Bacterial Communities of different Mediterranean Sponge Species

- Basic investigations for biotechnological sponge cultivation

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1 Abstract

The overall goal to use marine sponges and their associated microorganisms for the supply of bioactive natural products was the motivation for the investigation of bacterial communities of sponges in this work.

Marine sponges are considered one of the most prolific sources of new bioactive natural products. However, the combination of predominantly low concentrations of the bioactive compounds and the low growth rates of sponges in the sea results in a very low accessibility to these bioactive compounds for pre- and clinical trials by isolation from sponges. The biotechnological cultivation of sponges under controlled conditions is one possible way to allow for sufficient amounts of natural compounds for the investigations of their efficacy in pre- and clinical trials and for the subsequent development of novel drugs. However, sponges are difficult to cultivate. Two reasons can be considered to be responsible for the difficulties in the establishment of enduring sponge cultivation: (1) a deficiency in the supply of enough food to the sponges or (2) a loss of, for the sponges physiology and metabolism, essentially required sponge-associated microorganisms during sponge cultivation.

Successional changes in the bacterial communities associated with the mesohyl of *Aplysina aerophoba* sponges during the cultivation under different artificial conditions over a period of six months and 76 weeks, respectively have been investigated by use of denaturing gradient gel electrophoresis (DGGE). The cultivation conditions varied concerning the water temperature (20 ± 2°C and 25 ± 2°C) of the aquaria, additional illumination of one aquarium, and feeding of the sponges. Amplicons from DGGE separation of dominant colonizing or variably appearing sponge-associated bacteria were sequenced and aligned for taxonomical identification. In addition, secondary metabolites typically found in *A. aerophoba* were analyzed to investigate changes in the natural product profile during cultivation over a time period of six months.

The cultivation of sponges under any given condition did not lead to a depletion of their bacterial community in the course of the experiment. On the contrary, the distinctive set of associated bacteria was maintained in spite of a dramatic loss of biomass and morphological degradation of the sponges during the cultivation period. Generally, all sequences obtained from the DGGE gels were related to bacteria of four phyla: *Actinobacteria*, *Cyanobacteria*, *Proteobacteria*, and *Chloroflexi*. Despite the overall stability of the bacterial community in *A. aerophoba*, an unambiguous variability was detected for the *Cyanobacteria* "*Aplysina aerophoba* clone TK09". This variability was ascribed to the predominant light conditions. The analysis of the secondary metabolite pattern revealed that the concentration of a class of characteristic brominated compounds typically found in *A. aerophoba*, like aeroplysinin-1, aerophobin-1, aerophobin-2, and isofistularin-3, increased over the six months of cultivation. Altogether, the main consequence resulting from this study is that effects of varying bacterial communities on

sponges are negligible when cultivating them under conditions corresponding to their natural habitat. Thus, when examining the feasibility of growing sponges in aquaculture for the production of natural products the qualitative and quantitative aspects of the abiotic cultivation conditions and the supply with nutrients should be in the focus.

Due to the various and often chemically mediated interactions that occur between the microorganisms and their eukaryotic hosts and between the members of the epibiotic community, it is reasonable to expect that epibiotic communities on sponges obtain particularly high amounts of bioactive producing microorganisms. However, only little is known to date about the ecology of bacterial communities on the surface of sponges in contrary to the well-investigated tissue-associated bacterial communities of sponges. Thus, for the exploration of sponges and their epibiotic microorganisms as a source for new natural bioactive compounds, investigations of the diversity of sponge surface-associated bacteria provide a basis for the ecological understanding of sponge-microorganism associations and possibly initiate access to new bioactive compounds. Therefore, the second part of this work was focused on the investigations of bacterial communities associated with the surfaces of several Mediterranean sponge species in comparison to those associated with the sponges' mesohyl and other animate or inanimate reference surfaces as well as with those from bulk seawater.

DGGE analysis of PCR-amplified bacterial 16S rRNA genes obtained from the surfaces and tissues of the investigated sponge species demonstrated that the surface-associated bacterial communities were generally different from each other and to those of the corresponding tissue sample. Furthermore, the bacterial communities from sponges were different from those on reference surfaces or from bulk seawater. Additionally, clear distinctions in 16S rDNA fingerprint patterns between the bacterial communities from mesohyl samples of "high-microbial abundance (HMA) sponges" and "low-microbial abundance (LMA) sponges" were revealed by DGGE and cluster analysis. A dominant occurrence of particularly GC-rich 16S rDNA fragments was found only in the DGGE banding pattern obtained from the mesohyl of HMA sponges. Furthermore, sequencing analysis of 16S rDNA fragments obtained from mesohyl samples of HMA sponges revealed a dominant occurrence of sponge-associated bacteria. The bacterial communities within the mesohyl of HMA sponges showed a close relationship to each other and seem to be sponge-specific. In contrast, the bacterial community from the mesohyl of the LMA sponge species *Axinella polypoides* showed higher similarity to the bacterial community on its surface and to that on three other sponge species.

2 Zusammenfassung

Das langfristige Ziel der Gewinnung von bioaktiven Naturstoffen aus marinen Schwämmen und ihren assoziierten Mikroorganismen bildete die Motivation für die Untersuchung der bakteriellen Gemeinschaften von Schwämmen in der vorliegenden Arbeit.

Marine Schwämme gehören in der Naturstoffforschung zu den interessantesten Quellen neuer biologisch aktiver Naturstoffe. Ein kritisches Problem bei der Entwicklung neuer Arzneistoffe aus Naturstoffen mariner Schwämme, stellt dabei der Nachschub an Substanzen dar. Schon die für vor- und klinische Prüfungen benötigten geringen Substanzmengen können häufig nur unter großen Schwierigkeiten beschafft werden, da Wildbestände an Schwämmen diese in der Regel nicht nachhaltig liefern können. Nach einer erfolgreichen Zulassung als Arzneimittel steigt der Substanzbedarf gegebenenfalls noch drastisch. Die biotechnologische Kultivierung von wirkstoffproduzierenden Schwämmen unter kontrollierten Bedingungen stellt eine Möglichkeit dar, ausreichende Mengen an Sekundärmetaboliten, für die Untersuchung ihrer Wirksamkeit in Vor- und klinischen Studien und für die spätere Entwicklung neuer Arzneimittel, zur Verfügung zu stellen. Schwämme sind allerdings äusserst schwierig zu kultivieren. Für die Probleme bei der Etablierung einer dauerhaften Schwammkultivierung wurden zwei Hypothesen in Betracht gezogen: (1) ein Defizit in der Bereitstellung von ausreichenden Mengen an Nahrung für die Schwämme während ihrer Kultivierung oder (2) ein Verlust von für die Funktionalität der Schwämme möglicherweise essentiell wichtigen schwammassoziierten Bakterien.

Der Schwerpunkt des ersten Abschnitts dieser Arbeit lag auf Untersuchungen von Veränderungen in der bakteriellen Gemeinschaft, assoziiert mit dem Mesohyl der Schwammart *Aplysina aerophoba*, während der *ex-situ* Kultivierung unter verschiedenen Kultivierungsbedingungen in einem Zeitraum von 6 Monaten bzw. 76 Wochen mittels Denaturierender Gradienten Gel Elektrophorese (DGGE).

Die Kultivierungsbedingungen unterschieden sich hinsichtlich der Wassertemperatur, Belichtung und Fütterung der Schwämme. Die 16S rDNA der Amplifikate, die dominantes oder variables Vorkommen in den DGGE Gelen aufzeigten, wurden sequenziert. Die der Sequenzen entsprechenden Bakterien wurden durch Vergleich der Sequenzen mit einer Datenbank (Genbank) identifiziert. Zusätzlich erfolgte eine Analyse der Sekundärmetaboliten, die in *A. aerophoba* typischerweise vorkommen, um mögliche Veränderungen im Metabolitenmuster im Kultivierungszeitraum von 6 Monaten aufzuzeigen.

Die Populationsanalyse der schwammassoziierten Bakterien in *A. aerophoba* ergab, dass die Kultivierung der Schwämme zu keinen wesentlichen Verschiebungen innerhalb der bakteriellen Gemeinschaft führte. Im Gegenteil, trotz einer massiven

Degeneration der Schwammgeweben blieb die überwiegende Mehrheit der Bakterienarten im Gewebe erhalten. Die aus den DGGE Gelen erhaltenen 16S rDNA Sequenzen konnten zu vier unterschiedlichen Bakterienphyla zugeordnet werden: *Aktinobakterien*, *Cyanobakterien*, *Proteobakterien* und *Chloroflexi*.

Obwohl die bakterielle Diversität grösstenteils während der Kultivierung von *A. aerophoba* erhalten blieb, zeigte ein Cyanobakterium "*Aplysina aerophoba* clone TK09" ein variables Vorkommen. Die Variabilität im Auftreten dieses Bakteriums konnte auf unterschiedliche Belichtungsbedingungen während der *ex-situ* Kultivierung zurückgeführt werden. Die Analyse des Metabolitenmusters ergab, dass die Konzentrationen der bromierten Alkaloidverbindungen die typischerweise in *A. aerophoba* vorkommen wie Aeroplysinin-1, Aerophobin-1, Aerophobin-2 und Isofistularin-3 im Laufe der Kultivierung von sechs Monaten anstieg.

Aus diesen Ergebnissen resultiert, dass die Schwierigkeiten bei der dauerhaften Etablierung einer e*x-situ* Schwammkultivierung nicht an einem Verlust von für die Funktionalität der Schwämme wichtigen assoziierten Bakterien liegen. Das wesentliche Augenmerk bei der biotechnologischen Kultivierung von *A. aerophoba* sollte daher auf qualitativen und quantitativen Aspekten der abiotischen Kulturbedingungen und der Nährstoffversorgung liegen.

Aufgrund der zahlreichen und oft chemisch vermittelten Interaktionen, die zwischen den Mikroorganismen und ihren eukaryontischen Wirten und zwischen den Mikroorganismen untereinander auftreten, ist zu erwarten, dass epibiotische bakterielle Gemeinschaften auf Schwämmen eine besonders hohe Anzahl an wirkstoff-produzierenden Mikroorganismen aufweisen. Für die Erforschung der Schwämme und schwammassoziierten Mikroorganismen als Quellen neuer bioaktiver Naturstoffe schaffen Untersuchungen der bakteriellen Diversität auf der Oberfläche von Schwämmen eine Basis für das ökologische Verständnis der Wechselwirkungen zwischen Schwämmen und ihren assoziierten Bakterien und können den Zugang zu neuen bioaktiven Naturstoffen initiieren.

Der Schwerpunkt des zweiten Abschnitts dieser Arbeit lag auf Untersuchungen der bakteriellen Gemeinschaft auf der Oberfläche von verschiedenen Schwammarten im Vergleich mit der bakteriellen Gemeinschaft im Gewebe der Schwämme, sowie verglichen mit den Gemeinschaften auf anderen Referenzoberflächen und mit der im Meerwasser. Die DGGE Analyse der PCR-amplifizierten bakteriellen 16S rDNA der verschiedenen Oberflächen und Gewebeproben ergab, dass sich die bakteriellen Gemeinschaften auf der Oberfläche der Schwämme untereinander und im Vergleich zur der im Gewebe deutlich voneinander unterschieden. Des Weiteren unterschieden sich die bakteriellen Gemeinschaften der Schwämme deutlich von der der Referenzoberflächen oder der im Meerwasser. Zusätzlich zeigte die DGGE- und die Clusteranalyse klare Unterschiede in den bakteriellen Gemeinschaften aus dem Gewebe der bakterienreichen Schwämme im Vergleich zu der aus dem Gewebe der bakterienarmen Schwämme auf. In den

Gewebeproben der bakterienreichen Schwämme kamen, im Gegensatz zu allen anderen Proben, besonders GC-reiche 16S rDNA Fragmente vor. Zusätzlich ergab die Analyse der 16S rDNA Sequenzen, dass in den Gewebeproben der bakterienreichen Schwämme schwammassoziierte Bakterien dominant vorkommen. Die bakteriellen Gemeinschaften in den Gewebeproben der bakterienreichen Schwämme waren zueinander am ähnlichsten und scheinen schwammspezifisch zu sein. Im Gegensatz dazu, zeigte die bakterielle Gemeinschaft der Gewebeprobe der bakterienarmen Schwammart *Axinella polypoides* eine höhere Ähnlichkeit zu der bakteriellen Gemeinschaft auf seiner Oberfläche und zu der auf drei anderen Schwammarten.

3 Theoretical Background

3.1 Introduction

Natural products have a long history of use in the treatment and prevention of human diseases (Newman *et al.*, 2000). Traditionally most natural product-derived drugs were obtained from terrestrial plants. With the discovery of the unusual nucleosides spongothymidine and spongouridine in the marine sponge *Cryptotethya crypta* in the early 1950s (Bergmann and Feeney, 1950; Bergmann and Feeney, 1951) the potential of sea-derived natural compounds has been realized and the exploration of the marine environment has been initiated. These nucleosides were the basis for the synthesis of cytarabine (cytosine-arabinoside, Ara-C), the first marine sponge-derived anticancer agent and vidarabine (adenine-arabinoside, Ara-A) an antiviral drug (Newman and Cragg, 2004).

