more useful compounds from sponges. However, the role of the secondary metabolites or enzyme from sponge-associated bacteria in the ecological perspective was less dealt.

1.5. APPLICATION

The filtering capability of sponges might make them good candidates for bioremediation of wastewater which otherwise may cause serious environmental and health hazards, especially on aquatic biota. Sponge farming in organically polluted water was proposed by Pronzato et al. (1998). To use sponge-bacterial consortium to treat effluent in an eco-friendly manner, it is essential to understand the involvement of symbiotic bacteria associated with sponges in the degradation of these pollutants to non-toxic products and understand the effect of these pollutants on indigenous bacterial community in the sponge. In coastal areas especially intertidal region which is influenced by anthropogenic effect, sponge-associated bacteria might have been exposed to recalcitrant chemicals from industrial effluents. Considering the ability of bacteria to adapt the prevailing environmental conditions, these bacteria may develop rapid resistance to these compounds by degrading them into smaller and non-toxic products. Hence, bacteria from sponges inhabited on coastal areas should be explored to obtain potential bacteria capable of degrading toxic compounds such as industrial dyes.

1.6. *Cinachyra alloclada*

C. alloclada belongs to class: Demospongiae; Order: Spirophorida; and Family: Tetillidae. *C.alloclada* is a globular sponge with circular or oval cup-like depressions on the surface. The maximum size of this yellow coloured sponge reaches up to 7x8.5x7 cm. They are mainly found in coral habitats, rock pavements and hard bottoms, preferably regimented habitat and are often covered with sediment (Hooper and van Soest, 2002). The surface of the sponge is covered with protruding tips of radiating spicules. Long spicules are arranged in bundles radiating from the centre with tips extending beyond tissue surface (van Soest, 2001). Oscules are scattered and inconspicuous. It was reported that this sponge produces ceramides and tetillapyrone (Lakshmi et al., 2008). Chemostatic studies of this species from same study region have been carried out earlier (Mol et al., 2010; Devi et al., 2010; Wahidulla et al., 2011). Studies on associated microorganisms in

Cinachyra species are limited. Bacterial diversity of *Cinachyra* sp. from a different region in tropical water showed different community structure. The dominant bacterial phylum in sponge from Orpheus Island was Chloroflexi whereas Gulf of Mannar sponge showed the dominance of Firmicutes (Webster et al., 2013; Jasmin et al., 2015). From this background, tropical intertidal sponge, *Cinachyra alloclada* which was least explored for its bacterial association was studied in the present work.

1.7. OBJECTIVES OF THE STUDY

- To determine abundance and community structure of bacteria associated with sponge *Cinachyra alloclada*
- To study the functional role of sponge-associated bacteria
- To link phylogeny to function of bacteria associated with sponge

The bacterial abundance of intertidal sponge, *C. alloclada* was estimated by both culture-dependent and culture-independent methods. Different functions of *C. alloclada* associated bacteria such as degradation and uptake of organic and inorganic compounds, methane oxidation, nitrification, denitrification, sulfate-reduction and antagonistic interactions of Actinobacteria against heterotrophic bacteria from the sponge and ambient water were carried out. The diversity and functions of bacteria were linked based on the taxonomy and functions in the case of bacterial isolates and in the case of microbial consortia, OTUs, gene orthologs of KEGG pathway and functional activities were linked. Applications of sponge-associated bacterial functions such as treatment of aquaculture wastewater for particulate matter and dissolved nutrient removal and azo dye decolourisation were also studied.

1.8. SIGNIFICANCE OF THIS STUDY

The studies on sponge-associated bacteria mostly concentrated on their diversity from temperate and subtropical waters although high sponge cover was reported in the tropical region. The abundance of associated bacteria in sponges was studied by only a few researchers. It is essential to reveal the abundance and diversity of intertidal sponge-associated bacteria in the tropical region to understand global bio-geographical distribution and biodiversity, as these bacteria show spatial and species dependent variation. Among the functional roles, most of the studies on sponge-bacteria association

were extensively studied with reference to their biotechnological applications. Ecological role of sponge-associated bacteria needs to be further explored in detail. The role of sponge-associated bacteria in biogeochemical cycling mostly concentrated on nitrification. Most of the studies on the bacteria involved in nitrogen cycling were done in sponges inhabiting temperate regions and in deep and cold-water sponges using whole sponge tissues. Sulfur cycling in sponges was studied only in cold-water sponge, *Geodia barretti*. Most of the studies on sponge-associated bacteria were carried out with the whole sponge. However, studies showed that the heterogeneity in the internal environment of sponges may influence the bacterial abundance and their activities in different sections of the sponge. From Indian waters, studies related to sponge-associated microbes are very limited. Out of 332 species reported from the Indian waters (van Soest et al., 2012), only a few species have been studied and these studies have focused mainly on diversity (Feby and Nair, 2010; Jasmin et al., 2015) and bioactive compounds (Thakur and Anil, 2000; Selvin et al., 2004, 2009c; Skariyachan et al., 2014). In this work, next generation sequencing and novel culturing methods were used to further advance our understanding of the diversity of sponge-associated bacteria. Also, the metabolic potential of bacteria in carbon, nitrogen and sulfur cycle was studied in different sections of the sponge. This study will add to the ongoing effort to link sponge-associated bacterial diversity with function. This study also gives an insight into the ecological role of sponge-holobiont in the coastal marine environment as well as their industrial application.

CHAPTER 2

REVIEW OF LITERATURE

2.1. SPONGES

Sponges are the simplest and oldest metazoan (Ax, 1995; Müller, 1998 a, b), evolved approximately 600 million years ago. They were recognised as an independent metazoan lineage and coined the name "Porifera" by Robert Grant (Grant, 1836). Sponges are exclusively aquatic, sessile and filter feeding animals. Instead of organs or tissues, sponges possess cells that can move freely through the sponge matrix (mesohyl). The mesohyl contains a variety of cells, collagen and skeleton made of silicon dioxide or calcium carbonate. It is assumed that different cells have different functions, though their exact functions are not yet clear. The collencyte cells produce the spicules, which act as a skeleton. Archeocytes are ameboid and totipotent cells which can transform themselves into any other sponge cell type and they move freely between the skeletal elements (Ruppert et al., 1994). Through efficient pumping, sponges filter up to 1 litre of ambient water per hour and cm^3 body volume (Reiswig, 1971) with the help of microscopic opening for incurrent water and a few large sized pores called as ostia (5─50 μM) for excurrent water. The choanocyte cells through the beating of their flagella create a pressure difference and pump water. Ingestion of the particles from water takes place in the choanocyte chamber. Sponges feed on a variety of particulate food sources through phagocytosis of large food items such as phytoplanktons (Ruppert et al., 1994) (5─50 μm). Particles below 5 μm such as pico- and ultra-planktons from surrounding water are captured by choanocytes and digested by archaocytes (Ruppert et al., 1994; de Goeij et al., 2008). The long life history of sponges may be due to their ability to withstand changes in the environment and competing organisms (Muller, 2003) and partly because of the associated microorganisms. Eukaryotic microorganisms include dinoflagellates (Garson et al., 1998; Hill and Wilcox, 1998; Hanna et al., 2005), diatoms (Webster et al., 2004; Totti et al., 2005), microalgae primarily zoochlorellae (Saller, 1989; Sand-Jensen and Pedersen, 1994; Frost et al., 1997), fungi (Holler et al., 2000; Bugni and Ireland, 2004; Konig et al., 2006) and yeast (Maldonado et al., 2005).

Sponges grow in different sizes, may be soft or hard depending on the skeleton and internal minerals. The shape of sponge depends on the environment such as hydrodynamics, turbidity and light intensity. Sponges are classified into 4 classes (Figure

2.1). Each sponge species is adapted to particular hydrographic characteristics prevailing in the region such as illumination, current strength and physical turbulence.

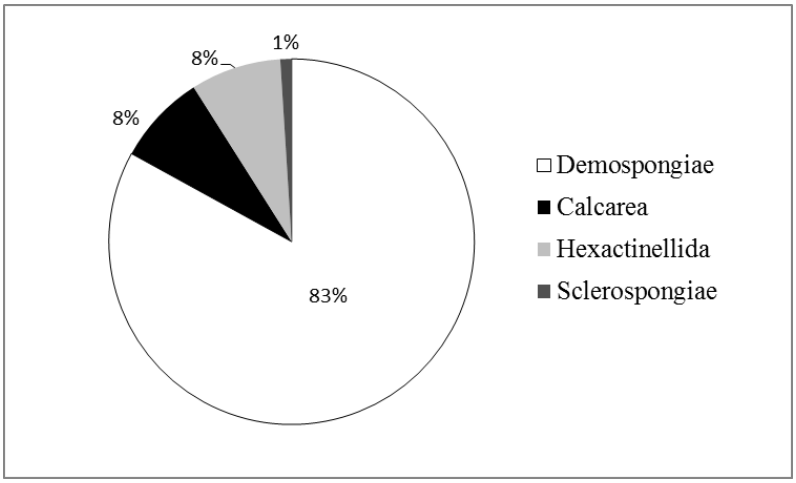


Figure 2.1. Percentage number of species in each class of Porifera

Sponges are grouped into four classes: Calcarea (calcareous sponges) with calcareous skeletal elements (spicules) and are exclusively marine sponges. Hexactinellida are glass sponges with siliceous spicules. Demospongiae (demosponges) comprise the majority (83%) of extant sponges with siliceous spicules, spongin (fibrous protein) or collagen fibres. Demospongiae (demosponges) is the largest and most diverse non-monophyletic class of the Porifera. Sclerospongiae have siliceous spicules and spongin. They also have an outer covering of calcium carbonate (Hooper and Van Soest, 2002). More than 8500 sponge species were identified and there was an exponential increase of species discovered since 1986 (van Soest et al., 2012) (Figure 2.2).

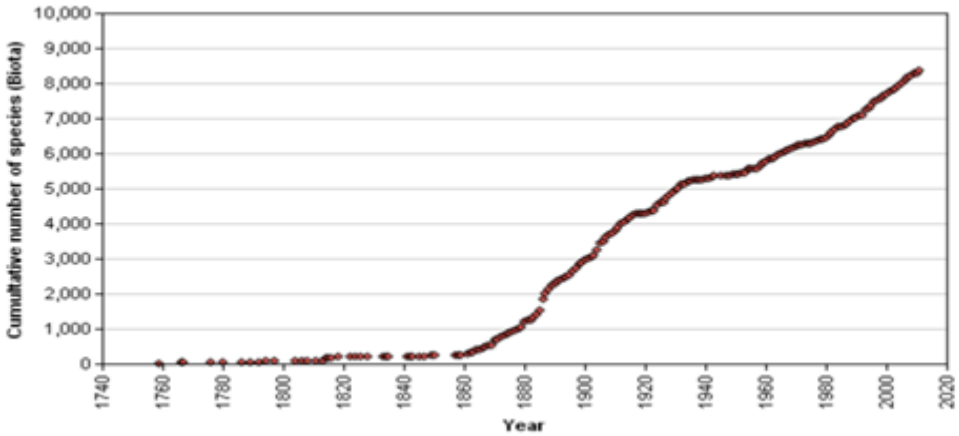


Figure 2.2. Number of sponge species discovered

2.1.1. SPONGE HABITAT AND THEIR DISTRIBUTION

Sponges are mostly present in tropical shallow waters but are also reported in extreme environments such as Artic waters (Dieckmann et al., 2005), deep-sea waters (Meyer and Kuever, 2008) and in alkaline lakes (Arp et al., 1996). Their habitats ranged from epilittoral to hadal depths and from rocks to mud bottom. Distribution of sponge species, genera and families recorded in each of the 232 marine eco-regions of the world are shown in Figure 2.3, as described by van Soest et al. (2012). Restricted distribution of sponges is due to limited swimming ability of larvae and occasional asexual reproduction, and their global distribution is mainly mediated by ship trafficking.

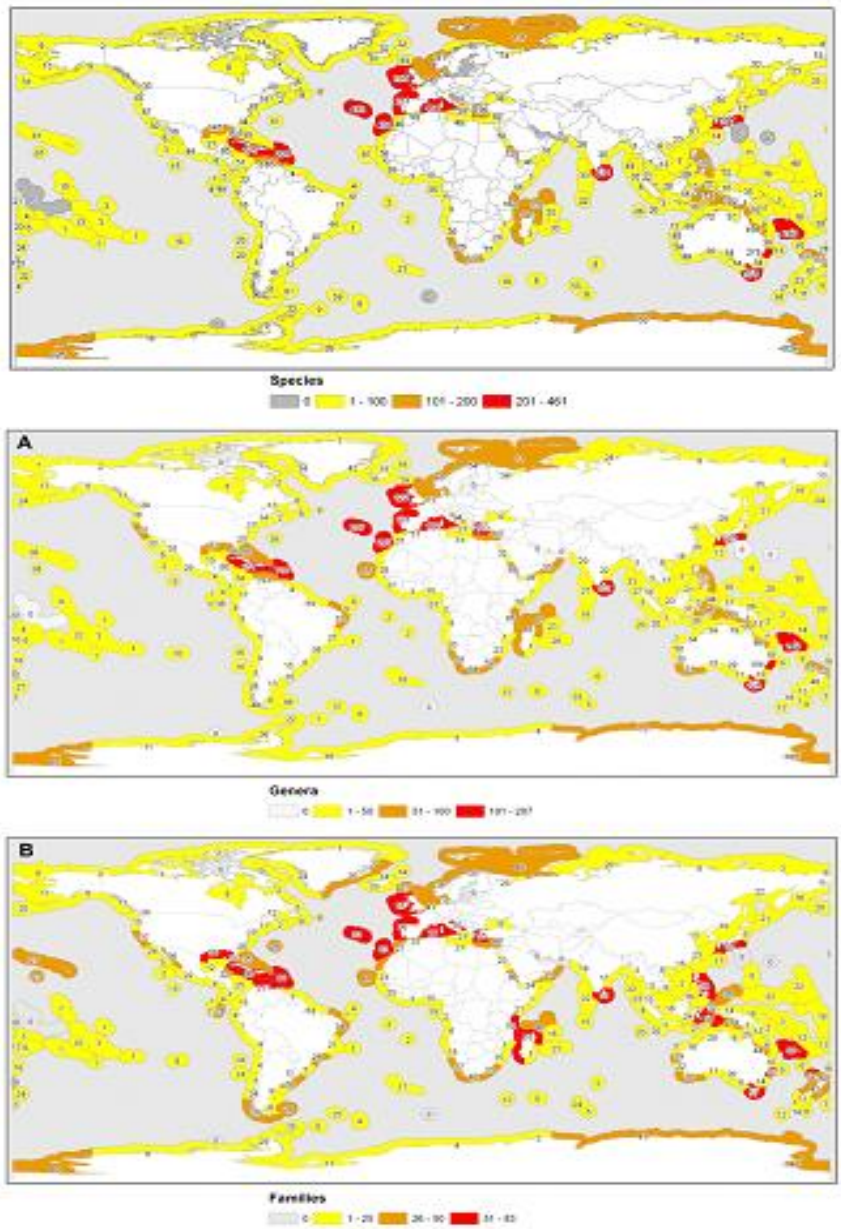


Figure 2.3. Global distribution of sponges

Sponges connect the benthic communities to open water nutrients and play a role in the health and the economics of marine systems. Ribes et al. (2012) found significant net excretion of phosphate from sponges, *Dysidea avara*, *Chondrosia reniformis* and *Apysina oroides* respectively, suggesting their important roles in the recycling of phosphorus in the marine environment. Bio-eroding sponges may compete successfully with other sessile organisms present on coral reefs, coralline bottoms and oyster beds, for nutrition. Some specific groups of fossil sponges and some recent sponges convert substrates such as coral rubble and pebbles into stable surfaces and form uplifted terrestrial habitats such as coral reef islands (Van Soest et al., 2012). Sponge-associated microorganisms may contribute significantly to organic production in oligotrophic habitats. The increase of seawater temperature, global climate change, and other prevailing environmental conditions are threatening many sponge species. The decrease in cyanobacterial abundance due to the change in temperature and irradiance (Webster and Taylor, 2012) can have an adverse effect on the growth of phototrophic sponges.

There is an increased interest in the cultivation of sponges as they are a source of a large number of bioactive compounds (Thakur and Muller, 2005) and these compounds cannot be synthesised chemically and/or their production is expensive. But the cultivation of sponges is difficult as the requirements of sponges are largely unknown. As sponges are sessile filter feeders, they are exposed to toxic compounds/ xenobiotics and can accumulate and withstand a wide variety of pollutants (Zahn et al., 1981; Patel et al., 1985; Carballo et al., 1996; Perez et al., 2002, 2003) and heavy metals (Philp, 1999; Cebrian et al., 2003; Perez et al., 2005). Gudimov (2002) and Gifford et al. (2007) suggested the use of filter feeders to remove pollutants from wastewater. Thus, sponges are considered as one of the most promising taxa among marine filter feeders for bioremediation strategies and can be used to mitigate pollution and bacterial contamination in coastal areas affected by urban sewage and industrial/agricultural discharge. Over the last 15 years, sponge mariculture has been suggested as a method for bioremediation as they have the ability to regenerate tissue and develop into functional individuals (Pronzato et al., 1998; Manconi et al., 1999). Mayzel et al. (2014) found differential uptake and accumulation of trace metals in 16 Red Sea coral reef sponge species and some of the metals were involved in skeletal fibres formation in sponges. Based on these data, it was proposed that sponges can be used in bio-monitoring for anthropogenic disturbances in an area.

The study of biology, ecology, taxonomy and chemistry of sponges is known as "spongology". There is an increased interest in sponogology in the last 2 decades. In the 9th world sponge conference held in Australia in 2013, the highest number (>50) of papers were on sponge diversity and biogeography and the next high number was on the sponge-associated microorganisms (Figure 2.4).

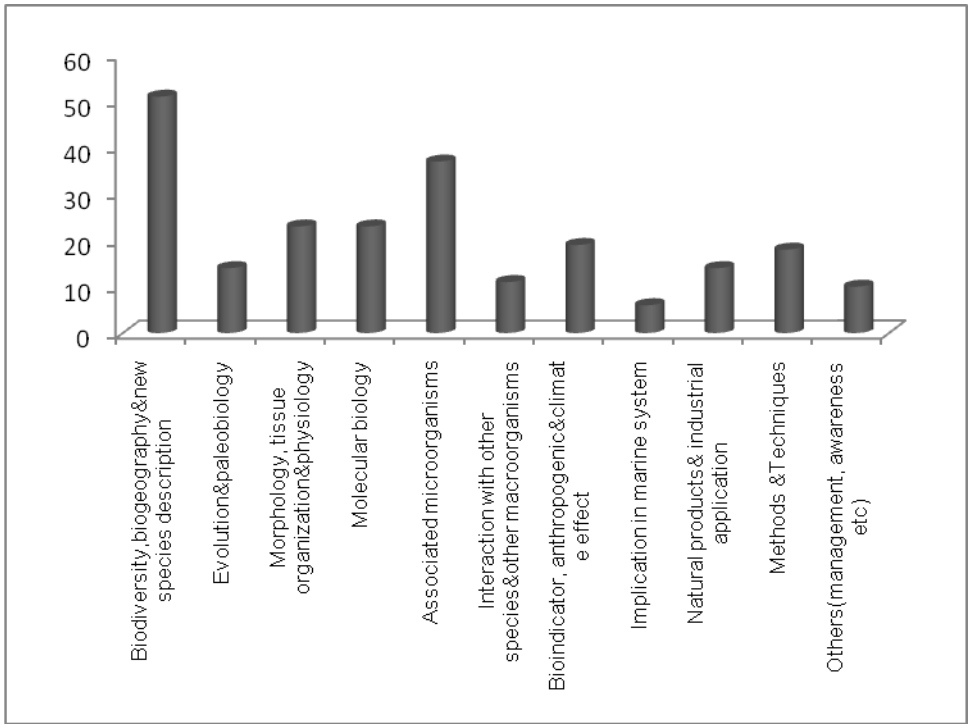


Figure 2.4. Number of papers presented in ninth world sponge conference

2.2. SPONGE -SSOCIATED MICROORGANISMS

Increased antibiotic resistance and the emergence of multidrug-resistant pathogenic bacteria necessitate the search for new antibiotics from different sources such as marine animals. This leads to the finding that sponges are the excellent source of bioactive compounds (Taylor et al., 2007; Kennedy et al., 2008). Evidences showed that most of these compounds have a bacterial origin (Hentschel et al., 2002; Kennedy et al., 2008; Lee et al., 2009), partly or wholly synthesised by sponge-associated bacteria. Consequently, a large number of studies have been carried out to understand the bacterial diversity associated with sponges and their host specificity to identify and exploit novel bioactive compounds (Jayatilake et al., 1996; Betancourt-Lozano et al., 1998; Perovic, 1998; Schmidt et al., 2000; Muller et al., 2004). Currently, sponges are considered as a reservoir of complex microbial communities that provide an area in marine microbiology and biotechnology as they harbour all the 3 domains of living organisms such as bacteria,

archaea and eukarya. They also harbour a large number of uncultured and elusive marine microorganisms that may play an important role in the chemistry of these animals (Hentschel et al., 2006). Sponge provides a protective and nutrient rich environment for growth, survival and for the extensive interaction of diverse groups of microbes (Mohamed et al., 2008a). These microorganisms act as a food source for the sponge (Reiswig, 1975a, 1979), mutualistic organisms (Wilkinson, 1983, 1992) and parasite or pathogens (Lauckner, 1980; Hummel et al., 1988; Webster et al., 2002). A large number of bacteria are present in the mesohyl and are estimated to account for about 40% of the sponge volume (Wilkinson, 1978b). Based on the abundance of associated microorganisms, sponges are divided into 2 categories; 1) High Microbial Abundance (HMA) sponges carry up to $10^8 - 10^{10}$ bacteria per gram of sponge wet weight and 2) Low Microbial Abundance (LMA) sponges carry $< 10^6$ bacterial abundance (Vacelet and Donadey, 1977; Hentschel et. al., 2003, 2006).

The electron microscopy and fluorescence *in situ* hybridization showed high bacterial density in the mesohyl region of different sponges from different locations (Santavy and Colwell, 1990; Althoff et al., 1998; Webster et al., 2001b). Webster et al. (2001b) found the patchy or even distribution of different bacterial phyla within the sponge body. For example, γ- proteobacterium and cytophaga /Flavobacterium were concentrated in the region surrounding the choanocyte chamber whereas β - Proteobacteria were found throughout the sponge. In another study by Manz et al. (2000) using widefield deconvolution epifluorescence microscopy (WDEM) combined with FISH, found that members of *Desulfovibrionaceae* were closely associated with micropores of *Chondrosia reniformis* from the Mediterranean Sea. Burlando et al. (1988) suggested that in the calcareous sponges, symbiont location in sponges may be related to ascon organisation. Vacelet (1975) and Wilkinson (1978a) proposed three broad types of microbial associates in sponges, based on electron microscopy and bacterial cultivation studies: sponge-specific microbes in the sponge mesohyl, sponge- specific bacteria occurring intracellularly, and nonspecific bacteria resembling those in the surrounding seawater in the water canals.

Taylor et al. (2007) proposed 3 scenarios for acquiring a high number of diverse bacteria by sponges. Scenario 1 deals with the Precambrian acquisition and recent colonization of bacteria which are maintained by vertical transmission, *ie*, from parent to offspring. The

second scenario is the vertical transmission from parent to offspring as well as environment symbiont transmission. The third one is the environmental acquisition of bacteria by selective absorption and specific enrichment of some of the bacteria which out-compete others whereas other bacteria will be utilized as food. The high rate of water pumping and efficient filtration system in sponges enable them to capture and enrich 1 in 20,000 bacteria from surrounding water (Pedros-Alio, 2012) to an estimated 10^{10} bacterial cells per day per ml (Hill, 2004). Immune system-like proteins and proteins rich in ankyrin and tetratricopeptide repeats found in these bacteria help to evade the sponge's digestive system (Muller and Muller, 2003a,b; Thomas et al., 2010) and proliferate within the sponge body. Based on the mode of transfer, Schmitt et al. (2012) divided sponge-associated bacteria into 3 groups: core, variable and species-specific bacteria. The core bacterial community is acquired by horizontal transfer of sponge-associated bacteria through seawater. Sponge-specific bacteria are mostly restricted to sponges or in sponges and corals and termed sponge & coral-specific microorganisms, acquired mostly by vertical transmission.

A high percentage of sponge-associated bacteria inhabited permanently in the mesohyl of some sponge species suggested a highly integrated interaction between the host sponge and associated microorganisms (Friedrich et al., 2001). However, bacterial community structure may undergo variation with the health of sponges, season and space. Gao et al. (2014) found that dominant species of sponge-specific *Candidatus Synechococcus spongiarum* in healthy sponges were shifted to sponge non-specific cyanobacterial clade in abnormal sponge tissues. However, the culturable bacterial community of sponge represents 0.10 – 0.23% (Friedrich et al., 2001) to 11% of the total bacterial community of sponge (Hentschel et al., 2001), depending on the sponge and the method used. Different methods were tried to increase the culturability of sponge-associated bacteria by formulating new media composition for oligotrophic bacteria present in the sponges which are prevented by the dominant copiotrophic bacteria when cultured on marine agar (Santavy et al., 1990) and by incorporating the extract of sponges in the culture media to cultivate fastidious bacteria from the sponge (Selvin et al., 2009a).

Though studies on microbial diversity started in late 1970's, sponge-associated microbial diversity is still a hot topic. In 2nd international symposium on sponge microbiology held in 2014, 36% of the papers presented were on microbial diversity (Figure 2.5). However, it

is equally important to analyse the nature of the relationship and biological functions of sponge-associated bacteria.

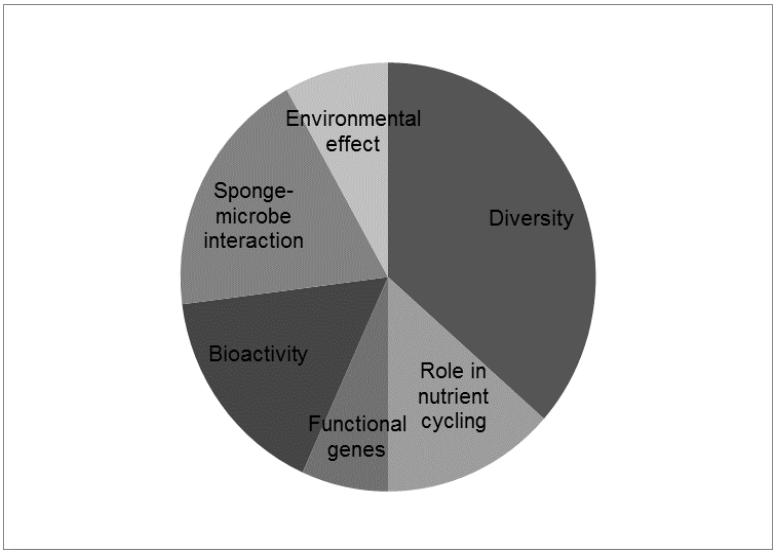


Figure 2.5. Papers presented in different categories in 2nd international symposium on sponge microbiology in 2014

2.2.1. INTERNATIONAL SCENARIO

2.2.1.1. Diversity

The sponge-microbial associations are the well-known feature of the phylum Porifera. Astonishing bacterial diversity within the same sponge and among different sponge species was revealed by using different techniques such as denaturing gradient gel electrophoresis, 16S rRNA gene sequencing, fluorescence *in situ* hybridization, pyrosequencing and next generation sequencing using Illumina technology (Webster et al., 2001a,b, 2004; Taylor et al., 2004; Li et al., 2006; Jeong et al., 2013; Li et al., 2014; Easson and Thacker, 2014; Kennedy et al., 2014; Gao et al., 2015; Jasmin et al., 2015; Luter et al., 2015). Bacteria with more than 30 phyla contribute to the high density of microorganisms in the sponge. Both Crenarchaota and Euryarchaota present in the sponge with a higher density of crenarchaota (Taylor et al., 2007). Many sponge-microbe associations studied from tropical and temperate zones involve diverse functional groups such as oxygenic and anoxygenic photosynthetic bacteria (Yurkov and Beatty, 1998), chemoheterotrophic bacteria (Wilkinson et al., 1981), methane-oxidizing bacteria (Vacelet et al., 1996), green sulfur and non-sulfur bacteria, nitrifying bacteria and denitrifying

bacteria belonged to the Acidobacteria, Actinobacteria. Armatimonadetes, Bacteroidetes, Caldithrix/Deferribacteres, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Halanaerobiales, Lentisphaeraea, Nitrospirae, Planctomycetes, α, β, γ, δ, ε, TA18- Proteobacteria, Spirochaetes, Verrucomicrobia and bacterial candidate phyla BD1-5, BRC1, Hyd24-12, NPL-UP2b OD1, OP1, OP3, OP11, SAUL, TM6 and TM7 (Simister et al., 2012a) and sponge-specific phylum Poribacteria (Webster and Hill, 2001; Webster et al., 2001b; Hentschel et al., 2002).

2.2.1.1.1. Spatial variation of sponge-associated bacteria

Several studies showed variation in abundance and diversity of bacteria associated with same sponge species inhabited in different geographic location and different sponges inhabiting in the same location. The variation was also observed in different studies. This might be because of the difference in methodology, for example, the bacterial community may change when the host sponge is maintained in aquaculture than that of the wild sponge (Mohamed et al., 2008a). However, transmission electron microscopy of sponge *Aplysina cavernicola* transplanted from lower depths to shallower depths revealed that the microbial community did not change. Another study by Taylor et al. (2005) observed spatial variability in bacterial community structure in the marine sponge *Cymbastela concentrica* from tropical and temperate waters using 16S rDNA-DGGE (denaturing gradient gel electrophoresis). The variation in *C. concentrica* - associated bacteria was higher than those of bacterioplankton variation. This suggests endemism attributed to host sponge association. Bacterial phylotypes associated with sponge *Chondrilla nucula* from two regions with distinct water masses (The Ligurian Sea and the Adriatic Sea) in the Mediterranean Sea belonged to Acidobacteria, γ-and δ-Proteobacteria which were closely related to other sponge-associated bacteria (Thiel et al., 2007). Althoff et al. (1998) found identical bacterial genera in sponges from different locations. Permanent members of sponge-specific bacteria shared among distantly related sponges from different, non-overlapping geographic regions include monophyletic cluster within Acidobacteria, β-Proteobacterium, Burkholderiacepacia and cluster of uncertain affiliation (Althoff et al., 1998). However, Thoms et al. (2003) found a significant difference in the bacterial community associated with different sponge species from the same region. Sponges from different oceans harboured closely related bacteria, distinct from other bacterial lineages

showing that they are specialised for residing within sponges (Hill et al., 2006). When bacterial communities on the surfaces of 2 species of sponge, *Mycale* sp. from Hong Kong and from Bahamas were compared using TRFLP, Hong Kong sponge showed the higher bacterial diversity and different bacteria from those on the Bahamas sponge suggesting species-specific, surface-associated bacterial communities. The different bacterial and fouling communities and their activities in sympatric sponges may reflect their habitat differences (Lee et al., 2007). Cyanobacterial symbionts in two congeneric and sympatric host sponges *Ircinia fasciculata* (higher irradiance) and *Ircinia variabilis* (lower irradiance) exhibited distinct habitat preferences correlated with irradiance. The difference in photosynthetic activity and their number in both the sponges suggest that ambient irradiance conditions may mediate the nature of sponge- Cyanobacteria symbiont relationships (Erwin et al., 2012). Some groups of bacteria might have stable association with sponges. For example, phylum Proteobacteria found in different sponges from the same or different geographic location may be attributed to their varied effects on sponge hosts (Burnett and Mckenzie, 1997; Stouthamer et al., 1999; Kalinovskaya et al. 2004; Groudieva et al., 2004). Friedrich et al. (1999, 2001) and Thoms et al. (2003) detected Bacteroidetes in two species of sponge *Aplysina sp.* from the Mediterranean Sea and from the coast of Marseille, France. Analysis of the deep sea sponge-microbiota revealed that these sponges shared a set of abundant OTUs that were distinct from the shallow water sponge bacterial community (Kennedy et al., 2014). The sponge microbial community of *Certeriosponge foliascens*, collected from inshore and offshore waters showed significant difference with increase in Cyanobacteria over Bacteroidetes between turbid inshore water and oligotrophic offshore water (Luter et al., 2015).

2.2.1.1.2. Temporal variation of sponge-associated bacteria

Studies showed that bacterial community structure associated with some sponge species show temporal variation. For example, *Halichondria panacea* carries a specific *Roseobacter* population with varying bacterial co-populations occurring seasonally between different sponge fractions (Wichels et al., 2006). However, most of the studies showed that some bacteria form a stable association with some host sponges unaffected by the changing environmental conditions. Sponge *Chondrilla nucula* from the Mediterranean Sea was shown to be associated with a stable and specific bacterial community regardless of sampling time and geographical region (Thiel et al., 2007).

Webster and Hill (2001) found that bacterial diversity of sponge, *Rhopaloeides odorabile,* collected from 4 different seasons from geographically distant habitats was dominated by an α-Proteobacterium. Erwin et al. (2012) studied bacterial community structure of 3 sympatric sponges, *Ircinia* spp. over 1.5 years in the Northwestern Mediterranean Sea during the large fluctuation in environmental variables. They found that dominant bacterial community (Proteobacteria, Cyanobacteria, Acidobacteria, Bacteroidetes and Chloroflexi) exhibited species-specific structure and stability. However, rare bacteria changed with seasons. Fiore et al. (2015) found that the thermal stress and ocean acidification has a significant effect on the functions and stability of the microbiota in Caribbean barrel sponge, *Xestospongia muta*, such as a decline in productivity. However, the environmental stress did not change the microbial community structure. Erwin et al. (2015) noticed a high degree of host specificity, low seasonal dynamics and low interannual variability among different LMA and HMA sponges from 6 orders.

2.2.1.1.3. Species variation of sponge-associated bacteria

Most of the sponge-associated bacteria are host specific. Though different sponge species harbour phylogenetically related bacteria, the dominant bacteria were generally different (Li et al., 2006). The taxonomically distantly related sponges, *Aplysina aerophoba* and *Theonella swinhoei* growing in different geographic regions harboured uniform microbial community which was different from that of seawater and sediments (Hentschel et al., 2002). Olson and McCarthy (2005) observed different banding pattern in DGGE of bacteria from 2 species of deep-water sponge *Scleritoderma* indicating that a far greater diversity of organisms exists in the sponges than that represented by the isolates. The similarity of 16S rRNA genes extracted from deep-water sponges *Scleritoderma* spp. to uncultivated microbial associates of sponges, *T. swinhoei* and *A. aerophoba*, suggests that sponges in phylogenetically disparate orders may carry similar bacterial communities (Olson and McCarthy, 2005). DGGE fingerprinting of the predominant bacteria associated with the sponges, *Dysidea avara, Craniella australiensis, Halichrondria* sp. and *Stelletta tenui* from the South China Sea showed different community structure. *C. australiensis* has the highest bacterial diversity with four bacterial phyla. *D. avara* associated bacterial community consist of two phyla and *S. tenui* and *Halichrondria* sp. harboured only one phylum. An electron microscopy study of 13 species of sponges was done by Vacelet and Donadey (1977). Two main aspects of the sponge- bacterial association were reported by

these researchers: 1) bacteria are numerous in massive sponges with high tissue density and 2) bacteria are scarce in well-irrigated sponge species with low tissue density. Fuerst et al. (1998) observed cells of a bacteria-like microorganism with a membrane-bounded nuclear region encompassing the fibrillar nucleoid within mesohyl tissue from five genera of marine sponges, *Astrosclera* sp., *Axinyssa* sp., *Jaspis* sp., *Pseudoceratina* sp. and *Stromatospongia* sp. Scanning and transmission electron microscopy studies of the symbiotic bacteria from six *Oscarella* spp. collected from the Mediterranean Sea and the Sea of Japan showed that mesohyl of adult sponges or intercellular space in embryos of each sponge species had a definite set of extracellular bacterial morphological types. This provides a good additional character for identification of *Oscarella* spp, which have no skeleton (Vishnyakov and Ereskovsky, 2009) and sponge identification was mainly carried out by spicule arrangement. Red Sea Demosponges, *Hyrtios erectus* and *Amphimedon* sp. showed the difference in the bacterial community in 2 sponges and one-third of the community was constituted of novel bacteria by culture-dependent and culture-independent studies (Radwan et al., 2010). *H. erectus* showed greater diversity and the bacterial community was dominated by δ-Proteobacteria whereas γ-Proteobacteria was the major component of the clone library of *Amphimedon* sp. A similar study on Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola* showed different phylogenetic groups like low and high G+C Gram-positive bacteria, α-Proteobacteria and γ-Proteobacteria (Hentschel et. al., 2001). Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were isolated from marine sponges *Suberites carnosus* and *Leucosolenia* sp. However dominant genus was different in these sponges (Flemer et al., 2012).

Red Sea sponges, *Hyrtios, Stylissa* and *Xestospongia* harboured 26 bacterial phyla, 11 of which were absent from the surrounding seawater and showed variation with host species but did not show geographic variation. These sponge-specific bacterial communities were resistant to environmental disturbance (Lee et al., 2011). Sponge species-specific association between sponges and bacterial communities were found among sponges around San Juan Island, Washington (Lee et al., 2009). The Greater similarity in sponge-associated bacterial communities in *Myxilla incrustans* and *Haliclona rufescens* suggests that there are stable specific associations of certain bacteria in these two sponges, while the bacterial communities in *Halichondria panacea* varied substantially among sites. An uncultured α-Proteobacterium and a culturable *Bacillus* sp. were unique to *M. incrustans*

while an uncultured γ-Proteobacterium was found exclusively in *H. rufescens*. Cleary et al. (2013) attributed the difference in bacterial composition between *Suberites* sp. and *Cinachyrella* sp. to different sponge species and habitat. *Suberites* sp. did not show habitat variation and the bacterial community was dominated by α-Proteobacterial taxa belonging to the order *Klioniellales*. *Cinachyrella* sp., in contrast, showed habitat variation and hosted markedly different bacterial communities (Cleary et al., 2013). Different individuals of taxonomically diverse Great Barrier Reef (GBR) sponge species harboured largely conserved bacterial communities with intra- species similarity ranging from 65─100%. These GBR sponge microbes were more closely related to associated-bacteria than to environmental communities. No relationship between host phylogeny and associated bacteria were found in sponges from different orders (Webster et al., 2013). A culture-independent study carried out by Zuppa et al. (2014) showed that 2 sponges *Ircinia muscarum* and *Geodia cydonium* from Naples showed different types of bacterial symbionts. Bacterial community in *I. muscarum* was dominated by Firmicutes whereas *G. cydonium* was dominated by γ-Proteobacterium. Kennedy et al. (2014) analysed the microbiota of four individual deep water sponges, *Inflatella, Lissodendoryx, Poecillastra, and Stelletta* together with surrounding seawater by pyrosequencing. The microbial communities of *Inflatella, Lissodendoryx* and *Poecillastra* were typical of low microbial abundance (LMA) sponges while *Stelletta* community was typical of high microbial abundance (HMA) sponges. It is known that phylogenetically closely related sponge species of the genus *Tethya* harbour phylogenetically very different phototrophic symbionts having similar roles (Sipkema and Blanch, 2010). One speculation for different bacterial communities in different sponges was that bacterial communities are selected by the host based on their role or presence in the surrounding seawater at the time of acquisition rather than on phylogeny. Consequently, higher intra-specific variation can be seen in associated bacteria that are acquired by horizontal transmission compared with vertically transferred bacteria.

2.2.1.1.4. Sponge-associated bacteria versus bacterioplankton

The studies showed that bacterial numbers in sponge exceed by approximately two orders of magnitude than those in seawater (Hardoim et al., 2009) whereas bacterial diversity was higher in the water than sponge (Turque et al., 2008). Microbial symbionts of biochemically active Australian Great Barrier Reef Dictyoceratid sponge, *Candidaspongia*

flabellata include sponge-specific Eubacteria and Cyanobacteria which are absent in the surrounding water column (Burja and Hill, 2001). Sipkema et al. (2015) also found different bacterial communities in 3 different sponge species and their ambient water. This could have implications for host health or for the extraction of bioactive compounds from symbiotic bacteria associated with sponges. Community structure of Western Atlantic demosponges, *Hymeniacidon heliophila* and *Polymastia janeirensis*- associated bacterial assemblages was phylogenetically different from that of the free-living bacterioplankton. On the other hand, some sediment-attached bacteria were found in the sponge bacterial community, indicating that sponges may incorporate bacteria together with sediment particles (Turque et al., 2008). Hill et al. (2006) and Lee et al. (2009) while comparing the bacterial diversity of sponges and ambient water. It was found that the bacterial communities in the water column consisted of more diverse bacterial ribotypes and were different from those associated with the sponges as found in tropical waters.

2.2.1.1. 5. Studies of sponge-associated bacteria in different regions

2.2.1.1.5.1. Sponge-associated bacteria in tropical water

Culture-dependent studies

Most of the studies in tropical sponge-associated bacteria were carried out on the sponges from Great Barrier Reef and China Sea. One of the earliest studies in this area on phototrophic sponge *Candidaspongia flabellata* showed highly diverse bacterial assemblages (Burja et al.,1999, Burja and Hill, 2001) with dominant groups constituting *Myxococcus xanthus* (α-Proteobacterium), *Photobacterium leiognathi* (γ-Proteobacterium), *Bacillus thermophilus* (High GC Gram-positive bacteria), *Curtobacterium citreum* (High GC Gram-positive bacteria), *Bacillus pumilus* (High GC Gram-positive bacteria) and *Escherichia coli* (γ-Proteobacterium). These bacteria were not detected in ambient seawater suggesting that they were sponge symbionts. Webster and Hill (2001) found that the culturable bacterial community of GBR sponge *Ropaloeides odorabile* was dominated by a single α –Proteobacterium strain. This is surprising as most of the studies showed diverse bacteria in sponges. The same strain was also observed in *Candidaspongia flabellata* from the same region suggesting that this bacterium may be transferred by horizontal transmission among GBR sponges and may play important roles

in this region. Culturing methods detected *Marinobacter, Micromonospora, Streptomyces* and *Pseudomonas* in the South China Sea sponge *Hymenacidon pervele* (Sun et al., 2010).

Culture-independent studies

In one of the earliest culture-independent studies by Webster et al. (2001b), microbial communities of GBR sponge *Ropaloeides odorabile* fell into Actinobacteria, Cytophaga-Flavobacterium group, low G+C Gram-positive group, α, β and γ-Proteobacteria, green sulfur and non-sulfur bacteria, and Planctomyces. Many of the clones from *R. odorabile* were Gram-positive bacteria as opposed to a finding of earlier culture-based studies which showed that only ~ 5% of marine bacteria are Gram-positive (Zobell, 1946). In another study by Hentschel et al. (2003), the Red sea sponge *Craniella austrialiensis*-associated bacterial community structure was similar to the results of Webster et al. (2001a) with Proteobacteria as the predominant component. A study conducted on the sponges *Suberitus tenui, Halichrondria* sp., *Dysidea avara and Craniella australiensis* from the same region showed the dominance of Actinobacteria, Bacillales, Sphingobacteria, Flavobacteria, α, β and γ-Proteobacteria (Li et al., 2006). In recent years, with the next generation sequencing method, pyrosequencing revealed high levels of bacterial diversity and species richness in sponges, and also site specificity of sponge-associated bacteria (Thomas et al., 2010; Lee et al., 2011; Simister et al., 2012a). The microbial community richness increased in the abnormal tissues of sponge, *Cartriospongia foliascens* than the healthy tissues, collected from Red Sea. Pyrosequencing profiles of 4 tropical sponges, *Rhabdastrella globostellata, Callyspongia sp., Rhaphoxya sp., and Acanthella cavernosa* showed that each sponge species possessed specific microbiota that was significantly different from other sponge species (Steinert et al., 2016), with Chloroflexi, Cyanobacteria and Proteobacteria as dominant phyla. They also found no significant difference in the bacterial community structure in sponges collected from the shallow and deep water. This emphasizes the influence of sponge identity on the associated bacteria. Luter et al. (2015) found that sponge, *Carteriospongia foliascens* was primarily composed of Proteobacteria and Cyanobacteria. Polonia et al. (2016) found that Proteobacteria dominated the *Stylissa cartei and S. massa* and ambient seawater, in Berau reef system, Indonesia.

2.2.1.1.5.2. Sponge-associated bacteria in subtropical water

Culture-dependent studies

One of the first and most cited studies on sponge-associated bacteria was carried out in subtropical waters by Wilkinson (1978 a). He found that sponges filter bacteria from water and the efficiency of filtration are not related to the existing bacterial populations in sponge tissue. He also found that the numbers of bacteria associated with the sponges are proportional to the sponge mesohyl density. Eleven taxonomically different sponges carry similar bacteria different from ambient water (Wilkinson et al., 1978 b). Culturable bacteria in Caribbean sponge, *Ceratoporella nicholsoni* accounted for 3 ─ 11% of the total bacteria inhabiting the sponge and 78% of the culturable bacteria were clustered into *Vibrio* sp., *Aeromonas* sp., and *Coryneform- or Actinomycete*-like sp. (Santavy et al., 1990). Lafi et al. (2005) found that culturable bacterial community of phylogenetically different sponges *Pseudoceratina clavata, Ceratoporella nicholsoni* and *Rhabdastrella globostellata* dominated by Firmicutes and α-Proteobacteria.

Culture-independent studies

Fieseler et al. in 2004 reported the presence of a previously unrecognised new candidate sponge-specific phylum Poribacteria from several sponge species from subtropical water but not from the environment. Two species of the sponge *Mycale such as M. adhaerens* from Hong Kong and *M. laxissima* from the Bahamas were dominated by α-Proteobacteria, γ-Proteobacteria, Bacteroidetes and Firmicutes (Lee et al., 2007). Another species of the same genus, *Mycale armata* carries phylotypes related to eight phyla with predominant α-Proteobacterial sequences (45.8%). Three bacterial phylotype groups (sequence from marine sponge, from sponges and other marine organisms and potential new phylotypes) were detected in this invasive sponge (Wang et al., 2008a). Associated bacterial assemblages of Brazilian sponges *Hymeniacidon* sp. and *Polymastia* sp. were phylogenetically different from that of bacterioplankton (Turque et al., 2008) and sponge *Clathria* sp. (Isaacs et al., 2009). Most of these bacterial phyla previously detected from sponges were found in northern Pacific sponge *Haliclona* sp. However, a few species formed stable association with this sponge (Sipkema et al., 2009). Sponge-specific β- and γ-Proteobacteria were abundant bacteria in this sponge. They were closely related to OTU found in other sponges, insects and amoeba. Aquaculture has a significant effect on the

bacterial community composition of the sponge when compared with the bacterial community of wild sponge *Clathria prolifera* maintained in aquaculture ponds (Isaac et al., 2009). They found that the dominant phyla in the wild sponge, such as Actinobacteria, Bacteroidetes, Cyanobacteria and Proteobacteria represented over 90% of the species diversity and their abundance decreased to 20% in the clones from sponge maintained in aquaculture ponds. The microbial community analysis of Caribbean fire sponge, *Tedania ignis* collected from different sites for 2 years showed that the dominant taxa were Chloroflexi and Crenarchaea and Proteobacteria in ambient water (Jouett et al., 2015). Fiore et al. (2015) found that Proteobacteria, Chloroflexi and Cyanobacteria dominated in the microbiota of Caribbena barrel sponge, *Xestospongia muta* that was exposed to thermal stress and ocean acidification.

2.2.1.1.5.3. Sponge-associated bacteria in temperate water

Culture-dependent studies

Most of the studies on the sponge - bacteria association were carried out in temperate water with comparatively less number of culturable bacteria. The bacteria associated with Aplysid family of sponges were affiliated with *Bacillus, Arthobacter, Micrococcus*, *Vibrio* and *Pseudoalteromonas* in the study of Hentschel et al. (2001). Olson and McCarty (2005) could cultivate only a small fraction of the bacterial community which was non-representative of sponge-associated communities from deep water sponge *Scleritoderma* sp. Lee et al. (2009) found that the culturable bacterial communities of sponges from same genus and species (congeneric and conspecific) showed large variations.

Culture-independent studies

Burnaldo (1988) and Althoff et al. (1998) by using electron microscopy detected bacteria in temperate water sponges *Clathria clavata* and *Halichondria panacea*. In 1999, Friedrich and his collegues with the help of electron microscopy and FISH documented the bacterial community associated with the sponge *Aplysina cavernicola*. The vast majority of mesohyl bacteria belonged to low G+C content. δ- Proteobacteria was most abundant, followed by the γ-Proteobacteria and representatives of the Bacteroides cluster. α- and β-Proteobacteria and Cytophaga/Flavobacterium clusters were not detected by FISH. Later, they proved that majority of sponge-associated bacteria resides permanently

in the *Aplysina aerophoba* mesohyl with the help of molecular techniques and aquarium experiment suggesting a highly integrated interaction between the host sponge and associated microorganisms. From sponges *Aplysina aerophoba* and *Theonella swinhoei,* 14 monophyletic, sponge-specific sequence clusters comprising three-fourth of all publicly available sponge-derived 16S rDNA sequences that were identified by Hentschel et al. (2002). Thoms et al. (2003) by transplanting sponges from 40 m depths to shallower water made a similar observation. Phylogenetically different sponges *Scleritoderma* spp, *Theonella* sp. and *Aplysina* sp. showed similar bacterial community (Olson and McCarthy, 2005). At the same time, the bacterial community associated with *Halichondria panacea* was diverse and variability was observed between different sponge fractions, sampling locations, and sampling dates (Wichels et al., 2006). However, sponge, *C. nucula* harboured diverse microbial community as found in other sponges in different habitats around the world (Hill et al., 2006; Thiel et al., 2007) and were dominated by bacteria (Acidobacteria, Bacteroidetes, and Cyanobacteria) that appeared to be sponge specialists. The most abundant phylum in the sponge *Haliclona simulans* library was also found to be Proteobacteria (86%), with the majority from the γ-Proteobacteria (77%) related to other sponge-associated bacteria and novel genera within the phyla Verrucomicrobia and Lentisphaerae (Kennedy et al., 2008). Gerce et al. (2009) found that Actinobacteria, Cyanobacteria, α-Proteobacteria, γ-Proteobacteria and Chloroflexi were stable predominant phyla in sponge *Aplysina aerophoba* and did not change with different cultivation conditions. Erwin et al. (2015) found that HMA sponges host more diverse microbial community than LMA sponges.

2.2.1.1.5.4. Sponge-associated bacteria in artic water

Culture-dependent studies

Only limited number of studies was carried out in cold water sponges. Dieckmann et al. (2005) with the help of Intact-Cell-MALDI-TOF mass spectrometry (ICM-MS) found that marine sponges *Isopsphle graei, Haliclona* sp. *1, Phakellia ventilabrum and Plakortis* sp. harbour *Alteromonas, Bacillus, Colwellia, Erythrobacter, Marinobacter, Marinococcus, Pseudoalteromonas, Pseudomonas, Roseobacter, Sphingomonas and Vibrio* sp.

Culture-independent studies

Vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* was demonstrated by Ereskovsky et al. (2005). Cold water sponge *Geodia barretti* hosted a stable homogeneous sponge-specific bacterial community in mesohyl which was not affected by sponge pathogens during cultivation (Hoffman et al., 2006). Jensen et al. (2008) found that Acidobacteria dominated the Artic reef sponge *Desmacidon sp.* library.

2.2.1.2. Functions

The *in situ* activity and function of the sponge-associated microbes have become a major research focus only in the last decade. The interaction between sponges and microorganisms occur in many forms. Sponge-microbial associations are often regarded as mutualistic symbiosis. Sponge provides a favorable environment to their microbial symbionts on the other hand, the sponge-associated bacteria are capable of carrying out a range of metabolic processes such as nutrient acquisition (Wilkinson and Garrone, 1979), supply of photosynthate (Wilkinson, 1983; Steindler et al., 2002), nitrification (Bayer et al., 2008a,b; Hoffmann et al., 2009; Schlappy et al., 2010), nitrogen fixation (Wilkinson, 1979; Mohamed et al., 2010), and sulfate reduction (Hoffmann et al., 2005b), stabilization of sponge skeleton (Rutzler, 1985), metabolic waste processing (Wilkinson, 1978a; Beer and Ilan, 1998) and production of secondary metabolites (Kennedy et al., 2007; Siegl and Hentschel, 2010). The interaction among bacteria is accomplished by the process of quorum sensing (Fuqua and Greenberg, 2002; Waters and Bassler, 2005). The production of quorum sensing molecule, N- aceyl homoserine lactone (AHL) by bacteria helps in establishing effective symbiosis with the sponge. It is also able to regulate the colonization and other important interactions among the sponge-microbiota. A large number of protein-protein interactions between host and bacteria help sponge to discriminate between food and symbiotic bacteria (Thomas et al., 2010).

2.2.1.2.1. Carbon cycling in sponges

There are three types of sponges based on their nutrition; photosynthetic, heterotrophic and mixotrophic. Most of the sponges are considered to be heterotrophic and obtain their nutrition by filtering and ingesting phyto- and bacterioplanktons from ambient water. Phototrophic sponges mainly found in tropical regions derive fixed carbon and / or

nitrogen from oxygenic autotrophs (cyanobacteria) through photosynthesis (Wilkinson and Fay, 1979; Rai, 1990; Sara et al., 1998) which may contribute up to 50% of sponge energy requirements and 80 % carbon requirements (Wilkinson, 1983; Cheshire et al., 1998). This also helps them to thrive in the low-nutrient, high-light areas on tropical reefs. DGGE and next generation sequencing method showed that bacterial phylum Chloroflexi was one of the dominant phyla in many sponges (Radax et al., 2012a; Simister et al., 2012a; Jeong et al., 2013). The members of this phylum carry bacteriochlorophyll and are considered to be involved in aerobic anoxygenic photosynthesis. Another group of bacteria capable of doing aerobic anoxygenic photosynthesis in the order, Rhodospirillales belonging to α-Proteobacteria were also detected in sponges (Lavy et al., 2014; Costa et al., 2013; Zan et al., 2013 and Gao et al., 2014). But no attempt was done in culturing these bacteria and/or to understand their function in sponges. However, van Duyl (2008) detected inorganic carbon fixation ability of sponge-microbial consortia associated with deep-water sponges, *Higginsia thielei* and *Rossella nodastrella*. As more than one species of phototrophic bacteria were isolated from sponges and inorganic carbon fixation was detected in phylogenetically distant sponges, the special microenvironment in sponges may provide suitable growth conditions for carbon fixing bacteria which may contribute a significant fraction to the carbon requirement of the host.

Some deep-sea cladorhizid carnivorous sponges do not possess aquiferous system which is the common feature of Porifera and get nutrition by preying on minute swimming organisms. It is believed that methanotrophs associated with them contribute a large portion of these sponges' nutrition (Vacelet et al., 1995, 1996; Vacelet and Boury-Esnault, 2002). Methanotrophs obtain their carbon source and substrate for energy production from a deep-sea mud volcano (Vacelet et al., 1996). 16S rRNA gene sequence affiliated with methanogenic archaea belonging to the phylum Euryarchaeota was also detected in sponges (Webster et al., 2001a) which may be responsible for producing methane within anoxic zones of sponges. Metagenomic analysis of sponges revealed the potential of sponge-associated microorganisms to utilize a variety of substrates. For example, genes codes for metabolism and transport of monosaccharides, polysaccharides, organic acids, sugar alcohol derivatives, amino acids, and amines were recovered from *Amphimedon queenslandica, Neamphilus huxleyi* and *Cymbastella concentrica* (Thomas et al., 2010; Gauthier et al., 2016), suggesting the roles of heterotrophic bacteria in sponge nutrition.

2.2.1.2.2. Nitrogen cycling in sponges

Sponge cells metabolize nitrogen in the organic matter and release ammonium (Brusca and Brusca, 1990). This ammonia will be utilized by nitrifying bacteria for energy. Genes code for nitrogen fixation, nitrification and denitrification were detected in sponges (Mohamed et al., 2008a). Wilkinson et al. (1984), Corredor et al. (1988) and Pile (1996) reported nitrification associated with marine sponges. Nitrification is a two-step process carried out by nitrifying organisms through chemolithotrophic pathway and convert ammonia to nitrate via the formation of nitrite (Kowalchuk and Stephen, 2001; Koops et al., 2001). Diaz and Ward (1997) found that the rate of nitrification (0 to 2650 nM g^{-1} h^{-1}) in sponges in tropical regions are two to four orders higher than that in coastal sediments which are considered as active nitrification sites. Net accumulation of nitrate was found without the loss of exogenous ammonium suggesting an efficient coupling between ammonia production and subsequent oxidation, before ammonia is released into the water (Diaz and Ward, 1997). This is the highest reported nitrification from benthic communities and was found in sponges associated with Cyanobacterial endosymbionts. This may be attributed to the ammonia production by Cyanobacteria through nitrogen fixation (Diaz and Ward, 1997). Cyanobacteria and nitrifying bacteria may form a mutualistic relationship as nitrification provides nitrate to Cyanobacteria. Bayer et al. (2008b) proved that nitrate excretion by temperate sponge, *Aplysina aerophoba* (up to 344 nM g^{-1} h^{-1}) is the product of microbial nitrification and the process is consistent with the aerobic condition in the sponge tissue. They found a seasonal variation in the nitrification process. In spring, sponges act as ammonium sink and in summer as ammonium excretion. Uncoupling of ammonia oxidation and nitrite oxidation was seen in sponges because of the differential response of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) to light intensity (Olson, 1981), changes in substrate concentration (Olson, 1981; Vanzella et al., 1990) or may be diverse nature of microbial communities inhabiting different sponges. The dissolved inorganic nitrogen released by nitrifying bacteria may contribute significantly to nutrient cycling in the marine environment (Diaz and Ward., 1997; Jimenez and Ribes, 2007). So, nitrification helps sponge to get rid of toxic ammonium and nitrite which could be deleterious.

Homologous genes associated with ammonia oxidation (ammonia monooxygenase, ammonia permease, nitrite reductase nitrite oxidase and urease) were detected in the

sponge, *C. symbiosum*. Anaerobic ammonia oxidizing bacterial (anammox) genes were also observed in this sponge (Jimenz and Ribes, 2007; Mohamed et al., 2010). Traditionally it was believed that only bacteria are carrying out the nitrification process. But now it is found that Thaumarchaeota which possesses the putative *amo* A gene, codes for ammonia oxidising enzyme and these archaea are able to oxidize ammonium in pure and enriched cultures (Konneke et al., 2005; Wuchter et al., 2006; Tourna et al., 2011). These archaeal symbionts show vertical transmission through larvae which imply host-microbe symbiosis (Sharp et al., 2007; Steger et al., 2008). Pitcher et al. (2011) and Radax et al. (2012a) found a correlation between Thaumarchaeota biomarker, archaeal *amo*A gene and nitrification rates with archaeal *amo* A genes dominating over bacterial *amo* A genes in sponges. So it is evident that both bacteria and archaea are carrying out nitrification. 16S rRNA gene sequences of AOB such as *Nitrosomonas*, *Nitrospira*, and *Nitrosococcus* related clade and Ammonia-oxidizing archaea (AOA), *Cenarchaeum symbiosum* and *Crenarchaon Candidatus Nitrosopumus* and NOB, *Nitrospina* sequences were obtained from sponges (Preston et al., 1996; Hentschel et al., 2002; Diaz et al., 2004; Hallam et al., 2006 a, b; Bayer et al., 2008a, b). Researchers found that high microbial abundant (HMA) sponges excrete nitrate and nitrite but low microbial abundant (LMA) sponges do not excrete or, excrete at a much-reduced amount (Jimmenez and Ribes, 2007; Yahel et al., 2007; Bayer et al., 2008a; Meyer and Kuever, 2007, 2008; Hoffmann et al., 2009). *Nitrospira* were reported in LMA and HMA sponges, but no activity was reported in LMA sponges indicating that they are metabolically inactive in LMA sponges. One sponge-specific archaea (1.1A Crenarchaota) carrying *amo* A gene was detected from sponges, but not found in sea water (Lee et al., 2003; Holmes and Blanch, 2007; Taylor et al., 2007; Mohammed et al., 2009; Steger, 2010; Sipkema and Blanch, 2010; Fan et al., 2012; Ribes et al., 2012; Yang and Li, 2012; Jouett et al., 2015). The nitrifying bacterial sequences from sponge showed a stable association of these microorganisms with sponges, the environment which is different from the surrounding water. The autotrophic bacteria such as nitrifying bacteria provide additional carbon source to the sponge.

It was believed that sponge tissue is saturated with oxygen because of enormous pumping activity. Hoffman et al. (2005a, b) with the help of oxygen microelectrodes proved that some regions of sponge tissues are anaerobic due to reduced pumping activity. Variation in ventilation exhibited in sponges when they stop pumping and become anoxic within 15

minutes. During anoxic condition, oxygen can be detected only in millimetre from the surface (Hoffman et al., 2008a). The anaerobic processes in sponges were first detected by Hoffman (2003) when they found out microbial sulfite reduction in cold-water sponge *Geodia barreti*. Denitrification and anammox remove nitrogen and recycle back to the atmosphere. Most of the denitrifying bacteria belong to β-Proteobacteria (Heylen et al., 2006). Till now, there are only 2 reports on denitrification activity in sponges (Hoffmann et al., 2009; Schlappy et al., 2010). Nitrification removes only 5 % of ammonia produced in anoxic condition by sponge and the rest will be released to the environment during ventilation. The nitrate produced by nitrifiers will be used in denitrification (Schlappy et al., 2010) or used by a sponge. Microbial community within the sponge may shift to aerobic and anaerobic processes in response to the occurrence of spatial and temporal oxic and anoxic condition within the sponge due to the variation in pumping behaviour of sponge. Schlappy et al. (2010) suggested that both LMA and HMA may host a diverse metabolic group of microorganisms and carry out both aerobic and anaerobic process in sponges. They determined nitrification and denitrification in *D. avara* and *C. reniformis* and found variation in the rates which was attributed to different microbial niches. The different microbial community at different parts of the sponge may be due to the variability in the availability of nutrients, oxygen and space and differential uptake and maintenance of microbes by sponge tissues (Schlappy et al., 2010). Nitrifying and denitrifying bacteria in the sponge create an optimized system for food utilization and storage under both aerobic and anaerobic condition. Hoffman et al. (2009) proved that nitrification, anammox and denitrification can occur simultaneously in the sponge. Denitrifying bacterial sequences (*nir S, nir K* and *nar* genes) in the sponge-associated microbes belonged to β- and γ- Proteobacteria and Bacteroidetes (Hoffmann et al., 2009; Yang and Li, 2012; Fan et al., 2012; Han et al., 2012; Zhang et al., 2013).

2.2.1.2.3. Sulfur cycling

The first anaerobic bacteria from sponge were isolated by Eimhjellen et al. (1967) from sponge *Halichondria panacea.* The bacterial isolates include *Chlorobium limicola, Thiocystis violacea* and a non-motile coccoid sulfur bacterium. Imhoff and Trupper (1976) suggested a close association between some species of sulfate reducing bacteria (SRB) and sponges as they found a relatively higher number of viable *Chromatium* cells (10^4 per litre) in the extract of *Euspongia officinalis* than in sea water. SRB might provide the

anaerobic condition inside the sponge by producing the hydrogen sulfide and photosynthetic electron for photosynthetic sulfur bacteria. Four genera/ groups of SRB: *Desulfovibrio* spp., *Desulforophalus* spp., *Desulfomicrobium* spp., *Desulfobacterium* spp., and members of the *Desulfosarcina/Desulfofaba* cluster were observed in sponges (Manz et al., 1998, 2000; Hoffmann et al., 2005a; Erwin et al., 2012; Dupont et al., 2014a, b; Gauthier et al., 2016). Sulfate reducing Archaea were also detected in sponges with specific oligonucleotide probes and metagenomics studies (Meyer and Kuever, 2008). These microbes reduce sulfate to sulfide, which is toxic to sponge cells. Anoxic zones in the tissues and high rates of microbial sulfate reduction of up to 1200 nmol $cm^{-3}d^{-1}$ were found in the cold water sponge, *G. barretti* (Hoffmann, 2003) which was comparable with sulfate reduction rate from other environments (Ravenschlag et al., 2000). Hoffmann et al. (2005a) suggested that *G. barretti* has developed effective buffer systems to prevent sulfide toxification. A steep decrease in oxygen was seen in the canals from *G. barretti* surface depending on the pumping activity, as high numbers of SRB belonging to the *Desulfoarculus/Desulfomonile/Syntrophus*-cluster in the choanosome of this sponge was detected by FISH. Analysis of lipid biomarkers indicates biomass transfer from associated SRB or other anaerobic microbes to sponge cells (Hoffmann et al., 2005a). The increase of sulfate reduction activity from the cortex toward the interior of the sponge is due to lower sulfate reduction or higher sulfide reoxidation in the cortex region. It has been hypothesised that a bacterial sulfur cycle exists in sponges in which both sulfur-oxidizing and sulfate reducing bacteria cooperate (Hoffmann et al., 2005a).

2.2.1.2.4. Antagonistic activity

Sponges are minimally affected by predators and fouling organisms, despite their high nutritional value and a lack of physical defences possibly due to sponges' chemical defences, by the production of natural products, most of which are originated from associated bacteria (Guarner and Malagelada, 2003). The competition among bacteria for space and nutrients inside sponge body may induce the production of natural products. Different secondary metabolites exhibiting antimicrobial and antifouling activity have been isolated from bacteria associated with sponges. Hentschel et al. (2001) found numerically abundant bacterial isolates with antimicrobial activity from the marine sponges *Aplysina aerophoba* and *Aplysina cavernicola*. Production of antimycobacterial antibiotics by symbiotic bacteria favours them in competition between sponge microbial

community members (Izumi et al., 2010). Antagonistic activity can confer a competitive advantage within a bacterial community by inhibiting sensitive isolates (Tait and Sutherland, 2002) and protect host sponge by inhibiting pathogens or fouling organisms (Gil-Turnes et al., 1989; Holmstrom et al., 1992).

2.2.1.3. Biotechnological application

The search for bioactive compounds from sponge started in 1950's (Bergmann and Burke, 1955) and the studies on these secondary metabolites showed rapid increase in last 2-3 decades ago (Sakai et al., 1986; Fredenhagen et al., 1987; Perry et al., 1988; Elyakov et al., 1991; Shigemori et al., 1992; Imamura et al., 1993; Williams et al., 1993; Arillo et al., 1993; Unson et al., 1993, 1994; Kobayashi and Ishibashi, 1993; Fusetani and Matsunaga, 1993; Hinde et al., 1994; Oclarit et al., 1993; Needham et al., 1994; Kobayashi and Kitagawa, 1994; Kobayashi et al., 1994; Kolachana et al., 1994; Miki et al., 1994). Sponge-associated bacteria gained importance as a source of bioactive compounds only when a significant similarity was found between those compounds isolated from sponges and those found in the terrestrial organism of entirely different taxa (Perry et al., 1988). Most of these compounds fall under antimicrobial agents such as antibacterial, antifungal and antiviral agents (Oclarit et al., 1993; Nagai et al., 2003; Bringmann et al., 2003, 2007; Laatsch, 2006; Blunt et al., 2007). Clinically important bioactive compounds from sponge-associated bacteria are given in Table 2.1.

Table 2.1. Bioactive compounds from sponge-associated bacteria

Sponge	Location	Bacteria	Compound	References
Homophymia sp.	Off Touho, New Caledonia	*Pseudomonas* sp.	2-undecyl-4-quinolone	Bultel-Ponce et al., 1999
Dysidea avara	Adriatic Sea	*Unidentified bacterium*	2-methylthio-1,4-naphthoquinone	Thakur et al., 2005
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005; Webster et al., 2002
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005
Haliclona sp.	Java Sea, Indonesia	*Unidentified bacterium*	Unidentified compound	Radjasa et al., 2007

Sponge	Location	Bacteria	Compound	References
Isodictya setifera	Antarctica	*Pseudomonas aeruginosa*	Cyclo-(L-proline-L-methionine)	Jayatilake et al., 1996
Aplysina aerophoba	Mediterranean coast, France	*Bacillus pumilus*	Pumilacidin containing β-hydroxy fatty- acid	Pabel et al., 2003
Dysidea sp.	Eastern Samoa	*Vibrio* sp.	Tetrabromo-diphenyl ethers	Elyakov et al., 1991
Aplysina aerophoba	Banyuls sur Mer	*Bacillus* sp.	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Micrococcus* sp.	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Enterococcus* sp.	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Arthrobacter* sp.	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Unidentified bacteria*	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*α-Proteobacteria*	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Vibrio halioticoli*	Unidentified compound	Zheng et al., 2000
Aplysina aerophoba	Banyuls sur Mer	*Pseudoalteromonas* sp.	Unidentified compound	Zheng et al., 2000
Homophymia sp.	Off Touho, New Caledonia	*Pseudomonas* sp.	2-nonyl-4-hydroxy-quinoline N-oxide	Bultel-Ponce et al., 1999
Suberea clavata	Great Barrier Reef,	*Salinospora* sp.	Rifamycin SV	Kim et al., 2005; Lafi et al., 2005
Suberea clavata	Great Barrier Reef,	*Salinospora* sp.	Rifamycin B	Kim et al., 2005, 2006
Craniella australiensis	China Sea	*Streptomyces* sp.	*Chitinase*	Han et al., 2009
Aplysina aerophoba	Mediterranean coast, France	*Bacillus subtilis*	Surfactin	Pabel et al., 2003
Aplysina aerophoba	Mediterranean coast, France	*Bacillus subtilis*	Iturin	Pabel et al., 2003
Theonella swinhoei	Palau	*Candidatus Entotheonella palauensis*	Theopalauamide	Bewley et al., 1998; Schmidt et al., 1998
Theonella swinhoei	Philippines	*Entotheonella palauenis*	Theonegramide	Bewley et al., 1994,1996

Sponge	Location	Bacteria	Compound	References
Acanthostrongylophora sp.	Indonesia	*Micromonospora* sp.	*Manzamine A*	Ang et al., 2000
Homophymia sp.	Off Touho, New Caledonia	*Pseudomonas* sp.	2-undecyl-4-quinolone	Bultel-Ponce et al., 1999
Stelletta tenuis	China Sea	*Alcaligenes faecalis*	Cyclo-(L-Pro-L-Phe)	Li, 2009
Dysidea avara	Adriatic Sea	*Unidentified bacterium*	2-methylthio-1,4-naphthoquinone	Muller et al., 2004
Hyrtios altum	Okinawa	*Vibrio* sp.	Trisindoline	Kobayashi et al., 1994; Braekman and Daloz, 2004
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Thakur et al., 2004, 2005
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005; Webster et al., 2002
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005; Webster et al., 2002
Hymeniacidon perlevis	Nanji Island, China Sea	*Pseudoalteromonas piscicida*	Norharman	Zheng et al., 2005; Zhang et al., 2009
Haliclona simulan	Ireland	*Pseudoalteromonas* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Halomonas* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Psychrobacter* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Marinobacter* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Pseudovibrio* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Streptomyces* sp.	Unidentified compound	Abrell et al., 1998
Haliclona simulan	Ireland	*Bacillus* sp.	Unidentified	Abrell et al.,
Mycale plumosa	Qingdao coast, China	*Saccharopolyspora* sp.	Metacyclo-prodigiosin	Liu et al., 2005

Sponge	Location	Bacteria	Compound	References
Xestospongia sp.	New Caledonia, southwest Pacific	*Micrococcus luteus*	2,4,4'-trichloro-2'-hydroxy-phenylether	Bultel-Ponce et al., 1998
Xestospongia sp.	New Caledonia, southwest Pacific	*Micrococcus luteus*	Acyl-1-(acyl-6'-mannobiosyl)-3-glycerol	Bultel-Ponce et al., 1998
Halichondria panacea	Adriatic coast, Croatia	*Microbacterium* sp.	1-O-acyl- 3-[R-glucopyranosyl-(1-3)-(6-O-acyl-R-mannopyranosyl)]-glycerol	Wicke et al., 2000
Acanthella acuta	Mediterranean Sea	*Bacillus pumilus*	GG11	Ramm et al., 2004
Acanthostrongylophora sp.	Indonesia	*Micromonospora* sp.	*Manzamine A*	Sakai et al., 1986; Dunlap et al., 2007
Theonella swinhoei	Hachijojima Island,Japan	*Uncultured bacterium*	Onnamide A	Piel et al., 2004; Grozdanov et al., 2007
Dysidea sp.	Eastern Samoa	*Vibrio sp.*	Tetrabromo-diphenyl ethers	Elyakov et al., 1991
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Thakur et al., 2003, Takur and Muller, 2005
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005; Webster et al., 2001
Homophymia sp.	Off Touho, New Caledonia	*Pseudomonas* sp.	2-undecen-1' -yl-4-quinolone	Bultel-Ponce et al., 1999
Fascaplysinopsis reticulata	Great Barrier Reef	*Pseudoalteromonas maricaloris*	Bromo-alterochromide A	Speitling et al., 2007; Blunt et al., 2009
Homophymia sp.	Off Touho, New Caledonia	*Pseudomonas* sp.	2-nonyl-4-hydroxy-quinoline N-oxide	Bultel-Ponce et al., 1999
Theonella swinhoei	Palau	*Unidentified bacterium*	Swinholide A	Bewley et al., 1996, 1998
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Webster et al., 2001

Sponge	Location	Bacteria	Compound	References
Aplysina aerophoba	Mediterranean coast, France	*Bacillus subtilis*	Surfactin	Pabel et al., 2003
Aplysina aerophoba	Mediterranean coast, France	*Bacillus subtilis*	Iturin	Pabel et al., 2003
Suberites domuncula	Northern Adriatic Sea	*α-Proteobacterium*	Unidentified compound	Takur and Muller, 2005; Webster et al., 2001

2.2.2. NATIONAL SCENARIO

A total of 332 sponge species were found in Indian waters (vanSoest, 2012). East coast of India, especially Gulf of Mannar is considered as one of the biodiversity hotspots and harbours 275 sponge species (Jasmin et al., 2015). Central east coast of India harbour 30 sponge species. West coast of India harbour 81 sponge species. Among these, 26 were from the central west coast and 55 from Lakshadweep (vanSoest, 2012). *Sigmadocia* and *Callispongia* were the dominant and most studied sponges in Indian waters (Ely et al., 2004; Anand et al., 2006; Rao et al., 2007; Gandhimathi et al., 2008; Boobathy et al., 2009; Dharmaraj and Sumantha, 2009; Feby and Nair, 2010; Feby, 2011; Satheesh et al., 2012; Arunachalam et al., 2013). Irrespective of the enormous diversity of sponges in Indian waters, studies on the sponge and their associated microorganisms are limited. Most of these studies were on their diversity, antagonistic activities and industrial applications.

2.2.2.1. Diversity

Studies on sponge-associated microbiota are mostly from Gulf of Mannar, East coast of India. Most of the studies on diversity are culturable diversity. The first study on the sponge-associated bacteria was by Thakur et al. (2004). The cultured epiphytic bacterium from intertidal sponge *Ircinia fuca* fell into *Bacillus* sp. *Pseudomonas* sp. *Enterobacteriaceae, Micrococcus* sp. *Vibrio* sp. *Staphylococcus* sp. *Staphylococcus* sp. and *Flavobacterium* sp. (Thakur et al., 2004). They studied the temporal variation of cultural epiphytic bacteria associated with *Ircinia remosa* and *Ircinia fusca*, from the west coast of India and found that the epibacterial population of the sponge was influenced by changes in the environment with time. The bacterial number increased from 10^5 to 10^7 CFU cm^{-2}, in January to October. Selvin et al. (2009a) formulated different media in order

to cultivate a maximum number of bacteria with emphasis on Actinobacteria and found enrichment with sponge extract, inducers and growth regulators enhance the retrievability of heterotrophic bacteria. They also found that *Marinobacer*, *Micromonospora, Streptomyces, and Pseudomonas*, were sponge-associated obligate symbionts in the sponge *Dendrilla nigra*, from the east coast of India. The diversity of culturable bacteria from the sponge *Callispongia diffusa* from Mumbai coast showed an association of *Pseudomonas aeruginosa, Escherichia coli, Vibrio parahaemolyticus and V. cholera* with this sponge. Feby (2011) studies the spatial and temporal variation of bacteria associated with *Sigmadocia fibulata and Dysidea granulosa*, from Lakshadweep and Gulf of Mannar. The culturable bacteria showed temporal variation with a higher number in warmer months (10^6 CFU g^{-1}). RFLP and DGGE analysis of the sponge showed the presence of Proteobacteria, Actinobacteria, Firmicutes and Chloroflexi. *Clathria procera, Sigmadocia fibulata and Dysidea granulosa* from Lakshadweep harboured 10^3 to 10^5 bacteria g^{-1}sponge tissue. And these bacteria were dominated by Acinetobacter (Gopi et al., 2012). Culture-independent study of bacteria was carried out in the sponge, *Cinachyra cavernosa* and *Halichondria pigmentosum*, from Gulf of Mannar by cloning. Core groups of *H. pigmentosum* showed close affinity to sponge-associated bacteria from other regions. γ-Proteobacteria, Chlororflexi, Planctomycetes, and Defrribacter formed core groups in *H. pigmentosum* whereas core group of *C. cavernosa* comprised of δ- and β- Proteobacteria (Jasmin et al., 2015). Selvin et al. (2010) suggested that sponge-associated bacteria can be typed as general and specific symbionts, habitat flora and antagonists based on the culturable community structure.

2.2.2.2. Function

Functional aspects of sponge-associated bacteria in Indian water were mainly dealt with enzyme production and antagonistic activity. The antagonistic activity of epibiotic bacteria of *Ircinina fusca* against bacteria in the vicinity showed that sponge promotes the growth of certain antibiotic producing strains (Thakur et al., 2004). However, the antagonistic *Bacillus* sp. showed a close association with the sponge *Ircinia fusca* and may be involved in the selective control of the bacterial epibiosis on the sponge surface. Four antibiotic producing bacteria were isolated from sponge, *Pseudoceratina purpurea* surface exposed to human pathogens and marine fouling organisms and this induction method enhanced their antimicrobial activity (Kanagasabhapathy and Nagata, 2008). Endosymbiotic

Actinomycetes from the sponge, *Callyspongia diffusa* showed potent antimicrobial activity against human pathogens. More than 50% Streptomycetes isolated from *Callyspongia diffusa, Mycale mytilorum, Tedania anhelans and Dysidea fragilis* showed antagonistic activity against bacteria and fungi (Dharmaraj and Sumantha, 2009). Gandhimathi et al. (2008) found that endosymbotic Actinomycetes from sponge *Callyspongia diffusa,* from Bay of Bengal exhibited antimicrobial properties. Twenty one percentages of bacterial isolates from sponges, *Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulata,* and *Mycale mnnarensis* showed antibiotic production against *Bacillus subtilis, Escherichia coli, Vibrio harveyi, V. parahaemolyticus and Candida albicans* (Anand et al., 2006). Feby and Nair (2010) screened bacteria associated with sponges, *Sigmadocia fibulata* and *Dysidea granulosa* and found that these bacteria are a source of multiple extracellular enzymes such as amylase, caseinase, gelatinase, urease, lipase and phosphatase. Acetyl cholinesterase inhibitors, which has a significant pharmacological role was produced by bacteria (10─42% isolates) associated with marine sponges, *Rhabdastrella globostella, Dragmacidon agariciforme, Leiodermatium pfeifferae, Fasciospongia cavernosa, Xestospongia testudinaria,* and *Siphonodictyon coralliphagum* collected from Bay of Bengal (Pandey et al., 2014).

2.2.2.3. Biotechnological Application

A few studies have been carried on bioprospecting of sponge-associated bacteria for industrial enzyme production, antifouling, antibacterial and antiplasmodial activities from Indian waters. Secondary metabolite production and their antioxidant properties were also studied (Valentin et al.,, 2011; Arunachalam et al., 2013). Antibacterial compounds have been isolated from *Streptomyces* spp. associated with the sponge, *Dendrilla nigra* from Vizhinjam and Kanyakumari (Selvin and Lipton, 2004; Selvin et al., 2004, 2009c). These researchers also purified 17 bioactive molecules from *D. nigra* - associated bacterium (*Nocardiopsis dassonvillei*) which showed anemiagenic, antiandrogenic, anti-inflammatory, antimicrobial, antioxidant, cancer-preventive, dermatitigenic, hemolytic, hypocholesterolemic and nematicide properties (Selvin et al., 2009c). Skariyachan et al. (2013) screened bacteria associated with 6 marine sponges from Gulf of Mannar. They characterized the secondary metabolites as quinones, alkaloids, flavonoids and flavonyl glycosides. A chromophore producing bacterium, *Pseudomonas* sp. associated with the sponge *Callyspongia* sp. showed inhibitory properties against methicillin-resistant

Staphylococcus aureus and fluorophore producing *Bacillus licheniformis* isolated from the sponge *Haliclona* sp., showed inhibitory activity against *Salmonella typhi.* A lipopeptide surfactant produced by *Bacillus licheniformis* isolated from the sponge *Acanthella* sp. from North Bay of Port Blair, Andaman was investigated by Lawrance et al. (2014). They could increase the production by three fold (11.78 g l^{-1}) by heterologously expressing the gene clusters (*sfp, sfp*O and *srf*A) in *Escherichia coli,* over original strain. An alkalophilic and relatively heat sensitive amylase enzyme produced by endosymbiont, *Halobacterium salinarum,* of *Fasciospongia cavernosa* collected from the peninsular coast of India was characterized by Shanmughapriya et al. (2009). In another study, Sheikh and Pattabhiraman (2015) found that sponge, *Bienna fortis* (Mhapan region, Maharashtra) - associated bacteria can degrade polycyclic aromatic hydrocarbons.

2.3. SPONGE, *Cinachyra* sp.

Sponges *Cinachyra* are mainly found in coral habitats, rock pavements and hard bottoms preferably regimented habitat and are often covered with sediment (van Soest, 2001). The surface of the sponge is covered with protruding tips of radiating spicules. Long spicules arranged in bundles radiating from the centre with tips extending beyond tissue surface (John, 1986). Sponge *Cinachyra* has been reported in extreme environments like hydrothermal vents, ridges and Antartica (Gomes et al., 2006; Danis, 2013). *Cinachyra* sp. collected from Okinawa, Japan harboured 4 Actinobacterial genera such as *Ilumatobacter, Ferrimicrobium, Acidimicrobium* and *Iamia*. This diversity was relatively less than the co-occurring sponges, *Ulosa* sp. and *Petrosa* sp. (Khan et al., 2012). A highly cytotoxic macrolide, cinachyrolide A, has been isolated from marine sponge *Cinachyra* sp (Fusetani et al., 1993) and, Lakshmi et al. (2008) isolated and characterized new ceramides 1 and 2, and tetillapyrone 3 from *C. cavernosa* from Gujarat Coast of Indian Ocean. Subcellular fraction of *Cinachyra* sp. from Monte Saldanha hydrothermal vent showed an abundant concentration of iron and zinc. The pattern of metal accumulation in this sponge was Zn>Fe>Mn>Ni>V>Cu>Cd>Ag. The metallothionein concentration in this sponge was 224.47±75.04 µg g^{-1} d.w. This high metal concentration might be due to the enrichment of metals in hydrothermal ecosystem and the pattern of the metal accumulation reflects the metal concentration in surrounding environment. The difference in the distribution of soluble and insoluble fraction of metals in the sponge and the presence of metallothioein demonstrate the ability of *Cinachyra* sp. to regulate their intracellular metal concentration

by different mechanisms of detoxification or accumulation of non-toxic forms (Gomes et al., 2006). The aqueous extract of Cinachyra alloclada is known to be active against some solid tumor cell lines and in the differential DNA repair assay (Warren, 1981). Bioassay guided purification have shown the activity to spread all over the chromatographed fraction and to be due to inseparable polyamines. 17-Z- tetracosenyl 1-glycerol ether was also reported from the sponge *Cinachyra alloclada* (Cardellina et al., 1983; Fusetani et al., 1993).

From this background, the present study was aimed to

- To determine the abundance and diversity of bacteria associated with sponge *Cinachyra alloclada*
- To study the functional roles of sponge associated bacteria
- To link bacterial phylogeny to function of bacteria associated with sponge

CHAPTER 3

MATERIALS & METHODS

3.1. SAMPLING SITE

Anjuna beach (15° 34' 0" N, 73° 44' 0" E) situated in North Goa (Figure 3.1) is a part of the 30 km long Goa coastline along the east coast of Arabian Sea.

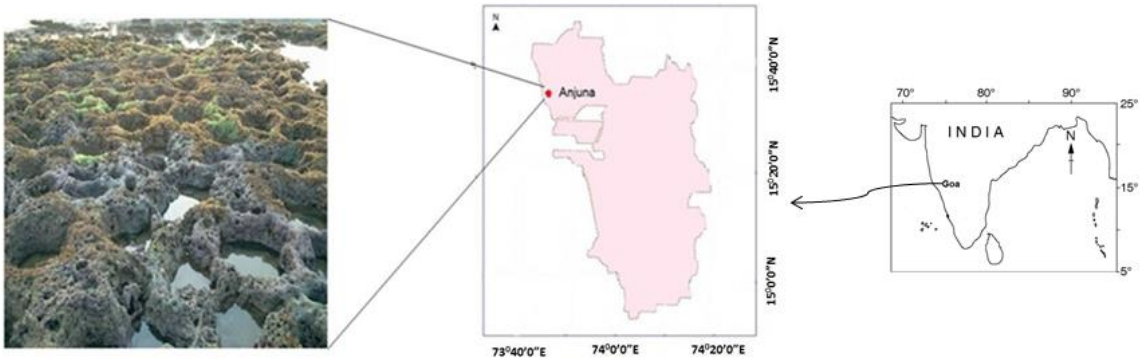


Figure 3.1. Sampling site

The large rock line along the beach is inhabited by different intertidal organisms such as sponges, ascidian, holothurian, crabs, gastropods and algae. Among the sponges, *Cinachyra alloclada* (Al-Fadhli, 2008), a globular sponge is the most abundant species in this region. It is attached to the intertidal rocks and invariably covered with a layer of sediment (Figure 3.2.). During low tide (less than 0.3 m tidal height), the rocks get exposed to the atmosphere.

Figure 3.2. *Cinachyra alloclada* attached to the intertidal rock

3.2. SAMPLE COLLECTION

C. alloclada specimens were collected during the low tide (Figure 3.3). A total of 11 collections were made in 2012 - 2013, except during the monsoon period, as the approach to this site was not possible. The collected sponges were immediately transferred to

sampling Whirlpak sterile bags to reduce handling contamination. Ambient water within a radius of 1 meter of the sponge location was collected prior to the collection of sponges.

Figure 3.3. Sample collection

For microbiological analysis, water samples were collected in Whirlpak sterile sampling bags and for nutrient analysis, water was collected in a 500 ml plastic bottle. Sampling bottles were rinsed well with ambient water before collection. The samples were transported to the laboratory in the icebox at 4 °C and processed immediately. The sponge samples for application study were transported to the laboratory in ambient water and maintained in running seawater aquarium. Flow chart of the workflow for sample analysis is given in Figure. 3.4.

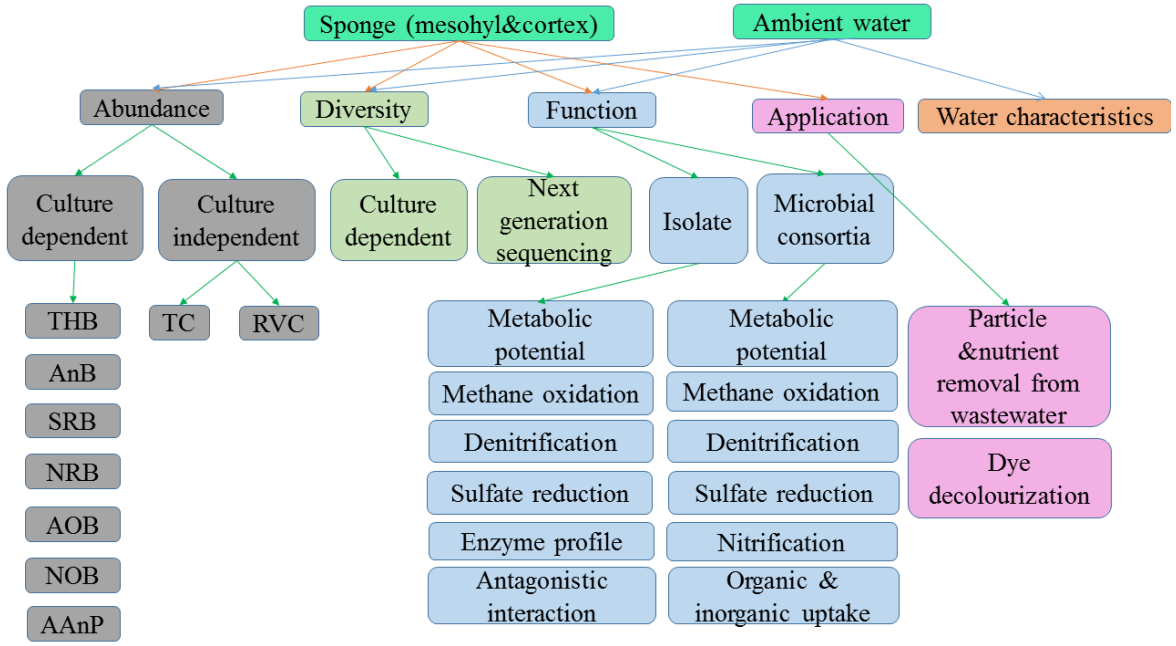


Figure 3.4. Flow chart of sponge and water analysis

3.3. AMBIENT WATER CHARACTERISTICS ANALYSIS

Temperature (ºC), salinity, pH, Eh (mv) and dissolved oxygen (mgl^{-1}) were recorded at the site using laboratory thermometer, refractometer (Atago, Germany), digital pH/Conductivity meter (Oakton PC testr), digital Eh probe (Oakton ORP testr) and digital DO meter (Oakton PC testr DO multimeter), respectively. Nutrients (ammonium, nitrite, nitrate and phosphate (µM)) were analysed using Continuous-Flow Autoanalyser (Hansen and Grasshoff, 1983). For suspended matter (mgl^{-1}), 500 ml of ambient water was filtered through pre-ashed, pre-weighed glass filter (Whatman GF/D). After filtration, the filter was washed with distilled water to remove the salts. The filter paper was dried and weighed until constant weight was obtained. The difference in the weights was considered as suspended matter. Labile organic matter such as carbohydrate, protein and lipid ($mg\ l^{-1}$) were estimated by using PSA method (Panagiotopoulos and Wurl (2009), Folin Ciocalteus method (Peterson, 1977) and extraction method (Bligh and Dyer, 1959), respectively.

3.4. MICROBIOLOGICAL ANALYSES

3.4.1. ABUNDANCE OF BACTERIA

The abundance of sponge-associated bacteria and ambient water were enumerated by direct counting and culture methods.

3.4.1.1. Sample processing

Upon reaching the laboratory, sponge specimens were rinsed with filtered (millipore membrane filter of pore size 0.22 μm), autoclaved seawater until the specimens were visibly free of debris and sediment. The cortex and the mesohyl tissues of the sponge were separated and cut into small pieces under aseptic conditions using a sterile scalpel and homogenised using sterilised mortar and pestle. Figure 3.5 shows the mesohyl and cortex region of *C. alloclada*. The homogenates were then serially diluted from 10^{-1} to 10^{-5} for enumeration. For enumerating the bacterial abundance in ambient water, the samples were serially diluted up to 10^{-2}. Sponge *C. alloclada* and the cross section of the sponge showing cortex and mesohyl region of the sponge is given in Figure 3.5.

Figure 3.5. Sponge *C. alloclada* cross-section showing cortex and mesohyl tissues

3.4.1.2. Enumeration

3.4.1.2.1. Direct counting Method

The total bacterial count and Viable counts were enumerated.

Total bacterial count

The total bacterial count was estimated by using fluorescent dye DAPI (4', 6 Diamidino-2-phenyl indole (Coleman et al., 1981). The homogenates of cortex and mesohyl were centrifuged. The supernatant of sponge homogenate and ambient water samples were fixed

with 2% paraformaldehyde and stored at 4°C until further analysis. For enumeration, the protocol of Friedrich et al., (2001) was followed. The supernatant and water samples were stained with DAPI (final concentration of 5 µg ml^{-1}). The stained homogenate was filtered through 0.22 µm polycarbonate membrane by applying vacuum. At least 20 different fields were counted using Nikon epifluorescence microscope with an excitation filter of 344 nm and emission at 450 nm. Total bacterial cells are expressed as the number of cells cm^{-3}.

Viable count

The viable bacterial count was evaluated using Boulos et al. (1999) protocol. Bac light Redox Sensor CTC viability Kit was used (Invitrogen). Healthy bacterial cells respiring via electron transport chain will absorb and reduce CTC (5-cyano-2, 3 ditolyl tetrazolium chloride) into an insoluble red fluorescent formazan product. The sponge homogenates and water samples were incubated with CTC (final concentration 5 mM) and filtered through 0.22 µm polycarbonate membrane by applying vacuum. CTC-stained cells were counted in 450/630 nm. The viable count is expressed as the number of cells cm^{-3}.

3.4.1.2.2. Culture-dependent (CFU) method

Organotrophic and autotrophic bacteria were enumerated as stated below. Organotrophic bacteria included aerobic and anaerobic heterotrophic bacteria, Actinobacteria, nitrate and sulfate-reducing bacteria. Autotrophic bacteria were ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and aerobic anoxygenic photosynthetic bacteria.

3.4.1.2.2.1. Organotrophic bacteria

Total heterotrophic bacteria (THB)

Serially diluted cortex and mesohyl tissue homogenates and ambient water samples were spread plated on Zobell Marine Agar (ZMA) and on different strengths (10%, 50% and 100%) of nutrient agar (NA). Apart from the above media, nutrient medium (10%, 50% and 100% NA and 100% ZMA) amended with 2.5% aqueous sponge extract were plated with sponge homogenates and the plates were incubated at 25±2 ^{0}C for 2 days. Bacterial colonies were counted and expressed as colony forming units (CFU) cm^{-3} of the sample.

Anaerobic bacteria (ANB)

Anaerobic agar (Himedia) was used to enumerate ANB using agar shake method. The inoculum (serially diluted homogenates of cortex and mesohyl and ambient water), mixed with the sterile medium was poured into test tubes up to the rim of the tube. The medium was overlaid by paraffin wax and paraffin oil (1:2, v/v), thereby providing a favourable condition for the growth of anaerobic bacteria. Sodium thioglycolate in the medium maintained low redox potential. The above procedure was carried out in an anaerobic chamber. The tubes were incubated at 25±2 ^{0}C for 2-3 weeks and bacterial colonies were counted and expressed as CFU cm^{-3} of the sample.

Sulfate-reducing bacteria (SRB)

Sulfate-reducing bacteria were enumerated in SRB selective medium (Himedia, India) by agar shake method. The addition of sodium thioglycolate and sodium sulfide helps in maintaining a low redox potential in the medium. The tubes were incubated at 25±2 ^{0}C for 2-3 weeks. Black colonies were counted and the number is expressed as CFU cm^{-3} of the sample.

Nitrate-reducing bacteria (NRB)

For NRB enumeration, agar shake method was used (Michotey et al., 2000). Serially diluted cortex and mesohyl sponge tissue homogenates and ambient water samples were inoculated into the selective NRB medium. The tubes were incubated at 25±2 ^{0}C for 2-3 weeks. NRB is expressed as CFU cm^{-3} of the sample.

Actinobacteria (AB)

Different Actinobacteria selective media such as marine sponge agar (MSA), casein starch agar (CSA), inorganic salt starch agar (ISP-4), International Streptomyces Project (ISP-7) and chitin agar (Selvin et al., 2009a) prepared in ambient water were used. Media were supplemented with 25 µg ml^{-1} cycloheximide to control fungal growth and 25 µg ml^{-1} nalidixic acid to inhibit Gram-negative bacteria. Serially diluted cortex and mesohyl tissue homogenates and ambient water were spread plated onto the above-said media and

incubated at 25±2 ^{0}C for 2-3 weeks. After incubation, the number of Actinobacteria was counted and is expressed as CFU cm^{-3} of the sample.

3.4.1.2.2.2. Autotrophic bacteria

Nitrifying bacteria

Aerobic nitrification involves 2 steps, 1) ammonia oxidation and 2) nitrite oxidation. The nitrifying group includes ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB).

Ammonium oxidising bacteria (AOB)

Ammonium oxidising bacteria from cortex and mesohyl tissue homogenates of sponge and ambient water was enumerated by modified MPN method (Elbanna et al., 2012) using phenol red as pH indicator. The inoculated tubes were incubated at 25± 2 ^{0}C for 2-3 weeks and the number of AOB was calculated based on McCready's table. The number of AOB is expressed as CFU cm^{-3} of sponge tissue and ambient water.

Nitrite-oxidising bacteria (NOB)

Nitrite oxidising bacteria was also enumerated by modified MPN method (Elbanna et al., 2012). The number of NOB is expressed as CFU cm^{-3} of sponge tissue and ambient water.

Phototrophic Bacteria (Aerobic anoxygenic photosynthetic bacteria, AAnP)

The 2 major culturable groups under this category are Chloroflexi and Rhodospirillales. The serially diluted sponge homogenates (cortex and mesohyl tissues) and ambient water were spread plated onto Chloroflexi and Rhodospirillales selective media (Rodina, 1972). The plates were incubated at 25±2 ^{0}C in dark for 2-3 weeks. The colonies were counted and are expressed as CFU cm^{-3} of the sample. Media compositions are given in the appendix.

3.4.2. DIVERSITY OF BACTERIA

A combination of culture-dependent and culture-independent methods would give a comprehensive assessment of diversity as culture-based methods cannot account for all organisms in the given sample. Bacterial diversity of sponge (mesohyl and cortex) and ambient water was estimated using both the methods in this study. In the culture-dependent method, the isolates were identified based on biochemical characteristics. The identification of the representative isolates was further confirmed using 16S rRNA gene

sequencing. For the culture-independent method, next generation sequencing (NGS) was carried out for bacterial diversity.

3.4.2.1. Culture-dependent method

For the culture-dependent method, sponge samples (cortex and mesohyl) and ambient water were processed as described under 3.4.1.1.

3.4.2.1.1. Bacterial isolation and purification

Four hundred and sixty nine bacterial colonies from mesohyl and cortex tissues of sponge and ambient water were isolated for diversity studies. For isolation, representatives of different morphotypes from all dilutions for a particular medium were selected. The isolates were purified by quadrant streaking on an agar plate (Stolp and Starr, 1981).

3.4.2.1.2. Phenotypic identification

Various phenotypic and biochemical tests (Table 3.1) were carried out as outlined by Gerhardt et al., (1981). The procedures are given in Appendix.

Table 3.1. Tests for phenotypic identification of bacterial isolates

Morphometric	**Biochemical**
Colony size	Gram staining
Colony shape	Motility test
Surface texture	Oxidase test
Elevation	Catalase test
Opacity	OF test
Swarming behavior	Citrate test
Production of slime	Triple sugar iron test
Pigmentation	Carbon utilization
Changing colour of the medium	Nitrate reduction

The isolates were identified by PIBWIN software and Bergey's manual (9^{th} edition).

In addition to the above tests, specific identification tests for AB, AAnP and SRB were carried out. The tests for AB were spore chain morphology, aerial mycelium colour, pigmentation, production of melanin and hydrogen sulfide, chitinase, cellulase, tyrosine

degradation, growth in sodium azide, in lysozyme, in different pH and at a different temperature, and antibiotic resistance. For AAnP, bacteriochlorophyll, utilisation of complex organic substrates (acetate, butyrate, citrate, fumarate, lactate, succinate and tartrate), growth factor utilisation (nicotinic acid, biotin, vitamin B3, vitamin B12, para-aminobenzoic acid) were tested. For SRB, apart from morphological characters, pigment characterization and carbon utilisation were tested.

3.4.2.1.3. Molecular identification

For molecular identification, representatives of bacteria belonging to *Bacillus, Vibrio, Staphylococcus, Brevibacterium, Alteromonas* and *Pseudoalteromonas* were selected.