In contrast to the terrestrial environment where plants by far exceed animals with regard to the production of bioactive natural products, the majority of marine natural products currently in clinical trials or under pre-clinical evaluation is produced by invertebrates, such as sponges, tunicates, molluscs or bryozoans (Proksch *et al.*, 2002). Sponges in particular are considered one of the most prolific source of new bioactive compounds (Blunt *et al.*, 2010 and its preceding versions). Since 2007 marine micro-organisms are on the advance as a source of natural products (Blunt *et al.*, 2009). The fact that nearly 10% of all currently known biologically active natural products are of microbial origin is demonstrating the potential of microorganisms as an emerging source for bioactive compounds (Berdy, 2005; Penesyan *et al.*, 2010). The naturally occurring associations of sponges with microorganisms increase the value of sponges as a presumable highly prolific source of natural products.

The combination of predominantly low concentrations of the bioactive compounds and the low growth rates of sponges in the sea result in a very low accessibility to these bioactive compounds for pre- and clinical trials by isolation from sponges. Thus, there is increasing interest in biotechnological production of marine sponge biomass to grant access to sufficient amounts of sponge metabolites of interest. However, in many cases it remains unclear whether the actual producers of the natural products are the sponges or the sponge-associated microorganisms.

An e*x-situ* cultivation of sponges (with their associated bacteria) and extracting the metabolites of the whole sponge is one possible way to provide a sufficient amount of the metabolites of interest. This would grant access to the metabolites without former determination of the actual producer. Thus, the cultivation of sponges to accomplish the purpose of supply of sufficient amounts of natural compounds has to fulfill certain conditions. First, the cultivation should not lead to a depletion of the associated

microorganisms since the actual producer of the compounds remains unclear in many cases. Second, the cultivation should not lead to a decrease in the concentration of the secondary metabolites of interest.

A stable microbial community of sponges during *ex-situ* cultivation is not only important for the retention of possible microorganism-derived metabolites, but is also important under the aspect that sponge-bacteria associations could be of major importance for the physiology and metabolism of the sponge (Vacelet and Donadey, 1977). Sponges are difficult to keep *ex-situ* (Hausmann *et al.*, 2006; Osinga *et al.*, 1997) and no enduring sponge cultivation could has been established yet. Two hypotheses were considered to be responsible for the difficulties in the establishment of enduring sponge cultivation: (1) a deficiency in the supply of an adequate amount of food to the sponges, or (2) a possible loss of associated bacteria that are essentially required for the physiology and metabolism of the sponge. Thus, for the recovery of natural products from cultivated sponges a survey of the diversity and stability of the associated bacterial community is crucial. Furthermore, the investigation of the stability of the secondary metabolites spectrum is necessary.

Several studies have demonstrated that animated surfaces represent a rich environment of epibiotic microorganisms that produce bioactive compounds (Chelossi *et al.*, 2004; Kanagasabhapathy *et al.*, 2005; Penesyan *et al.*, 2009). From a biotechnological perspective, the production of natural compounds, as effective competition and defense strategies by surface-associated microorganisms constitutes an unparalleled reservoir for the discovery of novel bioactive and therapeutic agents, with applications across medical, industrial and environmental settings (Egan *et al.*, 2008). However, the diversity of the bacterial communities on animated surfaces and the vast biotechnological potential of marine epibiotic microorganisms remain mostly unexplored (Egan *et al.*, 2008; Penesyan *et al.*, 2010).

Due to the various and often chemically mediated interactions that occur between microorganisms and their eukaryotic hosts, e.g., sponges and between members of the epibiotic community of microorganisms on the surface (Armstrong *et al.*, 2001; Egan *et al.*, 2008), it is reasonable to predict that epibiotic communities obtain particularly high amounts of bioactive producing strains (Penesyan *et al.*, 2010). Antimicrobial activity of bacteria isolated from the surface of sponges has been described for various sponge species (Chelossi *et al.*, 2004; Kanagasabhapathy *et al.*, 2004; Kanagasabhapathy *et al.*, 2005; Thakur *et al.*, 2004b). Chemically mediated interactions and communication between the microorganisms and their sponge hosts are likely to have a significant impact on the composition and function of surface microbial consortia. Differences between surface bacterial communities on different sponge species to that on reference inanimate surface bacterial communities have already been reported (Dobretsov *et al.*, 2005; Lee and Qian, 2004; Lee *et al.*, 2006b). These studies suggest that highly integrated interactions occur between sponges and their surface bacteria as within the mesohyl of

sponges. However, no comparisons between surface- and mesohyl-associated bacterial communities have been performed to date. Qualitative analyses of bacterial populations associated with the surface of Mediterranean sponge species, for investigating the relationship between surface- and mesohyl-associated bacterial communities can provide a basis for the ecological understanding of sponge-microorganism associations and possibly establish access to bioactive compounds.

3.2 Aim of the Work

With the increasing need for novel drug discovery, new sources and/or more sophisticated methods that provide access to natural compounds of interest have to be explored.

This work investigated bacterial communities of sponges with the overall goal to use biotechnologically cultivated sponges and sponge-associated microorganisms for the supply of natural products. The following two aspects were the focus of this work: (1) the investigation of the stability of the bacterial communities and the metabolites concentration during a long-term cultivation of sponges and (2) the investigation of differences of the diversity of bacteria on the surface of sponges in comparison to those in the mesohyl to serve as basis for the ecological understanding of sponge-microorganism associations and possibly initiate access to new bioactive compounds.

(1) Investigation of the stability of the bacterial communities and the metabolites concentration during a long-term cultivation of sponges.

There is increasing interest in biotechnological production of marine sponge biomass to produce a sufficient amount of sponge metabolites of interest. However, sponges are difficult to keep e*x-situ* and there are two possible reasons that can be considered to be responsible for the difficulties in sponge cultivation: (1) a deficiency in the supply of an adequate amount of food to the sponges or (2) a possible loss of associated bacteria that are essential for the physiology and metabolism of the sponge.

Successive changes in the bacterial community and possible correlations to the health conditions of sponges have been investigated over a period of 6 months and 76 weeks, respectively of cultivation of *A. aerophoba* sponges under completley artificial conditions by the use of denaturing gradient gel electrophoresis (DGGE). Different cultivation experiments in terms of water temperature, feeding, and illumination were carried out. In addition, the investigation of changes in the secondary metabolite pattern during cultivation of the sponges occurred.

The aim of this part of the thesis was to verify whether the bacterial community and secondary metabolites of sponges are stable during long-term cultivation under completely artificial conditions. The assessment of whether the loss of essentially required associated bacteria is responsible for the difficulties in the establishment of enduring sponge cultivation was the focus of this section.

(2) Investigation of differences of the diversity of bacteria on the surface of sponges in comparison to those in the mesohyl.

The microbial communities associated with mesohyls of various sponge species have been well investigated (see reviews Hentschel *et al.*, 2006; Taylor *et al.*, 2007). In contrast, neither the ecology of bacterial communities on animated surfaces, e.g., of sponges, nor the chemical and other functional traits of surface-associated bacteria were fully explored or understood (Egan *et al.*, 2008). Due to the various and often chemically mediated interactions that occur between the microorganisms and their eukaryotic hosts and between the members of the epibiotic community, it is reasonable to expect that epibiotic communities obtain particularly high amounts of bioactive producing microorganisms (Egan *et al.*, 2008; Penesyan *et al.*, 2010).

Bacterial communities on the surfaces of different Mediterranean “high-microbial abundance” (HMA) and “low-microbial abundance” (LMA) sponges in relation to their tissue bacterial communities have been investigated by the use of DGGE. Bacterial communities from the surfaces of inanimate substrata, seawater as well as different animate surfaces served as references. Sequencing analysis of 16S rDNA fragments obtained from the DGGE gel occurred to indentify variable occuring bacteria.

The aim of this part of the thesis was to investigate the diversity of bacteria on the surface of sponges in comparison to those associated with the mesohyl, to provide a basis for the ecological understanding of sponge-microorganism associations and possibly initiate access to bioactive compounds.

3.3 Sponge Physiology and Biology

Sponges (phylum Porifera) are simple organized multicellular animals that are subdivided into three classes based on their spicule structure: Calcarea (calcium carbonate skeleton), Demospongiae (spongin and/or siliceous skeleton), and Hexactenellida (silica glass skeleton) (Bergquist, 1978). All currently known sponge taxa are aquatic and there are an estimated 15,000 species. The class of the Demospongiae is the largest group of sponges, accounting for 85% of the recent species (Hooper and Van Soest, 2002). Sponges can be found in every type of aquatic habitats, from fresh to brackish to marine waters, and from deep polar oceans to tropical reefs (Bergquist, 1978).

Sponges are soft-bodied marine invertebrates that lack obvious physical defenses, although calcareous and siliceous spicules may provide some physical defenses. Adult sponges are sedentary and are anchored to a substrate, such as a rock or another organism. They are active filter feeders and feed on microbial plankton and detritus, within a size range of 0.1 to 50 µm, as well as dissolved organic compounds (Bergquist, 1978; Ruppert and Barnes, 1994).

a b

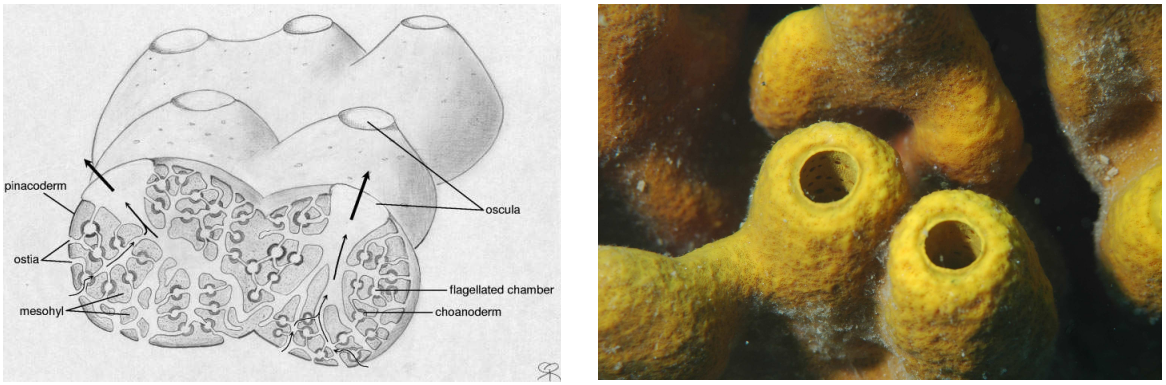

Figure 1a and b. Schematic (a) and photographic (b) representation of a sponge. Arrows in Figure 1a indicate the direction of water flow through the sponge (originally from Ruppert and Barnes, 1994, modified by Taylor *et al.*, 2007). Figure 1b shows a photograph of the sponge *Aplysina aerophoba* with pores on the surface and inside the osculi (photograph: A. Jaklin).

The unique body plan of sponges has been constructed around a system of water canals for efficient filtration of the normally nutrient-poor surrounding seawater. The water current enters the sponge body through numerous surface pores (ostia) located in the external body wall of the sponge, and leaves it through larger openings, so-termed osculi, mostly located at the top of the sponge body (Figure 1).

Water current is generated by planar beatings of flagella of specialized cells, so-called choanocytes (Figure 2), which are typically clustered in amounts of 50 to 100 cells in special parts of the canal system that are termed choanocytes chambers (Ruppert and Barnes, 1994).

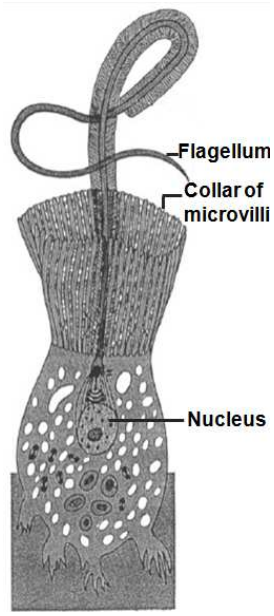

Figure 2. Schematic representation of a choanocyte (modified after Möhn, 1984).

The filter capacity of sponges is enormous. For different sponge species filtration rates from 0.044 to 0.221 mL water per mL sponge wet weight per s have been reported (Lynch and Philips, 2000; Reiswig, 1974). This corresponds for a sponge with a volume of 1 L to a filtration rate of seawater up to 19,000 L per day. Early studies of particle feeding in sponges indicated that as much as 96% of bacterial cells were removed from the inhalant seawater by the filtering activities of the sponge (Reiswig, 1971). These results were supported by the later application of more sophisticated techniques, in particular flow cytometry (Pile *et al.*, 1996; Pile *et al.*, 1997). The diet available may differ between sponges in different habitats, but generally

an unselective uptake of nutrient particles between 0.1 to 50 µm occurs. Large particles are directly phagocytized by sponge cells on the surface, smaller particles are taken up by sponge cells lining the canal walls (Bergquist, 1978), and even smaller particles down to bacterial size and below are trapped in the choanocytes chambers (Wehrl *et al.*, 2007) by a collar of microvilli that surrounds the flagellum of a choanocyte (Figure 2)

The food particles are passed on to amoeboid sponge cells so-termed archeocytes in which digestion then proceeds. Archeocytes probably act as storage centre for food reserves and it is generally assumed that the distribution of the nutrients to other cells of the organism occurs by mobile archeocytes. Furthermore, archeocytes are omnipotent and are capable of differentiating into any of the other sponge cell types (Ruppert and Barnes, 1994).

Sponges lack true tissues, such as muscles, conventional nerves and internal organs. Their anatomy is made up of different specialized cell types, organized in a relatively simple manner. The outer surfaces consist of pinacocytes (epithelial-like cells) which together assemble the pinacoderm. The inner matrix of sponges, the so-termed mesohyl, forms most of the sponge biomass. The mesohyl consists of different sponge cells embedded in a gelatinous proteinaceous matrix interlaced with skeletal material. Generally, all sponge cells are derived of the originally omnipotent archaeocytes that can differentiate to different sponge cells, such as contractile cells, to cells which produce skeletal material, or to cells with inclusions (Bergquist, 1978; Ruppert and Barnes, 1994).

The reproduction patterns of sponges can vary greatly, ranging from asexual reproduction by budding of body parts to sexual reproduction by fertilizing eggs. Many sponges are hermaphroditic usually producing eggs and sperms at different times to avoid self fertilizing. The release of clouds of sperm often occurs synchronic and is characteristic for most sponges. The fertilizing of eggs and the development to the larval stage mainly takes place within the body of the parental sponge. After release, the larvae of most sponges swim for a short period to find substrata suitable for settlement and metamorphosis to the adult sponge (Bergquist, 1978; Ruppert and Barnes, 1994).

3.4 Sponges, Natural Products and the Link to Biotechnology

Natural products have a long history of use in the treatment and prevention of human diseases (Newman *et al.*, 2000). Traditionally most natural products-derived drugs were obtained from terrestrial plants. The number of known compounds originating from a natural source exceeds the number of one million (Berdy, 2005). The majority (40 to 60%) of those compounds were derived from terrestrial plants. 20 to 25% of all known compounds from natural sources show bioactive properties (Berdy, 2005; Penesyan *et al.*, 2010).

The discovery of penicillin by Alexander Fleming in 1929 (Fleming, 1929) initiated the exploration of microorganisms as a source for natural products (Chin *et al.*, 2006; Tulp

and Bohlin, 2004). However, despite this relatively short history, nearly 10% of all currently known biologically active natural products are of microbial origin, demonstrating the potential of microorganisms as an emerging source for bioactive compounds (Berdy, 2005; Penesyan *et al.*, 2010).

More recently there has been a trend to explore marine environments on the search of new bioactive natural compounds (Tulp and Bohlin, 2004). The marine environment provides both biological and chemical diversity. Although marine species richness may only total 4% (estimated 500,000 marine species of estimated total 12,340,000 species) of global diversity (Benton, 2001), the diversity on higher taxonomical levels is higher in the marine environment than in the terrestrial (Benton, 2001; May and Godfrey, 1994). While 28 phyla of macroscopic organisms are terrestrial, 43 are marine. The contrast is higher on the class level. 90% of all known taxonomic classes are marine (Benton, 2001). Given that the majority of marine natural products currently in clinical trials or under pre-clinical evaluation is produced by marine invertebrates (Proksch *et al.*, 2002) this is demonstrating the potential of the marine environment as a source for bioactive compounds. Furthermore, compounds from marine environments often differ fundamentally from those from terrestrial sources. Unlike terrestrial organisms, marine organisms often produce halogenated secondary metabolites (Blunt *et al.*, 2010; Hay, 1996; Pauletti *et al.*, 2010). The majority of halogenated metabolites contain bromine and they are especially abundant in the marine environment, whereas terrestrial organisms preferably synthesize chlorinated compounds (Pauletti *et al.*, 2010). Until 2008 a total of 18,000 compounds have been discovered in marine organisms (Blunt *et al.*, 2008).

However, the majority of the annual marine natural product output every year is ascribed to sponges. They are responsible for more than 6,600 different natural compounds by now and every year hundreds of new compounds are being discovered in sponges (Blunt *et al.*, 2010 and its preceding versions). Figure 3 shows graphically the breakdown of discoveries of new marine natural products by phylum for the year 2001 (modified after Blunt *et al.*, 2003). Since 2007 marine microorganisms as a prolific marine source of natural products are on the advance (Blunt *et al.*, 2009). Therefore, the naturally occurring associations of microorganisms with sponges increase the value of sponges as a presumable highly prolific source of new bioactive natural products.

The compounds isolated from sponges show biological activity in various assays, such as anti-cancer related, anti-microbial, anti-oxidant, anti-viral, and anti-fouling assays to name a few (reviewed in Blunt *et al.*, 2005). The potential pharmaceutical application of these compounds led to an enormous interest in sponges in the last few decades. Historically the interest in the use of sponges has a long tradition and it has been reported that in ancient civilizations, such as in the Crete-Minoan culture (1900 to 1750 BC) sponges have been used for wall decorations. The use of bath sponges by Greeks and Romans was popular in the circum-Mediterranean area and the practice of using bath sponges spread out across Europe during Middle-Age and Renaissance (reviewed by

Pronzato and Manconi, 2008). Documents dating back to Hippocrates (460 to 377 BC) describe the application of sponges for the treatment of human diseases and injuries (Voultsiadou, 2007; Voultsiadou, 2010).

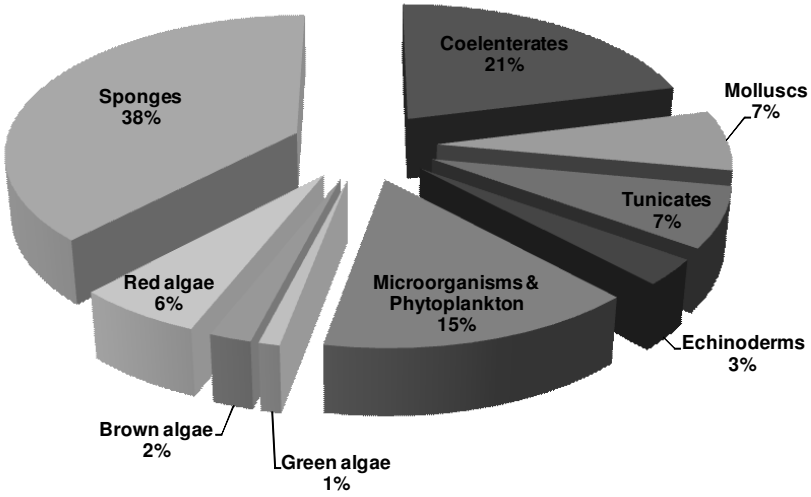

Figure 3. Distribution of marine natural products by phylum (modified after Blunt *et al.*, 2003).

A renewed pharmaceutical interest in sponges has started in the early 1950s by the discovery of the nucleosides spongothymidine and spongouridine in the marine sponge *Cryptotethya crypta* (Bergmann and Feeney, 1950; Bergmann and Feeney, 1951). Spongothymidine and spongouridine differ slightly from the nucleosides thymidine and uridine in that there is a hydroxyl (-OH, alcohol) group instead of a hydrogen (-H) in the sugar molecule. The sugars of thymidine and uradine are deoxyribose and ribose, respectively, where the sugar of spongothymidine and spongouridine is arabinose.

The nucleosides spongothymidine and spongouridine were the basis for the synthesis of cytarabine (cytosine-arabinoside, Ara-C), the first marine sponge-derived anticancer agent and vidarabine (adenine-arabinoside, Ara-A) an antiviral drug (Newman and Cragg, 2004). The structures of Ara-C and Ara-A are shown in Figure 4. Furthermore, after it was realized that biological systems would recognize the nucleoside base after modifications of the sugar moiety, chemists began to substitute the typical pentoses with acyclic entities or with substituted sugars, leading to the drug azidothymidine (AZT) a nucleoside analogue reverse transcriptase inhibitor used for the treatment of acquired immune deficiency syndrome/human immune deficiency virus (HIV/AIDS) or acyclovir, an antiviral drug (Laport *et al.*, 2009; Newman *et al.*, 2000).

The combination of predominantly low concentrations of the bioactive compounds and the low growth rates of sponges in the sea results in a very low accessibility to these bioactive compounds by isolation from sponges. The low accessibility of the natural compounds have often hampered investigations in pre- and clinical trials and the following establishment of this natural compound as a new drug.

a b

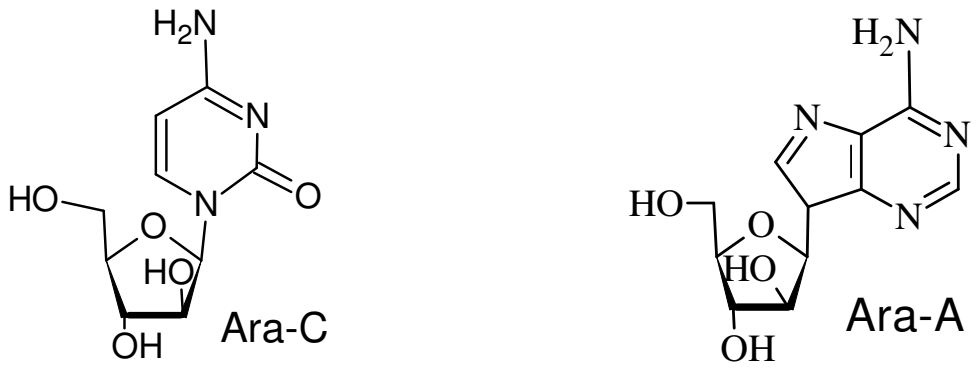

Figure 4a and b. Structures of the marine natural compounds Ara-C (a) and Ara-A (b).

For instance, halichondrin B (Figure 5a) originally isolated from the sponge *Halichondria okadai* by Hirata and Uemura (1986) showed exquisite anticancer activity and was highly prioritized for development as a novel anticancer therapeutic by the United States National Cancer Institute (NCI). For the supply of a sufficient amount (310 mg) of halichondrin B for initial preclinical trials, 1 t of the sponge species containing the highest halichondrin B concentration (400 µg/kg), *Lissodendoryx* sp., has been collected. It was estimated that after establishing halichondrin B as a pharmaceutical, an annual amount of 5 kg of this substance would be required. The estimated total biomass of *Lissodendoryx* sp. in the seas however amount to 300 t, thus, wild harvesting of the sponges cannot supply the demand of the compounds of interest (Munro *et al.*, 1999). The limited availability of halichondrin B has hampered the establishment of this natural compound as a new anticancer drug and encouraged efforts toward a chemical synthesis. The first total synthesis of halichondrin B has been reported by Aicher *et al.* (1992). However, total synthesis of halichondrin B requires no less than 100 chemical reactions (reviewed in Norcross and Paterson, 1995), but it is assumed that 30 steps is the maximum allowable for an economically feasible process (Sipkema *et al.*, 2005). However, in the case of halichondrin B it was possible to synthesize a structurally simpler analogue eribulin mesylate (E7389, Figure 5b), that retains the remarkable potency of the parent compound (Towle *et al.*, 2001; Zheng *et al.*, 2004). E7389 exert its effects via a tubulin depolymerizing antimitotic mechanism similar or identical to that reported for the parental halichondrin B.

On the website (http://clinicaltrials.gov) of the U.S. National Institutes of Health (NIH) currently 31 clinical trials with E7389 (Figure 6) were described. E7389 has already entered phase III clinical trial studies investigating the efficacy of this compound in metastatic breast cancer.