16S rDNA gene sequencing

Total genomic DNA was extracted by the phenol-chloroform method modified from Ausubel et al. (1987). The 16S rRNA gene was amplified using the primer pairs: 27F- AGA GTT TGA TCC TGG CTC AG and 1492R- TAC GGY TAC CTT GTT ACG ACTT (Lane, 1991). The bacterial DNA (0.5 µg) was amplified by the PCR in a total volume of 25 µl containing 0.5 U Taq DNA polymerase, 10 mM TAPS (tris methyl amino propane sulfonic acid) , pH 8.8, 3 mM $MgCl_2$, 10 pmol each of the two primers and 200 pM each of dATP, dCTP, dGTP and TTP. The amplification of DNA was carried out in a hot-air rapid thermocycler programmed for initial denaturation at 94 °C for 12 minutes, 30 cycles of denaturation at 94 °C for 1 minute, annealing at 45 °C for 45 seconds and extension at 72 °C for 90 seconds. A final extension of 20 minutes was carried out at 72 °C. The amplified DNA fragment (1.5 kb) was purified using the PCR purification Kit (Axyprep PCR cleanup kit), according to manufacturer's instruction. The purified PCR product was used for DNA sequencing. PCR amplification was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA, 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer and 3 U/µl of Taq polymerase in Taq buffer. The PCR conditions used were: initial denaturation at 95^{o}C for 5 minutes, 30 cycles consisting of denaturation at 95 ^{o}C for 1 minute, annealing at 50 ^{o}C for 1 minute and elongation at 72 ^{o}C for 2 minutes and a final extension at 72 ^{o}C for 10 minutes. Sequencing of the PCR products was carried out by ABS Biosystems, equipped at NIO, Goa. The sequences obtained were edited using DNA Baser and compared with sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.nlm.gov). The 16S rRNA gene sequences

were deposited in the EMBL database. The EMBL accession numbers of the sequences are given in the appendix.

3.4.2.2. Culture-independent method

Though culture-dependent methods have been employed to study the diversity earlier, it is now well established that the fraction of the bacterial community in any environment that can be detected by these methods is only less than 5%. A culture-independent method improves the resolution on the microbial diversity and makes a more accurate phylogenetic affiliation assessment for microbes in a complex community such as sponges. Therefore, culture-independent approach such as pyrosequencing of V6 region of 16S rRNA gene gives a holistic picture of the bacterial diversity present in the sponge.

3.4.2.1. Genomic DNA extraction

DNA of cortex and mesohyl (0.25 g) and ambient water was extracted by using MOBio soil DNA kit and MO Bio water DNA extraction kit, respectively, according to manufacturer's instruction. DNA content and purity were analysed with Nanodrop Spectrophotometer, (Nanodrop Inc) using elution buffer as blank, and the A_{260}/A_{280} ratio was calculated. DNA was stored at -20 ^{0}C until used as the template for PCR reactions.

3.4.2.2. Next generation sequencing

The DNA was used as a template for nested PCR to amplify the V6 region of 16S rRNA gene. Metagenomic analysis of V6 region of the total bacterial community from mesohyl and cortex and ambient water were carried out by using Illumina technology (Genotypic Technologies, Bangalore). The Illumina paired-end reads were quality checked using FastQC2. Raw DNA sequence reads were downloaded from Illumina Base Space platform and processed in Quantitative Insights into Microbial Ecology (QIIME v1.9.0) (Caparaso et al., 2010). Operational taxonomic units (OTUs) were assigned using uclust (Edgar, 2010) with 97% similarity. The sequences were aligned using the greengenes reference alignment (version 13.8) which contains 99322 sequences (DeSantis et al., 2006). Chimeric OTUs were detected and removed using the usearch (Edgar, 2010) algorithm in QIIME. The resulting alignment was used to create a phylogenetic tree with FastTree (Price et al., 2010) and a resulting OTU table. The paired-end sequences of all samples were submitted to NCBI Sequence Read Archives (SAMN06226897, SAMN06226899, SAMN06226901, SAMN06226903, SAMN06226904 and SAMN06226907).

3.4.3. FUNCTIONAL ROLE OF PROKARYOTES

The functions of prokaryotes of sponge and ambient water were studied using sponge-microbe consortia, ambient water and isolated bacteria from sponge and water. This sponge on an average filters 15-25 litres of ambient water per day per gram of sponge weight (Dahihande, personal communication). Prokaryotes which form more than 40% of the biomass of the sponge, release labile dissolved organic matter (DOM) from particulate matter. Dissolved organic matter partially fuels the nutritional requirement of the sponge. The functional role of prokaryotes associated with the sponge in the degradation of organic matter and uptake of organic and inorganic substrates, methane oxidation, nitrification, denitrification and sulfate reduction were investigated. Actinobacteria were tested for their role in the defence mechanism of the sponge.

3.4.3.1. Degradation of organic matter and uptake of organic and inorganic substrates

3.4.3.1.1. Metabolic potential

Metabolic potential of microbial consortia in mesohyl and cortex of sponge and ambient water were studied using Biolog Ecoplates. Biolog Ecoplate for the study of utilisation of 31 substrates under different categories like amines, amino acids, carbohydrates, carboxylic acids, polymers and other miscellaneous substrates, overcomes the drawbacks of the time consuming culture-based analyses or biochemical tests (Schutter and Dick, 2001; Preston-Mafham et al., 2002) and gives a better understanding of the metabolic potential and preferences of sponge-associated microbial consortia. Samples (sponge homogenate and ambient water) were inoculated into wells of the Biolog Ecoplates using Eppendorf multichannel pipettor. The microplates were then incubated at room temperature. During incubation, cells can utilise substrate in the well and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple colour. A cell density of 10^{5-8} cells ml^{-1} was required to develop colour in the Ecomicroplate (Preston-Mafham et al., 2002). The optical density was noted daily for 4 days with BMG Labtech fluorometer. The 0-day reading was subtracted from the remaining readings. The average well colour development (AWCD) is the substrate utilisation and was calculated using the following equation.

$$AWCD = \frac{\sum_{i=1}^{31}(Ci-R)}{31}$$

where *Ci* is the optical density (OD_{595}) of each sample well and *R* is the OD_{595} of control well (Garland and Mills, 1991). In order to test the difference in the utilisation of substrates, the data on the 4th day was analysed using the following equation.

$$X_{si}=C_i\text{-}R\,/\,AWCD$$

Where C_i is the optical density after substrate standardisation. $X_{si}=0$ indicates that there is no utilisation of the substrate by microbial consortia, $0<X_{si}<1$ indicates weak utilisation and $X_{si}>1$ indicates effective utilisation (Xi et al., 2005).

3.4.3.1. 2. Extracellular enzyme production

Bacterial isolates were tested for 6 polymer-degrading enzymes, *viz*. amylase, DNase, lipase, caseinase, gelatinase and urease. The bacterial cultures were spot inoculated onto respective media with substrates for each enzyme and incubated for 24 to 72 hours, depending on the growth rate of the bacteria at 25±2 ºC. After incubation, the plates were checked for the production of various enzymes and results were recorded.

3.4.3.1.3. Utilisation of 8 simple carbon sources

For simple carbon source utilisation, 96 well plates were used. Each well contained basal medium and a 1% simple carbon sources (glucose, arabinose, sucrose, raffinose, inositol, mannitol, glycerol or lactose) supplemented with phenol red indicator. Each well was inoculated with bacterial culture. The plates were incubated at 25±2 °C for 24 hours. Growth and colour change from red to yellow was noted.

3.4.3.1.4. Utilisation of organic carbon compounds under aerobic and anaerobic condition

Isolates from sponge (mesohyl and cortex) and ambient water were tested for their potential to utilise 46 organic compounds using Biolog GEN III and Biolog AN microplates (Biolog, Hayward, CA). For aerobic utilisation, the bacterial isolates were grown on BUG agar medium and then suspended in the special gelling inoculating fluid at the recommended cell density whereas for anaerobic, the isolates were suspended in anaerobic inoculating fluid according to manufacturer's instructions. About 100 µl of the cell suspension per well was inoculated into the microplates and incubated to allow the formation of the metabolic fingerprint. Colour formation was quantified spectrophotometrically, generating a carbon substrate utilisation pattern (CSUP). Based on chemical composition, the substrates of microplates were classified into different

categories (Amine/ amino acids, monosaccharides, oligosaccharides, sugar derivatives, carboxylic acids and other miscellaneous substrates).

3.4.3.1.5. Uptake of organic substrates

Heterotrophic uptake of simple molecules by the sponge-microbial consortia (mesohyl and cortex) and ambient water was measured by using ^{14}C-labeled glucose, glutamic acid and leucine (Wright and Hobbie, 1965). The substrates glucose and glutamate were added separately to sample volume of 30 ml in a narrow mouth 100 ml flask. To each of the flask 10, 50 and 100 µl of each substrate was added. The flask was tightly corked and incubated in the dark for 2 hours. The reaction was terminated by the addition of 0.5 ml of concentrated HCl. The samples were then filtered through 0.22 µm polycarbonate filter. The filters were rinsed with ASW (0.22 µm filtered). Dried filters were placed in scintillation vials. For leucine uptake, ^{14}C-leucine (20 nM final concentration) was added to 30 ml of sample (sponge mesohyl and cortex tissues (< 0.5mm) in ASW and ambient water) and incubated for one hour at an ambient temperature of 25±2 ^{0}C. The activity was stopped by the addition of 50% TCA for 5 minutes. The samples were then filtered through 0.22 µm membrane filter and rinsed with 5% cold TCA for 5 minutes followed by 80% cold ethanol. The dried filters were placed in scintillation vials filled with a scintillation cocktail. Radioactivity in filter samples was counted on a Beckmann Beta Scintillation Counter. Glucose and glutamic uptake was calculated by,

$$\text{Rate of uptake} = CA/\mu ct$$

Where C is the radioactivity of the filtered organism

A is the concentration of the added substrate

µ is the number of µCi added to the sample

c is the radioactivity of the 1µCi C^{14}

t is the time of incubation

Leucine uptake was calculated using the conversion factor of 3.1 kg C per mol of leucine incorporated (Simon and Azam, 1989).

3.4.3.1.6. Uptake of inorganic substrates

Inorganic uptake by mesohyl, cortex and ambient water microbial consortia were estimated using modified method of van Duyl et al. (2008). Into each vial which contained 1 g mesohyl or cortex tissues, 10 ml sterilised ASW containing 50 µCi ^{14}C-labeled sodium bicarbonate was added leaving negligible headspace to avoid degassing of CO_2. In the

case of ambient water, the vials were filled with ambient water to which 50 µCi ^{14}C-labeled sodium bicarbonate was added. All the vials were incubated in the dark at room temperature and terminated at 0 hour (control) and 4 hours with 0.22 µm filtered formaldehyde (2% final concentration). Subsequently, the samples were filtered through a 0.22 µm pore size polycarbonate filter and the free ^{14}C-labelled and unlabelled CO_2 molecules were fumed from the sample with concentrated HCl for 30 to 35 minutes. Radioactivity in filtered samples was counted on a Beckmann Beta Scintillation Counter.

3.4.3.2. Methane oxidation

Methane oxidation rate by bacterial isolates and sponge- microbial consortia was measured. The bacterial isolates in exponential phase were used for methane oxidation study. Aliquots of 100µl bacterial isolates, sponge homogenate or ambient water were dispensed into the 20 ml gas-tight vials containing 10ml of autoclaved ambient water. To this, 100 µl of methanogenic inhibitor (2-Bromoethanesulfonate) was added (Zinder et al., 1984) and 2ml of methane gas was injected into the vials. The vials were capped with butyl stoppers and the slurry was vortexed for 5 seconds. The vials were incubated for 4 days. Zero-day reading was taken on the same day. Methane was quantified using head space method on a Shimadzu 2010 Gas Chromatography (GC) equipped with a 30 m long mega bore (0.53 mm) GS-Q- coated quartz capillary column and flame ionisation detector (FID). The injector temperature was 100 ºC and detector temperature was at 150 ºC. Nitrogen was used as a carrier gas at a flow rate of 9 ml minute^{-1} and the oven temperate was run isothermally at 60 ºC. The GC was calibrated using reference mixture of methane standards (concentration range of 1─5 g l^{-1}) from M/S Alchemie Gases Ltd. The methane oxidation was measured by incorporation method as described by Bastviken et al. (2002) wherein the depletion of methane is quantified using the gas chromatography. The methane oxidation was measured as by Guerin and Abril (2007) as follows.

$CH_{4ox} = [CH_4] \times SCH_{4ox} \times [O_2]/([O_2]+Km\ (O_2)) \times I_z$

Where $[CH_4]$ is the concentration of methane

SCH_4ox is the specific oxidation of methane

$[O_2]$ is the concentration of oxygen

Km of oxygen for methane oxidation which varies from 0.14 to 58mmol L^{-1}

I_z is the inhibition of methanotrophic activity by light

3.4.3.3. Nitrification

Nitrification experiment was carried out according to Hoffman et al. (2009). Sponges of similar body mass (~16 g) which showed a visually healthy appearance were used for the experiments. For the nitrification experiment, mesohyl and cortex tissue and ambient water were placed in 500 ml of natural, unfiltered and autoclaved ambient water amended with 10 µM NH_4SO_4. Flasks with aerators and magnetic stirrers were placed in a temperature-controlled room at 25 °C in the dark. Water was sampled over a time-course of 24 hours and immediately frozen at -80 °C until nutrient analyses were carried out. Ammonium, nitrite and nitrate concentrations were measured using Continuous-Flow Autoanalyser (Hansen and Grasshoff, 1983).

3.4.3.4. Denitrification

Denitrification rate by microbial consortia from the sponge and ambient water and bacterial isolates was measured by the acetylene inhibition technique based on the inhibition of the conversion of nitrous oxide to Nitrogen gas (Sorensen, 1978). About 100 µl of sponge homogenate, ambient water or bacterial culture was transferred aseptically to sterile 20 ml headspace vials containing seawater. The vials were capped with butyl stoppers and vortexed for 5 seconds. The vials were purged with high purity nitrogen gas for 10 minutes to induce anaerobic conditions. Acetylene gas at 20 kPa (Bonin et al., 2002) was injected into the headspace to avoid nitrous oxide production by nitrification and its reduction by denitrification (Castro-Gonzalez and Farias, 2004). The vials were incubated in the dark for 2 days. At the end of the incubation period, bacterial activity in the vials was terminated by using 0.1 ml of 1M $HgC1_2$. Nitrous oxide in the headspace was analysed using a Shimadzu 2010 Gas Chromatograph fitted with an electron capture detector and Porapak Q column (Porapak Q 1/8" SS column, 3.05 m length, 80/100 mesh). The oven temperature and detector temperatures were 40 °C and 300 °C, respectively. High-purity nitrogen gas at a flow rate of 35 ml $minute^{-1}$ was used as a carrier gas. Total nitrous oxide in the vial was calculated based on the equation stated by Bleakley and Tiedje (1982) as follows.

$$M=Cg(Vg+Via)$$

where M=the total amount of nitrous oxide in the water plus gas phase

Cg=concentration of nitrous oxide in the gas phase

Vg=volume of gas phase

Vi= volume of liquid phase

a = Bunsen absorption coefficient

3.4.3.5. Sulfate reduction

Sulfate reduction activity was measured by using zinc acetate method. For microbial consortia, sponge homogenate and ambient water samples were inoculated into SRB liquid media. The media with inoculum was dispensed up to the rim of the test tube and sealed with paraffin oil. The medium without inoculum was kept as control. The tubes were incubated for 5 days under anaerobic condition. After incubation, the activity was arrested by 2% zinc acetate solution. To 1ml test solution, 5ml of 0.2% N, N-Dimethyl paraphenylene diamine sulfate and 0.25 ml of 10% ferrous ammonium sulfate were added and incubated for 10 minutes. OD_{670nm} was measured after making up the volume to 50 ml with boiled and cooled distilled water. The zero-day reading was subtracted from the 5^{th} day reading. The sulfide concentration was calculated using the formula,

[Sulfide] µM day^{-1} = OD/days of incubation x 78.04

3.4.3.6. Antagonistic interaction of AB against THB

Preparation of AB

AB (11 isolates) representing mesohyl and cortex were inoculated into ISP- 4 medium and incubated at 30 °C for 2 weeks for maximum growth. The cells were removed by centrifugation at 2000 *g* for 20 minutes. The supernatant obtained was filtered through 0.2 µm filter to remove the spores. The filtrate was used for antagonistic study against THB.

Preparation of THB suspension

THB consisting of 75 isolates from mesohyl, cortex and ambient waters were grown in Muller Hinton broth and incubated at 30 °C for 12-18 hours. The bacterial cells were pelleted by centrifugation at 8000 *g* for 10 minutes. The pellet was re-suspended in sterile saline solution and the bacterial density was adjusted to 10^6 cells ml^{-1} by comparing with McFarland standard.

Antagonistic assay

The antagonistic assay was performed according to Sarker et al. (2007) with modification. This assay used resazurin as growth indicator (McNicholl et al., 2007). This blue dye becomes pink when reduced to resorufin by oxidoreductases within viable cells. It is further reduced to colourless hydro resorufin. For the assay, 50 µl AB filtrate and 10 µl

resazurin (6.75 mg ml^{-1}) solution were added to the wells of a microtitre plate. To this, 30 µl Muller Hinton broth was added followed by 10 µl of THB suspension to reach a final bacterial density of 10^5 cells ml^{-1}. Three sets of control were used, *viz.*, 1) broad spectrum antibiotic ciprofloxacin, 2) without AB filtrate and 3) without THB in wells of the microtitre plate. The experimental plates were incubated at 25 °C for 24 hours. Any colour changes from blue to pink or colourless indicated growth. No change in blue colour indicated inhibition of bacterial growth by AB.

3.4.4. LINKING PHYLOGENY TO FUNCTION

Functions of the microbial consortia associated with the sponge (mesohyl and cortex) were analysed by comparing the orthologs of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway in KO databases. This database is a resource that integrates genomic, chemical and systemic functional information. In particular, gene catalogs from completely sequenced genomes are linked to higher-level systemic functions of the cell, the organism and the ecosystem. In KEGG, molecular-level functions are stored in the KO (KEGG Orthology) database and associated with ortholog groups in order to enable extension of experimental evidence in a specific organism to other organisms. Genome annotation in KEGG is ortholog annotaion, assigning KO identifiers (K numbers) to individual genes in the GENES database. The sequence similarity search against KEGG GENES is a search for most appropriate K numbers. Once K numbers are assigned to genes in the genome, the KEGG pathways maps and KEGG modules are automatically reconstructed, enabling biological interpretation of high-level functions (Kanehisa et al., 2011).

3.4.5. APPLICATION OF SPONGE-ASSOCIATED MICROBIOTA

3.4. 5.1. Aquaculture wastewater treatment

In order to test the efficiency of sponge-holobiont to filter and utilise the nutrients from the aquaculture wastewater, an aquaria experiment was set up. Healthy sponges were collected along with substratum and transported to the laboratory and acclimatised in ambient water for one month. After acclimatisation, the sponges in replicates were incubated in one litre of 50% and 100% shrimp aquaculture wastewater in aerated condition. One set of sponges were incubated in ambient water, as a control. Another set of control was set up without sponges. Aliquots of water samples and sponges were withdrawn at 0 day and 10^{th} day and changes in the total suspended matter (TSM), ammonium, nitrite, nitrate, phosphate and

silicate concentrations of the wastewater samples were measured as described in section **3.3**. Metabolic profiling of bacterial community was also analysed from wastewater and sponge, as described in section **3.4.3.1.1.**

3.4. 5.2. Azo dye decolourisation

Decolourisation assay

The bacterial isolates were inoculated into Zobell marine broth and incubated for 24 hours in a shaker incubator at 25 °C. The culture broth was centrifuged at 10000 *g* for 10 minutes. The pellet was resuspended in physiological saline and cell density to 10^6 cells ml^{-1} was adjusted and this was used as inoculum for decolourisation studies. The stock solutions of dyes, Amido black and Congo red (Himedia, India) were prepared (1 g l^{-1}). The above bacterial suspension (10 ml l^{-1}) was inoculated into Zobell marine broth containing 50 mg l^{-1} of the dye and incubated for 2 days under static condition, as azoreductase is oxygen sensitive. Sterile cell-free medium and sterile medium with heat-killed cells served as controls. All experiments were performed in triplicate. Decolourisation was monitored by measuring the absorbance of the supernatant at the maximum absorbance of the respective dye (Amido black at 618 nm and Congo red at 498 nm) and the absorbance of the respective control. The efficiency of decolourisation was calculated using the formula,

$$Decolourization\ efficiency(\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbace} \times 100$$

The bacterium which showed >50% decolourisation of both dyes was selected for detailed studies. It was identified as *Yangia pacifica* (Roseobacter clade) based on 16S rRNA gene sequencing (EMBL accession no: LK022801). Dye decolourisation study was carried out at different pH, temperature and concentrations of dye prior to the decolourisation assay. Growth was measured at 600 nm. The decolorisation potential was determined as mentioned above. For viable cell count, 2 ml of the culture broth was removed and incubated with a cocktail containing piromedic, pipemedic, nalidixic acid and yeast extract for 6 hours (Kogure et al., 1979) and fixed with formaldehyde (4% final concentration). The number of viable cells was counted (Hobbie et al., 1977) and expressed as cells ml^{-1}. Growth and decolourisation were monitored daily for 7 days. To understand the mode of decolourisation, clean glass pieces (6x6x1 mm) were incubated for 12 hours in the decolourisation culture flasks. The glass pieces were retrieved and subjected to stepwise dehydration in increasing ethanol concentration (30, 50, 70, 90 and 100%). The samples were then air dried, mounted on a stub and sputter coated with Au/Pd. The specimens were then visualized with a Scanning Electron Microscope (JEOL JSM-5800).

3.6. STATISTICAL ANALYSIS

The biological data were log transformed before statistical analyses. One way ANOVA and two-way ANOVA were performed to determine the significant variation of parameters in sponge and water and between different variables. Resemblance analysis between the variables was carried out with Bray-Curtis similarity measure for biological parameters and Euclidian distance for environmental variables. Cluster analysis was carried out with 5% significance level with SIMPROF test with 999 permutations. Alpha diversity in the culturable bacteria was measured using diversity indices such as Shannon-Wiener and Simpson (Multivariate statistical Package, Kovacs Computing). The significance of variation of alpha diversity in samples was tested using t-test. One way analysis of similarity (ANOSIM) was used to compare multiple parameters to test whether samples were related to each other within a group, more than to other groups. Non-metric multidimensional scaling (MDS) and hierarchical agglomerative clustering were carried out using Primer 6 software (Primer-E Ltd, UK) wherever required. Principal Component Analysis (PCA) and Principal Coordinate Analyses (PCO) were carried out to find out the major component driving the variation of bacterial community based on different traits. SIMPER analysis was carried out to check the similarity between data sets. To statistically evaluate the significance of the relationship, Spearman's rank pair-wise correlation was carried out (Statistica 8). Network analyses were carried out in Cytoscape. RELATE analysis was carried out to check the consistency between the small data set with the total data (PRIMER). The antagonistic potential of cortex and mesohyl tissues of the sponge was calculated as the ratio of the number of positive antagonistic interactions by AB and their total number in cortex and mesohyl, respectively. Metabolic diversity indices were calculated using the formula given below. The Shannon index (H') and Shannon evenness (*E*) were calculated using the following equation

$$H' = -\sum_{i=1}^{31} Pi \ln Pi$$

Where $Pi = Ci / \sum_{i=1}^{31} (Ci - R)$

$$E = \frac{H'}{\ln S}$$

Where *S* is the number of positive wells in each sample

Simpson index *(D)* was calculated as

$$D = \frac{1}{\sum_{i=1}^{31} [ni(ni-1)/Ni(Ni-1)]}$$

Where $ni = Ci - R$

$Ni = \sum_{i=1}^{31} (Ci - R)$

CHAPTER 4

ABUNDANCE & DIVERSITY OF BACTERIA

4.1. INTRODUCTION

Bacteria are the most important and abundant biological component of an ecosystem. Understanding the abundance and diversity of bacteria is very critical as they are the key players in biogeochemical cycling and food web dynamics (Cho and Azam, 1990; Pomeroy et al., 1991; Tian et al., 2000; Cotner and Biddanda, 2002; Azam and Malfatti, 2007; Fenchel et al., 2012). Most of the studies carried out on planktonic bacteria have shown that the environmental parameters largely dictate their abundance and diversity (Ghiglione et al., 2005, 2007; Subina et al., 2012; Malik et al., 2015). It is also equally important to explore bacteria associated with higher organisms to elucidate the ecological, physiological, and morphological attributes of these bacteria to the host as well as the ecosystem and to test their ability to carry out different functions. Studies on abundance and diversity of sponge-associated bacteria are still in its infancy. A few studies have demonstrated that sponges with similar ecological and evolutionary characteristics may share more similar microbes than those hosts with different characteristics (Thoms et al., 2003; Bjork et al., 2013; Kennedy et al., 2014). Some sponges maintain a stable bacterial community across spatial and temporal scales (Friedrich et al., 2001; Erwin et al., 2012) while others are affected by factors such as environmental changes, geographical variation (Webster and Hill, 2001; Taylor et al., 2005; Webster and Blackall, 2009), pollution (Lesser et al., 2016), transfer into artificial condition or cultivation (Mohamed et al., 2008b) or disease-related physiological changes (Webster et al., 2008). Planktonic bacteria also influence the associated bacteria in sponges as sponges filter a large volume of water and retain particles <60 µm, that include the bacterioplankton. Most of the sponge species, known as high microbial abundance (HMA) sponge or bacteriosponge harbour up to 10^{9-10} bacteria g^{-1} of sponge which constitutes up to 40% of their biomass (Hentschel et al., 2006). They possess a denser mesohyl, a more complex aquiferous system with narrower and longer water canals that allow slower filtration rates (Weisz et al., 2008). Yet, the factors that influence sponge-microbe association remain poorly understood (Noyer et al., 2014).

A large numbers of bacteria with diverse morphology have been reported from marine sponges (Reiswig, 1975b; Vacelet, 1975; Wilkinson, 1978a; Wikinson et al., 1981; Willenz et al., 1989; Santavy and Colwell, 1990; Dieckmann et al., 2005; Lafi et al., 2005; Olson and McCarthy, 2005; Sfanos et al., 2005). To understand the sponge- microbial relationship, their eco-physiological and environmental functions, and their potential applications, an

informative approach is to isolate the associated bacteria and test physiological and biochemical processes and also their taxonomic status. Cultivation provides access to genetic, biochemical and physiological characteristics of each microorganism that may not be revealed by molecular analyses. Microbes associated with physiologically and phylogenetically diverse sponges, *Ceratoporella, Aplysina, Ropaloides, Dendrilla, Dysidea, Sigmadocia etc.* from Indian Ocean, Mediterranean and Carribean region have been identified using cultivation-dependent methods and revealed bacterial community dominated by a single bacterium or diverse bacteria (Wilkinson et al., 1981; Santavy et al., 1990; Hentschel et al., 2001; Webster and Hill, 2001; Selvin et al., 2009a; Feby and Nair, 2010 and references therein). However, there is paucity of knowledge on the physiology of these bacteria because of the difficulty in getting their pure culture from the sponge. This is because majority of symbionts are unculturable and the culturable heterotrophic bacterial community in sponges constitute only 0.1–0.2% of the total bacterial community (Burja et al., 1999; Webster and Hill, 2001; Friedrich et al., 2001), which is consistent within the context of natural microbial ecosystems (Staley and Konopka, 1985; Amann et al., 1995). Hence, to get information on the actual microbial population in the sponge, electron microscopy has been used but this technique provided only morphological information (Manz et al., 2000; Usher et al., 2001). Methods using the 16S rRNA gene such as fluorescence *in situ* hybridization, amplified rRNA gene restriction analysis, terminal-restriction fragment length polymorphism and 16S rRNA sequencing-based phylogenetic analysis (Friedrich et al., 1999, 2001; Webb and Maas, 2002; Margot et al., 2002; Lee et al., 2003) make it possible to identify a dominant or a particular group of bacteria in the sponge (Amann et al., 1995). 16S rDNA clone library strategy (Webster et al., 2001a) is a better method to explore and compare bacterial community in sponges, but is a time-consuming method. Next generation sequencing of metagenome gives the holistic picture of the total bacteria present in the sponge. The studies have ranged from understanding a single group to total diversity of bacteria associated with sponges (Erwin et al., 2015; Gao et al., 2014, 2015). More than 30 bacterial phyla have been identified in sponges using next generation sequencing (Easson and Thacker et al., 2014; Luter et al., 2015; Gao et al., 2015). Most of these studies showed the dominance of Proteobacteria in sponges (Luter et al., 2015; Gao et al., 2015), though some other sponges showed the dominance of Firmicutes and Chloroflexi (Schmitt et al., 2012b; Erwin et al., 2015; Jasmin et al., 2015).

In this chapter, the abundance and diversity of bacteria associated with the intertidal sponge, *Cinachyra alloclada* by both culture-dependent and culture-independent methods will be discussed. The intertidal region acts as an interface between land and ocean and hence plays an important role in the exchange of various organic and inorganic matters between the two and acts as a buffering zone between them. Also, studying the physicochemical properties of water in this region is important for understanding the abundance, diversity, distribution and interactions of intertidal organisms and their activities. This would facilitate the role of these organisms in biogeochemical cycling and other processes. The materials and methods used for studying water characteristics and abundance and diversity of bacteria are given in Chapter 3 (Sections-3.2, 3.3.1. and 3.3.2).

4.2. RESULTS AND DISCUSSION

4.2.1. WATER CHARACTERISTICS

The ambient water characteristics are given in Table 4.1.

Table 4.1. Characteristics of the ambient water

Parameters	Values (Average ± SD)
Temperature (°C)	28.1±2.1
Salinity	34.6±1.7
pH	8.1±0.2
Eh (mv)	229±16
DO (mgl^{-1})	5.1±1.2
Nitrite (µM)	0.8±0.6
Nitrate (µM)	2.7±0.9
Ammonia (µM)	2.3±0.9
Phosphate (µM)	0.2±0.1
Suspended matter ($mg\ l^{-1}$)	33±4
Carbohydrate ($mg\ l^{-1}$)	6.4±3.1
Protein ($mg\ l^{-1}$)	2.2±0.7
Lipid ($mg\ l^{-1}$)	3.2±0.7

Samples were collected at the lowest tide of the month. Water temperature ranged from 25.9 to 32.0° C. Low temperature was noted during the morning sampling as compared to

afternoon and evening sampling. The higher average water temperature in the ambient water might be because of their close proximity to land and shallow depth which permits rapid temperature changes in response to atmospheric temperature (Williams, 1966), during low tide. Salinity and pH did not change significantly. Salinity was 34.6±1.7 and pH ranged from 7.9 to 8.3. Nitrate and ammonium concentrations in the ambient water were almost equal, ~ 3, and 12 times higher than nitrite and phosphate, respectively. The concentration of carbohydrate (6.4±3.1 mg l^{-1}) was higher than lipid (3.2±0.7 mg l^{-1}) and protein (2.2±0.7 mg l^{-1}) in the labile organic matter. Several studies have highlighted the role of water characteristics in regulating the distribution, growth and activities of many marine bacteria (Ghiglione et al., 2005, 2007; Subina et al., 2012; Malik et al., 2015). The water characteristics of Anjuna, Goa were within the same range as monitored in 2010 (Alkawri and Ramaiah, 2010; Khodse et al., 2010).

4.2.2. ABUNDANCE OF BACTERIA

4.2.2.1 Culture-independent bacterial abundance

Total and viable bacterial count

Total bacterial abundance in *C. alloclada* tissue varied between $8.0x10^8$ and $1.2x10^{10}$ cells cm^{-3} ($6.7x10^8$-$1.0x10^{10}$ g^{-1}). Viable count in sponge tissue was in the range of 10^8 to 10^9 cells cm^{-3}. The number of bacteria (total and viable) in the ambient water was 2─3 orders lower than that in the sponge tissue and this difference was highly significant ($p<0.001$). The cortex of the sponge harboured 2 times higher the total number of bacteria ($7.7±3.8x10^9$ bacteria cm^{-3}) than that in the mesohyl tissues, but the vice versa was in viable counts (Figure 4.1). However, this difference was not significant. The total bacterial abundance obtained is comparable with those reported in other sponges such as, *Tedania ignis* ($12.3 x 10^8$ cells g^{-1} to $5.0 x 10^9$ cells g^{-1}, Jouett et al., 2015), *A. aerophoba* ($6.4±4.6 x 10^8$ bacteria g^{-1}, Friedrich et al., 2001), *C. nicholsoni* (7.9-$11x10^{10}$ bacteria cm^{-3}, Santavy et al., 1990), and in *Rhopaloides odorabile* ($1.5x10^8$–$1.9x10^{10}$ cells ml^{-1} of sponge extract, Webster and Hill, 2001; Webster et al., 2001b). But, there are no reports on viable bacterial counts from sponges for comparison. However, from the same region, viable count of one order less than the total count has been reported in seawater and sediment (Gonsalves et al., 2011; Malik et al., 2015). In the Mediterranean sponges, higher number of bacteria has been reported in the mesohyl tissue than the surface tissues (Gerce et al., 2011) whereas in the sponge, *Polymastia janeirensis* which is attached to the sediment, higher bacterial density was at the base than the interior (Turque et al., 2008).

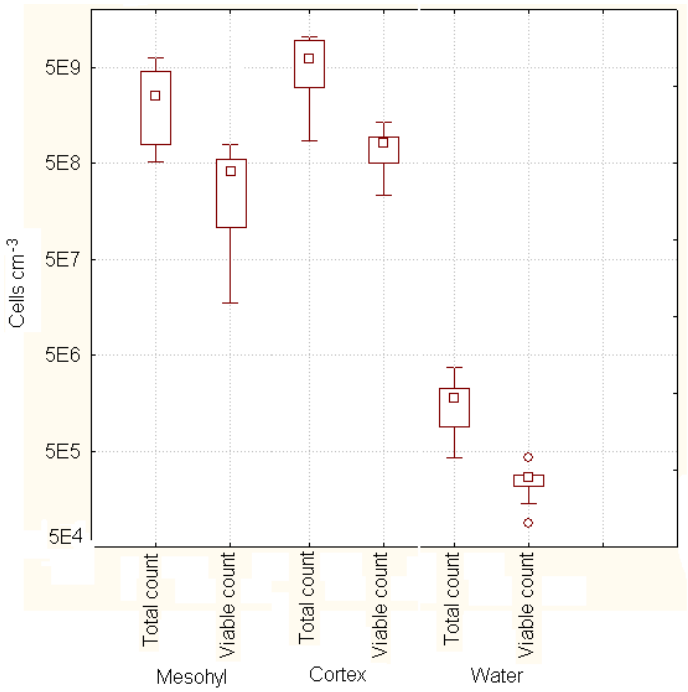


Figure 4.1. Box –whisker plot of total and viable bacteria in mesohyl, cortex and ambient water

The higher number of total bacteria in the cortex of *C. alloclada* might be due to the morphological and anatomical features. Unlike other sponges this sponge lacks the central canal. Also, being intertidal in habitat, this sponge is always covered with sediment which allows sediment-derived bacteria to invade into the cortex of the sponge. The presence of a high number of bacteria (10^{8-9} bacteria g^{-1}) in this species suggests that the tetillid sponge, *C.alloclada* comes under high microbial abundance (HMA) sponge (Hentschel et al., 2006). The microscopic view of bacterial cells (1000X magnification) are given in Figure 4.2.

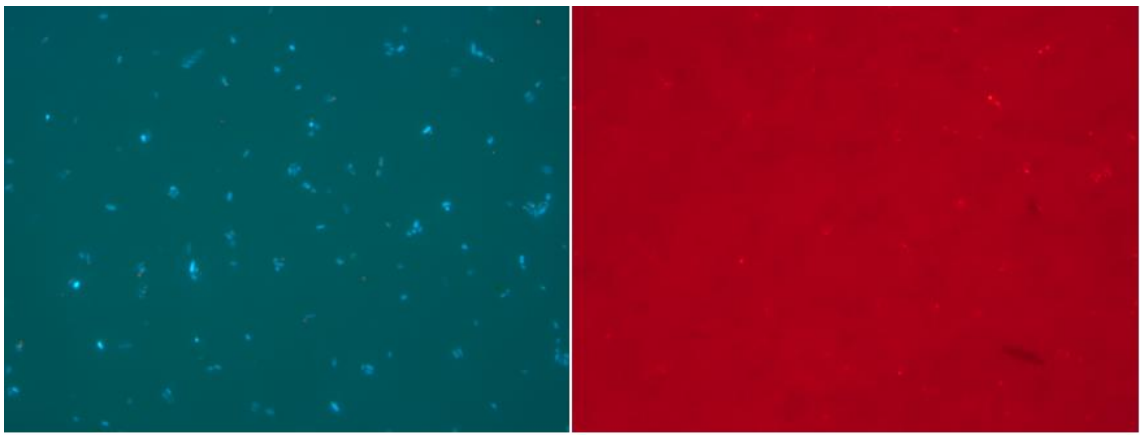


Figure 4.2. Total bacterial count (DAPI stained cells in blue) and viable count (CTC stained cells in red)

This high number may be due to the complex aquiferous system which increases the contact time between seawater and the bacteria, to utilise the particulate matter (Weisz et al., 2008). Also, bacteria filtered from ambient water by the sponge which can resist the host's digestive process and immune response can successfully inhabit sponges (Wilkinson, 1987). The electron microscopy and software assisted microscopy studies of HMA sponges showed that bacteria accounted for 20-70% of the sponge biomass (Vacelet, 1975; Willenz and Hartman, 1989; Ribes et al., 2012). This suggests that *C. alloclada* biomass might have been contributed significantly by the associated bacteria.

4.2.2.2. Culture-dependent bacterial abundance

4.2.2.2.1. Organotrophic bacteria: Organotrophic bacteria in this study comprises of total heterotrophic bacteria (THB), Actinobacteria (AB), anaerobic bacteria (AnB), sulfate-reducing bacteria (SRB) and nitrate-reducing bacteria (NRB).

Total heterotrophic bacteria (THB)

Different media with different nutrient concentrations were used for THB, as this group consists of bacteria with highly variable nutrient requirements [Marine agar (ZMA), Nutrient agar (100% NA), half strength nutrient agar (50% NA) and low strength nutrient agar (10% NA)].The above mentioned media were divided into 2 sets: one set amended with sponge extract in order to resemble as much as possible the nutrients that prevailed in the sponge for maximum retrieval of bacteria and other set of media without sponge extract. THB in the sponge tissue was 10^6–10^7 CFU cm^{-3} (Table 4.2).

Table 4.2. Abundance of THB in mesohyl and cortex of sponge and ambient water

		Bacterial abundance (CFU cm^{-3})			
		10% NA	50% NA	100% NA	100% ZMA
Mesohyl	Without extract	$2.8\pm2.6\times10^6$	$6.85\pm6.1\times10^6$	$4.0\pm2.1\times10^7$	$1.1\pm0.9\times10^7$
	With extract	$4.66\pm2.9\times10^7$	$5.0\pm4.5\times10^7$	$4.35\pm1.8\times10^8$	$7.3\pm1.7\times10^7$
Cortex	Without extract	$2.5\pm2.1\times10^7$	$3.4\pm3.3\times10^7$	$2.2\pm1.9\times10^7$	$2.4\pm1.8\times10^7$
	With extract	$2.0\pm1.7\times10^8$	$3.3\pm2.8\times10^8$	$1.8\pm1.4\times10^8$	$1.3\pm1.0\times10^8$
Water		$2.6\pm2.3\times10^4$	$1.1\pm0.8\times10^4$	$6.5\pm2.3\times10^3$	$2.5\pm2.4\times10^4$

The abundance of THB in the sponge and ambient water significantly varied with concentration of nutrients ($p<0.01$). In sponge, a higher number of bacteria were obtained in high nutrient media with higher retrievability ($4.0\pm2.1 \times 10^7$ CFU cm^{-3} or $3.3\pm1.8 \times 10^7$ CFU g^{-1}) from mesohyl region. In the cortex, retrieval of $3.4\pm3.3 \times 10^7$ CFU cm^{-3} ($2.8\pm2.7 \times 10^7$ CFU g^{-1}) occurred in half strength nutrient agar. This can be attributed to the prevailing nutrient-rich conditions in the sponge (Selvin et al., 2009a). Marine growth media supplemented with aqueous sponge extract increased culturability of bacteria in *C. alloclada* by one order and this gives a scope of higher retrieval of sponge-specific bacteria. The media supplemented with sponge extract gave one order higher bacterial number than media without sponge extract (Table 4.2). Similar observation was also made by Olson et al. (2000). In water, the maximum abundance of $2.6\pm2.3 \times 10^4$ CFU cm^{-3} was obtained in low strength nutrient agar as facultative oligotrophs are the predominant groups in coastal waters (Eguchi and Ishida, 1990). The high number of culturable bacteria in sponge was due to sequestration of seawater-derived bacteria that might have been enriched by the stable and nutritionally rich microhabitat in the sponge. The sponge may provide distinct microenvironments as ecological niches for different bacterial populations. The supplementation of host sponge extract might create a nutritional niche required for the growth of sponge-specific bacteria (Selvin et al., 2009a). From this study, it can be envisaged that the cultivation potential of sponge-associated bacteria could be increased considerably by the addition of sponge extract. Webster et al. (2001b) isolated previously uncultured bacteria by adding sponge extract to marine agar, however, it remains uncertain whether these are associated bacteria or symbionts and might perhaps be a small percentage of total microbial symbionts.

Selvin et al. (2009a) reported 10^5 CFU cm^{-2} (*ca* 10^7 cm^{-3}) bacteria in the sponge, *Dendrilla nigra* (east coast of India) whereas in sponges, *Sigmadocia fibulata* and *Dysidea granulosa* from Lakshadweep (west coast of India), Feby (2011) reported 10^6 to 10^7 bacteria g^{-1} of tissue. These numbers were also comparable with the earlier study from Great Barrier Reef (Webster and Hill, 2001). The difference in bacterial number has been reported in different sponge species such as the Caribbean sponge, *Ceratoporella nicholsoni* and the Mediterranean sponge, *Aplysina aerophoba* (Santavy et al., 1990; Friedrich et al., 2001). THB represented only a small fraction of the total count in *C. alloclada* (0.1─0.2%). Low representations of the total bacterial population have been reported in sponge *Aplysina aerophoba (*0.15%), *Rhopaloides odorabile* (0.1–0.23 %), and *Ceratoporella nicholsoni*

*(*3–11%) respectively (Santavy et al., 1990; Friedrich et al., 2001; Webster et al., 2001b). The abundance of THB showed a significant difference between the sponge cortex and the mesohyl. Sponge cortex harboured ~ 2x higher THB ($2.4\pm1.8x10^7$ CFU cm^{-3}) than the mesohyl ($1.1\pm0.9x10^7$ CFU cm^{-3}) ($p<0.001$). THB abundance of 10^5 CFU ml^{-1} in the seawater around Goa (west coast of India) has been reported earlier (Subina et al., 2012; Malik et al., 2015; Jain et al., 2014). ANOVA showed that THB in the ambient water ($2.5\pm2.4x10^4$ CFU cm^{-3}) was significantly different (2─3 orders, $p <0.001$) from that in the sponge. Such differences have been reported in sponges *S. fibulata* and *D. granulosa* from coral reefs of Lakshadweep (Feby, 2011) and *D. nigra* from the east coast of India (Selvin et al., 2009a).

Actinobacteria (AB)

AB is the major secondary metabolite-producing group in the bacterial domain. Studies on sponge-associated AB are limited compared to sediment and water as culturing of these bacteria in the laboratory to study the activity is a difficult task (Ward and Bora, 2006). The numbers of AB present in the mesohyl and cortex tissues of the sponge ranged between $1.9x10^2$ ─$1.3x10^4$ CFU cm^{-3} and $1.7x10^2$─$2.0x10^4$ CFU cm^{-3}, respectively and the difference was significant ($p<0.05$). Culture-independent studies have shown that AB is an important and a major component of the sponge's microbial assemblages (Webster et al., 2001a; Montalvo et al., 2005; Hentschel et al., 2006; Piel, 2006; Li and Liu, 2006). AB from water was detected only in ISP-4 and CSA media and was lower in number than sponge tissues (Figure 4.3). Sponge-associated AB was higher in ISP-4 and lower in MSA. Such variation in retrieval counts in different media has been reported by Selvin et al. (2009a). They found a higher abundance of AB associated with *D. nigra* in MSA ($3x10^1$ to $1x10^2$ CFU) than in CSA ($<2x10^1$CFU).

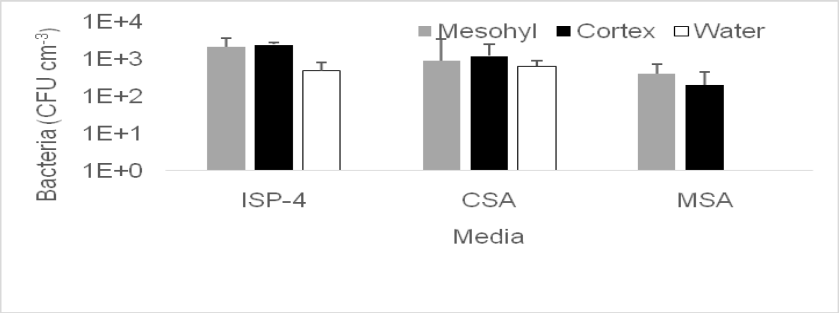


Figure 4.3. Abundance of Actinobacteria in different media

Anaerobic bacteria (AnB)

Anaerobic bacteria (AnB) in the sponge and ambient water were 10^5 and 10^2 CFU cm^{-3}, respectively (Table 4.3). AnB showed significant variation between mesohyl and cortex tissues of the sponge as well as between sponge and ambient water. Higher AnB was in the mesohyl tissues ($1.1\pm0.9x10^6$ CFU cm^{-3}) than cortex ($3.8\pm2.5x10^5$ CFU cm^{-3}) which was as high as THB. The reason might be attributed to the presence of anoxic zones in the sponges (Hentschel et al., 2003). Being an intertidal sponge, *C. alloclada* experiences alternative low and high tide in a day. During low tide, the sponge will be exposed to air and stops water filtration. Hoffmann (2003) and Hoffmann et al. (2005a, 2008) with the help of oxygen microelectrodes showed that the internal tissues of the sponge will be anoxic when sponge stops filtration.

Table 4.3. Abundance of AnB, SRB and NRB in sponge tissues and ambient waters

Bacteria (CFU cm^{-3})	Sponge		Water
	Mesohyl	Cortex	
AnB	$1.1\pm0.9x10^6$	$3.8\pm2.5x10^5$	$7.4\pm0.6x10^2$
SRB	$3.3\pm1.1x10^2$	$1.5\pm0.3x10^2$	$3.6\pm2.8\ x10^0$
NRB	$5.5\pm4.3x10^5$	$3.9\pm0.4x10^5$	$6.6\pm1.2x10^2$

Sulfate-reducing bacteria (SRB)

Sulfate-reducing bacteria (SRB) were mostly retrieved from the anaerobic environment (Hansen, 1994; Colleran et al., 1995), however, their presence in aerobic zones have been reported (Bharathi and Chandramohanan, 1990). Hoffman (2003) reported $2.2x10^{10}$ cm^{-3} SRB in the mesohyl of cold water sponge *Geodia barretti* by FISH which formed 7.6 % of the total bacterial community. However, they could not detect SRB in the cortex of *G.barretti*. SRB retrieved using culture method is given in Table 4.3. SRB in the mesohyl and cortex tissues were $3.3\pm1.1x10^2$ and $1.5\pm0.3x10^2$ CFU cm^{-3} respectively. This number was very low compared to that of cold-water sponge *G.barretti*. The low number may be due to the difference in techniques used, and also the sponge species. There was a significant difference in the number of SRB between sponge and ambient water with sponge harbouring one order higher number of SRB than water. SRB in the mesohyl tissue of *C. alloclada* accounted for 0.004% of THB. The difference in the proportion of SRB to total count in *G. barretti* and *C. alloclada* might be due to the difference in the oxygen concentration, habitat and sponge species. SRB prefer to be in the anoxic zone even though they can tolerate some amount of

oxygen. The survival of SRB in sponge cortex and ambient water might be due to the presence of anaerobic microniches (Jorgensen, 1977).

Nitrate-reducing bacteria (NRB)

Number of NRB in sponges has not yet been reported. NRB in the mesohyl and cortex tissues of the sponge, *C. alloclada* ranged from 2.4 $x10^5$ to $8.4x10^5$ and from $2.64x10^5$ to $1.2x10^6$ CFU cm^{-3} respectively. In ambient water, NRB number was 10^2 CFU cm^{-3}. There was a significant difference in the abundance of NRB between sponge and ambient water. The sediment cover on the sponge surface and particles in water might have provided the anaerobic microniches for cortex and ambient water bacteria, respectively. It has been reported that NRB abundance in the Arabian Sea water ranged between 10^3 and 10^7 CFU cm^{-3} (Fernandes et al., 2014). The Arabian Sea is also one of the important denitrification zones which accounts for ca 20% ocean denitrification (Codispoti et al., 2001). Detection of very low number of anaerobic bacteria in the ambient water (ANB, SRB, and NRB) might be due to the oxygenated condition in the rock pool, which was also supported by dissolved oxygen and Eh values of water. NRB in the sponge (10^5 CFU cm^{-3}) was 3 orders higher than that of water (Table 4.3).

4.2.2.2.2. Autotrophic bacteria

Under this section, both Chemotrophic (nitrogen cycle) and Phototrophic bacteria (carbon cycle) were studied. Under chemotrophic bacteria, nitrifying bacteria and under phototrophic bacteria, Chloroflexi and Rhodospirillales, which carry out aerobic anoxygenic photosynthesis were studied.

Nitrifying bacteria

There are two types of bacteria ammonia oxidising (AOB) and nitrite oxidising bacteria (NOB) which carry out nitrification in a sequence. The abundance of AOB and NOB in the sponge was $10^4 - 10^5$ and $10^3 - 10^4$ CFU cm^{-3}, respectively (Figure 4.4). The number of AOB in the cortex ($2.9\pm2.4x10^5$ CFU cm^{-3}) was one order higher than that of sponge mesohyl ($6.6\pm5.5x10^4$ CFU cm^{-3} tissue). Similarly, the number of NOB in cortex tissues of the sponge ($1.4\pm1.0x10^5$ CFU cm^{-3}) was 2 orders higher than the sponge mesohyl ($7.8\pm6.5x10^3$ CFU cm^{-3}). Sponges are favourable habitat for the survival of nitrifying bacteria as ammonia is released as a metabolic waste product (Brusca and Brusca, 1990). There are also reports on the release of nitrate from certain sponges (Corredor et al., 1988; Wang and Douglas, 1998).

These observations suggest that sponges may harbour nitrifying bacteria. Mohamed et al. (2010) detected the presence of genes for ammonia oxidation (*amoA*) in sponges, *Ircinia strobilina* and *Mycale laxissima* but NOB was not detected. However, RT-PCR did not show the expression of *amoA* gene which suggests that AOB were either not active or present in low level in these sponges in their study (Mohamed et al., 2010).

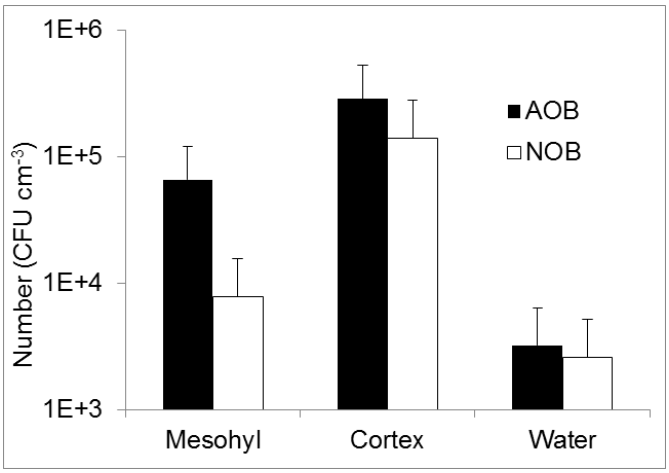


Figure 4.4. Abundance of AOB and NOB in sponge cortex, mesohyl and water

The higher number of nitrifying bacteria in sponge cortex might be related to the oxygen availability, as oxygen concentration is the major factor which regulates nitrification. Studies using immunofluorescence revealed the presence of AOB (3.5×10^2 cells ml^{-1}) and NOB (2.8×10^2 cells ml^{-1}) in seawater (Ward and Carlucci, 1985), but these numbers were one order less than those obtained in this study.

Phototrophic bacteria

Rhodospirillales

Rhodospirillales in the sponge ranged from $1.3x10^4$ to $9.0x10^5$ CFU cm^{-3} with one order higher number in the cortex than mesohyl (Table 4.4).

Table 4.4. Average abundance of phototrophic bacteria in sponge tissues and ambient waters

Bacteria (CFU cm^{-3})	Sponge		Water
	Mesohyl	Cortex	
Rhodospirillales	$5.3\pm3.7x10^4$	$5.5\pm2.4x10^5$	$4.0\pm1.1x10^3$
Chloroflexi	$5.5\pm3.5x10^4$	$4.8\pm0.8x10^5$	$3.1\pm1.7x10^3$

The abundance of *Rhodospirillales* in the ambient water ranged between 1.7×10^2 to 1.1×10^4 CFU cm^{-3} ($4.0\pm1.1x10^3$ CFU cm^{-3}) and was 1─2 orders less than the sponge ($p<0.001$). The presence of *Rhodospirillales* was detected in sponges, *Sarcotragus spinosulus* and *Ircinia variabilis* (Esteves et al., 2013). Studies on the abundance of *Rhodospirillales* in the South Pacific Ocean concluded that the abundance of *Rhodospirillales* in the ocean was as high as 1.9×10^5 cells ml^{-1} (Lami et al., (2007). The high abundance of these bacteria in the coastal and estuarine environment has also been reported (Cottrel et al., 2006).

Chloroflexi

Chloroflexi in the sponge was $5.6x10^3$ to $5.0x10^5$ CFU cm^{-3}. Chloroflexi also followed the same distribution pattern as that of *Rhodospirillales*, with an order higher number in the cortex than the mesohyl (Table 4.4), which might be related to the availability of sunlight. The abundance of Chloroflexi in the ambient water ranged between $3.3x10^2$ to 1.2×10^4 CFU cm^{-3}. Metagenomic analysis showed that Chloroflexi was the dominant phylum in sponges, *Ancorina alata* and *Stelletta maori* (Schmitt et al., 2011). These authors using Chloroflexi specific FISH probes showed that sponges, *A.alata* and S.*maori* harboured higher number ($1.2\pm0.03x10^6$ and $1.96\pm0.26x10^6$ cells cm^{-2}, respectively) of Chloroflexi than *C. alloclada*, whereas *Polymastia sp., R. topsenti*, and *T. stolonifera* harboured less number of Chloroflexi ($2.6\pm0.95x10^4$, 2.27 ± 1.04 $x10^4$ and $1.48\pm5.53x10^5$ cells per cm^{-2} of sponge tissue) than *C. alloclada*. The present study was based on the culturable method and it is known that only 0.01─0.1% of marine bacteria are culturable. Hence, it was assumed that *C. alloclada* may harbour higher number of Chloroflexi than *Polymastia sp., R. topsenti*, and *T. stolonifera.*

Spatial structural distribution

C. alloclada has clear differentiated cortex and mesohyl tissue. Different tissue types in sponge harbour different microbial community depending on the variation in nutrients, space and oxygen within the sponge tissues (Schlappy et al., 2010). Bacterial distribution in *C. alloclada* followed a general pattern (Figure 4.5). The order of culturable fractions of bacteria in mesohyl was THB> ANB> NRB> AOB> Chloroflexi>Rhodospirillales> NOB > SRB. In cortex tissues, the order was THB> Chloroflexi> Rhodospirillales> ANB> NRB> AOB> NOB > SRB. Photosynthetically active microorganisms were located in the outer,

light-exposed cortex tissue layer, as observed by Rutzler (1985) and Wilkinson (1992). Higher abundance of nitrifying bacteria in the cortex might be due to the availability of oxygen.

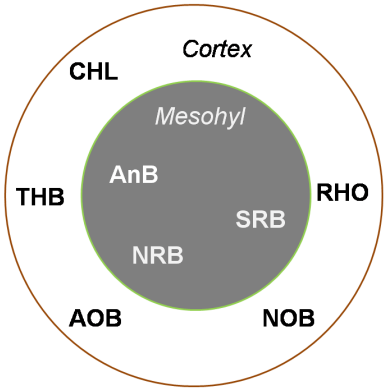


Figure 4.5. Spatial distribution of different physiological group of bacteria in the mesohyl and cortex of sponge. The bacteria were placed in mesohyl or cortex based on their higher abundance in the 2 sections

Heterotrophic bacteria populate the inner core of sponge, mesohyl. This pattern of bacterial distribution was suggested by Hentschel et al. (2003). Temporary spatial and temporal anoxic microniches form in the sponges in response to the variability in water filtration. These microniches activate the microbial processes by anaerobic bacteria or facultative anaerobes present inside the sponge (Hoffmann et al., 2005b). In mesohyl tissue of sponge, the anaerobic bacterial number was high and was comparable to THB due to comparatively less oxygen concentration in the interior tissues of sponge. Hence the differential distribution of bacteria was observed in sponge mesohyl and cortex tissues as well as in ambient water; the anaerobic bacteria formed the largest fraction in mesohyl and autotrophs in the cortex. Bacterial abundance in *C. alloclada* (Total count, Viable count and all culturable fractions of bacteria) was 1─3 orders higher than ambient water. It has already been reported that sponge harbour 2─3 orders higher number of bacteria than ambient water (Friedrich et al., 1999; 2001; Webster and Hill, 2001; Hentschel et al., 2001; 2003; Kefalas et al., 2003; Hardoim et al., 2009; Jouett et al., 2015), which was attributed to the nutrient rich condition and safe habitat in the sponge (Bultel-Ponce et al., 1998; Selvin et al., 2009c).

4.2.3. DIVERSITY OF BACTERIA

4.2.3.1. Culture-dependent method

A total of 469 bacterial morphotypes were isolated from mesohyl and cortex tissues of sponge and ambient water. Among these, 145, 132 and 171 isolates were from mesohyl and cortex of sponge and ambient water, respectively. The bacteria isolated from both mesohyl and cortex of sponge, *C.alloclada* were diverse. The phyla distribution of isolated bacteria from mesohyl and cortex of sponge and ambient water is shown in Table 4.5. This comprises a total of 50 genera in sponge (mesohyl-36, cortex-40) and 42 genera in ambient water coming under five phyla, viz., Proteobacteria (α, β, γ, δ), Firmicutes, Bacteroidetes, Actinobacteria and Chloroflexi. On the phylum level, the isolates from both sponge and ambient water were dominated by bacteria belonging to the Proteobacteria with 66.1 %, 77.9 % and 80.0 % of isolates from mesohyl, cortex, and ambient water, respectively. This is perhaps not surprising because demosponges are known to host a diverse range of culture-dependent and culture-independent bacteria (Taylor et al., 2007, Amer, 2015; Simister, 2012a). The next predominant group in sponges were Bacteroidetes (15.3% and 8.3 % in mesohyl and cortex, respectively) and Firmicutes (10.1% and 2.8 % in mesohyl and cortex, respectively). Firmicutes and Bacteroidetes were attached with sponges *Dysidea avara* and *Chondrilla australiensis* from China Sea (Li et al., 2006), *Aplysina cavernicola* from the Mediterranean Sea and the sponge *Aplysina aerophoba* from the coast of Marseille, France (Friedrich et al., 1999, 2001; Thoms et al., 2003). Phylum Actinobacteria has been found in sponges previously by Webster et al. (2001b), Hentschel et al. (2002), Montalvo et al. (2005) and Li et al. (2006). Among the Proteobacteria, the most abundant bacterial class was γ-Proteobacteria (35.6 % and 47.2 % in mesohyl and cortex, respectively). The predominance of this class in sponges has been reported from other geographical locations (tropical, subtropical, temperate and artic waters) and different demosponges (*Aplysina cavernicola*, *Rhopaloeides odorabile*, *Theonella swinhoei*, *Halichrondria panicea*, *Jaspis johnstoni* and *Plakortislita* sp. etc.) (Santavy and Colwell, 1990; Santavy et al., 1990; Althoff et al., 1998; Schmidt et al., 2000; Friedrich et al., 2001; Webster et al., 2001a,b; Webster and Hill, 2001; Thoms et al., 2003; Webster et al., 2004; Dieckmann et al., 2005; Kennedy et. al., 2008; Han et al., 2012 and references therein). Hence, it is clear that the γ-Proteobacteria is invariably

associated with sponges. However, the order of distribution of the phyla may differ in different host species and environment. The order of bacterial phyla in *C. alloclada* (present study) was Proteobacteria (γ>β>α)>Firmicutes>Bacteroidetes>Actinobacteria>Chloroflxi> δ-Proteobacteria whereas, in sponge, *Halichondria panacea* from temperate region, the order of phyla was γ- Proteobacteria>Bacteroidetes> α- Proteobacteria>Actinobacteria (Wichels et al., 2006). These types of differences are common among sponges as there are different types of association with bacteria (core bacteria or sponge-associated bacteria, acquired by horizontal transfer from seawater, variable bacteria present in the sponge and ambient water and sponge-specific bacteria, present in different sponges) (Schmitt et al., 2012a). Among these, some bacteria dominate sponge-bacterial community, for example, sponge *Rhopaloides odorabile* from different habitat was dominated by an α-Proteobacterium (Webster and Hill, 2001). Apart from the above-mentioned reasons, differences in media and culture conditions can also significantly influence the isolation and culturability of distinct bacterial isolates (Sipkema et al., 2011).

Table 4.5. Percentage of phyla distribution in sponge (mesohyl and cortex tissues) and ambient water

Samples	Proteobacteria				Firmicutes	Bacteroidetes	Actinobacteria	Chloroflexi
	α	β	γ	δ				
Mesohyl	10.2	18.6	35.6	1.7	15.3	10.1	6.8	1.7
Cortex	13.9	16.7	47.2	0.1	8.3	2.8	8.3	2.8
Water	17.8	17.8	42.2	2.2	8.9	7.2	2.2	1.7

Among 62 genera obtained in the sponge and ambient water, 21 genera were common in the sponge and ambient water and accounted for 75 % of total isolates (Table 4.6). Among the common genera, the most abundant ones were *Vibrio, Pseudomonas, Photobacterium and Bacillus. Bacillus* (11.7%) was the highest abundant genus in mesohyl whereas *Photobacterium was* the highest abundant genus (11.2%) in the cortex. In ambient water, *Vibrio* (20.4%) was the most abundant genus. The dominance of facultative oligotrophic bacteria *Pseudomonas* and *Vibrio* in the culturable fraction of sponge and water may also be because of their ability to grow in different concentration of media (Kjelleberg et al., 1987).

Table 4.6. Percentage of common culturable genera in mesohyl and cortex of sponge and in the ambient water

	Mesohyl	Cortex	Water
Vibrio	6.7	10.4	20.4
Pseudomoas	8.6	8.0	10.9
Photobacterium	3.7	11.2	11.4
Bacillus	11.7	4.8	3.8
Rubrivivax	6.1	3.2	3.8
Micrococcus	5.5	4.0	2.8
Nitrobacter	1.2	6.4	3.3
Actinoplanes	4.9	4.0	0.5
Nitrococcus	3.1	2.4	1.9
Pasteurella	4.3	2.4	0.9
Arthrobacter	5.5	0.8	0.5
Blastochloris	3.1	1.6	0.9
Plesiomonas	1.2	2.4	1.9
Rhodocyclus	3.1	1.6	0.9
Rhodoferax	3.1	1.6	0.9
Branhamella	1.2	0.8	2.4
Staphylococcus	3.1	0.8	0.9
Rhodoblastus	1.2	3.2	0.5
Rhodopseudomonas	0.6	3.2	0.5
Bordetella	0.6	1.6	0.5
Rhodomicrobium	0.6	0.8	0.9

This reiterates the earlier observation of Webster and colleagues (2004) that each sponge can be associated with unique bacterial communities. The abundance of certain genera has been reported by different researchers (Santavy et al., 1990; Muller et al**.,** 1995; Webster and Hill 2001; Dieckmann et al., 2005; Thakur et al., 2005; Kennedy et al., 2008; Mohamed et al., 2008b; Kennedy et al., 2009; Menezes et al., 2010; Bruck et al., 2012; Flemer et al., 2012; Esteves et al., 2013; Haber and Ilan, 2014). Shannon evenness indicates that *C. alloclada*-associated bacteria did not show the dominance of a particular genus, unlike in *R. odorabile* where a culturable symbiont NW001 (81-98%) dominated the bacterial community (Webster and Hill, 2001). Alpha diversity is the microbial diversity assessed within a sample. Shannon diversity index is being used to characterise species diversity in a community. Shannon diversity index showed that bacterial diversity was higher in mesohyl than cortex and ambient water (Table 4.7).

Table 4.7. Bacterial diversity indices in mesohyl, cortex and ambient water

	Shannon's method	
Sample	Index	Evenness
Mesohyl	1.745	0.936
Cortex	1.651	0.925
Water	1.673	0.879

Bacterial genera belonged to Actinobacteria (*Streptomyces, Kingella, Streptoverticillium* and *Yersinia*) and sulfate-reducing bacteria (*Desulfococcus* and *Desulfovibrio*) were retrieved to be sponge-associated bacteria, as they were detected from the sponge but not detected in ambient water. These bacteria have been reported earlier from sponges (Friedrich et al., 1999; Hoffmann et al., 2006; Li et al., 2006; Selvin et al., 2009; Izumikawa et al., 2010; Sala et al., 2013).

4.2.2.2. Culture-independent method

Screening of 16S rRNA gene markers from environmental DNA samples shifted research from bacterial culturing to direct estimation of bacterial community structure in the sponge. Next generation sequencing of the V6 region using Illumina technology has the ability to create multimillion reads of 16S rRNA gene sequences that target genus or species level differentiation (Chakravorty et al., 2007). After quality filtering, 295671 to 677020 16S rRNA reads were obtained from the sponge and ambient water, which belonged to 2978 to 3217 OTUs (Table 4.8).

Table 4.8. Next generation sequencing summary of microbial communities of sponge and ambient water

Samples	Number of reads	Number of OTUs	Shannon	Simpson	Chao1
Mesohyl	295671±44508	2978±124	6.69±0.85	0.95±0.04	3774±208
Cortex	197143±6075	3029±45	8.34±0.02	0.99±0.00	3835±134
Water	677020±197055	3217±304	6.63±0.02	0.97±0.00	4406±339

Rarefaction curves based on observed species were drawn using the QIIME alpha diversity pipeline at a 3% dissimilarity. On the left, a steep slope indicated that a large fraction of the species diversity remains to be discovered. The curves turned asymptotic with 25000

sequences and became flattened to the right indicating that the sequence depth of all the samples was the representative of the collected sample and more intensive sampling is likely to yield only a few additional species (Figure 4.6).

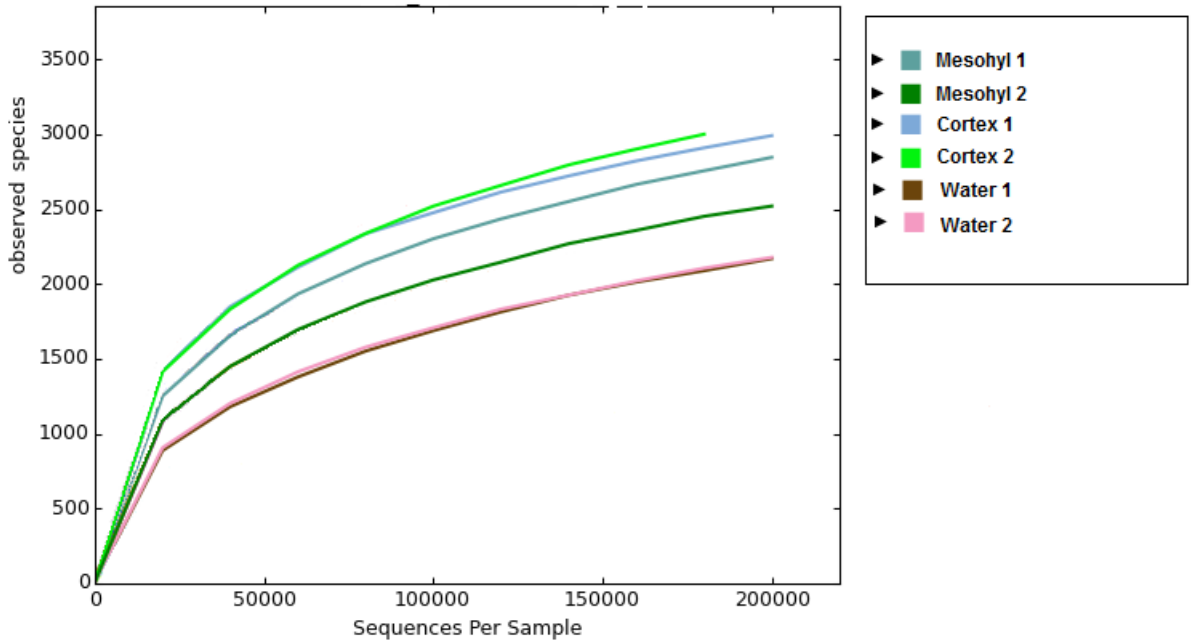


Figure 4.6. Rarefaction curve of samples

The rarefaction tables are the basis for calculating diversity metrices, which reflects the diversity within the sample based on the abundance of various taxa within a community. Shannon index ranged from 6.69 (mesohyl) to 8.34 (cortex) in sponge and 6.61 to 6.65 in ambient water. Simpson index represents the dominance of a particular bacterial group in the sponge and ambient water which ranged from 0.88 to 0.99 (0.95±0.04 and 0.99±0.00 in mesohyl and cortex, respectively) in sponge tissue and 0.97 in ambient water, (Table 4.8). Chao1 index based on the number of rare classes (OTU) found in the sample was higher in ambient water (4166 to 4646) than the sponge (1535 to 3922). The total number of OTUs was close to the Chao-based estimation of species richness. Shannon and Simpson indices showed that the prokaryotic diversity of sponge was higher than the ambient water (t-value 59.52, $p<0.001$ and t-value 268.75, $p<0.001$, for H' and D respectively). Hence, from the number of OTU and the diversity indices, it was clear that species diversity was higher in the cortex tissues of the sponge and rare species were more abundant in ambient water.

The cumulative dominance plot of dominant OTUs versus rank showed different OTU composition for sponge compared with water (Figure 4.7), as sharp increase of cumulative abundance in the first few OTUs was observed in water compared to sponge samples.

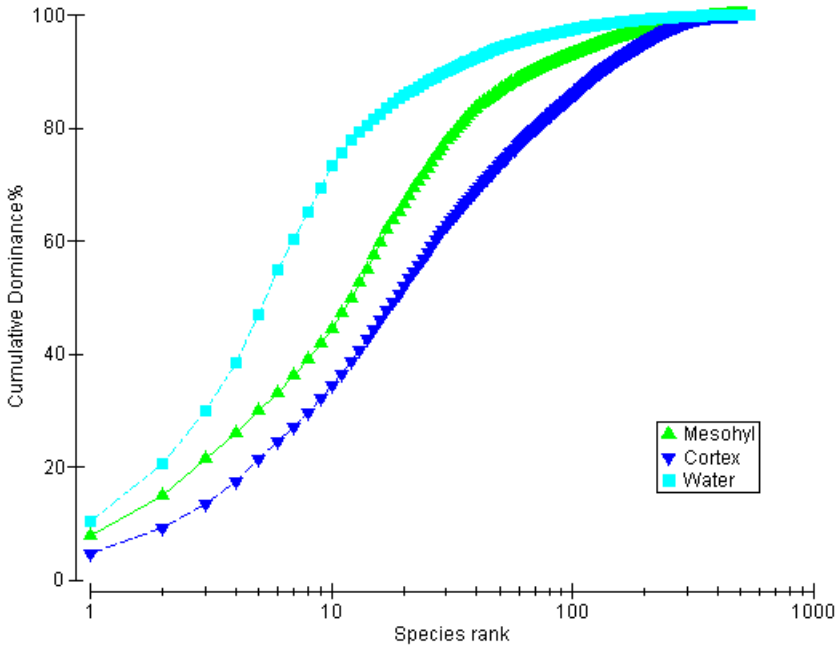


Figure 4.7. Dominance plot of 1000 most abundant OTUs in mesohyl, cortex and water

The identified sequences were assigned to 41 phyla, including 19 candidate phyla. A total of 38 phyla were present in the sponge, with 6 exclusive phyla (Figure 4.8).

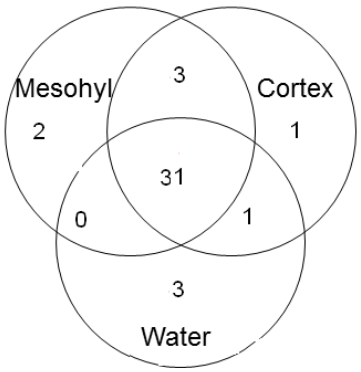


Figure 4.8. The common and exclusive phyla in mesohyl, cortex and ambient water

Taxonomic assignment of pyrosequencing reads at phylum level showed a total of 36 phyla in mesohyl and cortex of the sponge. A total of 31 phyla were found common in all the samples. This shows that the intertidal sponge, *C. alloclada* used in this study hosts

exceptional bacterial diversity, exceeding the OTU richness in other sponges studied (Easson and Thacker, 2014; Gao et al., 2015; Jasmin et al., 2015; Luter et al., 2015). However, the phyla whose abundance was >1% of total bacteria was <10. Among the 42 phyla in the sponge, 35 have been reported earlier from sponges (Amer, 2015). The sponge-specific phylum, Poribacteria (Fieseler et al., 2004) was not detected in *C. alloclada*. The diversity of *Cinachyra* sp. in the Indian Ocean and Great Barrier Reef showed the difference in the bacterial community composition from cohabiting sponges (Webster et al., 2013; Jasmin et al., 2015).

The order of dominant bacterial phyla in sponge and water was Proteobacteria>Bacteroidetes>Actinobacteria>Cyanobacteria>Verrucomicrobia>Planctomycetes>Acidobacteria>Chloroflexi>Gemmatinomonadetes>Nitrospira. Proteobacteria was the dominant phylum in all the samples (68.9%, 66.7% and 72.2% in mesohyl, cortex and ambient water respectively), as observed in culture-dependent analysis (Figure 4.9).

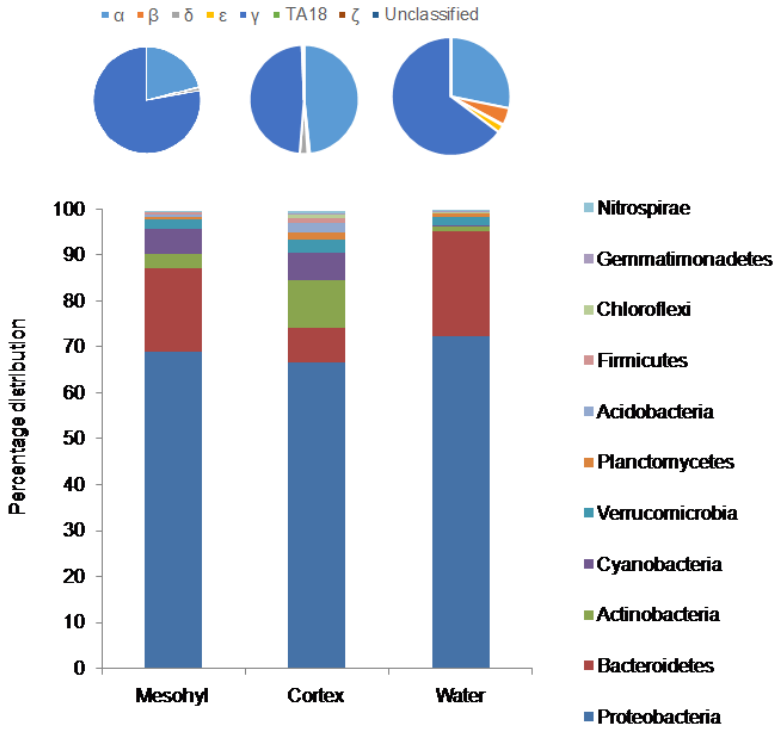


Figure 4.9. The distribution of phyla in mesohyl, cortex and ambient water. The stacked bars shows the dominant phyla and pie diagram shows different classes of Proteobacteria

Proteobacteria has already been reported as the dominant phylum in sponge by next generation sequencing (Schmitt et al., 2012a; Jeong et al., 2013; Li et al., 2014). However, the dominant phyla in *Cincahyra* sp. from Gulf of Mannar (GOM) and Great Barrier Reef (GBR) studied by cloning and DGGE analysis were different. GOM and GBR sponge-associated bacteria were dominated by Firmicutes and Chloroflexi, respectively. These studies showed lower diversity as compared to this study which might be due to the difference in techniques used. Although phylum Proteobacteria dominated in mesohyl, cortex and water, different classes of Proteobacteria dominated in different samples. For example, α-Proteobacteria dominated in cortex (32%) and γ-Proteobacteria dominated in mesohyl (53%) and ambient water (47%). Other classes of Proteobacteria such as δ-, β-, ζ-, ε-, and TA18 were also found in the sponge. The dominance of γ- and α-Proteobacteria in sponge was already established by next generation sequencing (Kennedy et al., 2014; Li et al., 2014). Bacteroidetes was the second most dominant phylum in mesohyl and water whereas in cortex, Cyanobacteria was the second most abundant phylum and Actinobacteria was also more abundant than Bacteroidetes in cortex. Other important phyla present in sponge were Verrucomicrobia and Acidobacteria. Similar patterns of phyla-level similarity and OTU-level differences have previously been reported for other sponge species such as *Aplysina aerophoba, A. cavernicola, Ircinia variabilis, Petrosia ficiformis, Pseudocortium jarrei, Lissodendoryx diversichela, Poecillastra compressa, Inflatella pellicula, Neamphius huxleyi* and *Stelletta normani* (Schmitt et al., 2012b; Simister et al., 2013; Luter et al., 2014; Kennedy et al., 2014; Li et al., 2014; Gao et al., 2015). The availability of higher amount of sunlight might have contributed to the higher abundance of α-Proteobacteria and Cyanobacteria in the cortex tissues of this intertidal sponge. The abundance of Planctomycetes was in the ordre of mesohyl> cortex> water, as most of the members of this group prefer anoxic or microaerophilic condition. Firmicutes followed the opposite trend and are mostly found in seawater. Chloroflexi and Nitrospirae were higher in cortex, as the former prefer sunlight and the latter prefer higher oxygen level.

Rare phyla in the sponge and ambient water (<1%) are shown in Figure 4.9. OP11 and GAL15 were present exclusively in mesohyl and cortex, respectively. AC1, NC10, KSB3 and WS6 were associated with sponge, but not in ambient water. SR1, PAUC34f, AncK and LCP-89 were found only in ambient water.

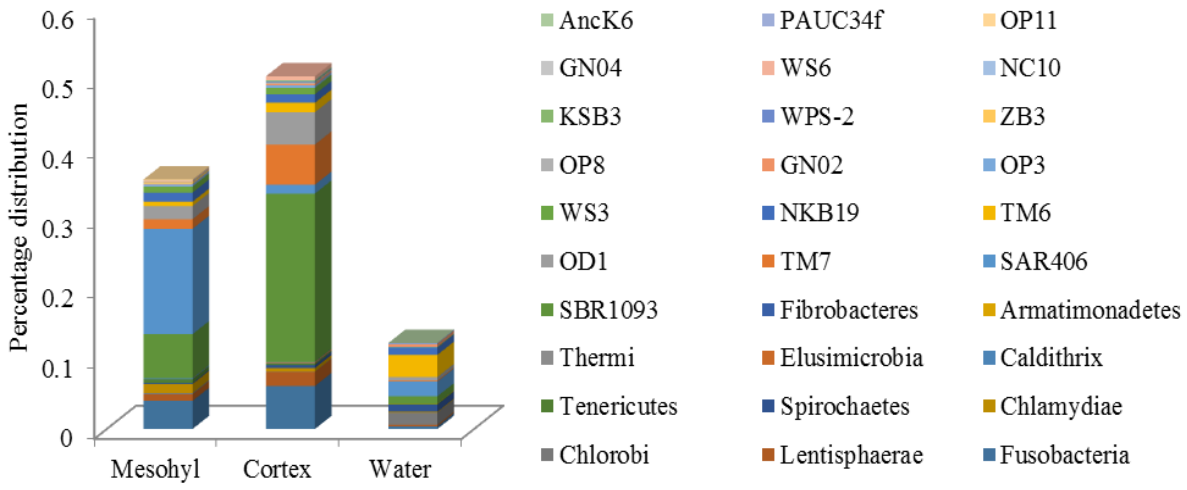


Figure 4.10. Rare phyla distribution in mesohyl, cortex and water

Oceanospirillales (17%), Rhodobacterales (19%) and Flavobacteriales (22%) were the dominant orders in mesohyl, cortex and ambient water, respectively. Rhodobacteriaceae (15%) and Flavobacteriaceae (19%) were the dominant families in the cortex and in ambient water (Figure 4.11). Alcanivoracaceae was the most abundant family in mesohyl (13%).

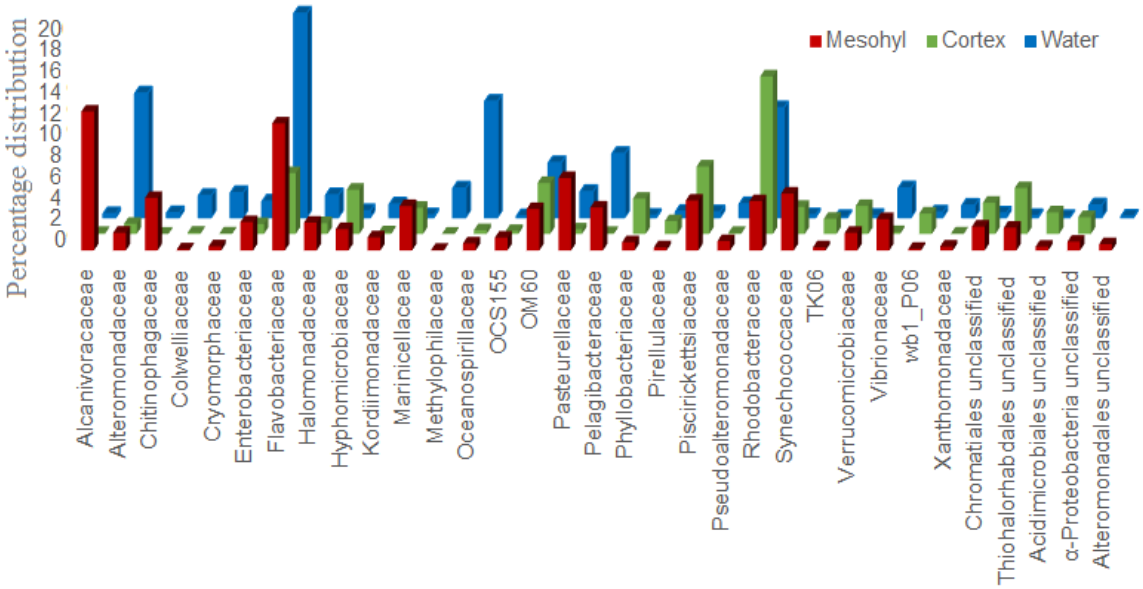


Figure 4.11. The phylogenetic affiliation of different families in mesohyl, cortex and ambient water, which showed >1% abundance in at least one sample

The difference in the abundance of pyrosequencing reads at the genus level for the samples has been further summarised in the heat map (Figure 4.12). A significant correlation was identified between distance matrices from the total dataset and the 40 most abundant OTUs (RELATE; Rho D 0.531, $p<0.001$), further supporting the consistency between the two data sets. The column of the left heat map show normalised relative abundance and provides a comparison of the difference of abundance between different genera in the same sample. This shows that a few genera occupied most of the reads in each sample and the predominant genera were different in each sample. *Alcanivorax* and unclassified Flavobacteriaceae were dominant in mesohyl, whereas it was unclassified Rhodobacteriacea in cortex and hence these groups could represent taxa specific to mesohyl and cortex of sponge. Flavobacteriaceae and Rhodobacteriaceae were associated with sponges (Lee et al., 2009; Yoon et al., 2013; Amer, 2015; Steinert et al., 2016). Although *Alcanivorax* was also detected from a few sponge species (Steger, 2010; Lavy et al., 2014), the dominance of this genus in any of the marine environment has not been reported yet. The right side of the heatmap show normalised relative abundance and provide a comparison of the abundance of same genus among different samples. There was a clear separation of the relative abundance of each genus in the samples. For example, *Photobacterium, Prochlorococcus, Methylophaga, Halomonas, Sediminibacterium* and *Alcanivorax* were more abundant in mesohyl and their abundance in other samples was relatively very few.

A similar pattern was noted in results of beta diversity analysis. Similarity percentage (SIMPER) analysis revealed that the average dissimilarity between bacterial communities of sponge and ambient water was 75 % and between mesohyl and cortex was 65%. γ- and α-Proteobacteria were accounting for the highest variation between mesohyl and cortex (40% and 22% dissimilarity). Specifically, OTU 793461 (*Alcanivorax dieselolei*, γ-Proteobacteria), which ranged from 9 to 85081 reads, was the primary drivers of dissimilarity between cortex and mesohyl (accounting for 10 % of total dissimilarity). The same OTU with OTU590090 (Alteromonadaceaea, γ-Proteobacteria) contributed the highest variation between ambient water and sponge samples (14.3 and 4.8 % dissimilarity by water with mesohyl and cortex, respectively). OTU590090 was higher in water (43324 to 74204 reads) than sponge (1 to 2101 reads). Gao et al. (2015) also reported that γ-Proteobacteria was the driving factor for the variation of bacterial communities associated with sponges from inshore and offshore locations.

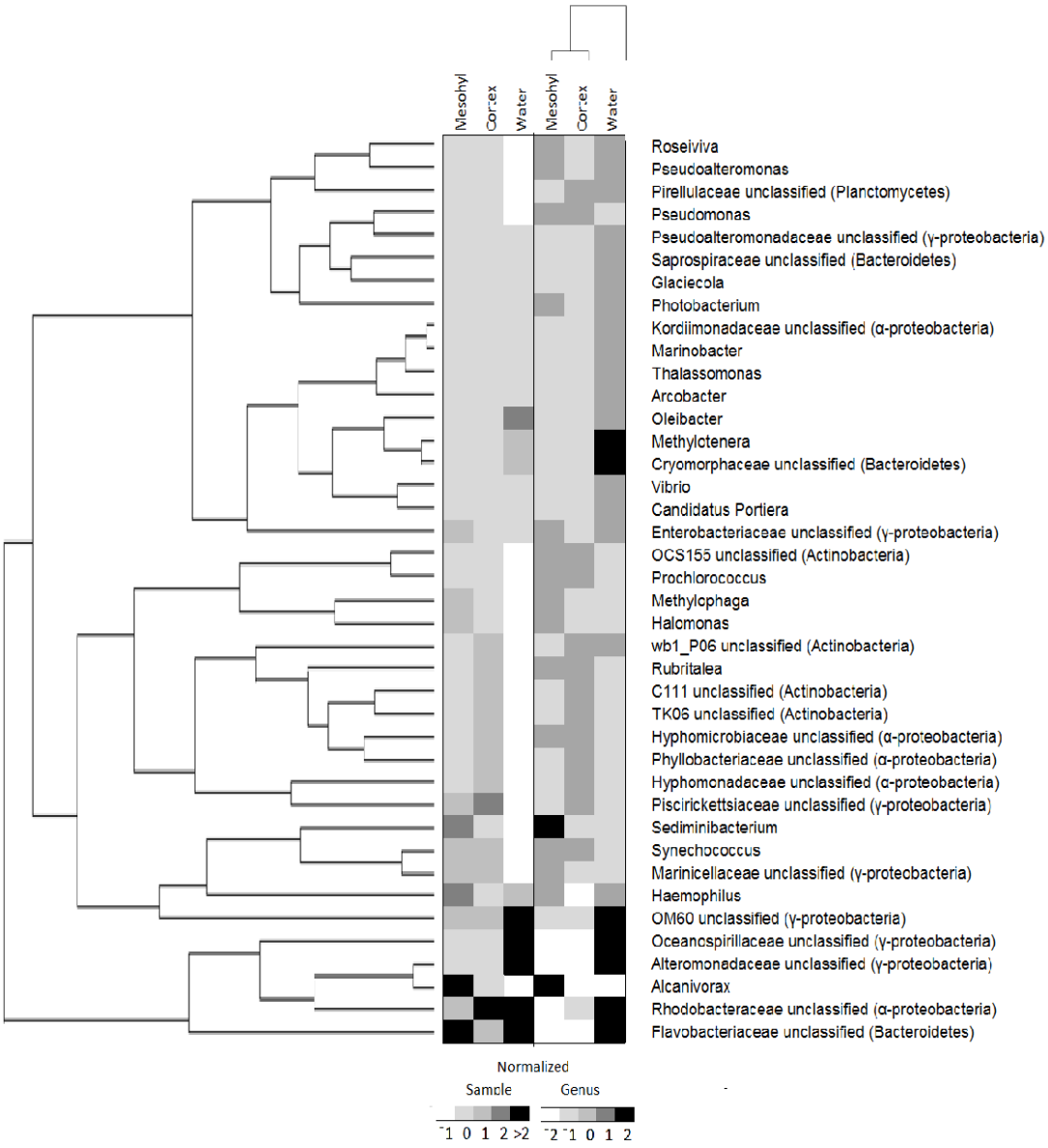


Figure 4.12. Heat map of abundant genera (>1% relative abundance in at least one of the samples) in mesohyl, cortex and water. The relative abundance was normalised in each sample (left side of heatmap) by values of each cell-average value of the column. The relative abundance of each genus in different samples (right side of heatmap) was normalised by subtracting the values of each cell-average value in each row. Dendrogram showed the similarity in relative abundance in genus and samples.

Distinct grouping of samples within the PCO ordination plot based on Bray-Curtis was evident (Figure 4.13). For example, to the right of the ordination plot, there was a general grouping of cortex community as this is the ecotone region between the seawater and surface sediment and mesohyl of sponge. It has already been established that ecotone community will

be more diverse than the other two neighbouring regions. This supports the significantly different alpha diversity among the samples as shown by t-test and SIMPER analysis. The overall level of similarity as determined by CLUSTER analysis in microbial composition at the OTU level was 20% and all the samples were significantly different (ANOVA, $p<0.001$). In addition, there was a significant difference in species diversity (ANOVA, $p<0.05$) and richness estimation between the samples. Bacterial phyla separated with 46 % of the total variation in community composition explained the first two axes. OTUs assigned to Planctomycetes, δ-Proteobacteria, Firmicutes and Acidobacteria contributed to most of the ordination, with case scores >0.45.

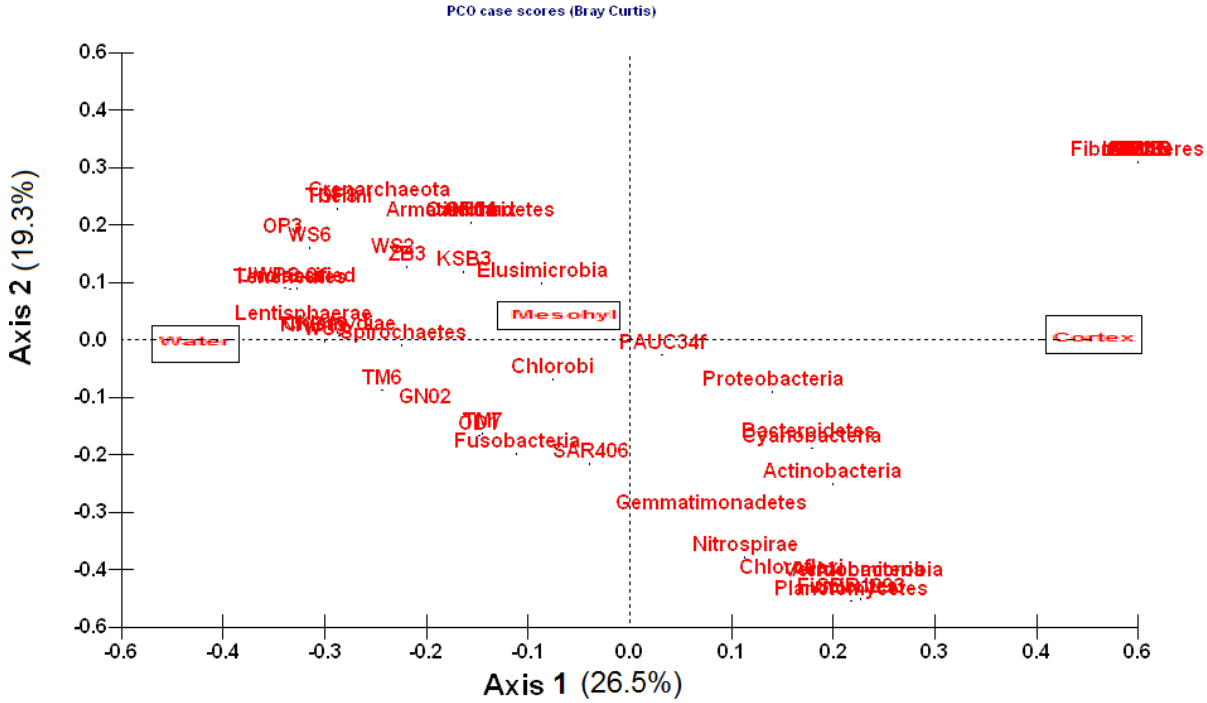


Figure 4.13. PCO analysis based on Bray-Curtis similarity of bacterial phyla with superimposed samples.

Conclusion

The presence of a high number of bacteria (10^{8-9} bacteria g^{-1}) in this species suggests that the intertidal tetillid sponge, *C.alloclada* comes under high microbial abundance (HMA) sponge. *C. alloclada* is an important reservoir of microbial diversity as it harbours at least 2900 OTUs. In the present study by both culture-dependent and culture-independent methods showed a higher diversity of bacteria in *Cinachyra* species was observed than those in previous studies. In both methods, Proteobacteria was the most dominant phylum. However,

culture-dependent studies showed higher diversity in mesohyl than cortex whereas vice versa was found in the culture-independent method. This might be because of the dominance of γ-Proteobacteria in the mesohyl which comprised of a higher number of culturable heterotrophic bacteria and α-Proteobacteria in the cortex with most of them requiring specialised media for growth. In the present study, samples could not be collected during the monsoon season. As seasonal variations are brought about mainly by the monsoon in tropical intertidal regions, water characteristics and sponge-associated microbial parameters did not show significant variation in different sampling time, as samples were not collected during monsoon due to logistic reasons. The general consensus is that sponge-microbial associations are largely stable over temporal scales including epibiont, culturable bacteria, and entire bacterial population over large seasonal shifts in the environmental characteristics (Webster and Hill, 2001; Thiel et al., 2001; Taylor et al., 2004, 2007; Erwin et al., 2012; White et al., 2012).

CHAPTER 5

FUNCTIONS OF BACTERIA

5.1. INTRODUCTION

Sponges harbour a large number of diverse bacteria with different metabolic capabilities to maintain a stable and balanced ecological niche. The rapid growth of sponge-associated microorganisms is advantageous for a thorough study of community interactions in the sponge. Association with different types of microbial consortia apparently enables the sponges to exploit different organic and inorganic carbon sources that are generally believed to be unavailable to these organisms (Simpson, 1984). The study of the functional roles of bacteria associated with sponges is still in its infancy. To understand the basic and applied ecological perspectives of the sponge-microbe interaction, the functions associated with the microbial consortia have to be elucidated. Schlappy (2008) suggested that different tissue types have different abilities in the uptake of nutrients and maintenance of sponge-associated microorganisms. It was suggested that associated methanotrophs might have contributed a significant portion of the host sponge's nutrition in the nutrient-depleted deep sea (Vacelet and Boury-Esnault, 2002). A deep sea sponge, *Cladorhiza* sp. lacks the aquiferous system and harbours methanotrophic bacteria that use methane from deep sea mud volcano (Vacelet et al., 1996; Vacelet and Boury-Esnault, 2002). Sponges can change the internal environment such as hypoxia/anoxia by water pumping (Hoffmann et al., 2005a, 2008) and create the microenvironment for denitrification and sulfate reduction. Denitrification and sulfate reduction in sponges were first reported in the cold water sponge, *Geodia barretti* (Hoffmann, 2003; Hoffmann et al., 2009). On the other hand, when sponges start pumping, their tissues become oxygenated and this facilitates nitrification and sulfur oxidation. Among the different functions associated with the sponge-microbe consortia, nitrification is the most studied. Studies on nitrification were mostly carried out on whole sponges from the temperate regions (Bayer et al., 2008a, b; Schlappy et al., 2010) and cold waters (Radax et al., 2012). The resulting byproducts of sponge metabolism such as ammonia could be toxic to the sponge holobiont. This could be mitigated by the nitrifying bacteria present in the sponge that would convert these to less-toxic nitrate. In addition, it would also provide carbon source to the sponge as 1 mol of CO_2 is fixed for every 9 mol of NH_4^+ oxidized to NO_3^- (Feliatra and

Bianchi, 1993). Particulate matter filtered from ambient water contains several organic compounds which are utilised by associated microbial consortia to provide nutrients to the sponge. *In situ* studies showed that the carbon flux in sponges was 29-1970 mg Cm^{-2} d^{-1} (Reiswig, 1974, 1975a; Pile et al., 1996, 1997; Pile and Young, 2006) and hence these are key players in the transfer of carbon from pelagic microbial food web to benthos.

The coastal ecosystems, including the intertidal regions, are the most geochemically and biologically active areas, as these ecosystems receive inputs of terrestrial organic matter and nutrients, which establish a variable nutrient gradient in time and space. The food sources that support intertidal sponges and associated microbial communities are not well understood. Thus, depending on the habitat and location, the abundance, diversity and functions of the microbial community in the sponge may differ. Further, it is also not clear whether the nutrition for sponges is solely dependent on the heterotrophic or autotrophic microbial consortia. Hence, an attempt has been made to study the autotrophic and heterotrophic ability of sponge–microbial consortia from mesohyl and cortex region of the demosponge, *C. alloclada*. The methodology used for this study is presented in Chapter 3 under section 3.3.

5.2. RESULTS AND DISCUSSION

5.2.1. DEGRADATION OF ORGANIC MATTER AND UPTAKE OF ORGANIC AND INORGANIC SUBSTRATES

Sponge metagenome revealed a large number of orthologs involved in the metabolism of nitrogen, methane, sulfur, amino acid, vitamin, and cofactor (Present study; Thomas et al., 2010; Li et al., 2013). Sponges filter 16-25 litres of ambient water per day and accumulate a large amount of organic matter within the choanocytic chambers. These organic matters, cell lysates and exudates by sponge are the nutrient sources for the associated bacteria (van Duyl et al., 2008). The source of organic matter for bacteria is mainly the ambient water. The distribution of resources varies spatially and temporally within the sponge. The utilisation of 31 different substrates in Ecomicroplates reflected the metabolic potential of the microbial consortia (Stefanowicz, 2006; Wang et al., 2010). The importance of growth indicates that the

colour responses produced in the microplates are a reflection of the functional potential rather than *in situ* functional ability.

Metabolic potential (AWCD) of microbial consortia associated with mesohyl, cortex and ambient water is shown in Figure 5.1. Metabolic potential of the microbial consortia of sponge (AWCD > 1.0) was more than that of the ambient water and other environments (Yang et al., 2013). The high potential associated with sponge microbial consortia is due to the high abundance of microbes (>10^9 cells cm^{-3}) in the sponge. Studies have shown that microbial numbers in sponge exceeds by approximately two orders in magnitude than that in ambient water (Present study; Webster and Hill, 2001; Hoffmann et al., 2005a; Hardoim et al., 2009).

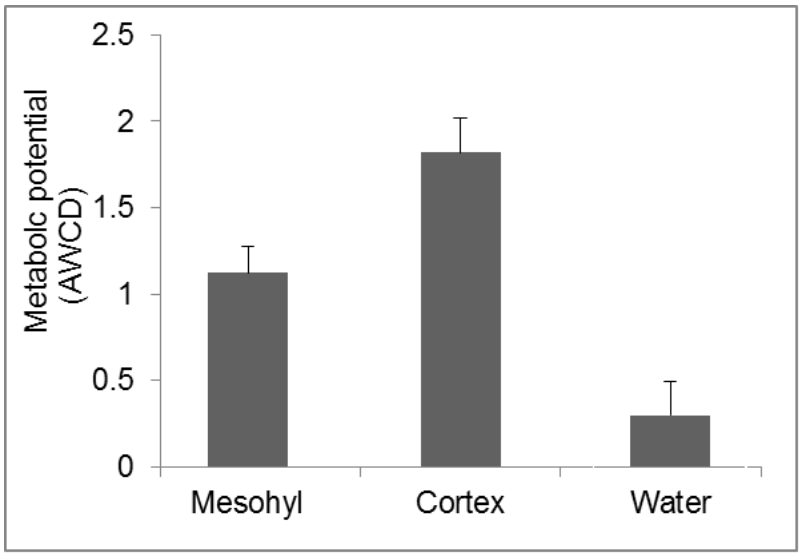


Figure 5.1. Metabolic potential of sponge microbial consortia and ambient water

There was a difference even in the categorywise utilisation of amines, amino acids, carbohydrates, carboxylic acids, polymers and other miscellaneous substrates between microbial consortia in mesohyl, cortex and ambient water ($p<0.05$). Ambient water microbial consortia effectively utilised 71% of the carbohydrates as against 43% of sponge microbial consortia (Table 5.1).