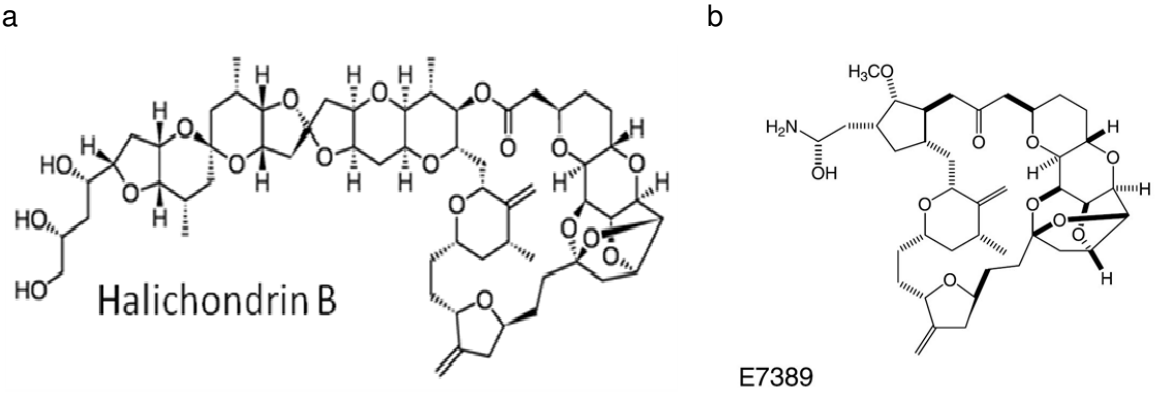

Figure 5a and b. Structures of the marine natural compounds halichondrin B (a) and its analogue E7389 (b).

Microtubules and microtubule-associated proteins are the major constituents of the mitotic spindle and thus, microtubules are extremely important in the process of mitosis and cell division (Jordan *et al.*, 1998). Drugs that affect the tubulin-microtubule equilibrium are effective anticancer drugs. The antimitotic agents act by targeting different sections, for instance, the colchicine, vinca or paclitaxel (taxane) binding sites of the tubulin heterodimer (Jordan and Wilson, 2004).

Whereas E7389 targets the vinca site of microtubules, another promising sponge-derived compound discodermolide that is being investigated in clinical trials targets the taxane sites. Discodermolide, a polyhydroxylated lactone was originally isolated from the marine sponge *Discodermia dissolute* (Gunasekera *et al.*, 1990). It was reported that discodermolide bound to microtubules more potently than Taxol™ (ter Haar *et al.*, 1996). Taxol™ (generic name paclitaxel) is a natural alkaloid isolated from the bark of a tree, *Taxus brevifolia* and is currently used to treat a variety of tumors, including ovarian, breast and non-small-cell lung cancers (Rodriguez-Antona, 2010). Discodermolide would have the advantage over paclitaxel that it also inhibits the growth of paclitaxel-resistant cells (Kowalski *et al.*, 1997). The limited availability of discodermolide also hampered the establishment of this natural compound as a new anticancer drug. In 2001 NCI awarded Kosan Biosciences, Inc. a grant to produce discodermolide by genetic engineering techniques (Newman and Cragg, 2004).

Other compounds originally isolated from marine sponges that entered clinical trials are the glycolipid KRN-7000 (cancer), the stereoids IPL-576092 (anti-asthmatic), IPL-512602 and IPL-550260 (both anti-inflammatory) and others are in the preclinical phase (Newman and Cragg, 2004). The only two sponge-derived pharmaceuticals that are actually on the market today are either products of microbial fermentation (Ara-A), or products of a facile chemical synthesis Ara-C (Sipkema *et al.*, 2005).

a b

Figure 6a and b. E7389, the analogue of halichondrin b (a) and the drug label (b) (source website of Developmental Therapeutics Program (DTP) of the NCI/NIH http://dtp.nci.nih.gov).

The limited availability of the natural compounds from the sponges led to other methods gaining attention in the last decade to obtain large quantities of sponge metabolites. However, before dwelling on the different possible ways to gain sponge-derived metabolites, it must be stressed that many bioactive natural products from marine sponges have striking similarities to the metabolites of their associated microorganisms (Proksch *et al.*, 2002), such as the phosphatase inhibitor okadaic acid originally assumed to be sponge-produced. Later it was shown that okadaic acid is produced by dinoflagellates of the genus *Prorocentrum* (Murakami *et al.*, 1982) and is now considered to be of dietary origin rather than a “true” sponge metabolite (Proksch *et al.*, 2002). Thus, metabolites extracted from sponge may actually not be produced by the sponges themselves, but by sponge-associated microorganisms, which are present in virtually all sponges. For production of these endosymbiotic metabolites, cultivation of the endosymbionts without their hosts may be easier than cultivation of the sponges (cells).

To gain access to sponge-produced metabolites of interest researchers have explored a range of possible ways:

1. Sponge cell culture.

The establishment of sponge cell culture with the aim of production of sponge-derived natural products has been problematic (Pomponi, 2006; Rinkevich, 1999). Sponge cell culture presents a unique purification challenge because, unlike higher metazoans, there are no areas of a sponge from which an aseptic inoculum can be obtained. To further complicate establishment of axenic cell cultures, many sponges host endosymbiotic microorganisms that may be released into an otherwise sterile culture if the

cells lyse (Pomponi, 2006). Even in cases primary cell cultures were obtained successfully, continuously dividing cell lines could not be maintained and sponge cell culture not be established yet (de Caralt *et al.*, 2007; Müller *et al.*, 1999).

2. Chemical synthesis of the metabolites or analogues.

Chemical synthesis is a useful method for providing sufficient quantities of compounds that might not be available in sufficient amounts from natural sources or through fermentation methods. However, chemical synthesis of natural products or analogues is in many cases no economically feasible alternative since the most interesting compounds are often structurally highly complex featuring numerous chiral centres (Proksch *et al.*, 2002; Sipkema *et al.*, 2005). For those compounds that are rich in centers of asymmetry, economically feasible strategies of chemical synthesis do not exist and are not likely to be developed in the foreseeable future (Proksch *et al.*, 2002).

3. Sea-based aquaculture: cultivation of sponges on designated areas in the sea.

Sponge aquaculture was first attempted in the Mediterranean during the 18th and 19th centuries for the production of bath sponges (Duckworth *et al.*, 1997). A new interest in aquaculture of sponges arose in the last decades, since it is one possible method that could supply sufficient and sustainable quantities of sponge metabolites for drug development. However, although some studies showed promising results in *in-situ* cultivation of sponges (Duckworth and Battershill, 2003; Müller, 1999), commercial sponge farming for production of metabolites has not established yet (Duckworth, 2009).

4. *Ex-situ* culture: cultivation of sponges under controlled conditions outside of the sea.

Ex-situ cultivation of sponges may be preferable to *in-situ* aquaculture, since it allows controlled cultivation conditions and the optimization of growth parameters, such as water temperature, oxygen demand, light levels and periods, food availability and the balance of the nutrients (Belarbi *et al.*, 2003; Osinga *et al.*, 1999b). *Ex-situ* cultivation of sponges can be performed in enclosed systems by using natural seawater or under completely artificial conditions by using artificial seawater (ASW). The cultivation of sponges under completely artificial conditions would allow a season- and location-independent continuous cultivation of sponges. In addition, as it stressed earlier, in many cases it is unclear whether the metabolites are produces by sponges themselves or by the associated microorganisms. A cultivation of the sponges (with their associated bacteria) would avoid the necessity of determining the actual producer of the metabolite of interest. Figure 7 shows the basins for the e*x-situ* sponge cultivation under artificial conditions at

the Institute of Process Engineering in Life Sciences, Section II: Technical Biology, at the Karlsruhe Institute of Technology (KIT).

The extraordinary capacity of sponges to regenerate after mechanical fragmentation or chemical dissociation of sponge cells (Wilson, 1907) allow the *ex-situ* sponge cultivation of sponge primmorphs, explants or whole sponges. Primmorphs are multicellular aggregates of the size of one to two mm obtained from dissociated sponge cell suspensions in which cell proliferation occurs and that can reorganize into a fully functional and structured sponge. Sponge explants are multicellular aggregates obtained from fragmented sponges in which cell proliferation occurs and that can reorganize into a fully functional and structured sponge.

However, sponges are difficult to keep e*x-situ* (Hausmann *et al.*, 2006; Osinga *et al.*, 1997). In their natural habitat, sponges are supplied with an almost unlimited amount of unfiltered seawater that provides food particles and removes waste products. In contrast, in a small enclosure, food sources will rapidly be depleted and waste products will accumulate (Osinga *et al.*, 1998). Furthermore, sponges are unselective filter feeders, their food consists of a mixture of living microorganisms, dead organic particles and dissolved organic matter (Osinga *et al.*, 1999b). Therefore, it may be difficult to create an artificial mixture of food particles that can cope with their nutritional demands. Thus, the question arose whether sponges can be grown on a microbial food source consisting of a single species (Osinga *et al.*, 1998). Furthermore, another worthwhile consideration is whether using a single species as a food source may create deficiencies in the sponges metabolism (Osinga *et al.*, 1999b) hampering growth of sponges or the production of natural compounds of interest. A study of Osinga *et al.* (2003) showed that sponges of the species *Pseudosuberites andrewski* fed with the diatom *Phaeodactylum tricornotum* can grow using a single microorganisms species as food source. However, it seems that not all feeds are equally adequate. Feeding the sponge species *P. andrewski* with the *Cyanobacteria Synechococcus* sp. and the microalgaes *Chlorella sorokiniana* and *Nannochloropsis* sp. a continuous growth could not be obtained (Osinga *et al.*, 2001b). It has been concluded in that study that qualitative aspects of feeding rather than quantitative aspects are the key to successful in vivo sponge culture.

A study from de Caralt (2003) also reveals a preference of the sponge *Corticium candelabrum* for to be fed with algae of the genus *Chlorella* sp. than with marine bacteria. The supply of an adequate food source is considered as one reason for the difficulty in the establishment of an enduring cultivation of sponges (Arndt, 1933; Osinga *et al.*, 1997; Osinga *et al.*, 1998; Osinga *et al.*, 1999b).

Furthermore, it has been assumed that sponge-bacteria associations are of major importance for the physiology and metabolism of sponges (Osinga *et al.*, 2001a; Vacelet and Donadey, 1977). Thus, another possible reason for the difficulty in cultivation of sponges is the displacement of essentially required bacteria. Although some studies showed promising results in cultivation of explants from different sponge species in closed

systems with natural or modified seawater (Hoffmann, 2003; Nickel and Brümmer, 2003; Osinga *et al.*, 1999a; Osinga *et al.*, 2003) and even in systems using artificial seawater (Hausmann *et al.*, 2006) an enduring cultivation of sponges for the production of natural compounds could not be established yet.

Figure 7. *Ex-situ* sponge cultivation basins at the Institute of Process Engineering in Life Sciences, Section II: Technical Biology, at the Karlsruhe Institute of Technology (KIT).

3.5 Chemical Defense in Sponges

Secondary metabolites in marine organisms, especially in sessile soft-bodied invertebrates, are often attributed a defensive function against environmental stress factors, such as predation, overgrowth by fouling organisms, competition for space, or ultraviolet radiation (Pawlik, 1993; Proksch, 1994). A protective function of sponge-derived metabolites against the invasion of pathogen bacteria after wounding the tissue of sponges of the genus *Aplysina* has been reported by Thoms *et al.* (2006). *Aplysina* sponges accumulate brominated isoxazoline alkaloids which include aerophobin-2, aplysinamisin-1 and isofistularin-3 as major constituents (Teeyapant *et al.*, 1993a; Teeyapant *et al.*, 1993b), presumably originating from 3,5-dibromotyrosine (Gopichand and Schmitz, 1979). The concentrations of the brominated compounds exceed 10% of the sponges dry weight (Teeyapant *et al.*, 1993a). X-ray microanalysis showed that these brominated metabolites are mainly stored in specialized sponge cells so-termed spherulous cells (Turon *et al.*, 2000).

Upon mechanical damage of cell compartments (e.g., wounding the sponge by predators) enzymatically catalyzed biotransformations of the brominated alkaloids aerophobin-2, aplysinamisin-1 and isofistularin-3 to the metabolites aeroplysinin-1 occur

(Figure 8), which in turn gives rise to the dienone verongiaquinol (Ebel *et al.*, 1997; Thoms *et al.*, 2006; Weiss *et al.*, 1996). Aeroplysinin-1 and verongiaquinol were shown to exhibit pronounced biological activities in various bioassays with marine organisms (bacteria, algae and molluscs) whereas their biogenetic precursors isofistularin-3 and aerophobin-2 were either inactive or exhibited only marginal activity (Weiss *et al.*, 1996).

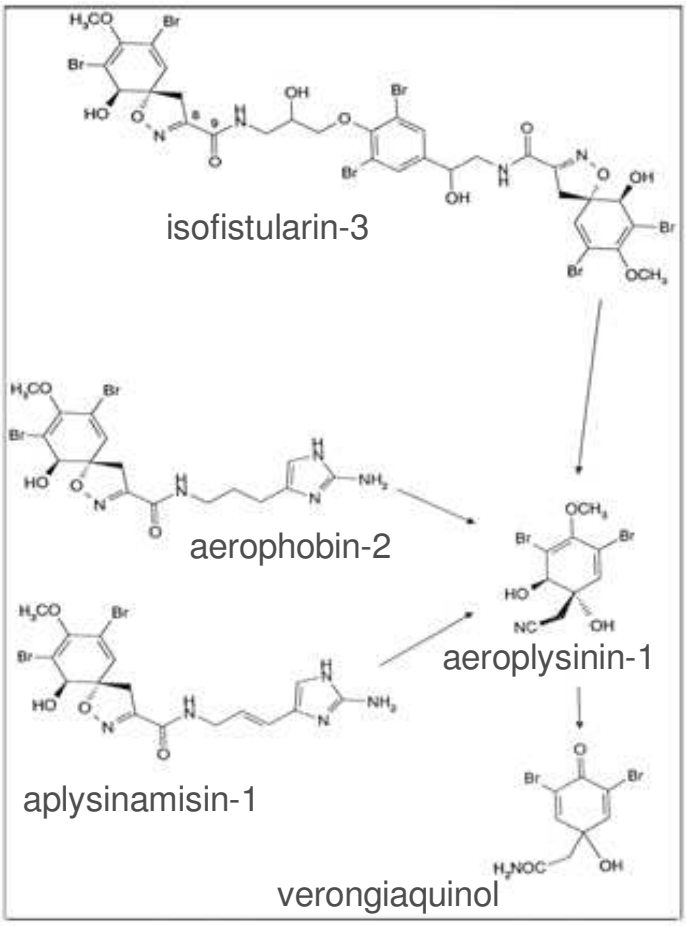

Figure 8. Wound induced bioconversion of brominated isoxazoline alkaloids in *Aplysina aerophoba* (modified after Ebel *et al.*, 1997).

3.6 Sponges and Mesohyl-associated Microorganisms

The evolution of the Metazoa from unicellular/colonial organisms occurred about 650 Ma ago in the pre-Ediacaran period (Morris, 1998) and marine sponges are widely considered to be the most primitive of the metazoans. The oldest isolated sponge spicules to date have been identified in thin-sectioned material from the Dengying Formation (Proterozoic: Sinian), “Shibantan” Member, Hubei Province (Steiner *et al.*, 1993) around 600 Ma ago. It is stated that sponge-bacteria symbioses have existed since the same period of time (Wilkinson, 1984). This would date such associations back to the Precambrian. Such long-term associations occurring between microorganisms and sponges can result in the evolution of adaptations and in a functional relationship between

them. Thus, sponges offer an excellent model to investigate invertebrate-microorganisms associations (Schmitt *et al.*, 2007a; Thakur and Muller, 2005) and in the past decade the number of studies in this field increased steadily (reviewed in Taylor *et al.*, 2007).

The question whether the associations between sponges and their microorganisms should be regarded as symbiosis has often been debated in the literature. The term “symbiosis” (from the Greek: σύν syn "with"; and βίωσις biosis "living") was originally coined by de Bary (1879) for the living together of two differently named organisms. In the association of two organisms the larger one is the host and it harbors the mutual partner - the symbiont, typically providing nourishment and shelter. Although the term symbiont is typically applied to mutualistic microorganisms, the term symbiosis is often used to include associates for which the full spectrum of effects on hosts is not known. However, the term symbiosis will be used hereafter in accordance with Hentschel *et al.* (2003) for an association between two or more organisms in which at least one organism benefits from the other. In contrast, commensally interactions are defined as interactions in which one organism simply uses the body of the other as a physical environment without any evidence of benefit or detriment. In pathogenic associations one partner benefits to the detriment of the other causing cell or tissue damage or even causes death of the host (Hentschel *et al.*, 2003; Steinert *et al.*, 2000). The exact nature of the sponge-bacteria associations remains unclear. Putative benefits of bacteria to their sponge hosts that have been proposed in the literature include involvement in the nutritional process, either directly by intracellular digestion or indirectly by translocation of metabolites in form of glycerol (Wilkinson, 1979) or glucose (Wilkinson, 1980). It has been also stated that associated microorganisms increases the sponges structural rigidity (Wilkinson *et al.*, 1981), or that bacteria participate in the host's chemical defense system against predators and biofouling by micro- and macroorganisms (Lee and Qian, 2003; Thakur *et al.*, 2003; Unson *et al.*, 1994).

In general, sponges are associated with diverse micro- and macroorganisms. As summarized by Taylor *et al.* (2007) sequences representing 16 bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Poribacteria*, *Proteobacteria (with the classes α-, β-, γ-,* and *δ-Proteobacteria*), *Spirochaetes*, *Verrucomicrobia*, *Lentisphaerae* and *Chlorobi*) and a new candidate phylum TM6, out of the 27 bacterial phyla described by Garrity *et al.* (2007) have been recovered from sponges. In addition, sequences of both major archaeal lineages (*Crenarchaeota* and *Euryarchaeota*) have been found associated with sponges (Taylor *et al.*, 2007). Furthermore, associations with diverse eukaryotic micro- and macroorganisms, for example, such as dinoflagellates, zoochlorellae (Frost and Williamson, 1980), macroalgae (Calcinai *et al.*, 2006), polychaetes (Magnino *et al.*, 1999) and other sponges (Wilcox *et al.*, 2002) have been described.

Early microscopic studies from (Vacelet and Donadey, 1977) revealed that sponges with dense tissues contain abundant, dense, and morphologically diverse microbial communities, while those with well-irrigated tissues contain few bacteria and typically only a single morphotype. These two groups of sponges that are distinguishable by the structure of their tissues and their associated bacteria were termed as “high-microbial abundance (HMA) sponges” and “low-microbial abundance (LMA) sponges” (Hentschel *et al.*, 2006). Table 1 shows two sponge species that are divided in HMA and LMA sponges. The concentrations of bacteria associated with LMA sponges reflect those of the ambient seawater. While typical seawater in the north-western Mediterranean Sea contains 5×10^5 cells per mL (Ribes *et al.*, 1999), the concentrations of associated microorganisms in HMA sponges exceed that of the surrounding seawater by two or three orders of magnitude (Hentschel *et al.*, 2003). Depending on the sponge species bacteria can amount 40% to 55% of the total volume of the sponge (Wilkinson, 1978). The estimated bacterial concentration of the sponge species *Aplysina aerophoba* amounts up to $6.4 \pm 4.6\times10^8$ bacteria g^{-1} of sponge tissue (Friedrich *et al.*, 2001). Similar bacterial amounts of 1.5×10^8 bacteria mL^{-1} to 8.3×10^9 bacteria mL^{-1} of sponge tissue within the mesohyl have been reported for the sponge species *Rhopaloeides odorabile* (Webster and Hill, 2001). Figure 9 shows transmission electron micrographs of a HMA sponge *Xestospongia muta* (a) that exhibits a high density of bacteria within the sponges mesohyl and in contrast a LMA sponge *Callyspongia vaginalis* (Figure 9b).

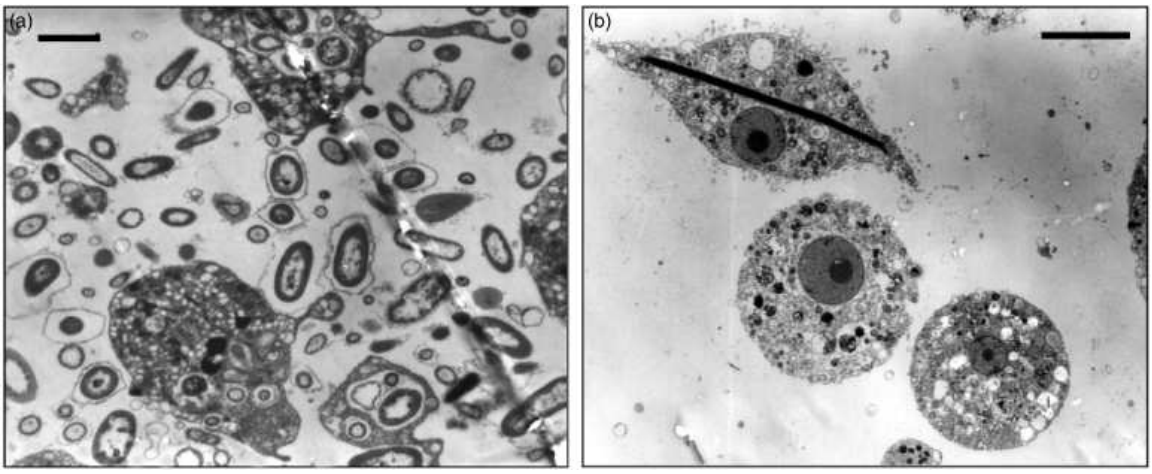

Figure 9a and b. Transmission electron micrographs of the high-microbial abundance sponge *Xestospongia muta* (a) and the low-microbial abundance sponge *Callyspongia vaginalis* (b). The size bars are 1.5 (a) and 5 mm (b), respectively (from Hentschel *et al.*, 2006).

The microorganisms are mainly found living free in the sponge mesohyl (Manz *et al.*, 2000; Vacelet and Donadey, 1977; Wilkinson, 1978), but some sponge species contain also intracellular bacteria within vacuoles of sponge cells (Vacelet and Donadey, 1977; Wilkinson, 1978). Bacteria in close association to choanocytes chambers and the

aquiferous system have also been described (Burlando *et al.*, 1988). Furthermore, bacteria were also observed within the nuclei of sponge cells (Friedrich *et al.*, 1999).

The hypothesis of a uniform microbial signature on phylum level associated with the mesohyl of phylogenetically and geographically distantly related sponges that is distinctly different from that of seawater as advanced by Hentschel and colleagues (2002) has been supported by Hill *et al.* (2006) or Lafi *et al.* (2005). Summarizing the data of Hentschel *et al.* (2002) and Hagstrom *et al.* (2002) it has been shown by Hentschel *et al.* (2006) that dominant groups of 16S rRNA gene sequences found in marine bacterioplankton belong to *α*- and *γ-Proteobacteria*. However, the most abundant 16S rRNA gene sequences in sponges are been affiliated with *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *γ-Proteobacteria*, and *δ-Proteobacteria*. In some cases, sponge-associated bacteria have been reported to be absent in environmental seawater (Fieseler *et al.*, 2004; Thiel *et al.*, 2007b; Wilkinson *et al.*, 1981). Thus, Taylor *et al.* (2004) discriminate between (1) specialist, (2) sponge-associated and (3) generalist bacteria of sponges. The clearly distinguishable composition of bacterial phylotypes in sponges in comparison to marine plankton or marine sediments indicates a highly integrated interaction between sponges and their microorganisms. However, the concept of a sponge-specific microbial community in different sponge species that is consistent in space and time is still debated.

Table 1. Bacterial density within the tissue of different sponge species.

Bacterial density	***Sponge species***	***Reference***
"High-microbial abundance sponges"	*Agelas oroides*	(Vacelet and Donadey, 1977)
	Chondrosia reniformis	(Schlaeppy *et al.*, 2010)
	Petrosia ficiformis	(Vacelet and Donadey, 1977)
	Geodia baretti	(Hoffmann *et al.*, 2005)
	Aplysina aerophoba	(Vacelet and Donadey, 1977)
	Ircinia wistarii	(Wilkinson, 1978)
Unknown bacterial density	*Tethya* sp.	
"Low-microbial abundance sponges"	*Chondrilla nucula*	(Thiel *et al.*, 2007a)
	Axinella polypoides	(Vacelet and Donadey, 1977)
	Crambe sp.	(Vacelet and Donadey, 1977)
	Dysidea avara	(Schlaeppy *et al.*, 2010)
	Oscarella lobularis	(Vacelet and Donadey, 1977)

Generally, there are three different strategies conceivable for sponges to obtain their associated (sponge-specific) bacterial community: (1) a vertical transmission of symbiotic bacteria between sponge generations via their embryos, (2) environmental (horizontal) acquisition, (3) the combination of vertical and horizontal symbiont transmission. The vertical transmission as a possible mechanism for the passage of

ancient bacteria between sponge generations has been proposed for different sponge species and their larvae (Enticknap *et al.*, 2006; Lee *et al.*, 2009; Schmitt *et al.*, 2007b; Sharp *et al.*, 2007). In contrast, Maldonaldo (2007) reported the occurrence of vertical transmission of microorganisms for some sponge species (*Haliclona caerulea*, *Corticium candelabrum*, *Chondrilla nucula*), but proposed an environmental acquisition of microorganisms for the sponge species *Petrosia ficiformis* by every new sponge generation. The environmental acquisition of microorganisms by sponges comprises the possibility that the sponge-associated microbial community appears to be sponge-specific, but that the specifity simply arises of the accumulation of rare seawater microorganisms by sponges filter activity. In fact these microorganisms can also be present in the ambient seawater, however at such low concentrations that the used methods failed to detect them. An interesting calculation by Hill (2004) supports the theory that the specifity of the sponge-associated bacterial community could arise by sponges filter capacity only. Assuming that a very rare bacterium is present in seawater at a concentration of only 1 cell ml^{-1} and a filtration capacity of a sponge of up to 19,000 L per day (Lynch and Philips, 2000; Reiswig, 1974), then the sponge could take in 1.9×10^{7} bacteria cells of these species per day (Hill, 2004). However, the combination of vertical and horizontal transmission as possible option for sponges to obtain their microorganisms has been proposed to occur in HMA sponges by Schmitt *et al.* (2008). However, the mechanisms of the (symbiont) bacterial acquisition actually occurring in sponges still remain unclear in many cases.