Table 5.1. Percentage utilisation of different categories of carbon compounds

Category	% utilization		
	Mesohyl	Cortex	Water
Amine	50	50	50
Amino acid	33	17	50
Carbohydrate	43	43	71
Carboxylic acid	44	11	22
Polymer	50	75	75
Others*	33	67	67

* esters and phosphorylated substrates

This may be due to their presence in large quantity in the ambient water as supported by other studies from the same region (Khodse et al., 2009; Fernandes, 2011) or that these microbial consortia may be requiring more substrates (They et al., 2013).

One or more of the substrates from each category were effectively utilised by both mesohyl and cortex microbial consortia with cortex utilising 27 to 30 substrates. Substrates such as malic acid, L-threonine, glycyl-L-glutamic acid and tween 80 were effectively utilised by both cortex and mesohyl microbial consortia, whereas L-serine was not utilised by mesohyl and was effectively utilised by cortex microbial consortia (Figure 5.2). Similarly, the utilisation of carbohydrate, D-xylose was different among different microbial consortia (ambient water-nil, cortex–weak and mesohyl –effective). Carboxylic acids produced during the sponge metabolism were utilised mainly by mesohyl microbial consortia. The simple organic carbons such as sugars and amino acids are quickly absorbed and provide nutrients to the microbial consortia (Maharning et al., 2009). This might be due to the abundance (Fernandes, 2011) and labile nature of these simple substrates and also the metabolic potential of majority of the microbial consortia to utilise such substrates.

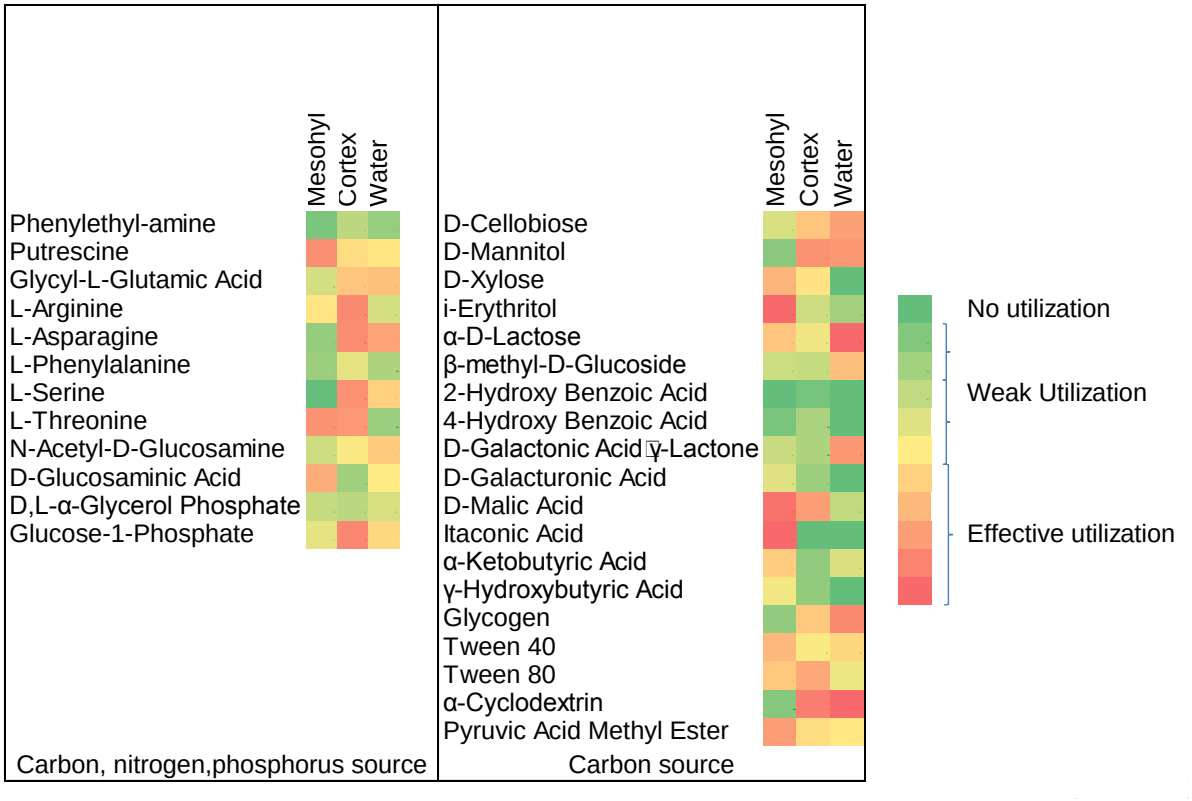


Figure 5.2. Heatmap of utilisation potential of single substrates by microbial consortia of sponge (mesohyl and cortex) and ambient water

Functional diversity indices based on metabolic potential showed significant differences ($p<0.001$) among cortex, mesohyl and ambient water with the highest value being recorded in the sponge cortex. H′ ranged from 1.46 to 2.61 with mesohyl having high value, reflecting the great metabolic diversity in the sponge (Table 5.2). Though H′ was low in the ambient water microbial consortia, D was high (0.063±0.000). Shannon functional diversity index (>2) in the sponge was more than that of the ambient water in the present study and that of other natural environments studied (Guckert et al., 1996; Roling et al., 2000; de Lipthay et al., 2004; Janniche et al., 2012). This shows that the microbial consortia associated with sponge exhibit high metabolic activity and metabolic diversity which may be required for their survival (Liu et al., 2012).

Table 5.2. Diversity indices: Shannon diversity (H'), evenness (E), Simpson's index (D) of the bacterial communities from sponge and ambient water

Sample	H'	E	D
Mesohyl	2.61±0.07	1.33±0.15	0.013±0.002
Cortex	2.51±0.03	1.00±0.01	0.003±0.000
Water	1.46±0.69	0.72±0.07	0.063±0.000

This metabolic potential is due to cell-free hydrolytic enzymes which convert the complex organic matters into simple nutrients (Arnosti, 2011), a means to acquire nutrition (Becquevort et al., 1998). Enzymatic profile of the bacteria showed that about 70% of the isolates were able to degrade biopolymers and out of which 48% showed multiple enzyme production. The percentage of bacteria producing the number of enzymes was higher in ambient water than in sponge ($p<0.05$). Extracellular enzymes from sponge-associated bacteria have been reported earlier (Santavy et al., 1990; Feby and Nair, 2010). There was no difference in the number of bacteria producing amylase in mesohyl and cortex (28.6 % and 28.4%, respectively) whereas lipase producing bacteria were higher in the cortex (15%) than in the mesohyl (12%) (Figure 5.3). In sponge *Fasciospongia cavernosa*, from the eastern peninsular coast of India, 27% of sponge-associated bacteria showed amylase activity (Shanmughapriya et al., 2009). Bacteria producing enzymes which degrade proteinaceous substances such as casein and gelatin were high in mesohyl (18% and 17%, respectively) than in the cortex whereas urease prodcing bacteria were high in the cortex (30%) (Figure 5.3).

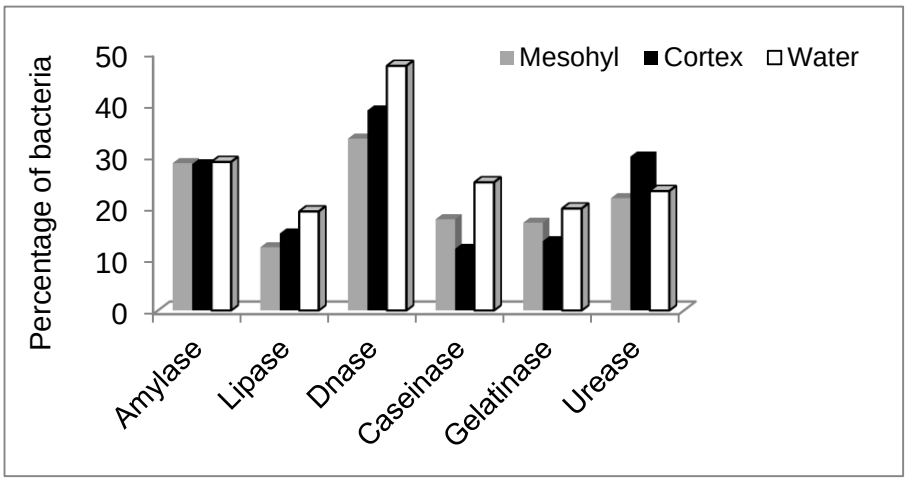


Figure 5.3. Percentage of hydrolytic enzymes producers

High activity of urease in the cortex can be attributed to high concentration of urea (1.4 nmol ml^{-1}) in these waters (Gopinath et al., 2002). Further, it may also be playing a protective role for sponge by degrading urea which is a toxic excretory product of the animal. The presence of bacteria with proteolytic and lipase activities in ambient water may be related to the degradation of some constituents of zooplankton, as lipid and protein are the most important fractions in zooplankton (Delong et al., 1993). DNase not only metabolises nucleic acid (DNA) but also provides phosphate to the sponge holobiont. About 4%, 3% and 5% bacteria from mesohyl, cortex and ambient water respectively produced 4 enzymes (Figure 5.4).

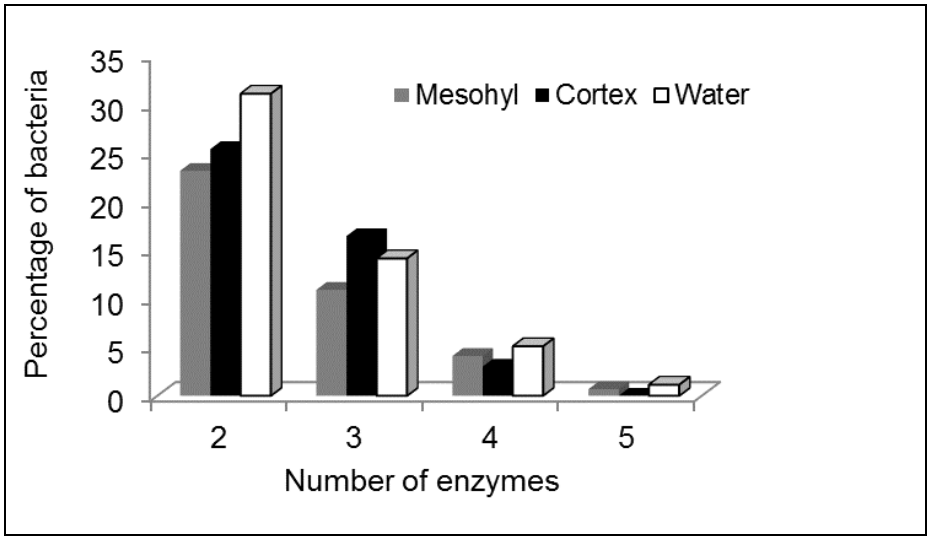


Figure 5.4. Percentage of multiple enzymes producers

In the mesohyl, amylase, DNase and urease (23% each) were produced by most of the single enzyme producing bacteria. Earlier reports have shown the presence of hydrolytic activity as well as multiple expression of enzymes among bacteria associated with *Ircinia dendroides* in the Mediterranean Sea, *Ceratoporella nicholsoni* in Caribbean sea and, *Sigmadocia fibulata* and *Dysidea granulosa* in the Indian Ocean (Santavy and Colwell, 1990; Lee et al., 2003; Feby and Nair, 2010). The bacterial community producing extracellular enzymes exhibit distinct biogeographic patterns with differing activity depending on location, season, water mass, and depth (Kan et al., 2006; Agogue et al., 2011; Gilbert et al., 2012; Hanson et al., 2012).

Although, ecological relevance of hydrolytic enzymes and their biotechnological application from marine microbes have been recognised, studies on enzymes from bacteria associated within the microhabitats of the sponges are rare (Wang, 2006). The associated microbial consortia survive on the nutrients filtered by sponges (van Duyl et al., 2008) and on the complex organic compounds from the sponge body (Hentschel et al., 2006). Many of the vital macromolecular compounds have to be converted into simple nutrients in order to uptake them. The intertidal sponges are exposed to varying concentrations and types of compounds including nutrients and other substances of anthropogenic origin from land run off, than the sponges from coral reefs and from the offshore water. Sponges being filter feeders, the bacteria associated with sponges are also exposed to a large amount of these particles accumulated in the intertidal seawater. Increased levels of available nutrients in the sponge lead to enhanced metabolic capabilities of associated bacteria. The differential production of enzymes in the cortex and mesohyl, suggests compartmentalization of associated bacteria in the metabolic activities of sponge, due to the difference in the accessibility of substrates. High utilisation seen in the cortex microbial consortia can be attributed to the habitat of this intertidal sponge as it is mostly covered with sediment which allows sediment-derived and ambient water microbial consortia to invade into the cortex of the sponge (Turque et al., 2008). Small sized dissolved components can be directly absorbed by the sponge and associated microflora. These enzyme activities allow bacteria to quickly exploit aggregate resources, which may reduce the carbon fluxes (Grossart et al., 2003).

Most of the bacteria utilised mono- and disaccharides irrespective of their habitat. Glucose was the most common sugar utilised by most of the isolates followed by sucrose (Figure 5.5). Sugar alcohols were utilised only by <40% bacteria and 3% bacteria did not utilise any of the studied simple carbon sources.

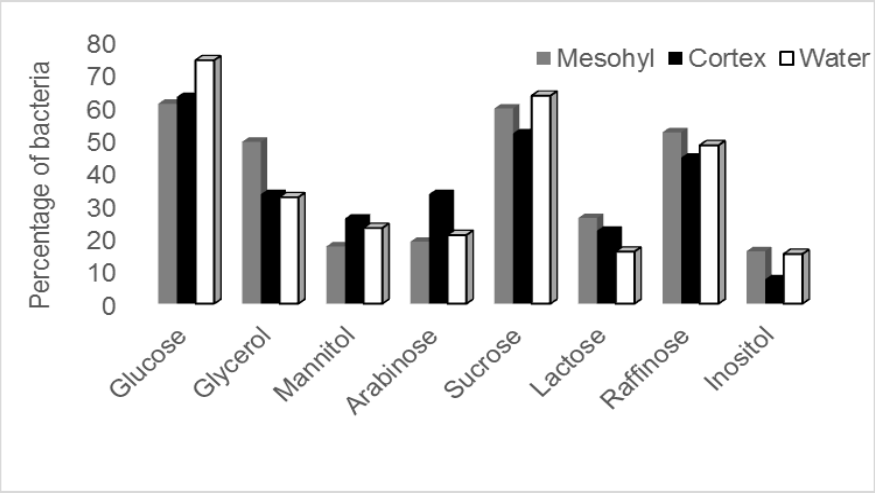


Figure 5.5. Percentage of bacteria utilising different carbohydrates

There was a significant difference in the utilisation of multiple carbon sources among bacteria from mesohyl, cortex and ambient water ($p<0.001$). Only 1% of bacteria from mesohyl and ambient water utilised all the carbon sources (Figure 5.6). Bacteria from cortex utilised < 5 carbon sources.

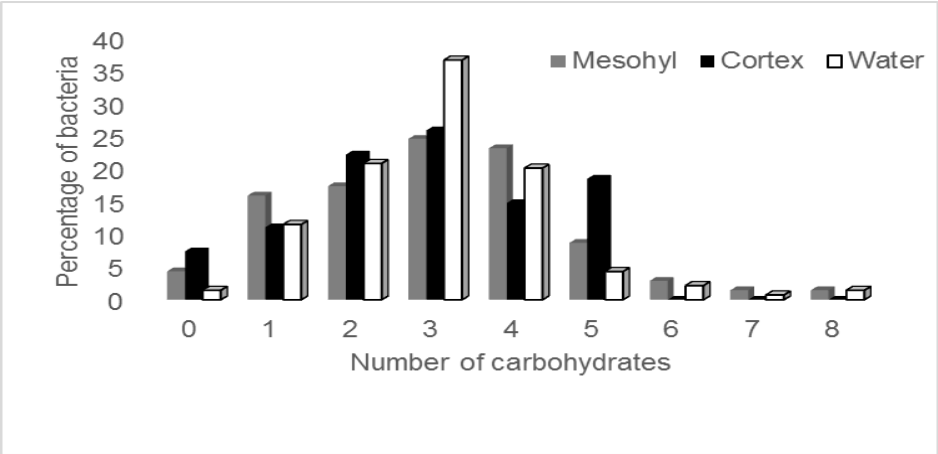


Figure 5.6. Percentage of bacteria utilising multiple carbohydrates

The utilisation (number and intensity of utilisation) of 46 organic substrates in 6 categories (Amine/ amino acids, monosaccharides, oligosaccharides, sugar derivatives, carboxylic acids and other miscellaneous substrates), using Biolog GENIII and AN microplates showed the differential utilisation of substrates under aerobic and anaerobic conditions as seen in Table 5.3.

Table 5. 3. Number of substrates utilised by bacteria

Number of substrates	Mesohyl		Cortex		Water	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1─10	0	20	33	43	14	0
11─20	8	50	17	0	14	50
21─30	54	20	8	43	14	0
31─40	31	10	25	14	14	0
41─46	8	0	17	0	43	50

More than 50% of the isolates from mesohyl utilised 21-30 organic substrates under aerobic condition and 11-20 substrates under anaerobic condition whereas 33% and 43% cortex bacteria utilised <10 substrates in aerobic and anaerobic conditions. More number of substrate utilisation was in mesohyl whereas high effective utilisation (intensity of utilization) was in the cortex. This differential utilisation in mesohyl may be attributed to the high culturable bacterial diversity, whereas in cortex it was high abundance which contributed high effective utilization (see Chapter 4). Ambient water bacteria utilised a higher number of substrates than sponge bacteria (Table 5.3). However effective utilization was less. This difference in the utilisation of substrates among bacteria from mesohyl, cortex and ambient water might be due to the difference in the availability of substrates in their natural environment. Ambient water bacteria interact with a large number of different substrates than the sponge-associated bacteria. In mesohyl, carboxylic acids and oligosaccharides were utilised by a higher number of bacteria (72% and 49%) under aerobic and anaerobic conditions, respectively. Most preferred substrates by bacteria from cortex were amine/ amino acid and monosaccharides, as the highest percentage of bacteria utilised these substrates under aerobic and anaerobic conditions (Table 5.4). This corroborated with the earlier results with sponge microbial consortia.

Table 5.4. Utilisation of different categories of substrates by bacterial isolates (Others include nucleoside, glycoside, ester and polymer).

Category	Mesohyl (%)		Cortex (%)		Water (%)	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Amine/amino acid	63	29	55	47	66	71
Monosaccharide	69	35	52	38	88	92
Oligosaccharide	64	49	47	40	63	72
Sugar derivative	63	42	51	46	71	78
Carboxylic acid	72	40	45	37	65	56
Others	54	32	40	40	65	70

The pattern of utilisation of each substrate under different categories by bacteria from mesohyl, cortex and ambient water under aerobic and anaerobic conditions are given in Figure 5.7.

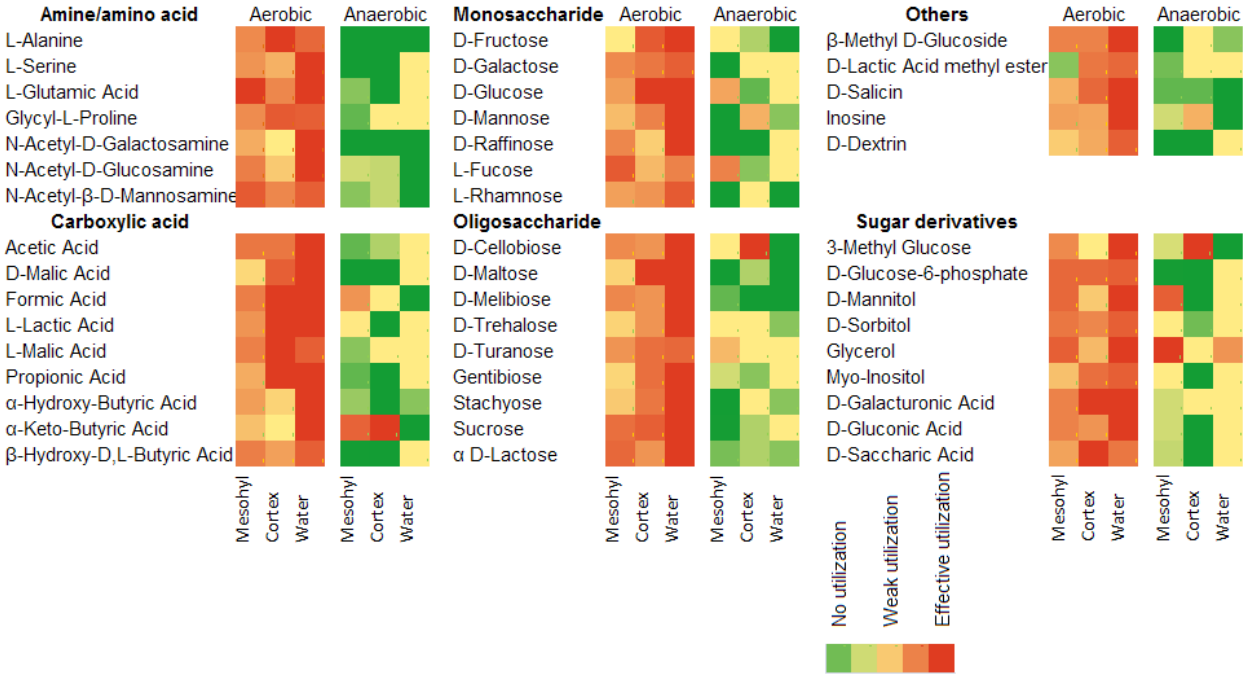


Figure. 5.7. Heat map of compound utilisation by bacteria isolated from mesohyl and cortex of sponge and ambient water

In mesohyl and cortex, 93% and 60% bacteria, respectively utilised more than 20 substrates under aerobic condition. However, the effective utilization of >20 substrates were shown by only 50% of these bacteria. Under anaerobic condition, bacteria from the sponge and ambient water utilised less than 20 substrates. When considering effective utilisation, none of the substrates was utilised by bacteria from all the 3 locations (mesohyl, cortex and ambient water). Though the number of substrates utilised by mesohyl bacteria was higher than the cortex bacteria under aerobic condition, the effective utilisation was higher in cortex bacteria. Mesohyl bacteria utilised simple substrates; glutamic acid, N-acetyl-β-D-mannosamine and fucose under aerobic condition and glycerol and mannitol under anaerobic conditions. Cortex bacteria showed wide metabolic capabilities under aerobic condition as they effectively utilised 11 substrates in 5 different categories (amino acids, carboxylic acids, mono and oligosaccharides and sugar derivatives). However, under anaerobic conditions, the cortex bacteria did not effectively utilise simple substrates like amine, amino acid and monosaccharides. Bacteria from ambient water effectively utilised about 70% substrates under aerobic conditions but did not show effective utilisation under anaerobic condition. These differences may be because of the differential utilization capability of facultatively anaerobic bacteria under aerobic and anaerobic conditions.

A higher number of mesohyl bacteria utilised carboxylic acids than cortex and ambient water bacteria. As sponge phagocytosis produce such complex molecules, sponge-associated bacteria may adapt to utilise these substrates. The sponge cells after phagocytosis also produce organic acids and sugar alcohols (Hentschel et al., 2006) which can be utilised by associated bacteria. It was found that bacterial symbionts in the sponge, *Amphimedon queenslandica*, harbour genes for the utilisation of amines, polymers, mono and oligosaccharides (Gauthier et al., 2016). They found that each symbiotic bacterium in sponge was equipped with a unique set of proteins that would permit them to utilise different substrates. To avoid competition, co-occurring bacteria in the host may utilise different substrates (Duperron et al., 2006; Kleiner et al., 2012) and thus show differential utilisation of compounds. However, cortex and ambient water bacteria showed less preference to complex molecules. This is because carbohydrates and other simple molecules were higher in the ambient water which can be easily accessible by bacteria. The differential utilisation

within the sponge may be due to the different microbial population on the sponge cortex and mesohyl tissues as explained in Chapter 4 and also as reported in other studies (Gerce et al., 2011; Yang and Li, 2012) or may be due to the difference in the uptake rate. Uptake rate using ^{14}C-labelled compounds of amino acids (glutamic acid and leucine) and the most used sugar glucose showed the difference in uptake by the bacteria. The uptake rate of glutamic acid was 250.7±78.7, 470.6±178.0, and 41.6±8.3 µg C cm^{-3} d^{-1} in mesohyl, cortex and ambient water, respectively. The uptake rate of leucine in sponge cortex (0.23±0.1 µg C cm^{-3} d^{-1}) was 2x higher than sponge mesohyl tissues (0.11±0.01 µg C cm^{-3} d^{-1}), though the difference was not significant. The uptake rate of glucose by mesohyl and cortex tissues of the sponge was 13.5±13.0 and 28.5±16.7 µg C cm^{-3} d^{-1}, respectively (Figure 5.8).

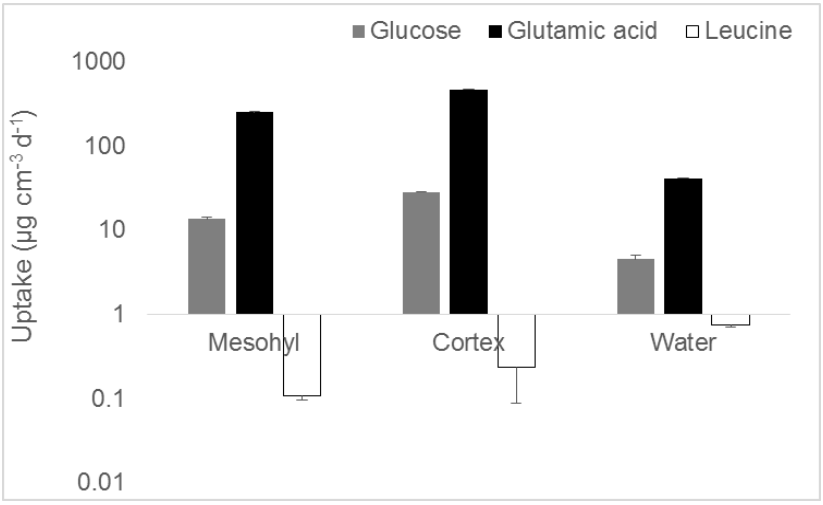


Figure 5.8. Uptake rate of glucose, glutamic acid and leucine

The uptake rate of glutamic acid was one and two orders higher than glucose and leucine, respectively. The glutamic acid uptake was reported to be 5 to 10 times higher than leucine uptake (Schwarz and Colwell, 1975). The differential uptake may be due to the difference in the preference of substrates by associated bacteria in the cortex and mesohyl, as discussed in the previous section. This differential uptake suggests that the difference in the availability of substrates may lead to physiological and genotypical adaptation of the community (Gocke, 1977a, b). The rates of uptake of substrates were also higher than the reported values in the seawater from west and east coasts of India (Ramaiah et al., 1996; Ram et al., 2003) and in the tissues of deep water sponges, *H. thielei* and *R. nodastrella* (0.03 to 0.11

nM cm^{-3} d^{-1}) (van Duyl et al. 2008). As *C. alloclada* is an intertidal sponge, the possibility is that the microbial consortia may have high uptake potential. Moreover, a high number of metabolically active bacteria (10^{8-9} viable bacterial cells) in the sponge would help in enhancing the heterotrophic activity (Hobbie and Crawford, 1969; Seki et al., 1975) than in that of the ambient water and deep water sponges (10^5 total bacterial cells). It has been reported that the choanocytes of sponges may also consume DOM (van Duyl et al., 2008). The ambient water along with DOM is pumped into the sponge and the DOM can either get incorporated into the microbial consortia in the sponge and/or in the sponge itself (Yahel et al., 2003). Incorporation in the sponge cell may be negligible as amino acids and simple sugars at nanomolar concentrations are primarily utilised by prokaryotic rather than eukaryotic cells (Kirchman et al., 1985). Therefore, it can be assumed that during this short time, protein synthesis and carbohydrate metabolism is performed predominantly by the sponge-associated microbial consortia (van Duyl et al., 2008).

On an average, inorganic carbon uptake in the sponge cortex and mesohyl tissues were 15.0±2.4 and 9.4 ± 1.1 µg C $cm^{-3}d^{-1}$, respectively with no significant difference between them (Table 5.5).

Table 5.5. Inorganic carbon fixation rate

Location	Production (µg C cm^{-3} d^{-1})
Mesohyl	9.4±1.1
Cortex	15.0±2.4
Water	0.20±0.07

The inorganic carbon uptake in sponge exceeded by 2 orders than that in the ambient water ($p<0.05$). The overall rate was higher than the deep water sponges, *H. thielei* and *R. nodastrella* (0.5 to 25 nM C $cm^{-3}d^{-1}$). This may either be due to 4 orders higher number of bacteria in *C. alloclada* than *H. thielei* and *R. nodastrella* or difference in the habitat. In *C. alloclada*, the inorganic carbon fixation might be contributed by both photosynthetic (Cyanobacteria, Chloroflexi, Rhobacteriales etc.) and chemosynthetic bacteria, (nitrifying and sulfur oxidizing bacteria), as these were detected in this sponge whereas in *H. thielei*

and *R. nodastrella,* the carbon fixation is mainly by major chemosynthetic organisms (Herndl et al., 2005). Another group which would have contributed to autotrophic production of DOM is Archaea (Konneke et al., 2005). The high number of photosynthetic symbionts in the cortex tissues of *C. alloclada* fixes inorganic carbon and the assimilated photosynthate may be passed actively or passively to sponge cortex cells and this could be taken up by associated bacteria (Lavy et al., 2014). Interestingly in *C. alloclada,* the autotrophic carbon fixation and heterotrophic glucose uptake were almost equal and was very similar to the deep sea sponges *H. thielei* and *R. nodastrella* (van Duyl et al., 2008).

Micro niches in the sponge may shape the bacterial community structure by the generation of carbon sources available for microbial growth as has been suggested for other symbiotic relationships (Berg and Smalla, 2009; Haichar et al., 2008), and bacteria might have developed metabolic adaptations to individual sponge species. Intertidal sponges often face unfavorable conditions. Bacteria associated with these sponges must have also evolved a wide variety of metabolic strategies to cope up with such a dynamic environment. These bacteria can survive where resources are diverse and compete for nutrients with other bacteria present in the host. The reason might be because of their easy uptake through the cell wall. Also, the increased levels of nutrients in the sponge helps increased metabolic capabilities of sponge-associated bacteria (Feby and Nair, 2010). The associated bacteria in *C. alloclada* were metabolically active and versatile as the sponge provided a stable and nutritionally rich habitat. The diverse suite of metabolic pathways may be indicative of the ability to live as well as to adapt to the complex sponge environment. Bacteria release labile DOM from POC that is consumed mainly by the bacterial community or the sponge (Reiswig, 1981). Alternatively, DOM in ambient seawater may also be used directly by benthic organisms (de Goeij and van Duyl, 2007) or via associated prokaryotes. It has been shown that proline is taken up by sponge-associated prokaryotes and is subsequently transferred to sponge cells (Wilkinson and Garrone, 1979). This study showed that *C. alloclada* use dissolved as well as particulate matter as a food source which would allow it to survive in areas with low levels of particulate organic carbon and in the intertidal region.

5.2.2. METHANE OXIDATION

Methane is an intermediate in the mineralisation of organic compounds (Dagley, 1978). Some marine invertebrates are able to live indirectly on methane (deChaine and Cavanaugh, 2005;

Cavanaugh et al., 2006; Dubilier et al., 2001). It was seen that the deep-sea sponge, *Cladorhiza* sp. harbour methanotrophic bacteria that are transferred from one generation to other via vertical transmission (Vacelet et al., 1996; Vacelet and Boury-Esnault, 2002). This suggests that methanotrophic bacteria form a stable association with this sponge (Vacelet et al., 1995, 1996; Vacelet and Boury-Esnault, 2002). As *Cladorhiza* sp. lacks the aquiferous system, it was suggested that associated methanotrophs might have contributed a significant portion of the host sponge's nutrition in the nutrient-depleted deep sea (Vacelet and Boury-Esnault, 2002). There was no indication whether methanotrophic bacteria are actively involved in methane oxidation in the sponge tissues or are simply associated with the sponge. In the present study, methane oxidation by microbial consortia in mesohyl was below detection level. However, in cortex tissues, the rate was 1.3±0.2 mM cm^{-3} d^{-1} (Table 5.6).

Table 5.6. Rate of methane oxidation by microbial consortia and methanotrophs

Sample	Microbial consortia (mM $cm^{-3}d^{-1}$)	Methanotroph (mM d^{-1})
Mesohyl	Nd	0.28±0.03
Cortex	1.3±0.2	0.36±0.03
Water	3.2± 0.3	0.43±0.04

Nd - Not detected

The rate of methane oxidation was 3 times higher in sponge ambient water (3.2± 0.3mM cm^{-3} d^{-1}). The number of methane-oxidising bacterial isolates accounted for 3.8% of total THB in *C. alloclada*. A higher number of methane oxidising bacterial isolates was found in the mesohyl tissues of sponge (32%) than cortex tissue (23%) and this difference was significant. The rate of methane oxidation ranged from 3.9±2.3 to 624.9±0.0 µM d^{-1} among bacterial isolates. The average rate of methane oxidation ranged from 8.6± 5.0 to 621.4 ±3.7 µM d^{-1}, 3.9 ±2.3 to 624.9 ±0.0 µM d^{-1} and 246.4 ±214.5 to 622.4± 1.6 µM d^{-1}, in mesohyl, cortex and ambient water, respectively. The rate of methane oxidation by isolates was one order less than the activity of whole microbial consortia of sponge tissues. The average rate of methane oxidation was less in sponge mesohyl, which might be related to the low oxygen concentration in the mesohyl.

The reported level of methane in the coastal waters off Goa was 2 nM and surface saturation was 110-2521 % (Lal et al., 1996; Jayakumar et al., 2001). There is an indication that methanotrophic bacteria are involved in methane oxidation in the intertidal sponge, *C. alloclada*, as OTUs of methanotrophic bacteria belonged to α-, β-, γ-Proteobacteria and Verrucomicrobia were detected. The functional gene analysis and metabolic properties inferred from 16S rRNA metagenomics data and process data show that the sponge-associated microorganisms are capable of methane oxidation (Taylor et al., 2007). However, at this juncture, it cannot be inferred that these methanotrophic bacteria were simply associated with the sponge in a transient and allochthonous manner or actively involved in the process of methane oxidation. Free-living methane oxidisers in sea water and sediment are directly exposed to methane. However, bacteria present in the sponge cannot access environmental methane. The sponge may provide the associated bacteria in the tissues with a stable environment, helping to buffer them against the temporal and spatial variability in the supply of electron donors and acceptors characteristic of the dynamic inner environment. Methane-oxidising bacteria occurring within the sponge tissue are therefore dependent on the diffusive flux of methane through water currents produced by the host. They may also help to sustain diverse microbial communities and higher organisms through the conversion of methane to organic compounds as seen in deep-sea vents (Semrau et al., 2010). Methane oxidisers may provide additional nutrition to the food chain in the sponge holobiont and help this sponge to dominate the intertidal fauna in the dynamic intertidal region.

5.2.3. NITRIFICATION

In sponge *C. alloclada*, nitrite and nitrate were produced in mesohyl and cortex indicating active nitrification by microbial consortia. High net production of ammonium and nitrite was found in mesohyl (5.16±4.56 μM cm^{-3} d^{-1} and 28.78±0.01 μM cm^{-3} d^{-1}, respectively), whereas high net production of nitrate was observed in cortex (115.10±10.70 μM cm^{-3} d^{-1}) which were 6X higher than mesohyl (20.42±10.56 μM cm^{-3} d^{-1}) (Table 5.7). The rate of production of nitrite and nitrate was 3 orders less in ambient water than sponge. There was a significant difference in the ammonium, nitrite and nitrate production among mesohyl, cortex and

ambient water ($p<0.05$). Average net production of ammonium was not significantly different between mesohyl and cortex. Unlike ammonium, the rates of nitrite and nitrate production between mesohyl and cortex were significantly different (Table 5.7).

Table 5.7. Production of ammonium, nitrite, nitrate and net nitrification in cortex and mesohyl of sponge and ambient water

	Ammonium	Nitrite	Nitrate	Net
	Net production rate (μM cm^{-3} d^{-1})			nitrification
Mesohyl	5.16±4.56	28.78±0.01	20.42±10.56	3.50±0.55
Cortex	2.38±0.02	4.32±0.82	115.10±10.70	8.52±1.2
Water	ND	0.02±0.02	0.53±0.01	0.02±0.02

The production of ammonium was not detected in ambient water. The ammonium production in sponge may be due to the activities of the photosynthetic community in the exposed tissues of the sponge (Perea-Blazquez et al., 2012a, 2012b) or decaying of some sponge tissues. The significant difference in the production of inorganic nitrogen in cortex and mesohyl may be due to the difference in their environment such as availability of nutrients, oxygen and space (Yang and Li, 2012). Nitrite being an intermediate is produced in a very small amount which has also been reported in other sponges (Schlappy, 2008). The anaerobic loss of nitrate through denitrification has not been taken into account which may be one of the reasons for the variability in the net nitrification. Photosynthetic community in the sponge may also uptake nitrate released by nitrifying bacteria (Fiore et al., 2013). There was significant variation in the net nitrification between mesohyl and cortex microbial consortia ($t = 5.0$; $p<0.05$). Net nitrification was higher in sponge cortex (8.52±1.2 μM cm^{-3} d^{-1}) than mesohyl microbial consortia (3.50±0.96 μM cm^{-3} d^{-1}). Net nitrification in sponges has been reported earlier (Bayer et al., 2008b; Hoffmann et al., 2009), suggesting that nitrification may be common in sponges, but the rates may be variable from 0.48 to 48.0 μM g^{-1} d^{-1} in different sponges (Corredor et al., 1988; Diaz and Ward, 1997; Jimenez and Ribes 2007; Bayer et al., 2008a, b; Southwell et al., 2008; Hoffmann et al., 2009; Schlappy et al., 2010; Radax et al., 2012a). The main benefit of nitrification in sponges lies within the efficient removal of waste products (Hoffmann et al., 2009). The bulk nitrification rate in this study may be considered as net rate, since part of the products might be used to fuel denitrification and anammox as

suggested by Hoffmann et al. (2009). The total net nitrification rates reported in this study were 2 to 12X higher than studies carried out with sponge explants and deep water sponges from temperate and cold water sponges (Bayer et al., 2008b; Hoffmann et al., 2009; Radax et al., 2012a). However, the net nitrification rate in this study (3.50 to 8.52 µM g^{-1} d^{-1}) was close to the reported value in the tropical sponge (Diaz and Ward, 1997) but 2-3 orders of magnitude higher than the estuarine sediment (Bernhard et al., 2007). Being an intertidal sponge, *C. alloclada* is exposed to the nutrient rich environment compared to the deep water sponges or aquarium maintained explant. The sponge actively and non-selectively filters bacteria and other organic sources from ambient water, which provide a significant amount of particulate organic nitrogen (Lesser, 2006; Trussell et al., 2006). The utilisation of nitrogen rich particulate organic matter results in the production of ammonium (Fiore et al., 2013), which fuels nitrification.

ANOSIM showed significant difference among different samples based on nutrient release and nitrification rate (Global R-0.165; $p<0.001$). It is therefore concluded that sponge, *C. alloclada* harbour nitrifying bacteria which are actively involved in nitrification. The differential activities of nitrifying bacteria in mesohyl and cortex may be due to the temporal and spatial occurrence of oxic and anoxic zones. This was achieved by controlling the pumping activity of sponge. The different nitrification rates in mesohyl and cortex may also be due to the difference in the number of nitrifying bacteria. This high number of nitrifying bacteria and their activity may help to get rid of ammonium and nitrite and may inhibit sulfate reducing and denitrifying bacteria.

5.2.4. Denitrification

Denitrification is a microbial process of nitrate reduction to nitrite, nitric oxide, nitrous oxide, and finally N_2 at limited oxygen concentration. Denitrification in animals was first described in the guts of benthic invertebrates (Stief et al., 2009). A few studies have been reported on denitrification process and the presence of genes *nir K* and *nos Z* which codes for enzymes involved in denitrification process in sponges from temperate region and cold-water sponges (Hoffmann et al., 2009; Schlappy et al., 2010; Yang and Li, 2012; Fiore et al., 2013; Li et al., 2014). Denitrification in sponges was first reported in cold water sponge, *Geodia barretti* (Hoffmann et al., 2009) and the rate of denitrification was 92 nM $cm^{-3}d^{-1}$. In *C. alloclada,* denitrification by microbial consortia measured in terms of nitrous oxide production in

mesohyl tissues (414±4 nM $cm^{-3}d^{-1}$) was *ca* 3 times higher than that in cortex tissues (152±1 nM $cm^{-3}d^{-1}$) (Table 5.8).

Table 5.8. Rate of denitrification by microbial consortia and denitrifying bacterial isolates

Sample	Microbial consortia (nM cm^{-3} d^{-1})	Denitrifying bacteria (nM d^{-1})
Mesohyl	414±4	0.02±0.01
Cortex	152±1	0.01±0.001
Water	Nd	Nd

Nd - Not detected

In Mediterranean sponges, *Chondrosia renifromis* and *Dysidea avara*, denitrification rates were 360 nM $cm^{-3}d^{-1}$ and 242 nM $cm^{-3}d^{-1}$ (Schlappy et al., 2010). The reason for the higher nitrous oxide production in mesohyl of *C. alloclada* than cortex might be attributed to the less oxygen availability or more available nitrate (Diaz and Ward, 1997). Denitrification rate by bacterial isolates ranged from 0.001 to 0.04 nM d^{-1}. Six percent of the mesohyl bacteria exhibited denitrification indicating that these denitrifiers may be sponge-specific. The nitrous oxide production was not detected in ambient water, because of the oxic condition in ambient water (DO-5.9 mg l^{-1}).

It has also been hypothesised that the production of nitrate and nitrite could facilitate denitrification in the interior hypoxic zones of the sponge (Southwell, 2007; Taylor et al., 2007). The source of nitrate for denitrification in the sponge may be either from ambient water or produced via nitrification in the sponge. Hoffmann et al. (2009) using stable isotope found that 74% nitrate was from ambient water and 26% from nitrification. In this sponge, nitrification would contribute to the nitrate production at a rate of ca 0.1 to 4 µM cm^{-3} d^{-1}. The temporal and spatial occurrence of oxic and anoxic zones within the sponge would favour both the nitrifiers and the denitrifiers and this suggests that the microbial community inside *C. alloclada* is able to deal with shifts from aerobic to anaerobic conditions inside the sponge. If nitrification and denitrification are tightly coupled, then variations in H_2S or O_2 concentrations may influence the rates of these processes and the net fluxes of dissolved inorganic nitrogen from the sponge. Since denitrification removes inorganic nitrogen from the environment, sponges may function as nitrogen sinks which is so far unrecognised in the ocean.

5.2.5. Sulfate reduction

Dissimilatory sulfate reduction is carried out by anaerobic sulfate reducing bacteria (SRB) that use sulfate as a terminal electron acceptor in the oxidation of organic matter, thus reducing sulfate stoichiometrically to sulfide. This activity is an important biogeochemical process that links the sulfur and carbon cycle. Sulfate reduction rate (SRA) in the mesohyl and cortex of *C. alloclada* by the microbial consortia was 46.0±2.8 and 34.3±6.8 (nM $cm^{-3}d^{-1}$), respectively. However, in water, the sulfate reduction rate (0.24±0.16 nM $cm^{-3}d^{-1}$) was 2 orders less than that in sponge (Table 5.9).

Table 5.9. Rate of sulfate reduction by microbial consortia and sulfate reducing bacteria

Sample	Microbial consortia (nM cm^{-3} d^{-1})	SRB (nM d^{-1})
Mesohyl	46.0±2.8	3.5 ± 0.1
Cortex	34.3±6.8	2.5 ± 1.4
Water	0.24±0.16	2.0±0.9

There was not much variation in the SRA activity in *C. alloclada* unlike *Geodia barreti*, a cold water sponge, where sulfate reduction rate showed wide variations (1-1200 nM $cm^{-3}d^{-1}$) which increased with depth. Sulfate reduction rate in the surface tissues of *G. barretti* (<200 nM $cm^{-3}d^{-1}$) was higher than that of the present study. The link between anatomy and microbial load and oxygen concentration has been attributed to the nutritional strategy of the sponge (Vacelet and Donadey, 1977; Hoffmann, 2003). SRA depends on the oxygen level present in the sponge as SRA corroborated with a decrease in oxygen level from the surface of sponge (Hoffman, 2003). The low rate seen in *C. alloclada* compared to *G. barreti* may be due to the difference in the width of the sponge. In *G. barretti* highest SRA was at 5 cm depth (increasing rates with depth). In case of *C. alloclada,* the radius of the sponge was only <2 cm. SRA also depended on the density of sulfate reducing bacteria (10^3 and 10^2 CFU cm^{-3} in the mesohyl and cortex, respectively) compared to 10^{10} cells cm^{-3} in *G. barretti*. The high abundance of SRB would have contributed to high SRA in the mesohyl. SRA by SRB from mesohyl ranged from 3.28 to 3.53 nM d^{-1} and by cortex bacteria was 0.52 to 2.98 nM d^{-1} (Table 5.9). Further, the active metabolism by associated aerobic microorganisms creates tissue anoxia inside the sponge. Under the anoxic condition, sponge cells will switch their metabolism to fermentation and the fermentation products will act as substrates for SRB

(Hoffmann, 2003). The toxic sulfide is likely to be removed by sulfur oxidising bacteria as OTUs belonging to *Thiobacillus, Chromatiales, Thiohalorhabdales* and *Thiotrichales* were found in *C. alloclada.*

5.2.6. Defence: Antagonistic interaction of Actinobacteria

Studies on antagonistic interaction in sponge is mainly on Actinobacteria (AB) against a wide range of organisms such as bacteria, fungi and parasites (Thakur and Anil, 2000; Selvin et al., 2004; Han et al., 2009; Vidgen et al., 2012). Most of these studies have examined the antagonism using primarily pathogens and have found up to 20% of sponge isolates to be inhibitory (Anand et al., 2006). In this study, 825 antagonistic interactions of AB against 75 heterotrophic bacteria (THB) from the same sponge and ambient water using 11 x 75 array of tests showed that all AB associated with sponge inhibited more than one THB. A total of 316 antagonistic interactions were exhibited and out of these, 157 interactions (49.7%) were against THB from ambient water (Figure 5.9).

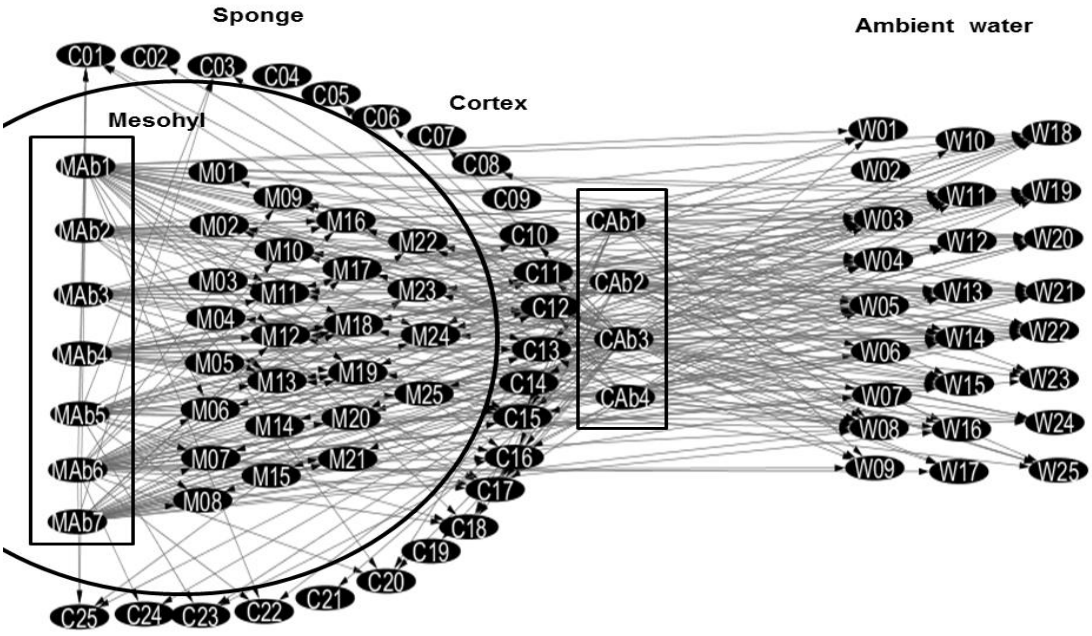


Figure 5.9. Network of total 316 antagonistic interactions by Actinobacteria from the mesohyl and the cortex (MAb and CAb, respectively) against heterotrophic bacteria from sponge mesohyl (M), cortex (C) and the ambient water (W). The nodes represent Actinobacteria and heterotrophic bacteria, and the directed edges represent the antagonistic relationship of the bacteria.

ANOSIM showed that antagonistic interactions with THB from ambient water was significantly higher than that with sponge associated THB (Global R=0.03, $p<0.05$). Multiple inhibitions or antagonism were also observed among AB. More than one AB inhibited 58 THB (Figure 5.10).

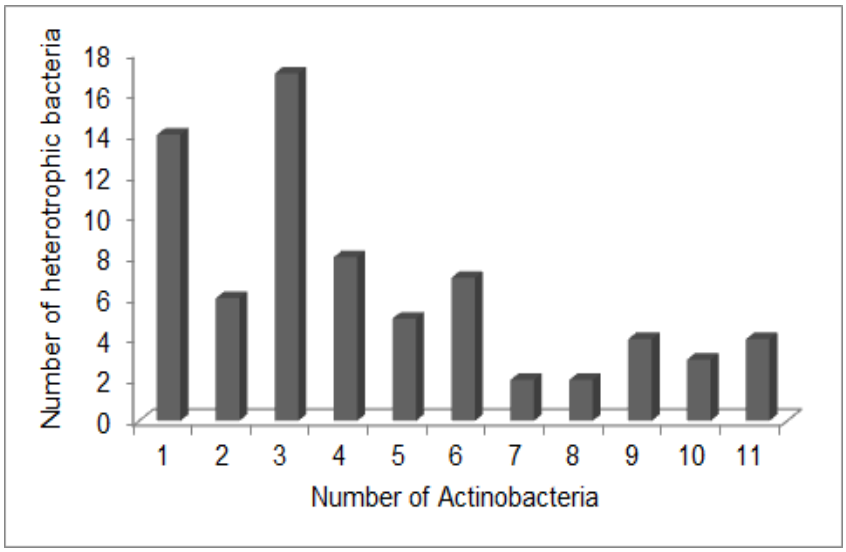


Figure 5.10. Multiple inhibition of Actinobacteria against heterotrophic bacteria

The order of inhibition of THB based on the source of isolation was, ambient water > cortex> mesohyl. Similarly, higher number of multiple inhibitions by AB was seen with THB from ambient water. Overall antagonistic potential of sponge was 28.7 and antagonistic potential of cortex was 35.0 which were higher than that of the mesohyl (25.1). Intra-antagonistic interaction showed that antagonistic potential of the cortex AB against the cortex THB was 10.3 and the mesohyl AB against the mesohyl THB was 5.3. This high interaction of AB might be because of their inherent potential to produce several antimicrobial substances (Selvin et al., 2004; Dharmaraj and Sumantha, 2009; Han et al., 2009). Antagonistic activity can confer a competitive advantage within a bacterial community by keeping sensitive isolates away from its niche (Tait and Sutherland, 2002).