Generally, assuming that a sponge-specific microbial community in sponges exists that is different from that of the ambient seawater, it is unknown yet how sponges regulate the composition and density of the associated microbial community within the tissue. A study of Wilkinson *et al.* (1984) revealed that sponges can discriminate between symbiotic bacteria and other seawater-derived (food) bacteria. While seawater-derived tritium-labelled bacteria fed back to the sponge host were phagocytized by sponges cells, sponge-derived (assumable symbiotic) bacteria passed through the sponges undisturbed. In that study it has been stated that this failure by sponges to phagocytize and digest sponge symbionts results by a masking of the bacteria by capsular sheaths. A similar conclusion that sponges are able to differentiate between “food bacteria” and their own bacterial symbionts has been reported by Wehrl *et al.* (2007). The existence of slime layer and sheets on sponge-associated bacteria has been reported by Wilkinson (1978) and Friedrich *et al.* (1999).

Another conceivable survival strategy of sponge-associated bacteria could rely on a high growth rate of the bacteria compensating the loss through phagocytosis by the host. The hypothesis that animal-associated communities in contrast to free-living communities are composed of r-selected organisms (fast growing organisms) has been stated by Ley *et al.* (2008). Whatever the underlying mechanisms for the maintenance of sponge-microorganism associations are, it is apparent that in many cases such associations are highly stable and even resistant to external disturbance. The stability of

the sponge-associated bacterial community has been investigated in a *in-situ* study (Thoms *et al.*, 2003) or in the majority of cases in e*x-situ* studies (Friedrich *et al.*, 2001; Klöppel *et al.*, 2008; Mohamed *et al.*, 2008a; Webster *et al.*, 2008a). Thoms *et al.* (2003) investigated whether transplanting of the sponge species *A. cavernicola* from its natural habitat into a more shallower (light-exposed) habitat that normally conforms to the requirements of another sponge species of the same genus *A. aerophoba* leads to shifts in diversity and abundance of sponge-associated bacterial community such as, e.g., photosynthetically active bacteria. However, the results showed a permanent association of the microorganisms and host sponges.

More studies focused *ex-situ* investigations of the stability of sponge-associated microbial consortia and occurred either by maintaining the sponges in basins with filtered or untreated natural seawater or under completely artificial conditions by using artificial seawater (ASW). No shifts in diversity and abundance of the sponge-associated bacterial community were observable in the sponge species *A. aerophoba* maintaining them in recirculating seawater aquariums upon starvation or antibiotic exposure for 11 days (Friedrich *et al.*, 2001). Permanent sponge-microorganism associations were also reported by Hoffmann *et al.* (2006). In that study they investigated the microbial community of *Geodia baretti* in explants cultivated in open systems with natural seawater. Altogether, these studies indicate a highly stable microbial community in seawater cultivated sponges, which suggests a highly integrated sponge-bacteria interaction irrespective of the predominant conditions of the experiments or the degree of extrinsic disturbances. In contrast to this, Klöppel *et al.* (2008) reported varying *Cyanobacteria* content while maintaining *A. aerophoba* in aquaria using ASW depending on the cultivation condition. In that study, the *ex-situ* cultivation of *A. aerophoba* under light-exposed conditions led to an increase of the *Cyanobacteria* content. In contrast, keeping the sponges under cryptic–dark conditions led to a reorganization of the sponge tissue, accompanied by a decrease of the cyanobacterial content. A variable bacterial diversity in sponges of the species *Mycale laxissima* cultivated under artificial conditions was also reported by Mohammed *et al.* (2008a). In that study the dominance of *α*- and *γ*-*Proteobacteria* decreased and the *Bacteroidetes* group was found to be enriched. *Actinobacteria* were not detected in *M. laxissima* in aquaculture. A study by Webster *et al.* (2008a) revealed the impact of elevated seawater temperature to the composition of the sponge-associated microorganisms in *R. odorabile* and the health of the sponges during a short-term *ex-situ* cultivation experiment of 28 days. Keeping the sponges at a temperature from 27 °C to 31 °C showed no differences in the composition of the bacterial community or the health of the sponges as compared to controls. The composition of the bacterial 16S rRNA clone library of the sponges kept at 27 °C was mainly composed of *Proteobacteria*, *Actinobacteria*, *Nitrospira*, *Acidobacteria,* and *Chloroflexi*. In contrast, at a temperature of 33 °C, sponges showed major tissue necrosis in addition to a loss of symbionts and the establishment of alien bacterial populations including potential pathogens. On the phylum level the 33 °C 16S rRNA clone library contained mainly

sequences from the *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. Further works of Webster *et al.* (2008b) revealed differences in the bacterial compositions of diseased and healthy *A. aerophoba* sponges collected out of their natural habitat. In that study only diseased sponges were found to contain sequences belonging to the *ε-Proteobacteria* and *Firmicutes*. In addition, diseased sponges showed a greater number of *Bacteroidetes* sequences, whereas the healthy sponges were dominated by *Chloroflexi* and *γ-Proteobacteria* sequences. Altogether, these studies imply a generally stable sponge-associated bacterial community in healthy sponges. In contrast, unhealthy sponges show a different bacterial community in comparison to that of healthy sponges. Especially bacteria of the *Firmicutes* and *Bacteroidetes* groups seem to be dominant in unhealthy sponges. Furthermore, the dominance of *Cyanobacteria* seems to be dependant of the illumination conditions during the cultivation of sponges.

3.7 Sponges and Surface-associated Microorganisms

In aquatic environments, all submerged living or inanimate surfaces are exposed to and colonized by living organisms ranging from microorganisms to invertebrates. The process of biofouling is composed of four phases: (1) biochemical conditioning, (2) bacterial colonization, (3) unicellular and (4) multicellular eukaryont fouling. This stages can overlap, be successional, or occur in parallel (Dobretsov *et al.*, 2006; Wahl, 1989). Furthermore, it is stated that the initial biofilm composed of bacteria and diatoms can inhibit or induce settlement of subsequent biofoulers (Dobretsov *et al.*, 2006). However, the surfaces of many benthic plants and invertebrates remain relatively free of macrofoulers. The relatively clean surfaces of benthic macroorganisms suggest that they are a possible source of natural product antifoulants, produced either by the basi- or the epibionts.

In contrast to the well-investigated bacterial communities in the mesohyl of sponges, much less is known to date about the ecology of bacterial communities on the surface of sponges. Most of the studies dealing with sponges surface bacteria focus the antifouling activity either of extracts isolated from sponges (Kelly *et al.*, 2003; Limna Mol *et al.*, 2009) or of the bacteria themselves (Chelassi *et al.*, 2004; Kanagasabhapathy *et al.*, 2005). However, for the ability to elucidate a rich array of novel bioactive compounds from sponges, the ecological understanding of marine microbial diversity on the surface of sponges plays a fundamental role.

The culturable epibacterial community on *Ircinia fusca* has been investigated by Thakur *et al.* (2004b) to evaluate the influences of environmental changes and a seasonally variable surface bacterial community has been reported for this sponge species. On the other hand, Lee *et al.* (2006a) showed a geographically and seasonally consistent bacterial surface community on *Mycale adhaerens*. Furthermore, the surface bacterial community on *M. adhaerens* has been reported as clearly distinguishable from that of a reference inanimate surface bacterial community (Lee and Qian, 2004; Lee *et al.*,

2006a). These results concerning different surface bacterial communities between sponges and inanimated reference samples are supported by a study of Dobretsov *et al.* (2005) for three other sponge species: *Haliclona cymaeformis*, *Haliclona* sp. and *Callyspongia* sp.. Taken together these studies suggest the occurrence of highly integrated sponge-surface bacteria interactions comparable to that occurring with sponges and their mesohyl-associated bacteria.

3.8 Culture-dependent and Culture-independent Techniques to Investigate Microbial Communities

A major challenge in the field of marine microbial ecology is to assess the diversity of a microbial community present in a defined habitat. The overall goal is to understand how microbial populations are able to adapt to a range of environmental parameters (or limitations) and yet influence marine microbiological processes.

The existence of microorganisms was discovered by the use of microscopes during the period from 1665 to 1683 by two members of The Royal Society, Robert Hooke (1635–1702) and Antoni van Leeuwenhoek (1632–1723). In Micrographia (Hooke, 1665), Hooke an English physicist presented the first published depiction of a microorganism, the microfungus *Mucor*. Reading the preface of Hookes Micrographia the significance of the invention of microscopes for the investigation of this whole new microbial world becomes clear.

"*By the means of Telescopes, there is nothing so far distant but may be represented to our view; and by the help of Microscopes, there is nothing so small as to escape our inquiry; hence there is a new visible World discovered to the understanding. By this means the Heavens are open'd and a vast number of new Stars and new Motions, and new Productions appear in them, to which all the ancient Astronomers were utterly strangers. By this the Earth it self, which lyes so neer to us, under our feet, shews quite a new thing to us, and in every little particle of its matter, we now behold almost as great a variety of Creatures, as we were able before to reckon up in the whole Universe itself.*" (in the Preface of Micrographia, Hooke, 1665)

Later, Leeuwenhoek a Dutch tradesman from Delft, often titled as "Father of microscopy" observed and described microscopic protozoa and bacteria which he originally referred to as animalcules. Van Leeuwenhoek became an expert in constructing extremely simple microscopes that magnified objects from about 25-fold to 250-fold. After that time until the 20th century the diversity of bacterial communities has been investigated using classical microbiological methods only basing on isolating and culturing the microorganisms.

It is generally accepted that cultivation methods recover often less than 1% of the total microorganisms present in environmental samples. The estimated recovery rate for the culturable fraction of bacteria from seawater, for instance, amount 0.001 to 0.1% of

the total amount of the seawater bacteria (Amann *et al.*, 1995). Also only a minor fraction of sponge-associated bacteria was culturable yet. The estimated amount of culturable bacteria isolated from sponges ranged between 0.1 to 0.23% of the total bacterial community (Friedrich *et al.*, 2001; Webster and Hill, 2001). A higher bacterial recoverability of 3 to 11% of the sponge-associated bacteria of the sponge species *Ceratoporella nicholsoni* by the use of a culture-based method has been reported by Santavy *et al.* (1990).

To overcome the deficiency of the culture-dependent methods in the bacterial recoverability from environmental samples new DNA based techniques for bacterial population analyses of environmental samples have been established. These culture-independent methods allow the identification of single bacteria species in sample material without the cultivation of the organisms, but also are appropriate for bacterial population analyses (Amann *et al.*, 1995; Liu *et al.*, 1997; Muyzer *et al.*, 1993; Ward *et al.*, 1990). Most of the experiments which have been carried out in this field so far are based on ribosomal gene sequences, which are used as phylogenetic markers (Woese, 1987). Ribosomal gene sequences are present in all organisms and they contain both variable and highly conserved regions which allow to distinguish between organisms on all phylogenetic levels. In addition, deposited ribosomal gene data in databases (Benson *et al.*, 2005; Cole *et al.*, 2009) can be used to compare them with DNA-sequences of unknown microorganisms allowing phylogenetic identification.

One culture-independent approach is the fingerprint method denaturing gradient gel electrophoresis (DGGE) first applied for the analysis of whole bacterial communities by Muyzer *et al.* (1993). This method uses PCR-amplified gene fragments coding for ribosomal RNA (rRNA) derived of whole microbial communities to generate an “image” of the community.

The principle of the DGGE relies on the separation of PCR-amplified 16S ribosomal DNA (rDNA) fragments of identical length, but different sequences due to their different melting behavior in a gel system containing a gradient of denaturant agents of formamide and urea. As a result, a banding pattern is obtained, which reflects the complexity of the microbial community. The reliability of the technique is very high. All species present in the community that are over approximately 1% of the total population can be detected by DGGE analysis (Muyzer *et al.*, 1993). This percentage of the recoverability of the bacteria from environmental samples by the use of DGGE is much higher than by the use of culture-dependant techniques. Furthermore, the use of DGGE allows the simultaneous analysis of numerous samples. By excising individual DGGE bands from the gel, eluting and reamplifying the 16S rDNA out of them, it is possible to get sequence information of single community members (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998). However, phylogenetic analyses of sequences obtained from DGGE gels are limited (Muyzer and Smalla, 1998). Only short fragments of up to approximately 500 bp can be separated in the DGGE gel (Myers *et al.*, 1985). This is only one third of the

total length of the 16S rDNA, hampering a precise phylogenetic analysis of sequence information obtained from DGGE gels and allowing affiliation on the phylum level only. Furthermore, bands that are located on the same height represent the same melting behavior, but not necessarily the same sequence/bacteria. Jackson *et al.* (2000) reported that DGGE could always separate sequences differing by a single base pair, but multiple sequence differences were not so easily resolved. Two sequences that differed by 2 base pairs showed identical migration in DGGE gels and could not be separated in a mixed sample, In addition, co-migration of several different 16S rDNA sequences, which have the same melting behavior and therefore the same position in the gel, leads to overlapping DGGE bands which cannot be sequenced directly (Sekiguchi *et al.*, 2001). This requires the excision of bands and the separation of the reamplified 16S rDNA fragments in a second DGGE gel, or the validation of the pureness of the sequence by sequencing. To sum up, the DGGE is a very valuable method to describe complex bacteria population in an environmental sample, but has also its limitations. If samples show different banding patterns, then the bacterial communities occurring in the samples are undeniably different. However, if samples show a similar banding pattern, then they may or may not contain similar bacterial communities and further analyses, e.g., sequencing are needed (Jackson *et al.*, 2000).

4 Results und Discussion

4.1 Chapter 1 Artificial Cultivation Effects on Sponge-associated Bacteria

The morphological, bacterial and secondary metabolite changes of 16 sponges of the species *A. aerophoba* upon long-term cultivation under artificial conditions over a time period of six months have been investigated. These results of artificial cultivation effects on sponge-associated bacteria were summarized in chapter 1 (section 4.1.1). The sampling of the sponges out of their natural habitat occurred by Dr. Matthias Voigt and the cultivation of the sponges occurred by Dr. Sebastian Rühle (both from the Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Karlsruhe Institute of Technology (KIT), 76131 Karlsruhe, Germany). The analysis of the secondary metabolites occurred by Dr. Annika Putz (Institute of Pharmaceutical Biology and Biotechnology, Universitätsstraße 1, Heinrich-Heine University, 40225 Düsseldorf, Germany).

After six months of cultivation 12 out of the 16 specimens have been disintegrated entirely. The four remaining sponge specimens have been cultivated over a time period of 54 and 76 weeks, respectively. The results concerning morphological and bacterial changes of these four specimens were summarized in the second part of chapter 1 (section 4.1.2.1 and 4.1.2.2).

4.1.1 Manuscript 1: Morphological, Bacterial and Secondary Metabolite Changes of *Aplysina aerophoba* upon Long-term Maintenance

Morphological, Bacterial and Secondary Metabolite Changes of *Aplysina aerophoba* upon Long-term Maintenance under Artificial Conditions

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Abstract

The aim of this study was to analyze successional changes in the bacterial community over a period of six months of cultivation of *Aplysina aerophoba* sponges under different artificial cultivation conditions by use of denaturing gradient gel electrophoresis (DGGE). The cultivation conditions varied concerning the water temperature (20 ± 2°C and 25 ± 2°C) of the aquaria, additional illumination of one aquarium, and feeding of the sponges. Amplicons from DGGE separation of dominant colonizing or variably appearing bacteria were sequenced and aligned for taxonomical identification. In addition, secondary metabolites typically found in *A. aerophoba* were analyzed to investigate changes in the natural product profile during cultivation.

The cultivation of sponges under any given condition did not lead to a depletion of their bacterial community in the course of the experiment. On the contrary, the distinctive set of associated bacteria was maintained in spite of a dramatic loss of biomass and morphological degradation during the cultivation period. Generally, all sequences obtained from the DGGE gels were related to bacteria of five phyla: *Actinobacteria, Cyanobacteria, α-Proteobacteria, γ-Proteobacteria,* and *Chloroflexi.* Despite the overall stability of the bacterial community in *A. aerophoba*, an unambiguous variability was detected for the *Cyanobacteria* "*Aplysina aerophoba* clone TK09". This variability was ascribed to the predominant light conditions. The analysis of the metabolic pattern revealed that the concentration of a class of characteristic brominated compounds typically found in *A. aerophoba*, like aeroplysinin-1, aerophobin-1, aerophobin-2, and isofistularin-3, increased over the six months of cultivation.

Introduction

Sponges (Porifera) are among the most prolific sources of newly discovered natural products with bioactive properties. They are characterized as benthic, sessile, and active filter feeders, which pump large amounts of water through their aquiferous channel system for nutrition and oxygen supply. They feed on microbial plankton and detritus within a size range of 0.1-50 µm, which are filtered out of the seawater and phagocytized by archaeocytes (Bergquist, 1978; Ruppert and Barnes, 1994; Van Soest, 1996). A large fraction of bacteria live permanently associated within some sponges (Friedrich *et al.*, 2001) and possibly are resistant to phagocytosis (Wehrl *et al.*, 2007; Wilkinson *et al.*, 1984). The microorganisms may make up 40% of the total biomass of some sponges (Wilkinson, 1978). Based on the abundance of sponge-associated bacteria, sponges have been classified into two groups, “high-microbial abundance sponges” and “low-microbial abundance sponges” (Hentschel *et al.*, 2006). The concentration of bacteria associated with “low-microbial abundance sponges” reflects those of seawater. While typical seawater in the north-western Mediterranean Sea contains 5×10^5 cells mL^{-1} (Ribes *et al.*, 1999), the concentrations of associated microorganisms in “high-microbial abundance sponges” exceeds that of the surrounding seawater by two or three orders of magnitude (Friedrich *et al.*, 2001; Hentschel *et al.*, 2003; Hentschel *et al.*, 2006). Especially sponges of the family Aplysinidae are known to contain large amounts of bacteria embedded in the sponge tissues. The estimated bacterial concentration of *A. aerophoba* amounts to $6.4 \pm 4.6\times10^8$ bacteria g^{-1} of sponge tissue (Friedrich *et al.*, 2001). Currently, it is unknown why some sponges harbor an enriched intrinsic microbial community compared to that of environmental seawater, while others species seem to harbor a more transient bacteria community only from the sponges filter activity.

A study of Hentschel *et al.* (2006) reveals that dominant groups of 16S rRNA gene sequences found in marine bacterioplankton belong to α- and γ-*Proteobacteria*. However, the most abundant 16S rRNA gene sequences in sponges are be affiliated with *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *γ-Proteobacteria*, and *δ-Proteobacteria*. In some cases, sponge-associated bacteria have been reported to be absent in environmental seawater (Fieseler *et al.*, 2004; Thiel *et al.*, 2007b; Wilkinson *et al.*, 1981). Thus, Taylor and coworkers (2004) discriminate between (1) specialist, (2) sponge-associated, and (3) generalist bacteria. The clearly distinguishable composition of bacterial phylotypes in sponges in comparison to marine plankton or marine sediments indicates a highly integrated interaction, presumably with benefits for one or both partners. For instance, *Cyanobacteria* are known to provide their sponge hosts with glycerol (Wilkinson, 1979). However, many intrinsic aspects of sponge-bacteria ecology still remain unclear.

Since marine sponges are a very rich source of new marine secondary metabolites, they are in the focus of natural products research (Blunt *et al.*, 2003; 2004; 2005; 2006; 2007). Often, a huge commercial potential is assumed mainly for

pharmaceutical applications (Garson, 1994; Osinga *et al.*, 1998; Osinga *et al.*, 1999b). Most frequently, however, it is difficult to identify the true origin of these sponge-derived secondary metabolites. In some cases, associated microorganisms are assumed to be the actual producers of the respective secondary metabolites (Unson *et al.*, 1994). In other cases, metabolites were produced by sponge cells directly (Garson *et al.*, 1998; Turon *et al.*, 2000) or possibly by a metabolic interplay between bacteria and sponge cells. A recent overview of the evolution, ecology, and biotechnological potential of sponge-associated microorganisms was given by Taylor *et al.* (2007).

There is a considerable scientific and commercial interest in the cultivation of sponges due to the huge pharmaceutical potential of sponge-derived natural products. However, sponges are notoriously difficult to keep in aquaria, and most sponges lose biomass and eventually die (Hausmann *et al.*, 2006; Osinga *et al.*, 1998; Osinga *et al.*, 1999b). One possible reason for this observation could be the loss of essentially required symbiotic microorganisms. The stability and specificity of sponge-associated microorganisms was studied by Thoms *et al.* (2003), Friedrich *et al.* (2001), Hoffmann *et al.* (2006), Mohamed *et al.* (2008a), Webster *et al.* (2008a; 2008b) and Klöppel *et al.* (2008). Thoms *et al.* (2003) investigated the shifts in diversity and abundance of the bacterial community in a field trial by transplanting *A. cavernicola* from its natural habitat into a habitat that normally conforms to the requirements of *A. aerophoba*. The results showed a permanent association of the microorganisms and host sponges. Friedrich *et al.* (2001) reported that the microbial community of *A. aerophoba* maintained in re-circulating seawater aquariums could not be cleared upon starvation or antibiotic exposure for 11 days. Permanent sponge-microorganism associations were also reported by Hoffmann *et al.* (2006). They investigated the microbial community of *Geodia baretti* in explants cultivated in open systems with natural seawater. Altogether, these studies indicate a highly stable microbial community, which suggests a highly integrated sponge-bacteria interaction irrespective of the predominant conditions of the experiments or the degree of extrinsic disturbances. In contrast to this, Mohamed *et al.* (2008a) reported increased diversity of the bacterial community in *Mycale laxissima* under artificial cultivation conditions. Furthermore, a study by Webster *et al.* (2008a) recently revealed the impact of elevated seawater temperature to the composition of the sponge-associated microorganisms in *Rhopaloeides odorabile* and the health of the sponges during a short-term cultivation experiment of 28 days. Keeping the sponges at a temperature from 27°C to 31°C showed no differences in the composition of the bacterial community or the health of the sponge as compared to controls. In contrast, at a temperature of 33°C sponges showed major tissue necrosis in addition to a loss of symbionts and the establishment of alien bacterial populations including potential pathogens. Further works of Webster *et al.* (2008b) revealed differences in the bacterial compositions of diseased and healthy *A. aerophoba* sponges collected out of their natural habitat. A varying cyanobacterial content depending on the cultivation conditions of *A. aerophoba* was reported by Klöppel *et al.* (2008). In that study the *ex-situ* cultivation of *A. aerophoba* under light-exposed conditions

led to an increase of the cyanobacterial content. In contrast, keeping the sponges under cryptic-dark conditions led to a reorganisation of the sponge tissue, accompanied by a decrease of the cyanobacterial content. In the meantime they reported an increased metabolite concentration of *A. aerophoba* kept under both cultivation conditions. However, the contribution of 16S rRNA gene sequences in sponges that can be affiliated with *Cyanobacteria* amount up to 4% of the total percentage of bacterial sequences found in sponges (Hentschel *et al.*, 2006). Thus, in order to further examine the potential of *ex-situ* long-term cultivation of marine sponges, it is crucial to determine how other bacteria phyla and in relation to the microorganisms the secondary metabolite spectrum change upon culturing. Due to the well-investigated microbial diversity of members of the Aplysinidae family (Friedrich *et al.*, 1999; Friedrich *et al.*, 2001; Hentschel *et al.*, 2001; Hentschel *et al.*, 2002; Klöppel *et al.*, 2008; Thoms *et al.*, 2003; Webster *et al.*, 2008b) and the secondary metabolites profile (Ciminiello *et al.*, 1997; Ebel *et al.*, 1997; Teeyapant *et al.*, 1993a; Teeyapant *et al.*, 1993b; Thoms *et al.*, 2004), *A. aerophoba* was chosen as a model organism. Characteristically, *A. aerophoba* contains brominated compounds, such as aeroplysinin-1, aerophobin-1, aerophobin-2, and isofistularin-3.

The present study was aimed at investigating successive changes in the bacterial community and in the secondary metabolite spectrum, over a period of six months of cultivation of *A. aerophoba* sponges using artificial seawater (ASW). Different cultivation experiments in terms of water temperature, feeding, and illumination were carried out.

Material and Methods

Sponge Collection and Cultivation

Sixteen specimens of *A. aerophoba* were collected by scuba diving in the Limski channel near Rovinj (Croatia). The sponges were collected from depths of 5 to 15 m, removed carefully with their substrate, and individually labeled. The individual specimens were placed separately into plastic buckets to avoid contact with air and brought to the surface. Until transportation to the laboratory, the sponges were kept in 2000 L flow-through basins with natural seawater at the Institute ”Ruđer Bošković“ in Rovinj (Zavodnik, 1995).