5.3. Conclusion

It is more likely that the intertidal ecosystem is fuelled mainly by particulate organic matter derived from terrestrial origin as well as from the oceanic water. The present study revealed the presence of high number of bacteria associated with the sponge (70%) producing extracellular enzymes that play a crucial role in biopolymer degradation like proteins, carbohydrates, lipids and nucleic acids thereby assisting the host in its nutrition and various metabolic processes. Functions of culturable bacterial communities associated with sponges were also variable and the rates were differing depending on the bacteria and location in the sponge. Functions associated with anaerobic or facultative bacteria were more in the mesohyl than cortex and aerobic bacterial process were higher in the cortex than mesohyl. Intertidal sponges experience alternate wet and dry period depending on the tide. Hence the accessibility of dissolved and particulate organic matter from surrounding water is not continuous and may not reach in ample amount to fulfill the required DOC for sponge-holobiont. Autotrophic microorganisms supplement the additional DOC. Though a part of DOC may be lost by respiration and the rest is stored as biomass which will be available to use for sponge host and /or associated microbial consortia (vanDuyl et al., 2008). It has been suggested that co-occurring bacteria avoid competition by utilizing different carbon and energy sources (Duperron et al., 2006; Kleiner et al., 2012). Apart from habitat structure, animal host interaction and local biogeochemical conditions, the function of heterogeneous bacterial communities were shaped by associated Actinobacteria as high antagonistic activity was seen in this sponge. Thus, these sponge holobionts not only utilise the carbon, nitrogen and phosphate for its survival but may also provide these simple compounds to the sponge. Sponge tissues provide a dynamic environment for associated bacteria, where resource distribution varies spatially and temporally. In addition, sponge provide a plethora of carbon and energy sources by filtering sea water, that significantly affect the population of microorganisms in a manner specific to the host. The diverse suite of metabolic pathways may be indicative of the ability to live as well as to adapt in the complex sponge environments. Thus, this study clearly demonstrates that multiple functions of associated bacteria help to balance the ecology of sponge *Cinachyra allocada*.

CHAPTER 6

LINKING BACTERIAL PHYLOGENY TO FUNCTION

6.1 Introduction

Studies on the microbial communities in the sponge and ambient water revealed that sponges harbour a high diversity of the microbial population that represents most of the phylogenetic groups o bacteria (Gao et al., 2015; Luter et al., 2015). Phylogenetically distant sponges from different geographical locations often share similar bacteria, which have not been detected in seawater (Hentschel et al., 2002; Taylor et al., 2007; Li, 2009). Many sponge-derived metabolites with bacterial origin have been reported (Hochmuth and Piel, 2009; Piel, 2009). There are some scattered studies available on the functional aspects of sponge-associated microorganisms, mainly dealing with antimicrobial activity.

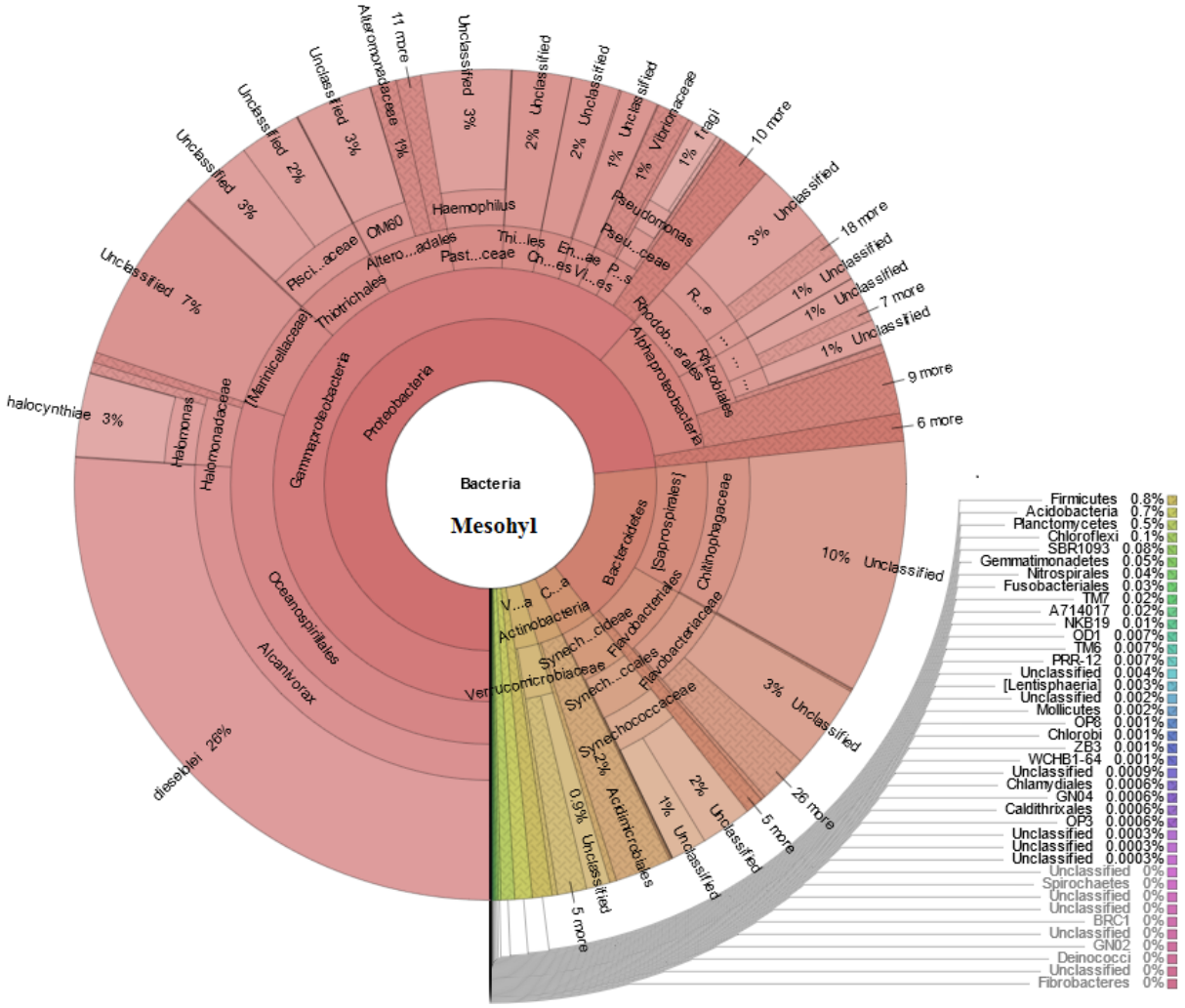
Studies linking the bacterial diversity to function in the sponge started in the early 2000s with the advent of 16S rRNA gene sequencing. Earlier studies were mostly concentrated on the antimicrobial activity and phylogenetic diversity of sponge- associated bacteria (Thiel and Imhoff, 2003; Chelossi et al., 2007; Durai et al., 2013; Handayani et al., 2015). With the introduction of metagenomic analysis, predicting the functions of sponge- associated microbiome based on the open reading frame of functional genes involved in various processes in the sponges, such as those involved in the metabolism, defence, cycling of carbon, nitrogen and sulfur etc. are being carried out (Kennedy et al., 2007; Thomas et al., 2010; Schmitt et al., 2012b; Radax et al., 2012; Nakashima et al., 2016). Associated microbes are now believed to be actively involed in providing nutrients to the sponge (van Duyl et al., 2008). However, the studies on actual processes involved are very limited except for nitrification (Schlappy et al., 2010; Radax et al., 2012). Nitrification process and diversity of bacteria carrying *amo A* genes were estimated in Mediterranean sponges (Bayer et al., 200b8a,b). Hoffmann et al. (2009) found various β- and γ-Proteobacteria responsible for denitrification in cold water sponge, *Geodia barretti*. However, the mere presence of the gene cannot reveal whether the specific function is actually being carried out in the sponge. Further, none of the studies showed the involvement of associated bacteria in multiple

activities such as biogeochemical cycling (both in aerobic and anaerobic conditions) of carbon, nitrogen and sulfur and in defence mechanism in any sponge species.

Metagenomic and culture approaches of the microbial community associated with the sponge *C. alloclada* indicated the presence of bacteria and archaea. Most of these species are known for their versatile metabolic properties and ecological functions. Details concerning the metabolic capabilities of the associated microbial communities are discussed in Chapter 5. From the information, it can be deduced that some of the identified bacterial groups may possibly be involved in the sponge's biology and linking the bacterial diversity to function in the sponges is imperative in understanding the role of associated microbes on the host biology. In this chapter, an attempt has been made in linking the OTUs, functional gene orthologs ad functional activities. However, interpretations of the functional potential of the identified microbial communities should be viewed with caution as the actual role and the functional potential can differ between species and within the same species.

6.2 RESULTS AND DISCUSSION

The phylogenetic analysis of 197143 to 295671 paired-end reads obtained from the duplicate samples of mesohyl and cortex tissues of the sponge in NGS analysis belonging to 2978 to 3029 OTUs showed 38 bacterial phyla with Proteobacteria as the predominant group. The Krona chart which allows comparison between bacterial OTUs from mesohyl and cortex based on the phylogenetic relationship is shown in figure 6.1a&b.


Bacteria
Mesohyl
Proteobacteria
Gammaproteobacteria
Alphaproteobacteria
Bacteroidetes
[Saprospirales]
Actinobacteria
Verrucomicrobiaceae
Thiotrichales
Oceanospirillales
Alcanivorax
dieselolei 26%
halocynthiae 3%
Halomonas
Halomonadaceae
Marinicellaceae
Unclassified 7%
Unclassified 3%
Unclassified 2%
Unclassified 3%
Alteromonadaceae 1%
11 more
Unclassified 3%
Unclassified 2%
Unclassified 2%
Unclassified 1%
Vibrionaceae 1%
Pseudomonas
4 agi
10 more
3% Unclassified
18 more
1% Unclassified
1% Unclassified
7 more
1% Unclassified
9 more
6 more
10% Unclassified
Chitinophagaceae
Flavobacteriales
Flavobacteriaceae
3% Unclassified
26 more
5 more
2% Unclassified
1% Unclassified
Synechococcaceae
2% Actinomicrobiales
0.9% Unclassified
5 more
Haemophilus
OM60
Pseu...ceae
Past...ceae
Altero...adales
Rhodob...erales
Rhizobiales
Firmicutes 0.8%
Acidobacteria 0.7%
Planctomycetes 0.5%
Chloroflexi 0.1%
SBR1093 0.08%
Gemmatimonadetes 0.05%
Nitrospirales 0.04%
Fusobacteriales 0.03%
TM7 0.02%
A714017 0.02%
NKB19 0.01%
OD1 0.007%
TM6 0.007%
PRR-12 0.007%
Unclassified 0.004%
[Lentisphaeria] 0.003%
Unclassified 0.002%
Mollicutes 0.002%
OP8 0.001%
Chlorobi 0.001%
ZB3 0.001%
WCHB1-64 0.001%
Unclassified 0.0009%
Chlamydiales 0.0006%
GN04 0.0006%
Caldithrixales 0.0006%
OP3 0.0006%
Unclassified 0.0003%
Unclassified 0.0003%
Unclassified 0.0003%
Unclassified 0%
Spirochaetes 0%
Unclassified 0%
Unclassified 0%
BRC1 0%
Unclassified 0%
GN02 0%
Deinococci 0%
Unclassified 0%
Fibrobacteres 0%

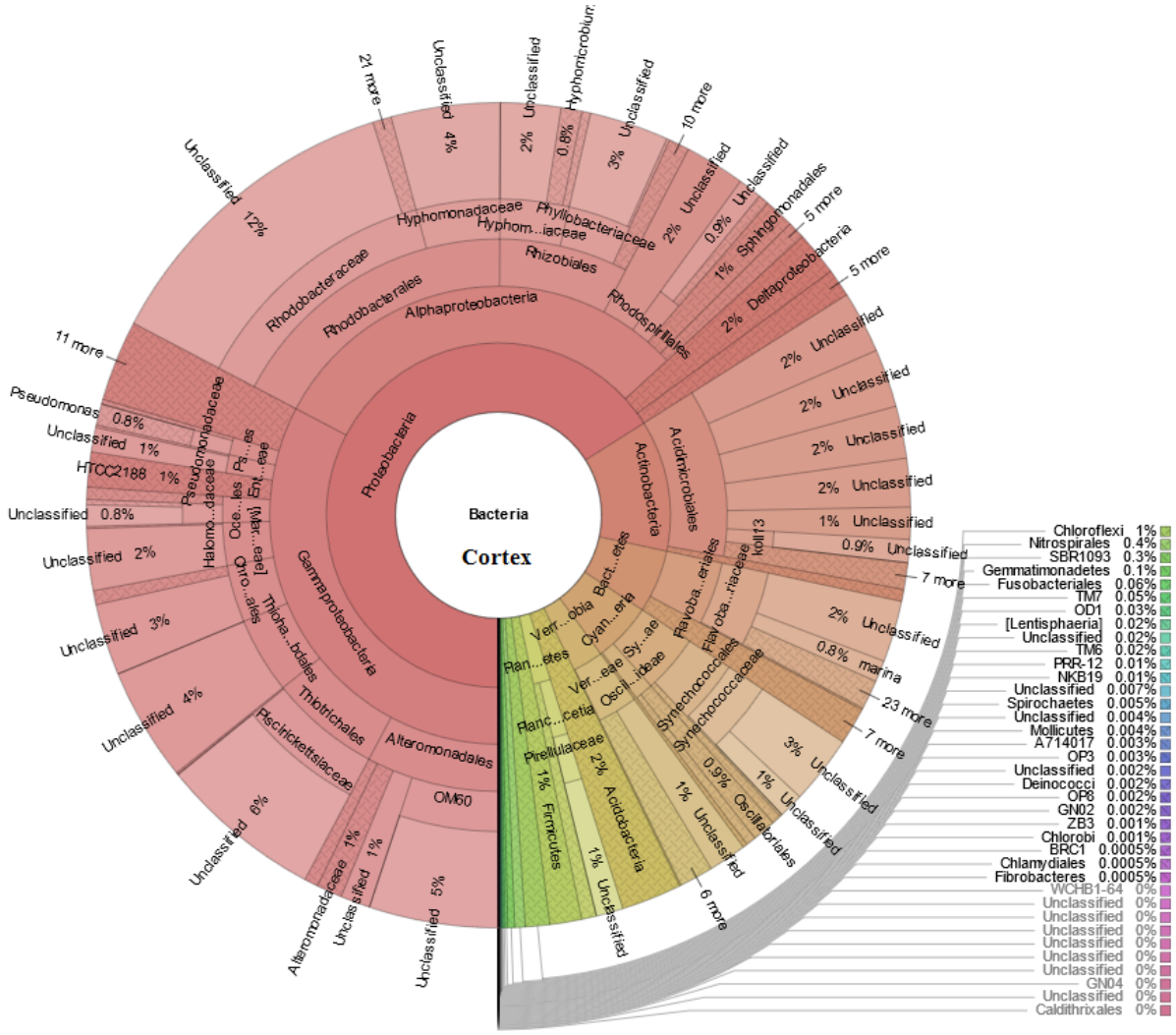


Figure 6.1b. Crona chart of bacterial diversity in the cortex of the sponge, *C. alloclada*

Functional profiling of microbial consortia associated with sponge was carried out by mapping the KEGG pathway ortholog ID (KO ID) with respect to OTU IDs and its corresponding KEGG pathway. Heatmap showing the relative abundance of bacterial genes from mesohyl and cortex tissue involved in different functions is shown in figure 6.2a&b. A total of 6119 KEGG pathway orthologs were found in *C. alloclada.* In general, they were involved in metabolism, genetic information processing, environmental information

processing, cellular processes, diseases and organismal systems, with higher number of genes in metabolism (1.3 –1.6 x10^8 copy number).

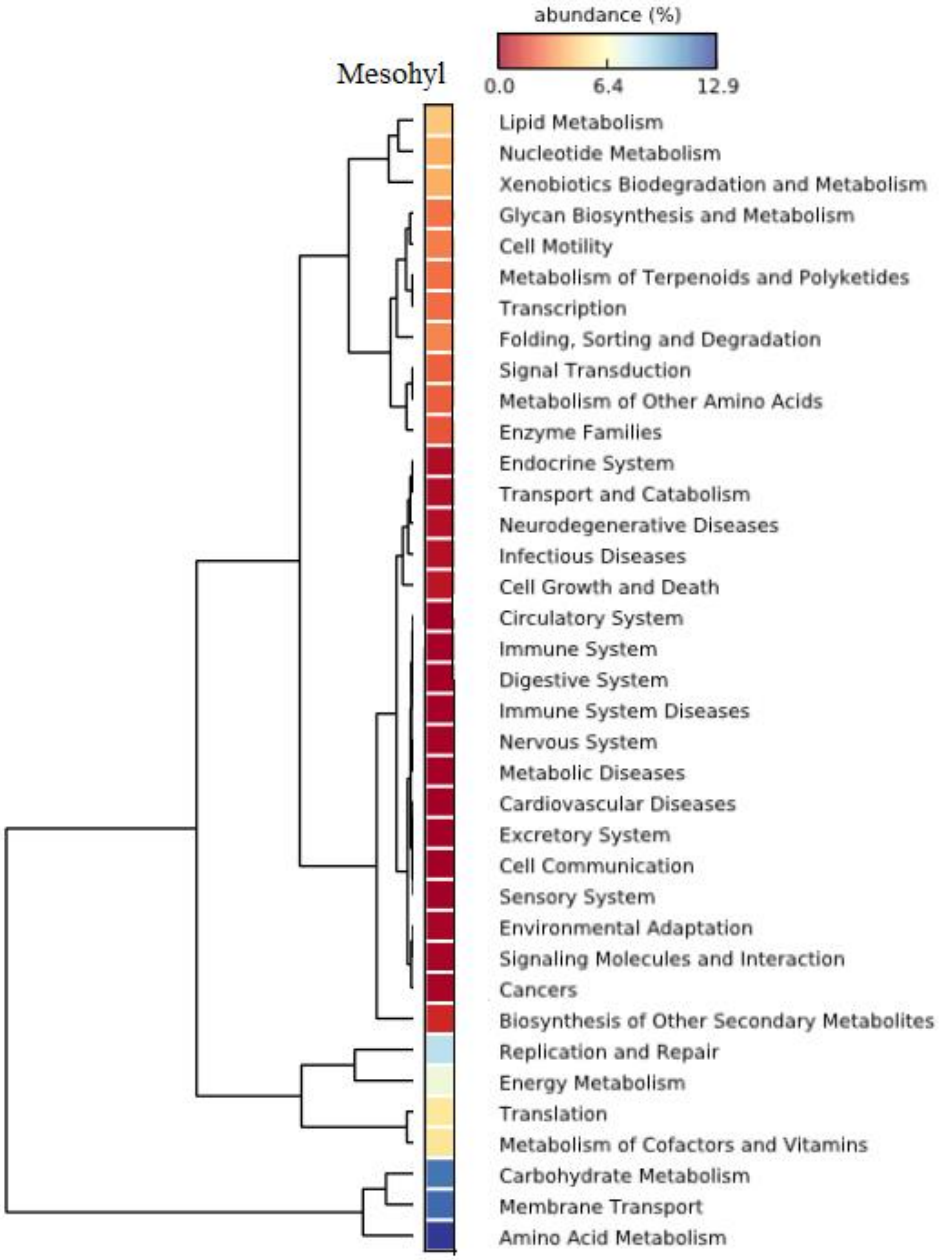


Figure 6.2a. Heat map showing the relative abundance of genes (copies per genome) involved in different functions in the mesohyl

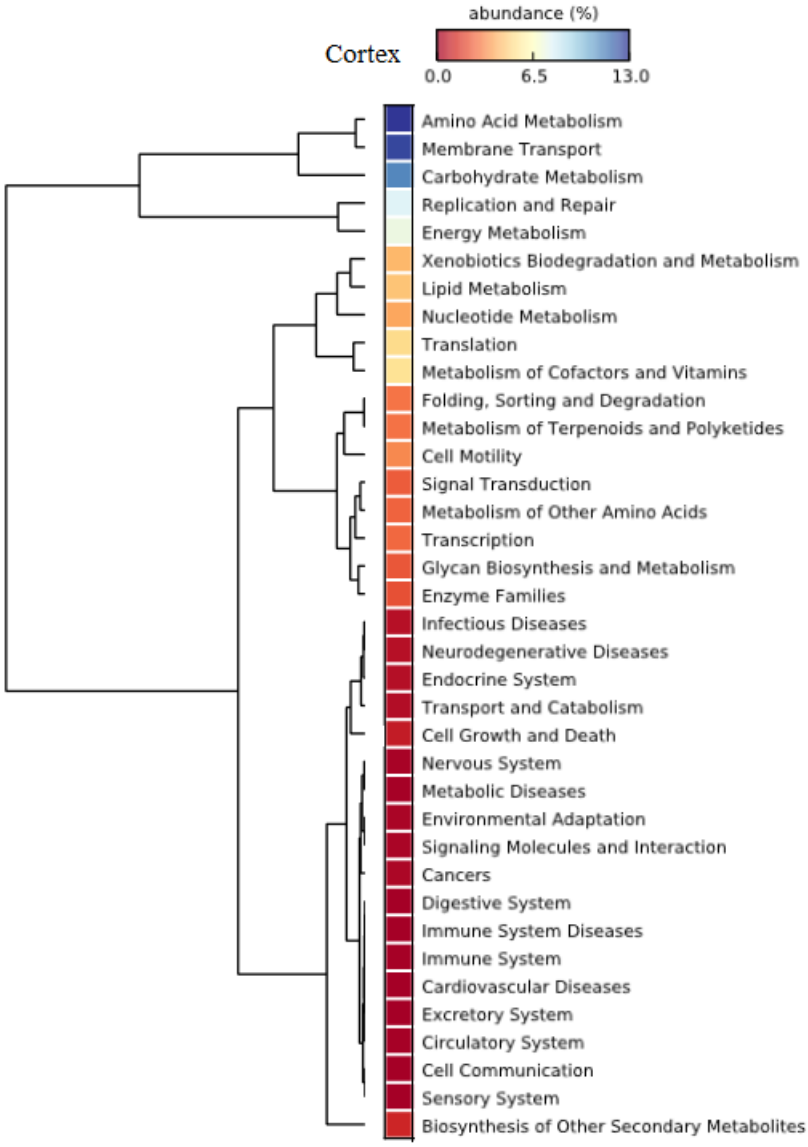


Figure 6.2b. Heat map showing the relative abundance of genes involved in different functions in cortex

In this chapter, an attempt has been made to link the bacterial phylogeny (OTU) to the functional genes and their activities, irrespective of their location in the sponge (Mesohyl and cortex). The KOs involved in the metabolism of amino acids, carbohydrates, lipids,

nucleotides, methane, nitrogen, sulfur, carbon fixation, secondary metabolites, terpenoid and polyketides, and xenobiotic degradation were studied further. Among the total predicted functions, top 5 KO IDs were selected for mapping with their corresponding OTU Ids (Table 6.1). Most of these KOs were involved in carbohydrate, amino acid, lipid, terpenoid, polyketide, and xenobiotic metabolism.

Table 6.1. KO IDs, the corresponding OTU numbers and their functions

KO ID	No. of OTUs	KEGG pathway
K00059	2883─2996	**Lipid Metabolism** (Fatty acid biosynthesis, lipid biosynthesis proteins, biosynthesis of unsaturated fatty acids)
K00626	2623─2711	**Carbohydrate Metabolism** (Butanoate metabolism, propanoate metabolism, glyoxylate and dicarboxylate metabolism, pyruvate metabolism), **Metabolism of Terpenoids and Polyketides** (Terpenoid backbone biosynthesis), **Lipid Metabolism** (Fatty acid metabolism, Synthesis and degradation of ketone bodies), **Xenobiotics Biodegradation and Metabolism** (Benzoate degradation), **Energy Metabolism** (Carbon fixation pathways), **Amino Acid Metabolism** (Lysine degradation, tryptophan metabolism, valine, leucine and isoleucine degradation)
K01692	2364─2421	**Metabolism of Terpenoids and Polyketides** (Limonene and pinene degradation, geraniol degradation), **Xenobiotics Biodegradation and Metabolism (**Caprolactam degradation, benzoate degradation, aminobenzoate degradation), **Carbohydrate Metabolism (**Butanoate metabolism, propanoate metabolism), **Lipid Metabolism (**Fatty acid metabolism), **Amino Acid Metabolism (**beta-alanine metabolism, lysine degradation, tryptophan metabolism, valine, leucine and isoleucine degradation)
K00799	1643─2626	**Xenobiotics Biodegradation and Metabolism** (Metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450), **Amino Acids Metabolism** (Glutathione metabolism)
K00249	1625	**Carbohydrate Metabolism** (Propanoate metabolism), **Lipid Metabolism** (Fatty acid metabolism), **Amino Acid Metabolism** (beta-alanine metabolism, valine, leucine and isoleucine degradation)

6.2.1 Carbon Cycle

6.2.1.1. Degradation and assimilation of organic compounds

Heterotrophs are known degraders of organic compounds. The degraded simple compounds may be assimilated by degraders or they will serve as nutrients for the host. In *C. alloclada*, heterotrophic bacteria were the major microbial biomass (10^{5-6} CFU cm^{-3}) and were distributed in 12 phyla (Actinobacteria, Acidobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaera, Planctomycetes, Proteobacteria, Spirochaetes and Verrucomicrobia). This consortium showed metabolic potential to degrade complex molecules and uptake of amines, amino acids, carbohydrates, carboxylic acids, polymers etc. and other miscellaneous substances. The genes codes for the amino acid, carbohydrate, lipid and nucleotide metabolism were obtained from *C. alloclada* metagenome. The number of orthologs involved in the amino acid metabolism ($1.3-1.6x10^8$ copy number) was higher than the genes code for proteins in carbohydrate, lipid and nucleotide metabolism ($2.9-3.6x10^7$, $1.1-1.3x10^7$ and $0.8-1.1x10^7$ copy numbers, respectively). The genes code for the metabolism of carbohydrates, amino acids, lipids and energy production have been reported in the metagenome of sponge *Cymbastella concentrica* (Thomas et al., 2010). The degradative nature of bacteria is because of their ability to produce an array of hydrolytic enzymes, which varied among different genera ($p<0.005$) (Figure 6.3). Most of the bacterial genera isolated from *C. alloclada* were carbohydrate degraders, thereby providing carbon source to the host. The dominant genera such as *Vibrio, Bacillus* and *Pseudomonas* were the main enzymes producers, which degrade carbohydrate, lipid, protein and DNA to provide carbon, nitrogen and phosphorus sources to the sponge holobiont, though variation was observed among different strains within each genus (Figure 6.3). In *Bacillus*, 33%, 20% and 40% of strains degraded lipid (Tween 20), casein and gelatin, respectively. It has been reported that *Vibrio, Bacillus* and *Pseudomonas* produce these enzymes (Janda et al., 1981; Focareta et al., 1991; Ali et al., 1999; Mehrotra et al., 1999; Baffone et al., 2001; Kwon et al., 1994; Heurlier et al., 2004; Dutta et al., 2004; Castro-Ochoa et al., 2005; Najafi et al., 2005; Venugopal and Saramma, 2006; Shanmughapriya et al., 2009; Molva et al., 2009; Mazotto et al., 2011). *Xanthomonas* and *Salinicola* showed similarity in their degradation potential as both produced only urea degrading enzyme.

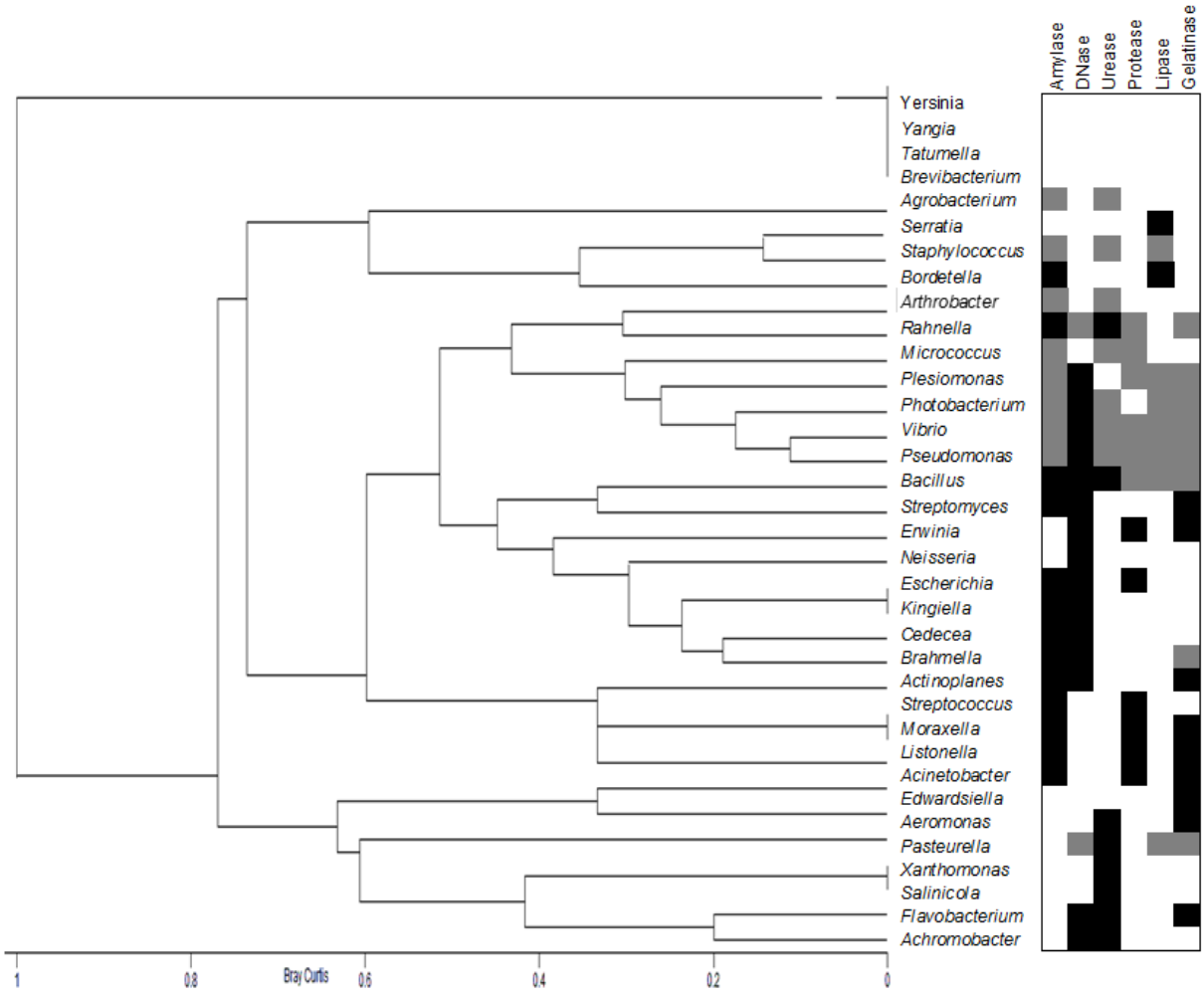


Figure 6.3. Production of extracellular enzymes by heterotrophic bacteria

■ production by all strains, ■ production by some strains, □ no production)

As these bacteria did not produce enzymes to degrade complex carbohydrates, they preferred simple sugars (Figure 6.4). Cao et al. (2012) have reported that *Salinicola* produces urease, but does not produce amylase and DNase, which is similar to the findings in the present study.A group of bacteria (*Salinicola, Flavobacterium, Xanthomonas, Pasteurella, Aeromonas, Achromobacter and Edwardsiella)* degraded only DNA, gelatin and urea. *Agrobacterium, Tatumella, Yangia* and *Yersinia* did not produce any enzyme. The absence of an extracellular enzyme production in these strains may be due to the presence of simple substrates which are readily available to the bacteria, as they utilise >6 simple carbon

compounds and hence there is no need to degrade complex molecules or for the production of alternative enzymes. *Alteromonas, Bordetella, Plesismons and Streptomyces* utilised all 8 simple carbon compounds. *Alteromonas* and *Streptomyces* strains have been reported (Lambert and Loria, 1989; Akagawa-Matsushita et al., 1992) to utilise these substrates. *Alcanivorax* can also utilise a large number of complex molecules (Yakimov et al., 1998; Wu et al., 2009). As these bacteria degraded carbohydrates, they might have a mechanism to take up simple mono- and disaccharides, which are the degradation products of complex carbohydrates.

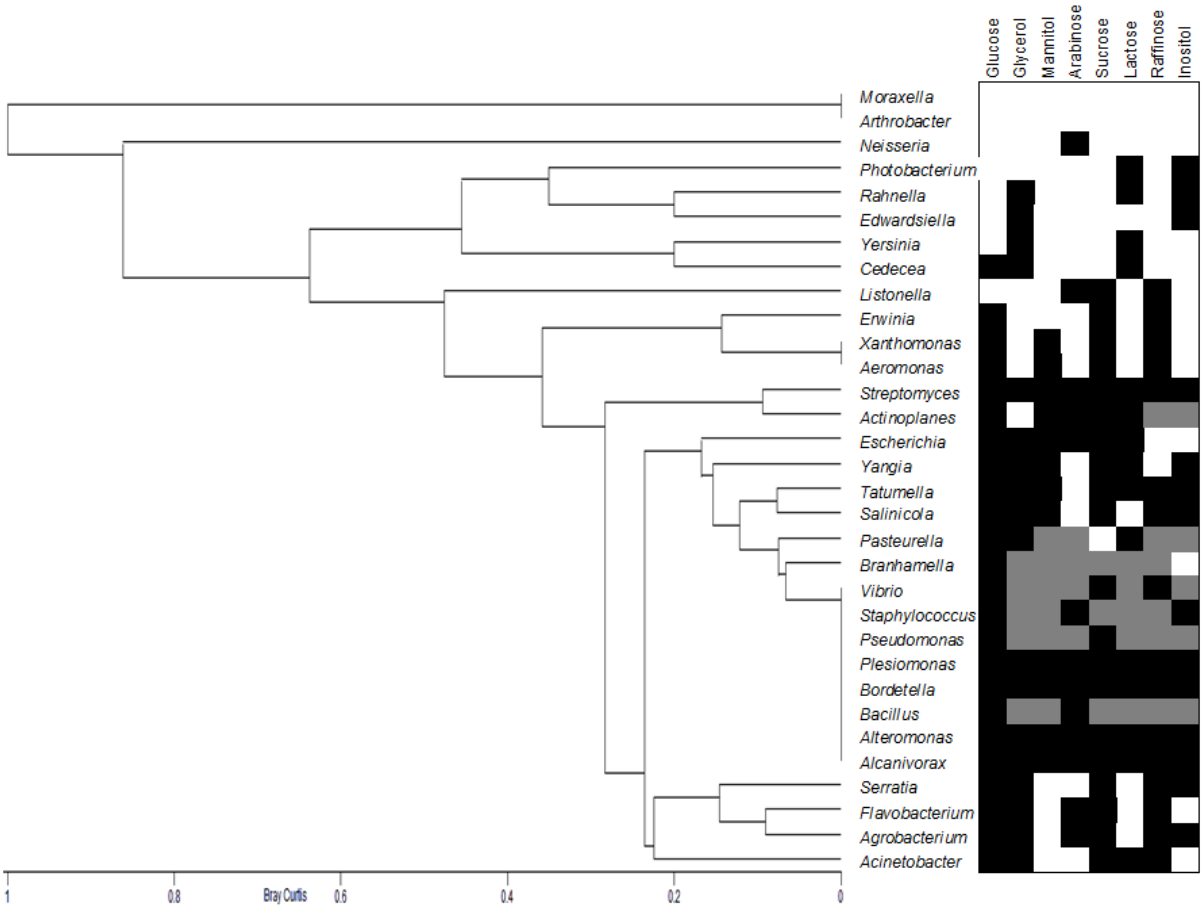


Figure 6.4. Utilisation of simple carbohydrates by bacteria (■ utilisation by all strains, ■ utilisation by some strains, □ no utilisation)

In general, *Bacillus, Pseudomonas, Staphylococcus* and *Vibrio* produced enzymes capable of degrading carbohydrates and utilised all the simple mono and disaccharides (Figure 6.4).

The differential utilisation of substrates showed that these bacteria not only utilised simple sugars but also utilised amino acids, carboxylic acids, polymers, sugar acids and alcohols (Table 6.2).

Table 6.2. Utilisation (%) of different category of substrates by different bacterial genera

	Amino acids	Carboxylic acids	Modified carboxylic acids	Modified sugar	Monosaccharide	Oligosaccharides	Other organic compounds	Polymers	Sugar acids	Sugar alcohols
Acinetobacter	67	14	0	13	14	9	0	25	50	25
Alcanivorax	89	100	88	100	100	100	86	75	83	100
Bordetella	78	100	100	100	100	100	86	50	100	100
Neisseria	100	71	75	88	100	73	71	100	83	75
Pasteurella	67	86	63	88	86	64	57	75	33	100
Photobacterium	33	43	50	38	14	36	29	0	17	50
Plesiomonas	89	57	88	75	57	73	29	75	83	75
Pseudomonas	89	71	75	63	86	82	100	75	100	50
Salinicola	67	86	75	75	86	55	57	25	50	100
Tatumella	78	71	75	75	71	82	57	50	17	100
Yangia	89	100	88	88	57	82	71	50	67	100
Bacillus	89	100	88	75	86	100	71	75	33	75
Staphylococcus	89	100	100	63	71	36	57	25	67	50
Vibrio	89	100	100	100	100	91	100	100	83	100

Genes involved in the utilisation of monosaccharides, polysaccharides, organic acids, sugar alcohol derivatives, amino acids, and amines were detected in sponge *Amphimedon queenslandica* bacterial genome (Gauthier et al., 2016). They also found genes for ATP-independent periplasmic type transporters which are essential for carboxylic acid import,

genes for ATP binding cassette transporters useful for carbohydrate, amino acid and peptide uptake, genes for *Liv*FGHMK cassette for branched-chain amino acid transport and genes for D-octopine dehydrogenase and opine oxidase for methyl group donors for carbon and nitrogen source.

Functional genes code for transport and metabolism of amino acids and their derivatives, proteins, carbohydrates and nucleotides, were obtained in deep water sponge, *Neamphilus huxleyi* microbiome with the highest percentage of amino acid metabolising genes (23%) followed by genes for carbohydrate metabolism (19%) (Li et al., 2014). They found 13 clusters of orthologous groups of amino acid transport and metabolism and 5 orthologs of carbohydrate transport and metabolism. Similar results were obtained in the present study. The rate of glutamic acid uptake by microbial consortia in *C. alloclada* (250 ─470 µg C cm^{-3} d^{-1}) was 10 to 20 times (13 ─ 28 µg C cm^{-3} d^{-1}) higher than glucose uptake. The genes involved in the metabolism of alanine, aspartatic acid and glutamatic acid were 2.8 ─ 3.3x10^6 copies and glycolysis/gluconeogenesis genes were 2.6 ─ 3.1x10^6 copies per genome. The genes codes for leucine, isoleucine and valine degradation were 2.5 ─ 3.1x10^6 copies per genome and the rate of leucine uptake by the sponge microbial consortia was 0.1 ─0.2 µg C cm^{-3} d^{-1}. Hence the differential uptake of these substrates may not be due to the difference in the gene copy number but may be because of their availability to microbial consortia of the sponge.

The production of multiple extracellular enzymes and utilisation of a number of carbohydrate substrates enable the bacteria to utilise a variety of substrates and these capabilities might have been one of the reasons for their dominance in the sponge and the ability to compete with other bacteria for nutrients. Most of the sponge-associated bacteria produced >50% extracellular enzymes and utilised more than half of the simple sugars tested thus showing the metabolic versatility of sponge-associated bacteria.

Heterotrophic bacteria can degrade complex organic compounds in POM filtered by the sponge to provide nutrition to sponge holobiont. This supply, however, may not be sufficient to support their nutritional requirement, as the availability of POM is not continuous and depend on the rate of filtration. Hence, the sponge may have to depend on other sources for

nutrition and therefore the autotrophic fixation might play an important role. A total of 770 bacterial OTUs which are reported to carry out CO_2 fixation were obtained from *C. alloclada*. These include, phototrophic bacteria such as Rhodobacterales (324 OTUs), Chloroflexi (182 OTUs), Cyanobacteria (100 OTUs), Rhodospirillales (67 OTUs) and Rhodocyclales (7 OTUs), sulfur bacteria such as Chromatiales (56 OTUs), Thiotrichales (170 OTUs), Thiohalorhabdales (29 OTUs) and Chlorobi (7 OTUs) and nitrifying bacteria such as Nitrospira (45 OTUs), Nitrospina (7 OTUs), and Nitrosomonas (2 OTUs). These bacteria have been reported in different sponge species (Bayer et al., 2008a,b; Hoffmann et al., 2009; Radax et al., 2012a). Around $1.1 - 1.3x10^7$ copies of genes which are involved in the carbon fixation were obtained from *C. alloclada* metagenome. These genes were as high as lipid metabolism related genes and ca half of the genes involved in the carbohydrate and amino acid metabolism. This high number of genes codes for proteins involved in carbon fixation might have contributed to inorganic carbon uptake of 9 to 16 µg C cm^{-3} d^{-1} in *C. alloclada*. This tropical intertidal sponge receives a large amount of sunlight and this plays an important role in deciding the bacterial community composition of this sponge. The gene involved in carbon fixation has been reported in sponges, *Cymbastela coralliophila, Stylissa* sp. and *Rhopaloeiede odorabile* (Fan et al., 2012). However, the rate of carbon fixation by these groups in the sponges has not been reported. A higher number of autotrophic bacteria in the cortex tissues (10^5 nitrifying and photosynthetic bacteria with dominant OTU belonging to Rhodobacteraceae in cortex), might be responsible for the high rate of carbon fixation (15 µg C cm^{-3} d^{-1}) observed. The contribution by nitrifying bacteria to carbon fixation in *C. alloclada* was 7.7 nM C cm^{-3} d^{-1} as 1 mol of CO_2 is fixed for every 9 mol of NH_4^+ oxidised to NO_3 (Feliatra and Bianchi, 1993).

6.2.1.2. Methane oxidation

According to the literature, methanotrophic bacteria belong to α-, β- and γ-Proteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia, Bacteroidetes, and Cytophagales (Lidstrom, 2006; Kolb, 2009). Methanotrophic bacteria and *pmoA* gene which codes for methane oxidation have been reported in sponges *viz.*, *Callyspongia* sp. and *Discodinium* sp. (Taylor et al., 2004; Kennedy et al., 2008; Thomas et al., 2010; Fan et al., 2012; Nakashima et al., 2016). The OTUs of *Methylophaga, Methylococcales Methylophilales Methylobacteriacea*

Methylocystaceae and *Methylacidiphilae,* (75 OTUs) which were reported to carry out methane oxidation, were found in *C. alloclada* metagenome. The genes for methane metabolism ($2.6 - 3.1 \times 10^6$ copies) were also detected in *C. alloclada,* which may contribute to the methane oxidation rate of 1.3 mM $cm^{-3}d^{-1}$. The rate of methane oxidation by the bacterial isolates in *C. alloclada* was 1–3 orders less than the rate of methane oxidation by the microbial consortia. The bacterial isolates from *C. alloclada,* which showed methane oxidation belonged to α- and γ-Proteobacteria and Firmicutes, with γ-Proteobacteria, constituting 63%. Among the different genera, *Bacillus* spp. showed a higher rate of methane oxidation compared to other bacterial isolates. The rate varied between different species of the same genus. For example, the rate of methane oxidation by different strains of *Bacillus* ranged between 9 and 625 μM d^{-1} (Table 6.3). The first methane-oxidizing bacterium reported was *Bacillus methanicus* (Sohngen, 1906).

Table 6.3. Methane oxidation rate by bacterial isolates

No	Bacterial isolate	Methane oxidation rate (μM d^{-1})
1	*Yangia pacifica*	563.651
2	*Bacillus safensis*	583.703
3	*Bacillus firmus*	8.646
4	*Staphylococcus* sp.	35.984
5	*Bacillus subtilis*	34.435
6	*Bacillus cereus*	621.458
7	*Bacillus cereus*	45.700
8	*Staphylococcus arlettae*	65.984
9	*Bacillus pumilus*	580.081
10	*Staphylococcus* sp.	90.601
11	*Vibrio* sp.	3.924
12	*Bacillus cereus*	622.409
13	*Staphylococcus pasteuri*	549.337
14	*Bacillus aryabhattai*	614.506
15	*Bacillus aquimaris*	63.186
16	*Bacillus pumilus*	624.900
17	*Alteromonas*	67.445
18	*Alcanivorax dieselolei*	246.404

Yangia pacifica, a recently identified bacteria whose only reported function was the production of polyhydroxy benzoate (Van- Thuoc et al., 2015), showed methane oxidation rate of 563±97 nM d^{-1}. *Alcanivorax dieselolei*, a known methanotrophic bacteria, showed a methane oxidation rate of 246±214 nM d^{-1}. Other important bacteria involved in the oxidation of methane in this study were *Staphylococcus* sp. and *Alteromonas* sp. However, the oxidation of methane by these bacteria has not been reported earlier. Although *Vibrio* sp. is known to be metabolically very versatile and shows different functional activities, in this study, it showed low methane oxidation rate (4 μM d^{-1}).

Along with the known methanotrophic OTUs in *C. alloclada* mentioned above, bacterial genera like *Bacillus, Alteromonas, Alcanivorax, Staphylococcus, Yangia* and *Vibrio,* might also be contributing significantly to the total methane oxidation in this sponge, as 182 OTUs of the above-mentioned genera were detected in *C alloclada*. Methane to these bacteria may be provided by the anaerobic metabolism inside the sponge as the presence of 10^{5-6} anaerobic bacteria and uptake of different substrates under anaerobic condition by bacterial isolates were detected in *C. alloclada*. Methane-oxidizing bacteria releases CO_2 (Semrau et al., 2010) which can be utilised by autotrophic microorganisms and the oxygen released can act as a terminal electron acceptor for methanotrophic bacteria. Hence, it is more likely that a mutual interaction exists among anaerobic, methanotrophic and autotrophic bacteria in *C. alloclada*.

6.2.2. Nitrogen cycle

Nitrification

Bacteria which carry out nitrification are divided into 2 groups viz. Ammonia-oxidizing bacteria which convert ammonium to nitrite and nitrite oxidising bacteria which convert nitrite to nitrate. There were 54 OTUs of nitrifying bacteria present in *C. alloclada* which belong to Nitrospira (45 OTUs), Nitrospina (7 OTUs), and Nitrosomonas (2 OTUs). These bacteria contributed to net nitrification rate of 3.50 to 8. 52 μM cm^{-3} d^{-1}. A lot of studies showed the presence of *amoA* gene, responsible for ammonium oxidation in sponges (Dobretsov et al., 2005; Meyer et al., 2008; Lee et al., 2009; Mohamed et al., 2010; Steger,

2010; Sipkema and Blanch, 2010; Schmidt et al., 2011; Lee et al., 2011; Fan et al., 2012; Ribes et al., 2012; Yang and Li, 2012; Fiore et al., 2013; Costa et al., 2013; Lavy et al., 2014; Gao et al., 2014; Amer, 2015; Jouett et al., 2015; Vijayan, 2015; Polonia et al., 2016;Gauthier et al., 2016; Steinert et al., 2016). As the denitrifying bacteria remove part of the nitrate produced by the nitrifying bacteria, this rate may increase by 0.2 to 0.4 µM cm^{-3} d^{-1}. However, the nitrate taken up by anammox and photosynthetic bacteria were not accounted in this study.

Denitrification

Denitrifying bacteria are a physiological group of bacteria distributed in different phylogenetic groups. In *C. alloclada*, OTUs of denitrifying bacteria belonging to Actinobacteria, Firmicutes, α-,β- and γ-Proteobacteria were detected (162 OTUs). They were affiliated to genera, *Alcaligenes, Pseudomonas, Staphylococcus, Hyphomicrobium, Bacillus, Paracoccus, Brevibacterium* and *Virgibacterium.* The genes for denitrification in α-, β- and γ-Proteobacteria were obtained in sponges (Yang and Li, 2012; Fan et al., 2012; Zhang et al., 2013). The bacteria harbouring *nir K, nos* and *nar* genes, which code for denitrification were detected in other sponges (Han et al., 2012; Li et al., 2014). The dominant denitrifying bacteria, *Thaurea* and *Ochrobacterium* found in some sponges (Hoffmann et al., 2009; Li et al., 2014) were not detected in *C. alloclada*. Bacterial isolates belonged to Firmicutes, Actinobacteria and α-Proteobacteria with Firmicutes constituting 60% of denitrifying bacteria. Denitrification rate by microbial consortia in *C. alloclada* was 152 to 414 nM cm^{-3} d^{-1}. This rate was 4 orders higher than individual bacterial strains. *Bacillus, Brevibacterium, Staphylococcus and Yangia* showed denitrifying activity. Denitrification by *Bacillus, Staphylococcus* and *Brevibacterium* has been reported earlier (Davies and Toerien, 1971; Denariaz, 1989; Constantin et al., 1996; Song, 2011; Yang, 2011; Priyadharsani, 2016). High rate of nitrous oxide production (~ 0.04 nM d^{-1}) in *C. alloclada* was seen in *B. cereus* and *S. arlettae*. Among *Bacillus* spp. the rate of denitrification ranged from 0.01 to 0.037 nM d^{-1} (Table 6.4), while *Yangia pacifica* showed very a low rate of 0.001 nM d^{-1}.

Table 6.4. Denitrification rate by different bacterial isolates

No.	*Bacteria*	Denitrification rate (nM d^{-1})
1	*Yangia pacifica*	0.001
2	*Bacillus safensis*	0.024
3	*Brevibacterium linens*	0.016
4	*Bacillus subtilis*	0.010
5	*Bacillus cereus*	0.037
6	*Bacillus cereus*	0.022
7	*Staphylococcus arlettae*	0.037
8	*Bacillus pumilus*	0.012
9	*Staphylococcus sp*	0.008
10	*Bacillus cereus*	0.014

The genes involved in the nitrogen metabolism for energy production in *C. alloclada* ranged from 1.9 to $2.3x10^6$ copies. In this sponge, it can be postulated that fermentative products excreted by sponges can be utilised by denitrifying bacteria (Hoffmann et al., 2005a) during anoxic condition. N_2, which is the final product of denitrification, will be the source for N_2-fixers like Cyanobacteria (100 OTU) and Rhizobiales (12 OTU) in *C. alloclada*. Similarly, NH_3 will be utilised by nitrifying bacteria at net nitrification rate of 145 to 355 nM cm^{-3} d^{-1}, producing 3.5 to 8.5 μM cm^{-3} d^{-1} of nitrate (Chapter 5, Section 5.2.3). This excess nitrate will be removed by denitrifying bacteria when the sponge tissue becomes anaerobic. Hence denitrifying bacteria and nitrifying bacteria mitigates the toxic metabolic waste of sponge.

6.2.3. Sulfur cycle

Sulfate reduction

Sulfate-reducing bacteria (SRB) (Imhoff and Truper, 1976; Manz et al., 1998, 2000; Hoffmann, 2003; Hoffmann et al., 2005a; Erwin et al., 2012; Dupont et al., 2014a, b) and Archaea (Meyer and Kuever, 2008) have been detected in sponges. In *C. alloclada*, these bacteria were detected by culture-independent and culture-dependent approach. SRB OTUs

(155) were affiliated to Nitrospira, δ- and ε-Proteobacteria. In *C. alloclada*, the presence of Desulfobacterales (80 OTU), Desulfomonadales (26 OTU), Desulfarculaceae (17 OTU), Thermodesulfovibrionaceae (11 OTUs) and Desulfovibrionales (8 OTUs) were detected. These SRBs from *C. alloclada* might be involved in the sulfate reduction (34.3 to 46.0 nM cm^{-1} d^{-1}). Genes involved in sulfur metabolism was detected in *C. alloclada* ($0.9–1.2x10^6$ copies). Culturable SRB belonged to *Desulfovibrio* sp. and *Desulfococcus* sp. The sulfate reduction by isolates ranged from 0.52 to 3.53 nM d^{-1}. However, no difference was observed in the activity of *Desulfococcus* and *Desulfovibrio*. The abundance of culturable SRB in *C. alloclada* was 10^{2-3} CFU cm^{-3}. Gauthier et al. (2016) detected genes codes for sulfate reduction in bacteria from coral reef sponge, *A. queenlandica*. SRBs are organoheterotrophs which gain energy by oxidation of fermentation products such as simple organic acids, alcohols and hydrogen. Mesohyl bacteria were able to utilise 43-44% of carboxylic acids and alcohols under anaerobic condition (Chapter 5, Section 5.2.1). The availability of carboxylic acids and alcohols released during the sponge cell phagocytosis might have provided the carbon source for SRB. In addition, due to the active metabolism of associated aerobic microorganisms, the anoxic condition will be created in the sponge when the sponge stops filtration. During anaerobic condition, sponge cells will switch their metabolism to fermentation and the fermentation products will be utilised by SRB. Sulfide produced by SRB during anoxia will be converted back to sulfur, sulfate, or thiosulfate by sulfur oxidising bacteria when sponge starts pumping (Hoffmann, 2003). Thus the toxicity of sulfide to sponges can be ruled out. A total of 260 OTUs of sulfur oxidising bacteria (*Thiobacillus, Chromatiales, Thiohalorhabdales* and *Thiotrichales)* were detected in *C. alloclada* in the present study and has been discussed in the previous section.

To summarise the linkage of bacterial diversity of *C. alloclada* to various functions in carbon, nitrogen and sulfur cycles, it was observed that the associated bacteria utilised most of the studied carbon sources either aerobically or anaerobically. Enzymatic degradation of polymers was seen among 45% of the bacteria. Overall the heterotrophs showed multiple functions, with *Bacillus* (50%) and *Staphylococcus* (20%) being prominent multiple functioning genera (Figure 6.5). Among these bacteria, 95% showed methane oxidation.

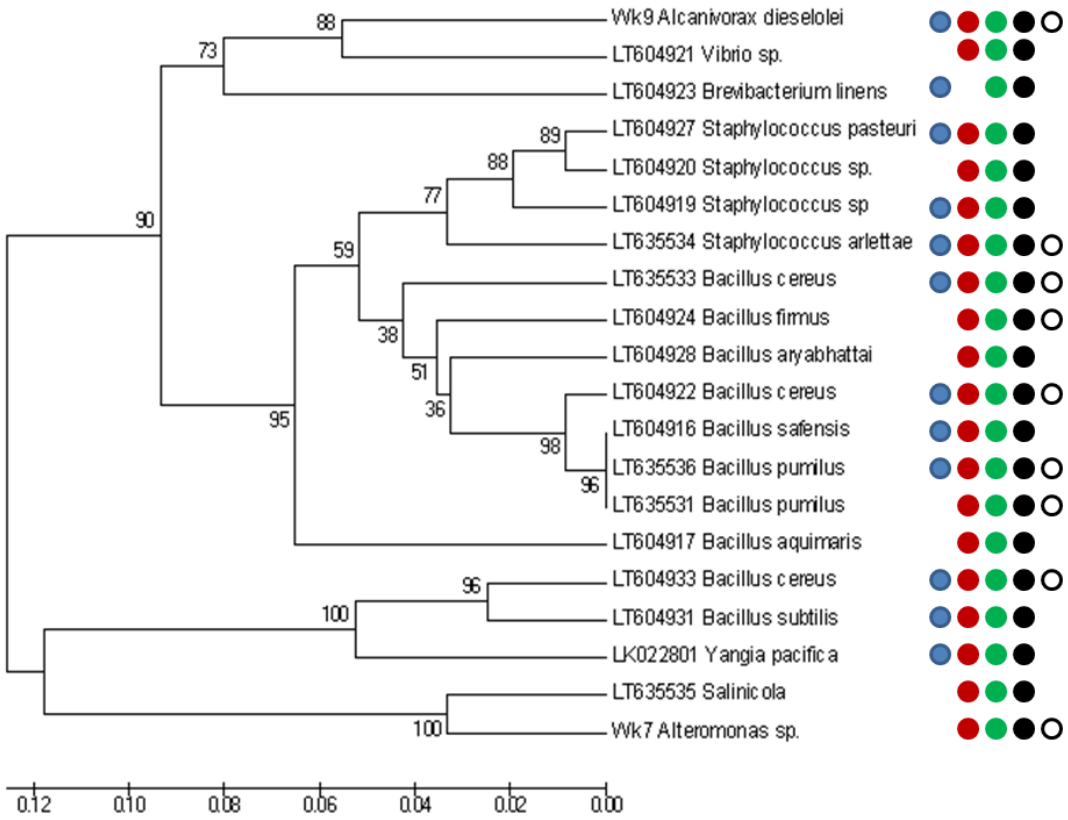
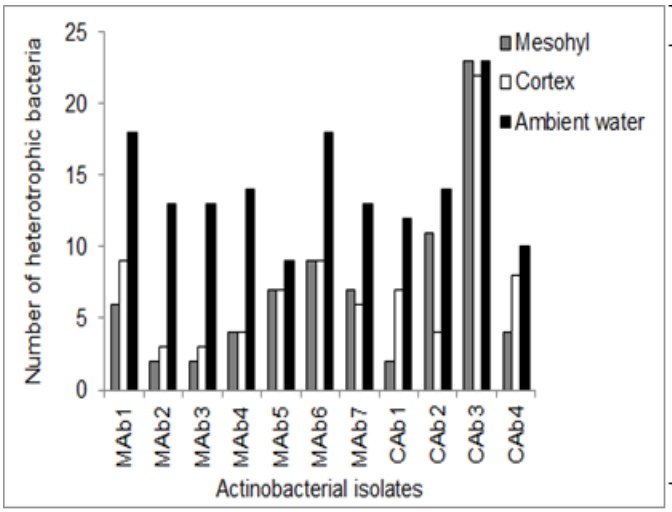


Figure 6.5. Phylogenetic tree of bacterial isolates showing multiple activities (- Denitrification, -Methane oxidation, aerobic carbon uptake, -anaerobic carbon uptake, - hydrolytic enzyme production)

6.3. Defence mechanism

Antagonism

Sponge-associated bacteria are known source of a variety of secondary metabolites. *C. alloclada* metagenome harboured 2.6 x10^6 –3.0x10^6 copies of genes for secondary metabolite production. Among 11 Actinobacteria tested for the antagonistic interaction against 75 heterotrophic bacteria from sponge and ambient water, *Streptomyces thermovulgaris*, isolated from the cortex of C. *alloclada* showed the highest number (68) of inhibition (Inhibition of 23, 22 and 23 bacteria from ambient water, cortex and mesohyl tissues, respectively), followed by *Streptomyces cyaneus* isolated from the mesohyl tissue (Figure 6.6).



Isolate	Bacteria
MAb1	*Actinoplanes conseffensis*
MAb2	*Streptoverticillum olevoreticuli*
MAb3	*Streptoverticillum olevoreticuli*
MAb4	*Streptomyces thermovulgaris*
MAb5	*Actinoplanes careuleus*
MAb6	*Streptomyces cyaneus*
MAb7	*Streptomyces thermovulgaris*
CAb1	*Streptomyces prasinosporus*
CAb2	*Streptomyces xanthochromogens*
CAb3	*Streptomyces thermovulgaris*
CAb4	*Actinoplanes careuleus*

Figure 6.6. Inhibition of heterotrophic bacteria by Actinobacteria

Bhattarai et al. (2006) found 50% of the marine antagonistic strains belonged to Actinobacteria (AB). This high interaction of AB might be because of their inherent potential to produce several antimicrobial substances (Selvin et al., 2004; Dharmaraj and Sumantha, 2009; Han et al., 2009), which would keep other bacteria away from its niche. A minimum number of inhibitions was observed by *Streptoverticillum olevoreticuli i*solated from the mesohyl tissue of the sponge which inhibited >50% bacteria from ambient water. The genes codes for antimicrobial proteins such as polyketides and non-ribosomal peptide synthetase were found in *C. alloclada* metagenome with a copy number of 6.1 $x10^6$– 7.4$x10^6$. It was reported that *Streptomyces* harbour these proteins and a high number of these genes may be the reason the high number of antagonistic interactions exhibited by these bacteria.

It was found that the antagonistic activity of sponge-associated AB dependent on the source of isolation as well as the taxonomic group. Mangano et al. (2009) found that sponge-associated AB tends to inhibit species of the same taxonomic group. The results suggest that host sponge may possibly exert some selective pressure on the associated bacteria and as a consequence, the bacteria associated with sponge may undergo some genetic changes in order to survive in the sponge environment, which may result in the variation of gene sequences compared to that of their counterparts in the water (Hoffmann et al., 2010). For instance, *Brahmella* sp, *Pseudomonas diminuta, Vibrio diazotrophicus, Vibrio metschnikovii, Vibrio*

nereis, and *Xanthomonas* sp. found in sponge as well as in ambient water were inhibited by a different number of AB, depending on their source. The inhibition of these bacteria by AB based on the source of isolation was in the order of ambient water>cortex> mesohyl. Similarly, sponge-associated *Pseudomonas stutzeri* was inhibited by a higher number of AB from cortex tissues than from mesohyl AB. The differential antagonistic activity of sponge-associated AB against different heterotrophic bacteria might be the reason for the absence of certain genera in mesohyl, cortex or ambient water. For example, *Aeromonas salmonicida* and *Agrobacterium* sp. which were isolated exclusively from mesohyl were inhibited by AB from cortex tissues and hence could not be detected in the cortex. Similarly, *Bacillus badius* was found only in cortex tissue and was inhibited by AB from mesohyl. *Acinetobacter* sp. and *Pseudomonas paucimobilis* isolated from mesohyl were inhibited by the cortex AB and those from the ambient water were inhibited by both mesohyl and cortex AB, and hence could not be detected in cortex tissues. Similarly, *Flavobacterium* sp. isolated from cortex was inhibited by mesohyl AB and that from ambient water was inhibited by mesohyl and cortex AB. The results suggest that antagonistic activity of AB associated with the sponge may provide an effective control mechanism of microbial populations in the intertidal sponge, *C. alloclada*. Mangano et al. (2009) found that the sponge *L. nobilis* associated bacterial community was more antagonistic against strains isolated from the same sponge species rather than against isolates from different sponge species. The reason might be conferring a selective advantage in the competition for nutrients in *L. nobilis* from oligotrophic Antarctic waters. The strains in *Moraxella* sp. and *Photobacterium* sp. isolated from the ambient water were inhibited by a higher number of AB than the strains from cortex tissues. It was observed that AB from both mesohyl and cortex tissues inhibited *Chromobacterium, Erwinia* and *Serratia* and hence these bacteria could not be detected in the sponge tissues. It is clearly seen that the inhibition against indigenous bacteria seems to be structured with maximum inhibition of bacteria in ambient sea water. Hoffman et al. (2010) suggested that sponge environment imposes strong selection on resident microflora, generally prohibiting habitation by planktonic strains.

The complex microbial processes in the sponge *C. alloclada* is shown in diagram 6.7

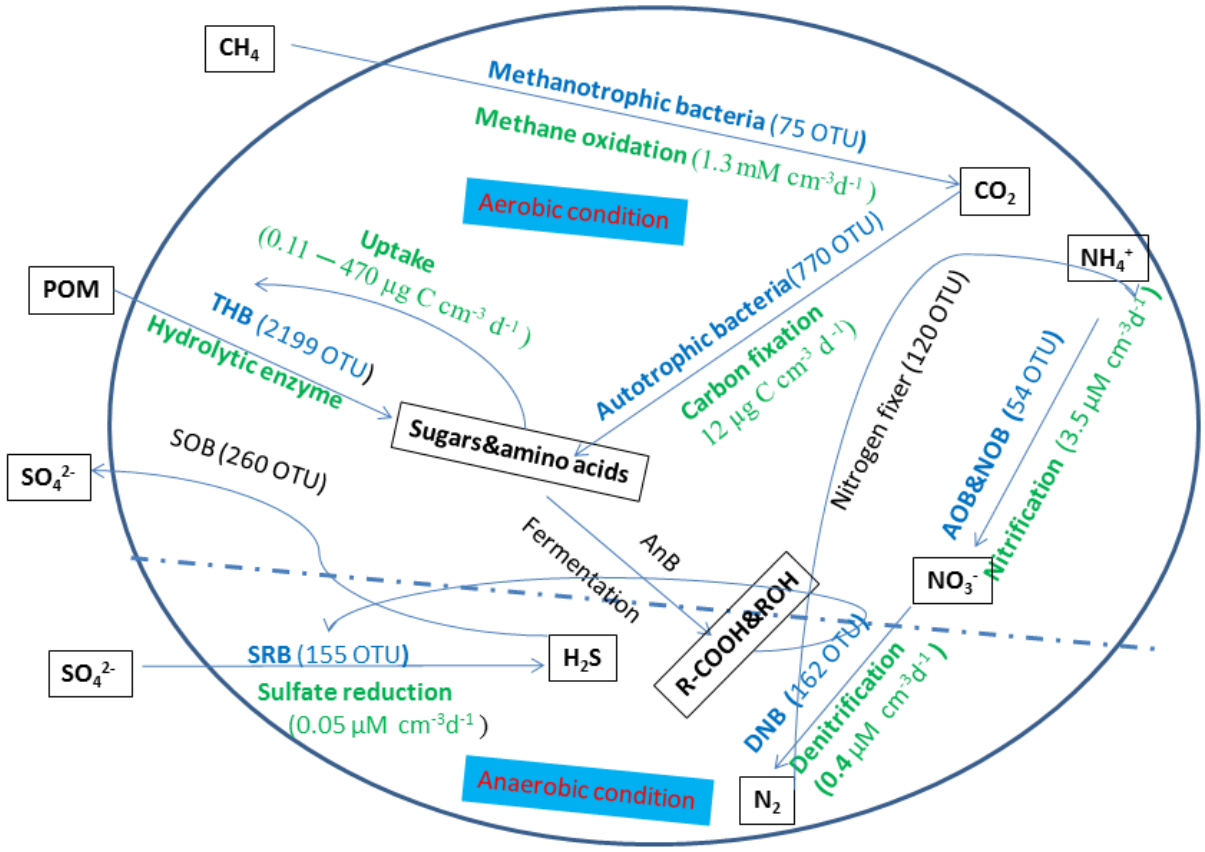


Figure 6.7. Linking microbial diversity and different microbial processes in *C. alloclada*

6.4. Conclusion

Associated microbes in *C. alloclada* are actively involved in the nutrient cycling in the sponge. It was found that various groups were responsible for multiple functions. This study demonstrated for the first time the multiple activities of the diverse microbial flora in carbon, nitrogen and sulfur cycling, associated with a single sponge. The associated Actinobacteria played an important role in structuring the bacterial community in this sponge.

CHAPTER 7

APPLICATIONS OF SPONGE-ASSOCIATED BACTERIAL FUNCTIONS

7.1. INTRODUCTION

C. alloclada, being a tropical intertidal sponge, often faces unfavourable conditions such as osmotic stress, drought, toxins, as well as temperature variations. Bacteria associated with these sponges might have evolved a wide variety of metabolic strategies to cope up with such a dynamic environment (Simister et al., 2012b). In the previous chapters, the potential of the sponge-associated bacteria to produce extracellular enzymes and their capability to degrade a variety of complex compounds and utilise simple molecules have been described. In this chapter, an attempt has been made to use *C. alloclada* to remove the high amount of ammonium, nitrite and nitrate and also solid particles from the aquaculture wastewater. A study was also carried out to test the potential of the associated bacterial isolates in degrading two azo dyes to remove these dyes from textile effluent.

Unpolluted water is an essential prerequisite for ecosystem conservation. The rapid pace of industrialisation in India in the last 2-3 decades has resulted in a surge of pollution, particularly in textile and aquaculture sectors which have been growing at a faster pace due to stability and market demand. However, the growth of such industries is also the cause for generation of large volume of wastewater. In aquaculture industry, the wastewater generated contains different organic and inorganic particles, which originate from the excretory products and leftover feed. The suspended solids present in the aquaculture effluent would cause an ecological imbalance in the receiving environment (Martinez-Cordova et al., 2009). Treating the waste water using chemical methods would in turn, add secondary pollutants to the aquatic environment. Similarly, during the last few decades, toxic dyes from the textile industry are being released in dangerous proportions into the marine environment in India. The effluent containing ≤1 ppm dyes holds apotential threat to the aquatic ecosystem (Aksu et al., 2007). Accumulation of dyes in the environment has prompted the development of various dye waste management strategies because chemical methods were not found to be economically feasible (Kumar et al., 2011). Therefore, bioremediation is the eco-friendly approach. Bivalves, microalgae and seaweed have been successfully used to reduce organic and inorganic compounds in aquaculture wastewater (Jones et al., 2002; Shpigel et al., 2005; Muangkeow et al., 2007).

Sponges have the ability to filter particles from water and can pump several times the volume of water than their body volume (Pile et al., 1996; Ribes et al., 1999). These organisms can accumulate metal ions in the tissue thus can be used as bioindicators of metal pollution (Selvin et al., 2009b). It is this filtering capacity of sponges that has led to the suggestion that they are able to reduce the high bacterial loads resulting from sewage discharges and wastewater from marine fish farms (Milanese et al., 2003; Fu et al., 2006; Stabili et al., 2006). Apart from the filtering capacity, they also harbour a large number of functionally diverse microorganisms and are referred to as microbial fermenters (Hentschel et al., 2006). Bacteria associated with sponges are also capable of utilising dissolved organic matter from the ambient water (Frost, 1987; Ribes et al., 1999). A large number of bioactive compounds have been isolated from sponges and their associated microorganisms with more than 200 new metabolites being reported each year (Blunt et al., 2007)

7.2. RESULTS AND DISCUSSION

7.2.1. TREATMENT OF AQUACULTURE WASTEWATER WITH SPONGE

The physicochemical characteristics of shrimp aquaculture wastewater are given in Table 7.1.

Table 7.1.Physico-chemical characteristics of waste water

Parameters	Values
Temperature (°C)	25
pH	8
Salinity	10.0
DO (mg l^{-1})	8.0
ORP (mv)	141.0
Nitrite (µM)	3.69
Nitrate (µM)	7.53
Ammonium (µM)	ND*
Phosphate (µM)	167.79
Silicate (µM)	443.7
TSM (g l^{-1})	0.63

*not detected

High levels of nitrite and nitrate were recorded. A reduction in the dissolved nutrients and total suspended matter in both 100% and 50% waste water from shrimp aquaculture when treated with *C. alloclada*, for 10 days. The rate of removal of suspended matter in 50% waste water was higher than 100% wastewater (Table 7.2).

Table 7.2. Removal of suspended matter and nutrients from aquaculture wastewater

	Rate of removal	
	100% waste water	50% waste water
TSM (mg l^{-1} d^{-1})	14.29±12.12	21.43±2.02
Ammonium (µM d^{-1})	-0.53±0.75	0
Nitrite (µM d^{-1})	0.81±1.14	0.65±0.91
Nitrate (µM d^{-1})	0.44±0.11	1.04±1.27
Phosphate (µM d^{-1})	29.97±17.85	20.76±29.19
Silicate (µM d^{-1})	10.00±1.23	Not measured

The sponge-associated bacteria live on the POM filtered by sponges and the organic compounds from the sponge body by secretion of the different extracellular hydrolytic enzymes as discussed in Chapter 5. It is assumed that the low uptake of particulate matter might be because of the clogging of ostia of sponge with particulate matter or may be depending on the uptake efficiency of the sponge.

Ammonium concentration was below detection level in 50% waste water. This might be because of the escape of ammonia from wastewater. However, an increase in the ammonium concentration (0.53 µM d^{-1}) was detected in 100% wastewater. This might be because of the degradation of excretory products of shrimps. Nitrite and phosphate removal was higher in 100% waste water whereas nitrate removal was higher in 50% waste water. *C. alloclada* also harboured 10^{5-6} ammonia oxidising and nitrate-reducing bacteria and 10^{3-4} nitrite oxidising bacteria. Reduction in the nitrite and nitrate might be attributed to the uptake by denitrifying bacteria and photosynthetic bacterial community present in the sponge (Fiore et al., 2013).

Phosphate may be utilised by sponge-associated bacteria whereas silica can be utilised by sponge itself for spicule biosynthesis and by diatoms. The removal of total suspended matter from 100% wastewater by sponge was 14.29±12.12 mg l^{-1} d^{-1}, as can be seen from Figure 7.1.

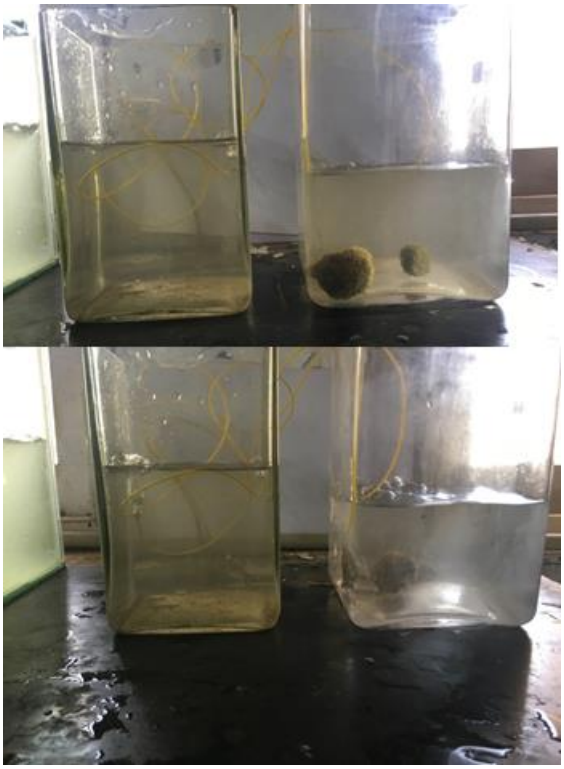


Figure 7.1. Aquaculture wastewater before (top) and after the treatment with sponge

Doucette et al., (1998) and Jasti et al. (2005) suggested that after several generations, sponge and bacteria may develop a dynamic balance. Once this balance is altered, it will either benefit or decline the bacterial growth (Wang et al., 2010). In this study, the sponge collected from seawater was introduced into aquaculture wastewater. The organic-rich waste water may provide nutrients for bacterial growth and the toxic compounds may hinder the bacterial growth. The metabolic activity of microbial consortia of sponge and wastewater were compared before and after 10 days of the experiment. Average well colour development (AWCD) in the Ecoplates increased in samples during incubation due to the metabolic activity of the bacterial community, as reported elsewhere (Yang et al., 2013). AWCD in

Ecoplate did not vary in the wastewater indigenous community before and after the experiment. This showed that the indigenous community is well balanced. Surprisingly, AWCD of the bacterial community in sponge also did not differ before and after the experiment. This may be because of the utilisation of organic dissolved nutrients in the wastewater by the indigenous community which makes them unavailable to the sponge. The balance between the sponge and bacteria was not altered and thus no significant difference was noted. The highest AWCD was observed in sponge microbiota before the experiment (Figure 7.2) though there was no significant difference observed in AWCD between the samples.

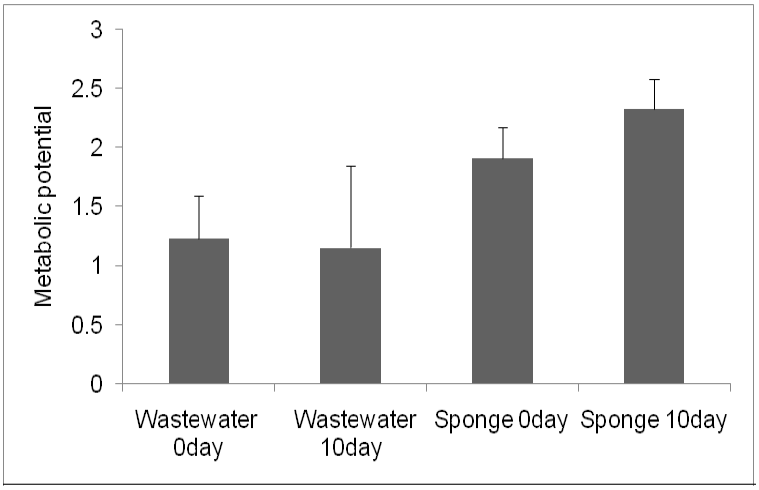


Figure 7.2. Metabolic potential of microbial consortia from waste water and sponge before (0 day) and after (10 day) experiment

The highest effective utilisation was observed in waste water. The number of substrates utilised increased in the sponge incubated in wastewater after the experiment (Figure 7.3), due to the availability of a large variety of substrates in wastewater. The reduction in the number of substrates utilised by indigenous bacteria in wastewater might be because of the shift in the bacterial community which can use a variety of substrates, to the community which utilise only a narrow range of substrates.

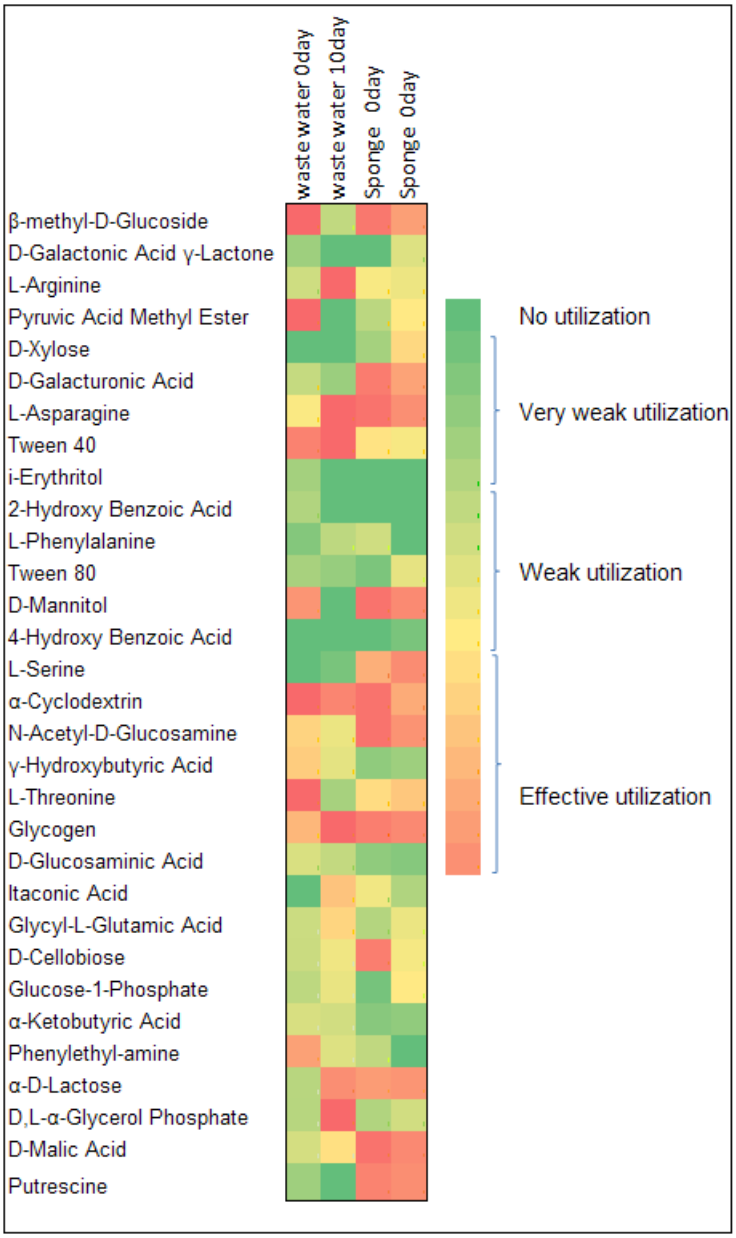


Figure 7.3. Heatmap of single substrate utilisation by microbiota from waste water and sponge before (0day) and after (10 day) experiment

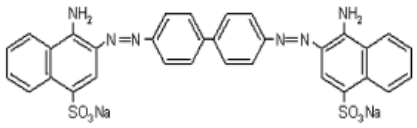
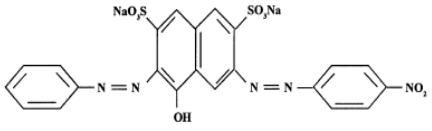
It was expected that when wastewater was added to the sponge holobiont, the balance between the sponge and bacteria would be altered and the bacterial diversity might be increased or decreased. Under stress of pollutants, the activities of sponge-associated bacteria

may produce some enzymes for relieving pollutant toxicity as found in soil microorganisms (Muratova et al., 2009). From this study, it can be concluded that *C.alloclada* is a good candidate for treating aquaculture wastewater to reduce particulate matter as this sponge can filter 14 mg l^{-1} d^{-1} of suspended matter and retain it within its body. *C. alloclada* can also remove dissolved nutrients, which in higher amount is deleterious to the environment. And this sponge-associated microbial flora was not altered in the metabolic abilities when subjected to wastewater.

7.2.2. AZO DYE DECOLOURISATION BY ASSOCIATED BACTERIAL ISOLATES

Amido black and Congo red are the azo dyes used in textile industries which are recalcitrant and are categorised as a health hazard (Jalandoni-Buan et al., 2010; Mittal et al., 2013). Characteristics of azo dyes are given in the table below (Table 7.3).

Table 7.3. Characteristics of dyes Congo red and Amido black

Dye	Structure	Chemical formula	MW ($gmol^{-1}$)	Color Index	Λmax (nm)
Congo red		$C_{32}H_{22}N_6Na_2O_6S_2$	696.7	22110	498
Amido black		$C_{22}H_{14}N_6Na_2O_9S_2$	616.5	20470	618

Screening of sponge-associated bacteria for azo dye decolourisation

A total of 75 bacterial isolates were screened for azo dye decolourisation. It was seen that 23.7% of the bacteria showed decolourisation and out of this, 33% showed decolourisation for both Amido black (AB) and Congo red (CR) dyes. The decolourisation efficiency for AB

(7─77% and 8-77% by cortex and mesohyl bacteria respectively) was higher than CR (1 and 4─62% by cortex and mesohyl bacteria respectively), in 2 days (Table 7.4).

Table 7.4. Decolourisation efficiency of bacteria from sponge

Decolorization efficiency (%)		
	AB	CR
Cortex	7 -77	1
Mesohyl	8-77	4-62

This high potential to degrade complex dye molecules by sponge-associated bacteria might be because of the presence of large copy numbers of genes involved in xenobiotic degradation and metabolism in *C. alloclada* metagenome ($8.9x10^6$ ─$1.1x10^7$). The relatively low decolourisation of CR may be due to its high molecular weight, structural complexity and an additional azo bond (Isik and Sponza, 2003). Azo dye colour reduction is a ubiquitous capacity of many microorganisms under anaerobic conditions (Razo-Flores et al., 1997), as azoreductase is oxygen sensitive (Chang and Lin, 2000). However, some specific strains of aerobic bacteria have developed the ability to reduce the azo group by special oxygen-tolerant azoreductase (Kulla, 1981). Thus, it appears that aerobic, microaerophilic and anaerobic niches within the sponge augment the development of strains with specialised oxygen-tolerant azoreductase.

On initial screening, *Y. Pacifica* which is an anaerobic strain showed 77% and 61% decolourisation of AB and CR, respectively. These values were comparatively higher than that reported for *E. coli* and *Pseudomonas* sp. under microaerophilic condition within 5 days (Isik and Sponza, 2003). *Y. pacifica* was first reported from the sediment of China Sea (Dai et al., 2006). Very limited studies have been carried out on this species (Van-Thuoc et al., 2015). The bio-decolourisation of dye depends on the organism, its growth conditions and concentration of the dye (Babu et al., 2013; Mittal et al., 2013). *Y. pacifica* decolourised 96.7% and 96.2 % of 50 mg l^{-1} of AB and CR, respectively at 6 g l^{-1} nutrient medium, 37±2°C pH 9 and 8, respectively (Table7.5). Growth related decolourisation of 4-chloro-2-nitrophenol

has been reported for *Bacillus subtilis* (Arora, 2012) which are similar to that of the present strain.

Table 7.5. Dye decolourisation efficiency and growth of *Y. pacifica*

	CR		AB	
	0 day	7 day	0 day	7 day
Decolourisation potential (%)	0	96.2±1.9	0	96.7±1.3
Growth (Cells ml^{-1})	3.54±0.2x10^{6}	1.77±0.1x10^{7}	3.54±2.0x10^{6}	8.25±2.40x10^{7}

SEM image showed no AB adherence on the cell or pellet whereas CR was visible on the surface of bacterial cells and a red color was seen on the pellet (Figure 7.3). Based on this, it can be deduced that the decolorization of AB was by degradation while that of CR was more by adsorption.

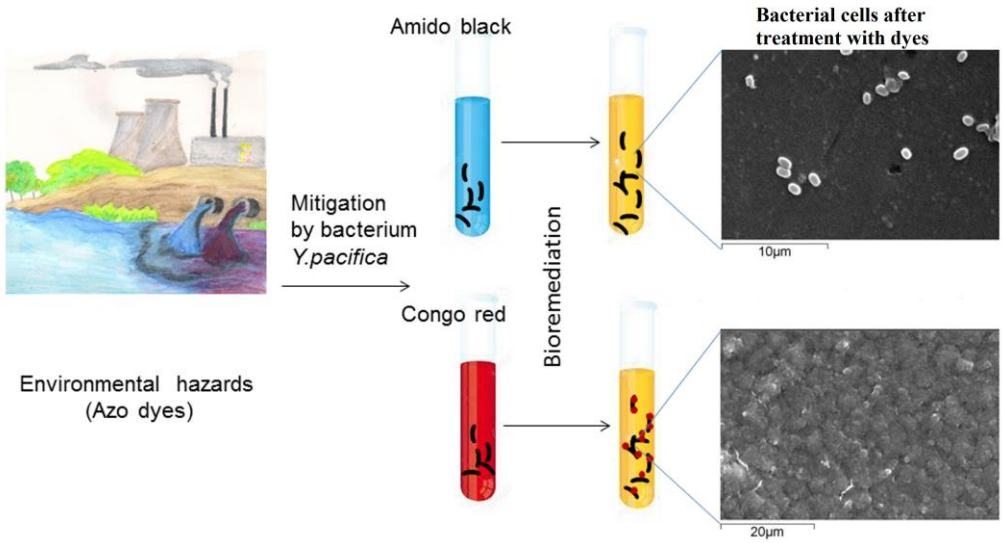


Figure 7.4. Schematic diagram shows the decolourisation of azo dyes, Amido balck and Congo red by *Y. pacifica*

Conclusion

The main advantage of using this intertidal sponge is that it is adapted to varying nutrient conditions and pollutants. Also, a large number of diverse microbial communities associated with this sponge may have resilience capability during adverse conditions as the bacterial flora of the sponge did not vary when exposed to aquaculture waste water. The present study reiterates biotechnological potential of sponges. This study revealed that *C.alloclada* associated bacteria are promising bio-prospecting source for bioremediation of pollutants especially dyes from textile industries and aquaculture wastewater treatment.

CHAPTER 8

SUMMARY & CONCLUSIONS

8.1. INTRODUCTION

Sponges are the oldest group of metazoans as they have originated in Precambrian era. More than 8500 species of sponges have been recorded (van Soest et al., 2012). They are sessile filter feeders that pump a large volume of water through a unique and highly vascularized canal system (Hentschel et al., 2002). Sponges harbour complex microbial community such as bacteria, archaea, diatoms, dinoflagellates and fungi (Fuerst et al., 1999; Manz et al., 2000; Hentschel et al., 2002; Konig et al., 2006), with bacteria being the predominant one (Vacelet, 1975; Hentschel et al., 2002). Most of the sponge species, known as high microbial abundance (HMA) sponge or bacteriosponge harbour up to 10^{9-10} bacteria g^{-1} of sponge which constitutes up to 40% of their biomass (Hentschel et al., 2006) whereas low microbial abundance sponge (LMA) harbour $<10^{6}$ bacteria g^{-1} of sponge. Sponge-bacteria is classified into three ecological groups, *viz*. core bacterial community (Acquired by horizontal transfer of microorganisms from seawater), sponge-specific bacteria (Restricted to sponge or sponge and coral specific organisms, acquired mostly by vertical transmission) and variable bacterial community (Schmitt et al., 2012). The diversity of associated bacteria may vary among sponges depending on the species and environmental conditions. Sponge-bacterial association thus provides an ideal niche to study the associated microbes.

Most of the studies on sponge-associated bacteria are on their diversity, biogeography and production of bioactive compounds (Schmidt et al., 2000; Kennedy et al., 2007; Taylor et al., 2007; Siegl and Hentschel, 2010) from temperate and subtropical waters or from coral reefs. These studies have clearly demonstrated the need to study the community structure of sponge-associated bacteria from different geographical regions in order to understand the ecological implications of sponge-microbe interactions. From Indian waters, studies related to sponge-associated microbes are very limited. Out of 332 species reported from the Indian waters (van Soest et al., 2012), only a few species have been studied and these studies have focused mainly on diversity (Feby and Nair, 2010; Jasmin et al., 2015) and bioactive compounds (Thakur and Anil, 2000; Selvin et al., 2004, 2009c; Skariyachan et al., 2014). In the recent years, though our overall knowledge on sponge –microbe interaction has improved, the functions of sponge-associated microorganisms have been overlooked while investigating their diversity or the production of bioactive compounds. Therefore, understanding the diversity of microorganisms associated with sponges is necessary to delineate the diverse

metabolic functions of the sponge. The research carried out in this thesis has aimed to answer some fundamental questions about the abundance, diversity and functions of sponge-associated bacteria. This research not only addresses these questions but also links the diversity of different physiological groups of bacteria and their functions which contribute to the host sponge physiology. The results obtained add further information to the existing knowledge about bacterial community structure of marine phylum Porifera (Sponges), their functions in nutrition, biogeochemical cycling and defence. Conclusively, the subject matters of the thesis have been divided under the following heads**.**

- **The abundance of bacteria:**

Bacterial abundance was estimated by 2 approaches.

Culture-independent method

- Total bacteria and viable bacteria were enumerated by using epifluorescence microscopy (Friedrich et al., 2001; Boulos et al., 1999).

Culture-dependent method (CFU)

- Organotrophic and autotrophic bacteria were enumerated by using selective media. Organotrophic bacteria included aerobic and anaerobic heterotrophic bacteria, Actinobacteria, nitrate- and sulfate-reducing bacteria. Total heterotrophic bacteria (THB) were enumerated on Zobell Marine Agar and different strengths (10%, 50% and 100%) of nutrient agar. Anaerobic bacteria and sulfate-reducing bacteria (SRB) were enumerated on Anaerobic agar and SRB selective medium (Himedia, India), respectively by agar shake method. Nitrate-reducing bacteria (NRB) were enumerated by agar shake method (Michotey et al., 2000). Different selective media such as marine sponge agar (MSA), casein starch agar (CSA), inorganic salt starch agar (ISP-4), International Streptomyces Project (ISP-7) and chitin agar (Selvin et al., 2009a) were used to enumerate Actinobacteria. Autotrophic bacteria were ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and aerobic anoxygenic photosynthetic bacteria (AAnP). Nitrifying bacteria such as Ammonium oxidising bacteria and Nitrite-oxidising bacteria were enumerated by

MPN method (Elbanna et al., 2012). AAnP was enumerated according to Rodina (1972).

- **Diversity of bacteria**

Culture-dependent method

- Bacterial isolates (469 bacterial colonies from mesohyl and cortex tissues of sponge and ambient water) obtained from different media were identified by phenotypic and biochemical tests. Representative strains were identified by molecular characterization.

Culture-independent method

- Metagenomic analysis of V6 region of 16S rRNA gene of the total bacterial community from mesohyl, cortex and ambient water were carried out using Illumina technology. The data were analysed in QIIME pipeline for assigning OTU IDs and to estimate bacterial diversity.

- **Functions of bacteria**

Culture-independent method

- Degradation of organic matter and uptake of organic and inorganic matter
  Metabolic potential of microbial consortia in mesohyl and cortex of sponge and ambient water were studied using Biolog Ecoplates. Heterotrophic uptake of simple molecules by these microbial consortia was tested by using ^{14}C-labelled substrates (glucose, glutamic acid and leucine). Inorganic uptake by mesohyl, cortex and ambient water were also estimated using ^{14}C-labelled sodium bicarbonate.
- The rates of different microbial processes in carbon, nitrogen and sulfur cycles such as methane oxidation, nitrification, denitrification and sulfate reduction by microbial consortia associated with the sponge and ambient water were also studied. The rate of methane oxidation by microbial consortia was measured by headspace method using Gas Chromatography. Nitrification rate was measured by incubation experiment with NH_4SO_4. Denitrification rate was measured by the

acetylene inhibition technique based on the inhibition of the reduction of nitrous oxide to nitrogen gas by Gas Chromatography and sulfate reduction activity was measured by zinc acetate method.

Culture-dependent method

- Bacterial isolates from sponge (mesohyl and cortex) and ambient water were tested for their potential to utilise 46 organic compounds using Biolog GEN III and Biolog AN microplates (Biolog, Hayward, CA). They were also were tested for the production of 6 polymer-degrading enzymes, *viz*. amylase, DNase, lipase, caseinase, gelatinase and urease by the degradation of respective substrates incorporated in the nutrient media. Methane oxidation, denitrification and sulfate reduction activity by bacterial isolates were also tested as described above.
- Antagonistic interaction of Actinobacteria against heterotrophic bacteria from the sponge and ambient water was tested using resazurin as growth indicator (Sarker et al., 2007).

Linking bacterial diversity to function

- The paired-end reads (NGS) obtained from sponge mesohyl and cortex were analysed in QIIME (QIIME v1.9.0) pipeline for phylogenetic analysis. Functional analysis of the metagenomic data from mesohyl and cortex of sponge was carried out by comparing the orthologs of KEGG pathway (KO). Phylogeny of the microbial community was also linked with different tested functions of bacteria associated with *C. alloclada*. The phylogeny of bacterial isolates was also linked to their functional activities.

Application

- The aquaculture wastewater was treated with sponge-holobiont to filter and utilise the nutrients from the aquaculture wastewater. An aquaria experiment was set up with live sponge holobiont and changes in the total suspended matter (TSM), ammonium, nitrite, nitrate, phosphate and silicate concentrations of the wastewater samples were measured.
- Sponge-associated bacteria were screened for their potential to decolourise Azo dyes, Amido black and Congo red.

8.2. THE CONCISE DESCRIPTION OF OVERALL FINDINGS

8.2.1. ABUNDANCE

- Total bacterial abundance in *C. alloclada* tissue varied between $8.0x10^8$ and $1.2x10^{10}$ cm^{-3} ($6.7x10^8$-$1.0x10^{10}$ cells g^{-1}) and the viable count was in the range of 10^8 to 10^9 cells cm^{-3}.
- The cortex of the sponge harboured 2 times higher the total number of bacteria ($7.7\pm3.8x10^9$ bacteria cm^{-3}) than that in the mesohyl tissues, but the vice versa was in viable counts.
- THB in the sponge tissue was 10^6–10^7 CFU cm^{-3}. The abundance of THB in the sponge and ambient water significantly varied with concentrations of nutrients ($p<0.01$). In sponge, a higher number of bacteria (4.0 ± 2.1 $x10^7$ CFU cm^{-3}) were obtained in high nutrient media whereas, in the cortex, higher retrieval of 3.4 ± 3.3 $x10^7$ CFU cm^{-3} bacteria occurred in half strength nutrient agar. This can be attributed to the prevailing nutrient-rich conditions in the sponge. The media supplemented with sponge extract gave one order higher bacterial number than media without sponge extract. The sponge may provide distinct microenvironments as ecological niches for different bacterial populations.
- AnB is as high as THB in mesohyl and one order less than the cortex. NRB and SRB abundances were 10^5 and 10^2 CFU cm^{-3}. Anaerobic bacteria such as AnB, NRB and SRB were higher in mesohyl than the cortex. Actinobacteria.
- Autotrophic bacteria such as AOB, NOB and AAnP were higher in the cortex and were 2-3 order less than THB in mesohyl and cortex.
- The order of culturable fractions of bacteria in mesohyl was THB> ANB> NRB> AOB> Chloroflexi> Rhodospirillales> NOB > SRB. In cortex tissues, THB> Chloroflexi> Rhodospirillales> ANB> NRB> AOB> NOB > SRB. Photosynthetically active microorganisms were located in the outer, light-exposed cortex tissue layer. Higher abundance of nitrifying bacteria in the cortex might be due to the availability of oxygen. Heterotrophic bacteria populate the inner core of sponge, mesohyl. In mesohyl tissue of sponge, the anaerobic bacterial number was high and was

comparable to THB due to comparatively less oxygen concentration in the interior tissues of sponge.

- The presence of a high number of bacteria (10^{8-9} bacteria g^{-1}) in this species suggests that the tetillid sponge, *C.alloclada* comes under high microbial abundance (HMA) sponge. The number of bacteria in the ambient water was 2─3 orders lower than that in the sponge tissue which can be attributed to the nutrient rich condition in the sponge.

8.2.2. DIVERSITY

Culture-dependent bacterial diversity

- A total of 469 bacterial morphotypes were obtained from mesohyl, cortex and ambient water. Among these, 145, 132 and 171 isolates were from mesohyl, cortex and ambient water, respectively.
- The bacteria isolated from both mesohyl and cortex of sponge, *C.alloclada* were diverse. This comprises a total of 50 genera in the sponge (mesohyl-36, cortex-40) and 42 genera in water coming under five phyla, *viz*., Proteobacteria (α, β, γ, δ), Firmicutes, Bacteroidetes, Actinobacteria and Chloroflexi. The order of bacterial phyla in *C. alloclada* in the present study was Proteobacteria (γ>β>α)> Firmicutes> Bacteroidetes> Actinobacteria> Chloroflxi> δ-Proteobacteria.
- Among 62 genera obtained in the sponge and ambient water, 21 genera were common in the sponge and ambient water and accounted for 75 % of total isolates. *Bacillus* (11.7%) was the highest abundant genus in mesohyl whereas *Photobacterium* was the highest abundant genus (11.2%) in the cortex and *Vibrio* was abundant in ambient water. Shannon diversity index showed that Culturable bacterial diversity was higher in mesohyl (H'-1.745) than cortex (H'-1.651) and ambient water (H'-1.673).

Culture-independent bacterial diversity

- 295671 to 677020 16S rRNA reads were obtained from the sponge and ambient water, which belonged to 2978 to 3217 OTUs. Diversity was higher in the cortex (H'-8.34)

than mesohyl (H'-6.693) and ambient water (6.63). Chao1 index based on the number of rare classes (OTUs) in the sample was higher in ambient water (4166 to 4646) than the sponge (1535 to 3922).

- The identified sequences were assigned to 42 phyla, including 22 candidate phyla. A total of 38 phyla were present in the sponge, with 6 exclusive phyla. A total of 36 to 37 phyla were detected in mesohyl and cortex of the sponge, respectively. A total of 30 phyla were found in all the samples. However, the phyla whose abundance was >1% of total bacteria was <10. The order of dominant bacterial phyla in sponge and water was Proteobacteria> Bacteroidetes> Actinobacteria> Cyanobacteria> Verrucomicrobia>Planctomycetes>Acidobacteria>Chloroflexi>Gemmatinomonadetes > Nitrospira.
- *Alcanivorax* and unclassified Flavobacteriaceae were the dominant genera in mesohyl, whereas it was unclassified Rhodobacteriacea in the cortex.
- The average dissimilarity between bacterial communities of sponge and ambient water was 75 % and between mesohyl and cortex was 65%. γ- and α-Proteobacteria were accounting for the highest variation between mesohyl and cortex (40% and 22% dissimilarity). OTU 793461 (*Alcanivorax dieselolei*, γ-Proteobacteria), which ranged from 9 to 85081 reads, was the primary drivers of dissimilarity between cortex and mesohyl, accounting for 10 % of total dissimilarity. The same OTU with OTU 590090 (Alteromonadaceaea, γ-Proteobacteria) contributed the highest variation between ambient water and sponge samples (14.3 and 4.8 % dissimilarity by water with mesohyl and cortex, respectively).
- Cortex and mesohyl of sponge harbour different microbial community depending on the variation in nutrients, space and oxygen in the sponge tissues. In the present study, both culture-dependent and culture-independent methods showed a higher diversity of bacteria in *Cinachyra* species than previous studies.

8.2.3. FUNCTIONS

- The utilisation of 31 different substrates in Ecomicroplates reflected the high metabolic potential of the microbial consortia which was more than that of the ambient

water and other environments. There was a difference even in the category wise utilisation of amines, amino acids, carbohydrates, carboxylic acids, polymers and other miscellaneous substrates between microbial consortia in mesohyl, cortex and ambient water ($p<0.05$). One or more of the substrates from each category were effectively utilised by both mesohyl and cortex microbial consortia with cortex utilising 27 to 30 substrates.

- 70% of the bacterial isolates were able to degrade biopolymers and out of which 48% showed multiple enzyme productions, which degrades starch, lipid, casein, gelatin, urea and DNA, to provide carbon, nitrogen and phosphorus source. There was a significant difference in the percentage of degradative enzyme production in *C. alloclada* and ambient water ($p<0.05$).
- Most of the bacteria utilised mono- and disaccharides irrespective of their habitat. Glucose was the most common sugar utilised by most of the isolates followed by sucrose. There was a significant difference in the utilisation of multiple carbon sources among bacteria from mesohyl, cortex and ambient water ($p<0.001$). Only 1% of bacteria from mesohyl and ambient water utilised all the carbon sources. These bacterial isolates showed the differential utilisation of substrates under aerobic and anaerobic conditions. In mesohyl, carboxylic acids and oligosaccharides were utilised by a higher number of bacteria (72% and 49%) under aerobic and anaerobic conditions, respectively. Most preferred substrates by bacteria from cortex were amines/ amino acids and monosaccharides.
- Microbial consortia in mesohyl, cortex and ambient water showed differential heterotrophic uptake of ^{14}C-labelled compounds of amino acids (glutamic acid and leucine) and the most used sugar glucose, with the highest uptake by microbial consortia from the cortex. The uptake rate of glutamic acid was 250.7±78.7, 470.6±178.0, and 41.6±8.3 µg C cm^{-3} d^{-1} in mesohyl, cortex and ambient water, respectively and this rate was one and two orders higher than glucose and leucine, respectively. Inorganic carbon uptake measured by using radiolabeled sodium bicarbonate in the sponge cortex and mesohyl were 15.0±2.4 and 9.4 ± 1.1 µg C

$cm^{-3}d^{-1}$, respectively with no significant difference between them. The inorganic carbon uptake in sponge exceeded by 2 orders than the ambient water ($p<0.05$).

- Methane oxidation by microbial consortia in cortex tissues was 1.3±0.2 mM cm^{-3} d^{-1}. However, the rate in mesohyl was below detection level. The rate of methane oxidation was 3 times higher in sponge ambient water (3.2± 0.3mM cm^{-3} d^{-1}) than the sponge. The rate of methane oxidation by isolates was 1─3 orders less than the activity of whole microbial consortia of sponge tissues. The rate of methane oxidation ranged from 3.9±2.3 to 624.9±0.0 µM d^{-1} among bacterial isolates. The average rate of methane oxidation by bacterial isolates ranged from 8.6± 5.0 to 621.4 ±3.7 µM d^{-1}, 3.9 ±2.3 to 624.9 ±0.0 µM d^{-1} and 246.4 ±214.5 to 622.4± 1.6 µM d^{-1}, in mesohyl, cortex and ambient water, respectively.
- There was significant variation in the net nitrification between mesohyl and cortex microbial consortia ($t = 5.0$; $p<0.05$). Net nitrification was higher in sponge cortex (8.52±1.2 µM cm^{-3} d^{-1}) than mesohyl microbial consortia (3.50±0.96 µM cm^{-3} d^{-1}).
- In *C. alloclada*, denitrification by microbial consortia measured in terms of nitrous oxide production in mesohyl tissues (414±4 nM $cm^{-3}d^{-1}$) was ~ 3 times higher than that in cortex tissues (152±1 nM $cm^{-3}d^{-1}$). Denitrification rate by bacterial isolates ranged from 0.001 to 0.04 nM d^{-1}.
- Sulfate reduction rate (SRA) in the mesohyl and cortex of *C. alloclada* by the microbial consortia was 46.0±2.8 and 34.3±6.8 (nM $cm^{-3}d^{-1}$), respectively. However, in water, the sulfate reduction rate (0.24±0.16 nM $cm^{-3}d^{-1}$) was 2 orders less than in sponge. SRA by SRB from mesohyl ranged from 3.28 to 3.53 nM d^{-1} and by cortex bacteria were 0.52 to 2.98 nM d^{-1}.
- Antagonistic interactions of AB against 75 heterotrophic bacteria (THB) from the same sponge and ambient water using 11 x 75 array of tests (825 tests) showed that all AB associated with sponge inhibited more than one THB. A total of 316 antagonistic interactions were exhibited and out of these, 157 interactions (49.7%) were against THB from ambient water. Multiple inhibitions or antagonism were also observed among AB. More than one AB inhibited 58 THB. The order of inhibition of THB

based on the source of isolation was ambient water > cortex> mesohyl. Similarly, a higher number of multiple inhibitions by AB was seen with THB from ambient water.

- Functions of microbial consortia and culturable bacteria associated with sponges were variable and the rates were differing depending on the bacteria and location in the sponge.

8.2.4. LINKING BACTERIAL DIVERSITY TO FUNCTION

- A total of 6119 KEGG pathway orthologs were found in *C. alloclada.* In general, they were involved in metabolism, genetic information processing, environmental information processing, cellular processes, diseases and organismal systems, with a higher number of genes in metabolism ($1.3 - 1.6 \times 10^8$ copy number).
- In *C. alloclada*, heterotrophic bacteria were the major microbial biomass (10^{5-6} CFU cm^{-3}) and were distributed in 12 phyla (Actinobacteria, Acidobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaera, Planctomycetes, Proteobacteria, Spirochaetes and Verrucomicrobia). This consortium showed metabolic potential to degrade complex molecules and uptake of amines, amino acids, carbohydrates, carboxylic acids, polymers etc.. The number of orthologs involved in the amino acid metabolism ($1.3-1.6 \times 10^8$ copy number) was higher than the genes in carbohydrate, lipid and nucleotide metabolism ($2.9-3.6 \times 10^7$, $1.1-1.3 \times 10^7$ and $0.8-1.1 \times 10^7$ copy numbers, respectively). The degradative nature of bacteria is because of their ability to produce an array of hydrolytic enzymes. The dominant genera such as *Vibrio, Bacillus* and *Pseudomonas* were the main enzymes producers, which degrade carbohydrate, lipid, protein and DNA to provide carbon, nitrogen and phosphorus sources to the sponge holobiont and utilised all the simple mono and disaccharides. they also utilised amino acids, carboxylic acids, polymers, sugar acids and alcohols.
- A total of 770 bacterial OTUs which are reported to carry out CO_2 fixation were obtained from *C. alloclada*. There were $1.1 - 1.3 \times 10^7$ copies of genes which are involved in the carbon fixation was obtained from *C. alloclada* metagenome. This

high number of genes codes for proteins involved in carbon fixation might have contributed to inorganic carbon uptake of 9 to 16 µg C cm^{-3} d^{-1}

- In *C. alloclada*, 75 OTUs of methane oxidising bacteria were detected. The bacterial isolates from *C. alloclada*, which showed methane oxidation belonged to α- and γ-Proteobacteria and Firmicutes. The genes for methane metabolism (2.6 ─3.1x10^6 copies) were also detected in *C. alloclada*, which may contribute to the methane oxidation rate of 1.3 mM $cm^{-3}d^{-1}$. The rate of methane oxidation by the bacterial isolates in *C. alloclada* was 1─3 orders less than the rate of methane oxidation by the microbial consortia. Among the different genera, *Bacillus* spp. showed a higher rate of methane oxidation (625 µM d^{-1}) compared to other bacteria.
- There were 54 OTUs of nitrifying bacteria present in *C. alloclada* which belong to Nitrospira (45 OTUs), Nitrospina (7 OTUs), and Nitrosomonas (2 OTUs). These bacteria contributed to net nitrification rate of 3.50 to 8. 52 µM cm^{-3} d^{-1}. OTUs of denitrifying bacteria belonging to Actinobacteria, Firmicutes, α-,β- and γ-Proteobacteria were detected (162 OTUs). Denitrification rate by microbial consortia in *C. alloclada* was 152 to 414 nM cm^{-3} d^{-1}. This rate was 4 orders higher than individual bacterial strains. The high rate of nitrous oxide production (~ 0.04 nM d^{-1}) in *C. alloclada* was seen in *B. cereus* and *S. arlettae*. The genes involved in the nitrogen metabolism for energy production in *C. alloclada* ranged from 1.9 to 2.3x10^6 copies. The fermentative products excreted by sponges can be utilised by denitrifying bacteria during anoxic condition. Similarly, NH_3 will be utilised by nitrifying bacteria at net nitrification rate of 145 to 355 nM cm^{-3} d^{-1}, producing 3.5 to 8.5 µM cm^{-3} d^{-1} of nitrate. This excess nitrate will be removed by denitrifying bacteria when the sponge tissue becomes anaerobic.
- SRB OTUs (155) were affiliated to Nitrospira, δ- and ε-Proteobacteria. In *C. alloclada*, the presence of Desulfobacterales (80 OTU), Desulfomonadales (26 OTU), Desulfarculaceae (17 OTU), Thermodesulfovibrionaceae (11 OTUs) and Desulfovibrionales (8 OTUs) were detected and culturable bacteria belonged to *Desulfovibrio* sp. and *Desulfococcus* sp. These SRBs from *C. alloclada* might be involved in the sulfate reduction (34.3 to 46.0 nM cm^{-1} d^{-1}). Genes involved in sulfur

metabolism was detected in *C. alloclada* (0.9─1.2x10^6 copies). The sulfate reduction by isolates ranged from 0.52 to 3.53 nM d^{-1}.

- Sponge-associated bacteria are known source of a variety of secondary metabolites. *C. alloclada* metagenome harboured 2.6 ─3.0x10^6 copies of genes for secondary metabolite production. Among 11 Actinobacteria tested for the antagonistic interaction against 75 heterotrophic bacteria from sponge and ambient water, *Streptomyces thermovulgaris*, isolated from the cortex of C. *alloclada* showed the highest number (68) of inhibition (Inhibition of 23, 22 and 23 bacteria from ambient water, cortex and mesohyl, respectively), followed by *Streptomyces cyaneus* isolated from the mesohyl. The genes codes for antimicrobial proteins such as polyketides and non-ribosomal peptide synthetase were found in *C. alloclada* metagenome with a copy number of 6.1─ 7.4x10^6. It was reported that *Streptomyces* harbour these proteins and a high number of these genes may be the reason the high number of antagonistic interactions exhibited by these bacteria.
- Associated microbes in *C. alloclada* are actively involved in the nutrient cycling in the sponge. Overall the heterotrophs showed multiple functions, *Bacillus* (50%) and *Staphylococcus* (20%) being prominent multiple functioning genera. This study demonstrated for the first time different microbial processes in carbon, nitrogen and sulfur cycling in a single sponge species.

8.2.5. APPLICATION

- When shrimp aquaculture wastewater was treated with live *C. alloclada*, for 10 days, a reduction in the dissolved nutrients such as nitrite, nitrate, phosphate and silicate was observed. Total suspended matter in wastewater was removed at a rate of 14.29±12.12 mg l^{-1} d^{-1}.
- A total of 75 bacterial isolates were screened for azo dye decolourization. It was seen that 23.7% of the bacteria showed decolourization and out of this, 33% showed decolourization for both Amido black (AB) and Congo red (CR) dyes. The potential bacterium, *Y. pacifica* decolourized 96.7% and 96.2 % of 50 mg l^{-1} of AB and CR,

respectively at 6g l^{-1} nutrient medium, 37±2°C pH 9 and 8, respectively. The present study reiterates biotechnological potential of sponges.

8.3. **CONCLUSIONS AND FUTURE PERSPECTIVES**

The present study revealed that *C. alloclada* is an HMA sponge which harbour $>10^9$ bacteria cm^{-3} and exhibit high bacterial diversity with 40 bacterial phyla. Sponge tissues provide a dynamic environment for associated bacteria, where resource distribution varies spatially and temporally. In addition, sponge provides nutrient sources by filtering sea water that significantly affects the population of microorganisms in a manner specific to the host. The presence of a high number of bacteria associated with the sponge producing extracellular enzymes showed that they play a crucial role in biopolymer degradation like proteins, carbohydrates, lipids and nucleic acids thereby assisting the host in its nutrition and various metabolic processes. Functions of microbial consortia and culturable bacteria associated with sponges were also variable. Functions associated with anaerobic or facultative bacteria were more in the mesohyl than cortex and aerobic processes were more in cortex tissues. It has been suggested that co-occurring bacteria avoid competition by utilising different carbon and energy sources (Duperron et al., 2006; Kleiner et al., 2012a). Apart from habitat structure, animal host interaction and local biogeochemical conditions, the function of heterogeneous bacterial communities were shaped by associated Actinobacteria as high antagonistic activity was seen in this sponge. Thus, these sponge holobiont not only utilise the carbon, nitrogen and phosphorus for its survival but may also provide these simple compounds to the sponge. To conclude, this study clearly demonstrates that multiple functions of associated bacteria help to balance the ecology of sponge *Cinachyra alloclada*. Associated microbes in *C. alloclada* are actively involved in the nutrient cycling in the sponge. It was found that various groups were responsible for multiple functions. This study demonstrated for the first time the multiple activities of the diverse microbial flora associated with this sponge. *C.alloclada* associated bacteria are promising bio-prospecting source for bioremediation of pollutants especially dyes from textile industries and aquaculture wastewater treatment. This work will add to the ongoing search of linking bacterial diversity to function associated with sponges.

Considerable research effort is currently being directed towards understanding the dynamics of the bacterial population associated with sponges. Still, some questions remain unanswered. Next generation sequencing technologies combined with varied heterologous expression systems are expected to open up the large unexplored reservoir of functional roles of yet uncultivated sponge-associated microbes. Future studies on the associated bacteria among diverse sponges and targeting host-bacteria interactions will improve our understanding of the selective pressures that shape these communities. This also reveals the occurrence and exchange of generalist versus specialist microbial communities in the sponges. Additional studies on the temporal variation of bacterial diversity as well as different functions carried out by sponge-associated bacteria under natural conditions will help in determining natural variation in the sponge microbial consortia and its consequences for host-symbiont dynamics. Further, such studies create a baseline to define shifts in the bacterial community structure and function with respect to environmental factors. The metagenomic analysis provided valuable information on metabolic functions such as primary metabolism, adaptation to anaerobic conditions, resistance to antibiotics, and even potential interaction with the host through eukaryotic-like proteins. Such information can be useful in planning novel culture conditions. Similarly, information about the conditions in the bacterial natural environment, for example, the physicochemical conditions inside sponge mesohyl would help to design better culture conditions which result in the culture of more number of previously uncultured bacteria from the sponge. In situ bacterial culture (Kaeberlein et al., 2002; Ben-Dov et al., 2009) or gradual acclimatisation of bacteria to the artificial culture medium (Bollmann et al., 2007; Taylor et al., 2011), may also help to increase the culturability of sponge-associated bacteria.

Research also should focus on the sponge genome projects. The studies on sponge genome will help to detect possible metabolic pathways absent in the sponge host, and these functions may be fulfilled by associated bacteria. Future studies on sponge microbiology should also focus on developing suitable assays and indicators for assessing sponge health and determining the disease causing agents. These studies would also enhance our understanding of the mechanisms by which sponges differentiate between associated bacteria, food and pathogens. A more detailed description of complex functions of bacteria associated with *C.*

alloclada needs to be established in future studies. Factors shaping the structure and functions of these bacterial communities should be elucidated in detail and with sponges from different habitats and geographic locations. As mesohyl and cortex showed the difference in the community structure and function, research should be extended to understand the variability of diversity and functions associated with intracellular bacteria which are assumed to be sponge-symbionts. The need and encouragement for sponge-microbiology research continue to increase with its implications ranging from ecology to host-symbiont interaction and evolution to natural product discovery. Lastly, the present study establishes a foundation for more detailed metagenomic, functional, proteomics and metabolomics analyses that will provide an insight into the detailed structure and specific function of complex microbial microcosm in the sponge.

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APPENDIX

CULTURE MEDIA COMPOSITION

1. Nutrient agar

Peptic digest of animal tissue	- 5.0 g
Sodium chloride	- 5.0 g
Beef extract	- 1.5 g
Yeast extract	- 1.5 g
Agar powder	- 15 g

2. Half strength nutrient agar

Peptic digest of animal tissue	- 2.5 g
Sodium chloride	- 2.5 g
Beef extract	- 0.7 g
Yeast extract	- 0.7 g
Agar	- 15 g

3. Desi strength nutrient agar

Peptic digest of animal tissue	- 0.5g
Sodium chloride	- 0.5 g
Beef extract	- 0.1 g
Yeast extract	- 0.1 g
Agar powder	- 15 g

4. Nutrient broth

Peptic digest of animal tissue	- 5.0 g
Sodium chloride	- 5.0 g
Beef extract	- 1.5 g
Yeast extract	- 1.5 g

5. Zobell marine agar (marine agar 2216)

Peptic digest of animal tissue	- 5.00 g
Yeast extract	- 1.00 g
Ferric citrate	- 0.01 g
Sodium chloride	- 19.45 g
Magnesium chloride	- 8.80 g

Sodium sulfate	- 3.24 g
Calcium chloride	- 1.80 g
Potassium chloride	- 0.55 g
Sodium bicarbonate	- 0.16 g
Potassium iodide	- 0.08 g
Strontium chloride	- 0.034 g
Boric acid	- 0.022 g
Sodium silicate	- 0.004 g
Sodium fluorate	- 0.0024 g
Ammonium nitrate	- 0.0016 g
Disodium phosphate	- 0.008 g
Agar	- 15.0 g
pH	-7.6±0.2

6. Nitrate reducing bacteria selective medium

Sodium lactate	-1.00 g
Sodium acetate	-1.00 g
Sodium succinate	-1.00 g
Potassium nitrate	-0.101 g
Nutrient broth	-0.01%
pH	-8.1 ± 0.2

7. Sulfate reducing bacteria

Yeast extract	- 1.0 g
K2HPO4	- 0.2 g
Sodium acetate	-1.0 g
Sodium lactate	-8.0 ml
Trace element	-1.0 ml
Ferrous sulfate (1 g/10 ml)	-5.0 ml
Sodium thioglycolate (6/100ml)	-5.0 ml
Sodium sulfide (5g/100 ml)	-2.5 ml
Agar	-8.0 g

pH	-7.8-8

8. Anaerobic agar

Peptic digest of animal tissue	- 5.0 g
Sodium chloride	- 5.0 g
Beef extract	- 1.5 g
Yeast extract	- 1.5 g
Sodium thiglycolate(6/100ml)	- 5.0 ml
Agar powder	- 15.0 g

9. Ammonia oxidizing bacteria selective medium

di -Potassium Hydrogen Orthophosphate	- 0.10 gm
Anhydrous Magnesium Sulfate	-0.10 gm
Anhydrous Iron Sulfate	-0.10 gm
Anhydrous manganese sulfate	-0.10 gm
Sodium molybdate	-0.05 gm
Ammonium sulfate	-2 mM
pH	-7.5 – 8.

10. Nitrite oxidizer media

$NaNO_3$	-2.0 g
NaCl	-0.50 g
$MgSO_4.7H_2O$	-0.05 g
KH_2PO_4	-0.15 g
$CaCO_3$	-70 mg
$(NH)_4\,MoO_4.4H_2O$	- 0.05 mg
pH	-8.9

11. Rhodosprillales selective media

$NaHCO_3$	-3.90 g
KH_2PO_4	-0.30 g
KCl	-1.0 g
$CaCl2.2H_2O$	-0.05 g

$MgCl_2$. $6H_2O$	-3.50 g
Na_2SO_4	-1.0 g
NaCl	-100 g
Proline	-5 mM
Acetate	-2 mM
K3-citrate	-10 mM
Na-glutamate	-5 mM
pH	-7.0
Trace element solution	-1.0 ml
Vitamin solution	-1.0 ml

Trace element solution/litre

$FeCl_2.4H_2O$	-1800 mg
$CoCL_2.6H_2O$	-250 mg
$NiCl_2.6H_2O$	-10 mg
$CuCl_2.2H_2O$	-10 mg
$MnCl_2.4\ H_2O$	-70 mg

Trace element solution/litre

$FeCl_2.4H_2O$	-1800 mg
$CoCl_2.6H_2O$	-250 mg
$NiCl_2.6H_2O$	-10 mg
$CuCl_2.2H_2O$	-10 mg
$MnCl_2.4\ H_2O$	-70 mg
$ZnCl_2$	-100 mg
H_3BO_3	-500 mg
$Na_2MoO_4.2H_2O$	-30 mg
$Na_2SeO_3.2H_2O$	-30 mg

12. Chloroflexiselective medium

Nitrilotriacetic acid	- 0.100 g
$CaSO_4.7H_2O$	-0.060 g
$MgSO_4.7H_2O$	-0.100 g

NaCl	-0.008 g
Na_2HPO_4	-0.007 g
KH_2PO_4	-0.036 g
Glycylglycine	-0.500 g
Yeast extract	-0.5 g

Phenotypic and biochemical characterization of THB

Various features of colony morphology such as pigmentation, surface texture, size, shape, production of slime, elevation, opacity, swarming behavior, and changing color of the medium were recorded. For various biochemical tests, actively growing culture was used. For all biochemical methods, the protocols outlined by Gerhardt et al., (1981) were used.

1. Gram staining

   A smear of bacterial culture was prepared in physiological saline, air dried and heat fixed. Primary staining was done with crystal violet followed by application of Gram's iodine (mordant). The slide was decolourized and secondary staining was done with safranin and observed under Olympus light microscope. Violet or purple colour indicates Gram positive bacteria and pink colour indicates Gram negative bacteria.

2. KOH test (string test)

   Bacterial culture was emulsified with 3% KOH solution. Formation of string indicated positive reaction for Gram negative bacteria. No string was formed in Gram positive bacteria.

3. Motility test

   Bacterial cultures were stabbed onto the mannitol motility agar medium in a test tube and incubated for 24 hours. Motile bacteria showed a spreading growth from the stabbed line.

4. Oxidase test

   A strip of filter paper was dipped in oxidase reagent and to the moist paper, bacterial culture was smeared. Blue colouration within 10 seconds indicated positive reaction.

5. Catalase test

Bacterial culture mixed with 30% hydrogen peroxide resulted in effervescence was regarded as positive reaction.

6. Oxidative/ Fermentative test (OF test)

   Bacterial culture was inoculated into the OF medium in the test tube till the rim of the tube. Change of green colour of the medium to yellow colour only on top of the tube indicated oxidative reaction and only in bottom of the tube indicated fermentative reaction. Change of colour in the entire length of tube was recorded as oxidative and fermentative reaction. Blue colouration on the top of the tube indicated alkaline reaction.

7. Citrate test

   Bacterial culture was inoculated by stab and streak method. Change of colouration from green to blue indicated citrate utilization.

8. Triple sugar Iron test

   This is used to test the ability of bacteria to ferment glucose, lactose, sucrose and produce hydrogen sulfide. The medium contained 1% lactose and sucrose and 0.1% glucose. Phenol red, the acid-base indicator was incorporated in the medium to detect carbohydrate fermentation. Bacterial culture was inoculated by stabbing the center of butt and streaking the slope of the slant. The tubes were incubated for 24 hours at 25 °C. The colour of both slant and butt was observed.

9. Methyl red test

   Glucose utilization was assessed by the production of acid in MR-VP broth. Methyl red indicator was incorporated in the medium to detect glucose utilization and positive isolates produced red colour and negative reaction was marked by yellow colouration in the medium.

10. Voges – Proskauer test

    VP medium was inoculated and incubated for 24 hours at 25 °C. After incubation, Baritt reagents A and B were added. Formation of dark red colour was recorded as positive reaction.

11. Indole test

Peptone broth was inoculated and incubated for 24 hours at 25 °C. After incubation, indole reagent was added, and formation of cherry red coloured ring at the interface regarded as positive reaction.

12. Carbon utilization

Bromo cresol purple was used as the indicator in the basal medium devoid of carbohydrate. A spectrum of carbohydrate sources such as monosaccharides, disaccharides, trisaccharides and polysaccharides like adonitol, arabinose, cellobiose, dextrose, ducitol, fructose, galactose, inositol, inulin, maltose, mannitol, mellibiose, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose were employed. Change of colour from purple to yellow indicated positive reaction and negative reaction was indicated by no colour change.

13. Nitrate reduction

The medium used was basically a nutrient broth supplemented with 0.1% potassium nitrate as the nitrate substrate. Following incubation after inoculation with bacterial culture, the ability of organisms to reduce nitrate to nitrite is determined by the addition of two reagents. Solution A, which is sulfanilic acid, followed by solution B, which is alpha-naphthylamine. A cherry red colour in the medium indicated positive result.

14. Growth on Mac Conkey agar

Sterile Mac Conkey agar plates were inoculated with the bacterial cultures and were kept for incubation at 25℃ for 24-48 hours. Following incubation, the plates were observed for growth of bacterial colonies. Pink colour denoted the lactose fermenting bacteria and cream colour indicated non lactose fermenting bacteria.

Phenotypic and biochemical characterization of Actinobacteria

1. Gram staining

Gram staining was done as described early.

2. Spore chain morphology

A sterile Z - shaped rod was placed on sterile filter paper in a petri dish and a sterile microscopic slide on the Z- rod. The solid ISP-4 medium ($1cm^2$) was cut and placed

on the centre of a microscope slide. The four sides of the agar were inoculated with Actinobacterial spores using a needle. A sterile cover glass was aseptically placed on the upper surface of the agar cube and pressed gently. The filter paper was moistened using sterile distilled water. The plates were kept for incubation at 25℃ for 2 weeks. The spores formed were observed by wet mount technique.

3.Colony morphology

Aerial mass colour

The colour of the mature sporulating aerial mycelium (white, grey, green, red, brown, black) grown on ISP-4 medium was recorded.

Reverse side pigment the colour of substrate mycelium was observed from the reverse side of the colony.

4. Melanin production

Presence or absence of melanoid pigments on medium was tested by stab and streak the culture. Production of greenish brown or black diffusible pigment or distinct brown pigment indicates the positive result. No characteristic change indicates negative result.

5. Chitinase production

Chitinase assay was performed using colloidal chitin agar prepared in sea water (Hsu and Lockwood. 1975). The isolates were inoculated on 4% of chitin agar plates and incubated for 7 days at 25℃. A clear zone around the colony indicated chitinase activity.

6. Hydrogen sulfide production

Triple sugar iron agar medium was used to check the hydrogen sulfide production by the bacteria. The ferrous ammonium sulfate in the media reacts with hydroden sulfide produced by the bacteria to form ferrous sulfide resulting the formation of black precipitate.

7. Citrate utilization

Citrate utilization test was done as described earlier. Results were observed after a week.

8. Growth at pH 6

The growth on ISP-4 broth at pH 6 after incubation at 25° for 3 days was noted.

9. Growth at temperature 37° C

The growth on ISP-4 broth at pH 6 after incubation at 37℃ for 3 days was noted.

10.Tyrosine degradation

If the organism produces tyrosine decarboxylase, the pH increases and form highly alkaline condition. The pH indicator bromothymol purple shows a colour change from purple to dark purple due to the alkaline pH.

11. Growth in sodium azide

Sodium azide is an inorganic compound which is highly toxic. The growth of Actinobacteria in medium with sodium azide was noted.

12. Growth in lysozyme

The growth of Actinobacteria in medium with lysozyme was noted.

13. Nitrate reduction

Nitrate reduction was done as described early

14. Antibiotic resistance

Antibiotic resistance of Actinobacteria was tested using Kirby Bauer method. Using an aseptic technique, bacterial culture was spreaded on the surface of the Muller Hinton agar (MHA) plates. The antibiotic disc was gently placed on the surface of the plate. The disc was gently tapped with the forceps to fix it in position. The plates were incubated at 25 °C for 48 hours. The antibiotics used were given in appendix.

Phenotypic and biochemical characterization of AAnP

1. Gram staining
2. KOH test (string test)
3. Oxidase test
4. Catalase test
5. Oxidative/ Fermentative test (OF test)
6. Bacterial chlorophyll

Bacterial culture in PPES II medium was incubated at room temperature for 3 to 4 days. After sufficient growth was obtained, the cultures were centrifuged at 10,000

rpm for 3 minutes to pellet down the cells. To the pellet, 3 to 4 ml of 90% acetone was added and incubated for 1 to 2 hours following which the tubes were again centrifuged at 10,000 rpm for 3 minutes. The absorbance spectrum of supernatant at the wave length range of 350-1050 nm and absorbance at 375 nm were noted.

7. Carbon utilization

The test was performed to investigate the growth of the bacterial isolates on different carbon sources like acetate, butyrate, citrate, fumarate, lactate, succinate and tartarate. 96 well plates were used to perform the tests. Basal medium without any carbon source supplemented with an indicator (phenol red) and carbon source was added to the wells such that the final concentration of the carbon source in each well is 1%. Actively growing bacterial culture was inoculated to the wells and incubated at 25 °C for 3 days. The plates were observed for growth and colour change from red to yellow.

8. Growth factor utilization

Vitamins like nicotinic acid, biotin, vitamin B3, B12, and para-aminobenzoic acid (PABA) are some of the basic growth factors required for the growth of AAnP. The test was performed in 96 well plates. The medium without any growth factors and were added with each growth factor in the wells followed by the addition of bacterial culture. The growth of these isolates in medium without growth factors was also analyzed. The plates were incubated in the dark for 25 °C for 3 to 4 days. Following incubation the plates were observed for growth.

Extra cellular enzyme production

All the bacterial isolates (THB, AB, NRB, PAB) were screened for the production of various complex molecule degrading enzymes. Twenty four hour old bacterial cultures were spot inoculated onto respective media and incubated for 24 to 72 hour depending on the growth rate of the bacteria at 25±2℃. After incubation the plates were checked for the production of various enzymes and results were recorded.

1**.**Amylase

Nutrient agar supplemented with 0.2% soluble starch were used to screen for amylase production. Presence of clear zone around the culture in the blue background was taken as positive result.

2. DNase

DNase test agar base (Himedia, India) contains 0.2% DNA in final concentration. A characteristic blue precipitate forming on bacterial colony when plate was flooded with 0.1% toluidine blue solution, registered as positive reaction.

3. Lipase

Nutrient agar was supplemented with 0.01% of calcium chloride and 1% of Tween 80 (Oleic acid). The presence of halo around the colony by the formation of calcium soaps with oleic acid was regarded as positive reaction.

4. Caseinase

Skim milk (5%, final concentration) incorporated in nutrient agar were used as the screening medium. Clear zone around the bacterial colony after incubation indicated hydrolysis of casein.

5. Urease

Christensen agar base (Himedia) was supplemented with 40% urea solution was used. A change in colour of the yellowish medium to a pink colouration around the bacterial culture was regarded as positive reaction.

6. Gelatinase

Nutrient agar with 0.4% gelatin was used as media to detect gelatinase activity. Gelatinase positive isolates exhibited a clear zone around the colony when flooded with 15% mercuric chloride solution.

Table S1. Bacterial isolates from mesohyl region of the sponge

Isolate no	Isolate ID	Isolate no	Isolate ID	Isolate no	Isolate ID	Isolate no	Isolate ID
M001	*Micrococcus*	M039	*Aeromonas*	M077	*Vibrio*	M114	*Nitrococcus*
M002	*Brevibacterium*	M040	*Vibrio*	M078	*Pseudomoas*	M115	*Nitrococcus*
M003	*Yangia*	M041	*Actinoplanes*	M079	*Pseudomoas*	M116	*Nitrococcus*
M004	*Vibrio*	M042	*Actinoplanes*	M080	*Vibrio*	M117	*Nitrosomonas*
M005	*Staphylococcus*	M043	*Actinoplanes*	M081	*Pseudomoas*	M118	*Nitrobacter*
M006	*Vibrio*	M044	*Streptoverticillum*	M082	*Photobacterium*	M119	*Rubrivivax*
M007	*Vibrio*	M045	*Actinoplanes*	M083	*Staphylococcus*	M120	*Rhodoblastus*
M008	*Photobacterium*	M046	*Streptoverticillum*	M084	*Streptococcus*	M121	*Blastochloris*
M009	*Photobacterium*	M047	*Actinoplanes*	M085	*Micrococcus*	M122	*Rhodocyclus*
M010	*Bacillus*	M048	*Streptomyces*	M086	*Staphylococcus*	M123	*Blastochloris*
M011	*Arthrobacter*	M049	*Actinoplanes*	M087	*Bacillus*	M124	*Rhodoblastus*
M012	*Bacillus*	M050	*Actinoplanes*	M088	*Vibrio*	M125	*Rubrivivax*
M013	*Photobacterium*	M051	*Actinoplanes*	M089	*Vibrio*	M126	*Rhodoferax*
M014	*Bacillus*	M052	*Streptomyces*	M090	*Agrobacterium*	M127	*Blastochloris*
M015	*Acinetobacter*	M053	*Streptomyces*	M091	*Rahnella*	M128	*Rhodocyclus*
M016	*Plesiomonas*	M054	*Nitrosococcus*	M092	*Pasteurella*	M129	*Rubrivivax*
M017	*Pseudomoas*	M055	*Nitrosomonas*	M093	*Rahnella*	M130	*Blastochloris*
M018	*Pseudomoas*	M056	*Nitrosococcus*	M094	*Bacillus*	M131	*Rhodopseudomonas*
M019	*Bacillus*	M057	*Rhodoferax*	M095	*Vibrio*	M132	*Rhodomicrobium*
M020	*Pasteurella*	M058	*Rubrivivax*	M096	*Rahnella*	M133	*Rhodocyclus*
M021	*Pseudomoas*	M059	*Rubrivivax*	M097	*Pasteurella*	M134	*Rhodocyclus*
M022	*Micrococcus*	M060	*Rhodoferax*	M098	*Pasteurella*	M135	*Rhodoferax*
M023	*Streptococcus*	M061	*Rubrivivax*	M099	*Arthrobacter*	M136	*Pseudomoas*
M024	*Bacillus*	M062	*Blastochloris*	M100	*Bacillus*	M137	*Vibrio*
M025	*Arthrobacter*	M063	*Rubrivivax*	M101	*Staphylococcus*	M138	*Vibrio*
M026	*Bacillus*	M064	*Rubrivivax*	M102	*Salinicola*	M139	*Xanthomonas*
M027	*Micrococcus*	M065	*Rubrivivax*	M103	*Micrococcus*	M140	*Branhamella*
M028	*Streptococcus*	M066	*Plesiomonas*	M104	*Arthrobacter*	M141	*Listonella*
M029	*Staphylococcus*	M067	*Pseudomoas*	M105	*Arthrobacter*	M142	*Pseudomoas*
M030	*Streptococcus*	M068	*Branhamella*	M106	*Arthrobacter*	M143	*Rhodocyclus*
M031	*Staphylococcus*	M069	*Pseudomoas*	M107	*Acinetobacter*	M144	*Rubrivivax*
M032	*Vibrio*	M070	*Pseudomoas*	M108	*Bordetella*	M145	*Providermia*
M033	*Vibrio*	M071	*Micrococcus*	M109	*Kingella*	S1	*Desulfovibrio*
M034	*Vibrio*	M072	*Bacillus*	M110	*Kingella*	S6	*Desulfovibrio*
M035	*Vibrio*	M073	*Bacillus*	M111	*Neisseria*	S7	*Desulfovibrio*
M036	*Vibrio*	M074	*Pseudomoas*	M112	*Nitrobacter*	S8	*Desulfovibrio*
M037	*Vibrio*	M075	*Bacillus*	M113	*Nitrococcus*	S10	*Desulfococcus*
M038	*Pseudomoas*	M076	*Pseudomoas*				

Table S2. Bacterial isolates from cortex

Isolate no	Isolate ID	Isolate no	Isolate ID	Isolate no	Isolate ID
C001	*Rhodoblastus*	C047	*Nitrobacter*	C093	*Tatumella*
C002	*Rhodocyclus*	C048	*Streptoverticillum*	C094	*Cluster 6*
C003	*Rhodoferax*	C049	*Actinoplanes*	C095	*Erwinia*
C004	*Rubrivivax*	C050	*Actinoplanes*	C096	*Erwinia*
C005	*Rhodobium*	C051	*Actinoplanes*	C097	*Bacillus*
C006	*Rhodoblastus*	C052	*Streptomyces*	C098	*Plesiomonas*
C007	*Blastochloris*	C053	*Streptomyces*	C099	*Bacillus*
C008	*Rhodopseudomonas*	C054	*Actinoplanes*	C100	*Escherichia*
C009	*Rhodopseudomonas*	C055	*Actinoplanes*	C101	*Escherichia*
C010	*Rhodopseudomonas*	C056	*Streptomyces*	C102	*Yersinia*
C011	*Pseudomonas*	C057	*Streptomyces*	C103	*Pseudomonas*
C012	*Vibrio*	C058	*Nitrosomonas*	C104	*Vibrio*
C013	*Streptococcus*	C059	*Nitrosomonas*	C105	*Photobacterium*
C014	*Alteromonas*	C060	*Nitrosomonas*	C106	*Photobacterium*
C015	*Micrococcus*	C061	*Nitrosomonas*	C107	*Plesiomonas*
C016	*Vibrio*	C062	*Rubrivivax*	C108	*Vibrio*
C017	*Phenon 5*	C063	*Rubrivivax*	C109	*Photobacterium*
C018	*Streptococcus*	C064	*Rhodoblastus*	C110	*Pasteurella*
C019	*Lactobacillus*	C065	*Rhodocyclus*	C111	*Photobacterium*
C020	*Streptococcus*	C066	*Pseudomonas*	C112	*Micrococcus*
C021	*Streptococcus*	C067	*Photobacterium*	C113	*Micrococcus*
C022	*Vibrio*	C068	*Photobacterium*	C114	*Vibrio*
C023	*Vibrio*	C069	*Vibrio*	C115	*Nitrobacter*
C024	*Flavobacterium*	C070	*Photobacterium*	C116	*Nitrococcus*
C025	*Providermia*	C071	*Vibrio*	C117	*Nitrobacter*
C026	*Vibrio*	C072	*Vibrio*	C118	*Nitrobacter*
C027	*Vibrio*	C073	*Pseudomonas*	C119	*Rhodomicrobium*
C028	*Vibrio*	C074	*Xanthomonas*	C120	*Rhodopseudomonas*
C029	*Photobacterium*	C075	*Bacillus*	C121	*Rhodoblastus*
C030	*Vibrio*	C076	*Photobacterium*	C122	*Rhodoferax*
C031	*Vibrio*	C077	*Pseudomonas*	C123	*Blastochloris*
C032	*Vibrio*	C078	*Kingella*	C124	*Pseudomonas*
C033	*Vibrio*	C079	*Micrococcus*	C125	*Pseudomonas*
C034	*Vibrio*	C080	*Pseudomonas*	C126	*Branhamella*
C035	*Moraxella*	C081	*Photobacterium*	C127	*Salinisphaera*
C036	*Nitrosomonas*	C082	*Vibrio*	C128	*Neisseria*
C037	*Nitrobacter*	C083	*Photobacterium*	C129	*Xenorhabdus*
C038	*Nitrobacter*	C084	*Vibrio*	C130	*Listonella*
C039	*Nitrospina*	C085	*Photobacterium*	C131	*Phenon 10*
C040	*Nitrosococcus*	C086	*Staphylococcus*	C132	*Nitrobacter*
C041	*Nitrosomonas*	C087	*Micrococcus*	S2	*Desulfococcus*
C042	*Nitrosomonas*	C088	*Staphylococcus*	S3	*Desulfovibrio*
C043	*Nitrococcus*	C089	*Staphylococcus*	S4	*Desulfovibrio*
C044	*Nitrosomonas*	C090	*Bordetella*	S5	*Desulfovibrio*
C045	*Nitrospira*	C091	*Pseudomonas*	S9	*Desulfococcus*
C046	*Nitrococcus*	C092	*Arthrobacter*		

Table S3. Bacterial isolates from ambient water

Isolate No	Isolate ID	Isolate No	Isolate ID	Isolate No	Isolate ID	Isolate No	Isolate ID
W001	*Rubrivivax*	W044	*Pasteurella*	W087	*Pasteurella*		
W002	*Vibrio*	W045	*Photobacterium*	W088	*Aeromonas*	W130	*Vibrio*
W003	*Flavobacterium*	W046	*Photobacterium*	W089	*Pasteurella*	W131	*Alteromonas*
W004	*Serratia*	W047	*Vibrio*	W090	*Alteromonas*	W132	*Vibrio*
W005	*Vibrio*	W048	*Plesiomonas*	W091	*Nitrobacter*	W133	*Xanthomonas*
W006	*Nitrosomonas*	W049	*Branhamella*	W092	*Nitrobacter*	W134	*Pseudomonas*
W007	*Rhodoferax*	W050	*Edwardsiella*	W093	*Nitrospina*	W135	*Flavimonas*
W008	*Cluster 7*	W051	*Photobacterium*	W094	*Nitrospira*	W136	*Vibrio*
W009	*Photobacterium*	W052	*Photobacterium*	W095	*Nitrobacter*	W137	*Pseudomonas*
W010	*Serratia*	W053	*Agrobacterium*	W096	*Sphingomonas*	W138	*Vibrio*
W011	*Nitrococcus*	W054	*Agrobacterium*	W097	*Acinetobacter*	W139	*Nitrobacter*
W012	*Cluster 10*	W055	*Pseudomonas*	W098	*Vibrio*	W140	*Nitrococcus*
W013	*Cluster 10*	W056	*Pasteurella*	W099	*Sphingomonas*	W141	*Photobacterium*
W014	*Pseudomonas*	W057	*Alteromonas*	W100	*Vibrio*	W142	*Nitrosomonas*
W015	*Pseudomonas*	W058	*Rahnella*	W101	*Vibrio*	W143	*Rubrivivax*
W016	*Pseudomonas*	W059	*Pseudomonas*	W102	*Nitrobacter*	W144	*Rubrivivax*
W017	*Photobacterium*	W060	*Neisseria*	W103	*Vibrio*	W145	*Rhodomicrobium*
W018	*Pseudomonas*	W061	*Pseudomonas*	W104	*Nitrobacter*	W146	*Vibrio*
W019	*Vibrio*	W062	*Cluster 7*	W105	*Sphingomonas*	W147	*Pseudomonas*
W020	*Pseudomonas*	W063	*Pseudoalteromonas*	W106	*Pseudomonas*	W148	*Vibrio*
W021	*Listionella*	W064	*Erwinia*	W107	*Sphingomonas*	W149	*Pseudomonas*
W022	*Plesiomonas*	W065	*Plesiomonas*	W108	*Vibrio*	W150	*Vibrio*
W023	*Vibrio*	W066	*Photobacterium*	W109	*Vibrio*	W151	*Rhodomicrobium*
W024	*Branhamella*	W067	*Alteromonas*	W110	*Sphingomonas*	W152	*Rubrivivax*
W025	*Pseudomonas*	W068	*Staphylococcus*	W111	*Photobacterium*	W153	*Rubrivivax*
W026	*Acinetobacter*	W069	*Micrococcus*	W112	*Photobacterium*	W154	*Rubrivivax*
W027	*Bordetella*	W070	*Arthrobacter*	W113	*Chryseomonas*	W155	*Rhodoblastus*
W028	*Photobacterium*	W071	*Photobacterium*	W114	*Vibrio*	W156	*Nitrococcus*
W029	*Photobacterium*	W072	*Micrococcus*	W115	*Serratia*	W157	*No match*
W030	*Vibrio*	W073	*Pasteurella*	W116	*Vibrio*	W158	*Vibrio*
W031	*Photobacterium*	W074	*Photobacterium*	W117	*Rubrivivax*	W159	*Moraxella*
W032	*Alcanivorax*	W075	*Staphylococcus*	W118	*Nitrococcus*	W160	*Nitrosomonas*
W033	*Vibrio*	W076	*Vibrio*	W119	*Sphingomonas*	W161	*No match*
W034	*Pasteurella*	W077	*Plesiomonas*	W120	*Nitrococcus*	W162	*No match*
W035	*Micrococcus*	W078	*Chromobacterium*	W121	*Rubrivivax*	W163	*Pseudomonas*
W036	*Photobacterium*	W079	*Vibrio*	W122	*Rhodopseudomonas*	W164	*Sphingomonas*
W037	*Photobacterium*	W080	*Acinetobacter*	W123	*Rhodoferax*	W165	*No match*
W038	*Photobacterium*	W081	*Vibrio*	W124	*Phenon 5*	W166	*Rhodoferax*
W039	*Photobacterium*	W082	*Pasteurella*	W125	*Sphingomonas*	W167	*Blastochloris*
W040	*Photobacterium*	W083	*Micrococcus*	W126	*Pseudomonas*	W168	*Rhodocyclus*
W041	*Photobacterium*	W084	*Pasteurella*	W127	*Sphingomonas*	W169	*Rubrivivax*
W042	*Photobacterium*	W085	*Branhamella*	W128	*Vibrio*	W170	*Rhodobium*
W043	*Vibrio*	W086	*Chromobacterium*	W129	*Sphingomonas*	W171	*Phenon 21*

Table S4. Bacteria showed different functional activities and their EMBL accession numbers

	Nearest neighbour	Accession No
1	*Yangia pacifica*	LK022801
2	*Salinisphaera hydrothermalis*	LK022802
3	*Pseudoalteromonas rubra*	LT604915
4	*Bacillus safensis*	LT604916
5	*Bacillus aquimaris*	LT604917
6	*Bacillus sp.*	LT604918
7	*Staphylococcus sp.*	LT604919
8	*Staphylococcus sp.*	LT604920
9	*Vibrio sp.*	LT604921
10	*Bacillus cereus*	LT604922
11	*Brevibacterium linens*	LT604923
12	*Bacillus firmus*	LT604924
13	*Bacillus sp.*	LT604925
14	*Aeromonas sp.*	LT604926
15	*Staphylococcus pasteuri*	LT604927
16	*Bacillus aryabhattai*	LT604928
17	*Staphylococcus pasteuri*	LT604929
18	*Bacillus megaterium*	LT604930
19	*Bacillus subtilis*	LT604931
20	*Vibrio sp.*	LT604932
21	*Bacillus cereus*	LT604933
22	*Bacillus sp.*	LT604934

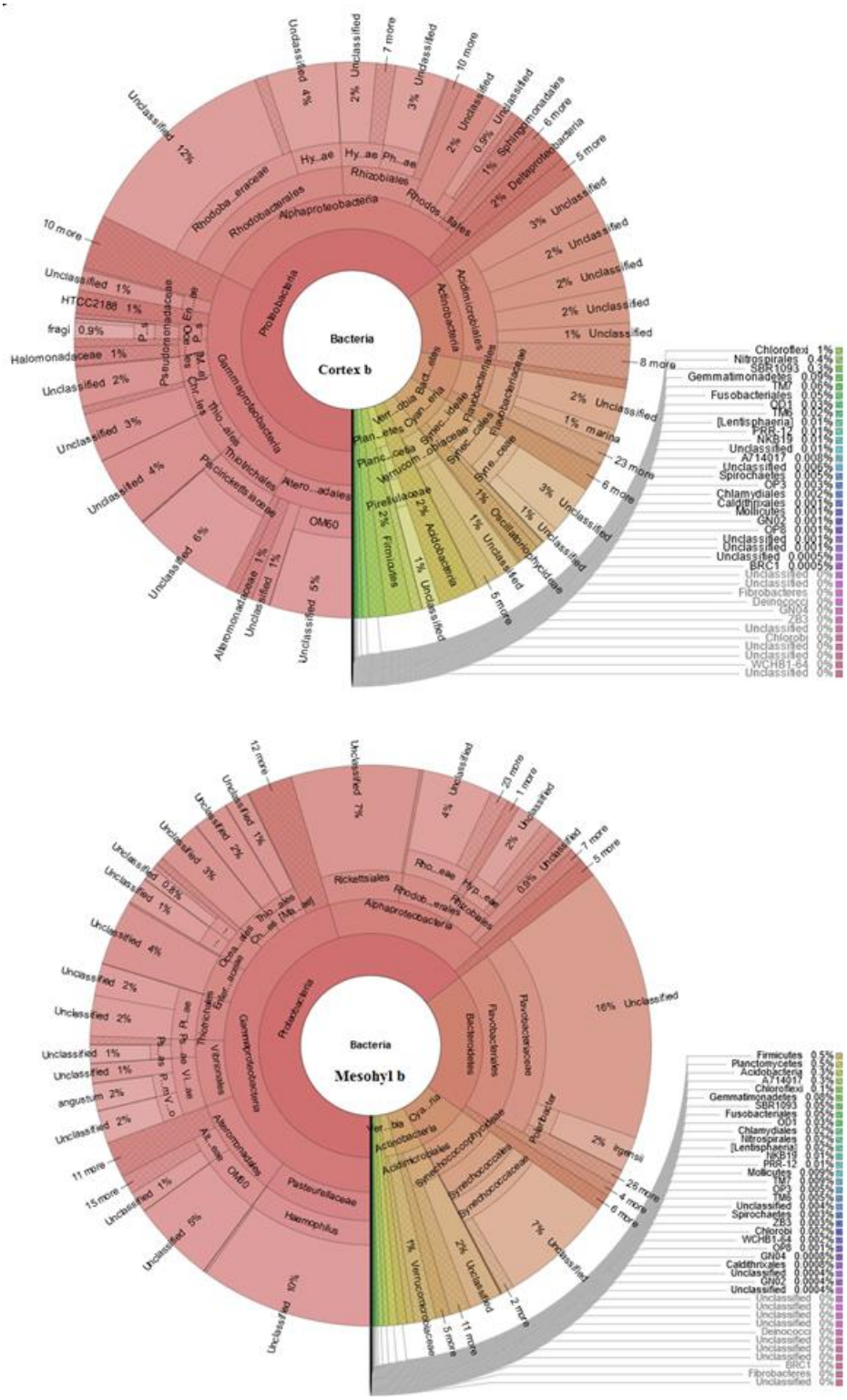

Figure 1.Crona chart of bacterial diversity in the duplicate samples of cortex and mesohyl