Afterwards, the specimens were kept in six 96 L aquaria for 6 months using artificial seawater (ASW). For the preparation of the ASW, sea salt (Tropic marin, Dr. Biener GmbH, Wartenberg/Angersbach, Germany) adjusted to a salinity of 36.6 was used. Different biotic and abiotic cultivation parameters in terms of water temperature, feeding, and illumination were employed (Table 1). Four aquaria were kept at an ambient temperature of 20 ± 2°C and two at 25 ± 2°C. The sponges in one of the last-mentioned aquaria kept at a temperature of 25 ± 2°C were fed with two marine planktonic bacteria. The sponges in the remaining aquaria were kept without any additional feeding. Five mL ethanol per week was administered in three of the four aquaria running at the ambient

temperature of 20 ± 2°C. The addition of ethanol is a simple method routinely applied by reef aquarists to counterbalance the nitrogen and phosphate input from living microorganisms, as it serves as a carbon source for microorganisms, in order to achieve a balanced ratio of carbon to nitrogen and phosphate. Additionally, a daylight spot light (250 W, Gewiss GmbH, Merenberg, Germany) was placed over one of the four basin running at the ambient temperature of 20 ± 2°C. The other basin was exposed to natural daylight. Each aquarium system was equipped with a cooler (Titan 500, Aqua Medic, Bissendorf, Germany), an ultraviolet lamp sterilizer (55 W, Troptronic, Lüdenscheid, Germany), and an ozonized air flotation filter (Certizon Ozonisator, Sander Aquarientechnik, Uetze-Eltze, Germany) for the physico-chemical cleaning of the water.

Table 1. *Aplysina aerophoba* cultivation conditions.

Specimen	*Water temperature*	*Feeding/EtOH*	*Light*
49-52	20 ± 2°C	No feeding	-
53-56	25 ± 2°C	No feeding	-
57-60	25 ± 2°C	Feeding	-
10	20 ± 2°C	Approx. 5 mL EtOH / week	-
24	20 ± 2°C	Approx. 5 mL EtOH / week	- (+)
11 and 17	20 ± 2°C	Approx. 5 mL EtOH / week	+

The health status and morphological changes of the cultivated sponges were visually assessed at regular intervals at least once a week. The criteria to identify morphological changes during the cultivation of the sponges were i) changes of the color, e.g., surface discoloration from yellow-brown to black points, to highly necrotic tissue; ii) changes in the appearance of the surface, e.g., appearance of the spongin skeleton or overgrowth by epibiotic algae; iii) changes in the shape of the body, e.g., degeneration of the sponges as reflected by the formation of reduction bodies, so-called “buds”; iv) the condition and shape of the oscula. Open and closed oscula indicate the pumping activity of sponges and can therefore be used as an indicator of the health status of sponges.

Food Organisms

To investigate whether feeding of the sponges with live marine planktonic bacteria has any effect on sponge physiology, two strains, *Janibacter limosus* (Hel1), an *Actinobacterium*, and *Halomonas variabilis* (Hel4), *a γ-Proteobacterium*, (both received by courtesy from Dr. Irene Wagner-Döbler, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany), were regularly added to the respective basin. The bacteria were grown on ZoBell medium (containing 0.5% peptone, 0.1% yeast extract, 250 mL autoclaved deionized water, and 750 mL sterile filtered ASW at a final salinity of 3.5) in an incubation shaker (Multitron, Infors, Bottmingen, Germany) at 130 rpm and a temperature

of 20°C. Harvesting of the cells by centrifugation occurred every two days. The pellet was resuspended in 1 L ASW. The concentration of bacteria was measured using a cell counter (Casy TT, Schärfe System, Reutlingen, Germany) and adjusted daily to about 6.25×10^6 cells mL^{-1} in the basins.

Sampling of Tissue and Seawater and DNA Extraction

For the extraction of bacterial DNA, cube-shaped samples of the sponges tissue with the edge length of 0.5-1 cm were taken with an EtOH-sterilized scalpel blade, rinsed three times in sterile seawater and immediately frozen in liquid nitrogen. All samples were stored at -20°C. Within the period of six months, five tissue samples each were taken from twelve specimens of *A. aerophoba*. Sampling took place 2 weeks, 14 weeks, 15 weeks, 20 weeks, and 23 weeks after removal of the specimens from their natural habitat. To allow the sponges acclimatization the first sampling of the tissue occurred after one week of cultivation under artificial conditions. To investigate whether the removal or the ensuing transportation had caused changes in the bacterial community, samples were taken from two more *A. aerophoba* sponges directly after removal from the natural habitat on board the research vessel. The respective samples are termed “specimen 10, 0 hour” and “specimen 11, 0 hour”. Further sampling of these specimens took place 2 hours and 11 weeks later. In all DGGE gels, the reference samples used originated from the specimens 17 and 24, which were sampled 4 and 24 hours after harvesting, respectively. A second sampling of these reference sponges was carried out 12 weeks later. Sponge health was assessed visually during sampling.

For DNA extraction, the tissue was ground using a sterile mortar and pestle submerged in liquid nitrogen. Genomic DNA was extracted by using a commercial extraction kit (Fast DNA Spin Kit for soil, Qbiogene, Heidelberg, Germany) according to the manufacturer’s instructions and stored at -20°C.

Reference samples of seawater were taken at different sampling sites in and near the Limski canal near Rovinj. One seawater sample was taken at the place of origin of the sponges, the Limski canal (Lk09) and two were taken next to two offshore islands off Rovinj: Island Figarole (Fg01) and Island San Giovanni (Sg01) to investigate potential regional differences. An autoclaved, modified glass bottle (Duran, Schott AG, Mainz, Germany) was filled with 500 mL of seawater at the sampling site by SCUBA diving. The water was cooled on ice on the way back to the laboratory and immediately filtered through a membrane filter (ME 24/21 ST, ETO, 0.2 µm, Schleicher & Schuell, Whatman, Dassel, Germany). Fixation was performed with 60% ethanol. The filter was frozen in liquid nitrogen and stored at -20°C until further processing. For extraction of the DNA, the filter was placed in 1 mL sterilized deionized water. For all molecular analyses, DNAse/RNAse-free water (LiChroSolv®, Merck, Darmstadt, Germany) was used. Hot and cold alternating treatment was performed by putting the vial first into liquid nitrogen and

then into a water bath at a temperature of 50°C to release the DNA into the water. 10 µL of this undiluted solution were directly used for the PCR.

Denaturing Gradient Gel Electrophoresis (DGGE)

The universal primers 341F (5´-CCT ACG GGA GGC AGC AG-3´) with the GC-clamp (5´-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-5`) spanning *Escherichia coli* positions 341-357 and 518R (5´-ATT ACC GCG GCT GCT GG-3´) spanning *E.coli* positions 518-534 were used for amplification of the variable V3 region of the 16S rDNA of bacteria (Muyzer *et al.*, 1993). All primers used were purchased from MWG-BIOTECH AG (Ebersberg, Germany). PCR was performed using a thermocycler (Mastercycler, GeneAmp PCR-System 9700, Applied Biosystem, USA) as follows: One activation step for the polymerase (HotStar Taq^{TM}, Qiagen, Hilden, Germany) for 15:00 min at 95°C, 30 cycles of the initial denaturation for 1:00 min at 95°C, annealing for 1:00 min at 54°C, and elongation for 1:30 min at 72°C, followed by a final elongation step for 10:00 min at 72°C. The PCR mix contained 10 µL of 1:10 diluted DNA template, 1.25 U polymerase, 1 µL dNTP´s (0.2 mM final concentration per vial and dNTP) (GE Healthcare, Buckinghamshire, United Kingdom), 0.2 µL of each primer (20 pM final concentration per vial), and 5 µL of a reaction buffer (10x, Qiagen, Hilden, Germany), the total volume being 50 µL.

DGGE was performed on a 10% (w/v) polyacrylamide gel in 1x TAE running buffer (Tris 40 mM, acetic acid 20 mM, EDTA 1 mM, pH 8.5) with a 30% to 70% gradient of formamide and urea using a DGGE-DCode System (Bio-Rad, Munich, Germany). 100% denaturant solution corresponded to 7 M urea and 40% (v/v) formamide. Gradient optimization was performed using first 0% to 100% formamide and 30% to 70% urea. The conditions chosen for electrophoresis were 6 hours at 60°C and a voltage of 150 V. Gels were stained for 15 min in DNA dye solution (SYBR® Green, Sigma, Steinheim, Germany) and visualized using a gel documentation device (Lumi-Imager F1, Roche, Mannheim, Germany).

Sequencing and Taxonomical Affiliation

DNA bands were excised from the DGGE gels and stored over night in 40 µL water (LiChroSolv®, Merck, Darmstadt, Germany) at 4°C. 10 µL of the eluted product was used as a template for a second PCR with the primer pair GC 341F and 518R. After amplification, 15 µl of the PCR product were purified by 5 U of SAP (Sigma, Steinheim, Germany) and 2 U of Exo I (BioLabs, Ipswich, USA) with SAP-buffer (25 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) for 1 h at 37°C and 15 min at 75°C to remove primers and unincorporated dNTP´s. Sequencing of PCR products based on the Sanger reaction was performed using the reverse primer 518R and the BigDye Terminator Cycle Sequencing Ready Reaction Chemistry (Applied Biosystems, Foster City, USA). The

sequencing reaction started with a denaturing step at 96°C for 5 min, followed by 25 cycles of the initial denaturation for 10 s at 96°C, annealing at 50°C for 5 s, and was terminated with an extension reaction at 60°C for 1 min using a thermocycler (Mastercycler, GeneAmp PCR-System 9700, Applied Biosystem, USA). Subsequently, unincorporated dye terminators of the sequencing reaction were removed using the Dye Ex™ Spin Kit 2.0 (Qiagen, Hilden, Germany). Fragment separation and sequencing analysis were performed on the ABI PRISM® Genetic Analyser 310 (Applied Biosystem, Foster City, USA). The resulting sequences were compared to 16S rDNA gene sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using the Blast (Basic Local Alignment Search Tool) algorithm (Altschul *et al.*, 1990).

Cluster Analysis

To evaluate the DGGE banding patterns, the total numbers of bands per lane and the amount of common bands of pairwise lanes were counted. To count the bands, the Lumi-Imager software (Boehringer, Mannheim, Germany) was used, which automatically determines dark bands in a bright background using the intensity of contrast. Manual correction of the counting was performed whenever necessary to avoid the counts of accidental spots.

The similarity of the bacterial communities was visualized in dendrograms compiled by hierarchical cluster analysis (Clarke, 1993; Clarke and Warwick, 2001b) using the Primer E software (Version 5, Primer E Ltd, Plymouth, United Kingdom). Similarity based on common bands was calculated using the Bray-Curtis index. Cluster analysis was performed with complete linkage and without any standardization or transformation of the data. The similarity of samples is expressed in percent.

High performance Liquid Chromatography

The metabolite pattern of *A. aerophoba* was analyzed by high performance liquid chromatography (HPLC) as described by Thoms *et al.* (2003). Liquid nitrogen-frozen sponge tissue was lyophilised, ground with a mortar and extracted exhaustively over night with methanol (50 ml per 100 mg sponge tissue). After centrifugation 1.5 ml of the extract were transferred into 2 ml reaction tubes. Methanol was evaporated via vacuum centrifugation (Speedvac SPD111V Savant, Thermo Fisher Scientific GmbH, Dreieich, Germany). The dried samples were redissolved in 450 µl HPLC methanol and directly submitted to HPLC analysis.

Extracts were analyzed using an HPLC system coupled to a photodiode array detector (Dionex, Idstein, Germany; column prefilled with Eurospher C-18, 5 µm, 125×4 mm, Knauer, Berlin, Germany). Routine detection was at 254 and 280 nm. A solvent system consisting of 0.02% phosphoric acid at pH 2 (A) and methanol (B) at a gradient increasing linearly from 10% to 100% B within 25 min and hold for 10 min at

100 % B was used for compound separation. Compounds were identified by liquid chromatography-mass spectrometry (LC-MS) and by comparison of their retention times and mass spectra with those of authentic standards that had been isolated and fully spectroscopically identified before (Teeyapant *et al.*, 1993a; Teeyapant *et al.*, 1993b). All compounds were quantified by HPLC using calibration curves obtained for the respective isolated substances.

Results

Morphological Changes of the Sponges

The morphological appearance of the sponges at the beginning of the experiment corresponded to their natural habitus, as exemplarily shown by a photograph of specimen number 11 (Figure 1a). Following the six months of cultivation the individual sponges showed morphological changes to a greater or lesser extent depending on the cultivation conditions. The morphological changes included an irreversible discoloration of the tissues, from the natural color to black in some areas. The loss of biomass was considerable and the spongin skeleton was widely apparent. The sponge body showed deformations in the form of buds and no oscula were visible. Some sponges were overgrown by algae. The morphological degradation of the sponges is exemplarily shown in Figure 1b by means of a photograph of specimen number 11 after five month of cultivation kept at a water temperature of 20 ± 2°C and with an additional dosage of ethanol and illumination.

After six months of cultivation, the twelve sponges kept at a temperature of 25 ± 2°C or without additional ethanol dosage were entirely degraded. Interestingly, feeding had no positive effect on the health status of these sponges (data not shown). However, the four sponges cultivated in a temperature range of 20 ± 2°C with an additional ethanol dosage exhibited much less tissue degradation in the same time period as compared to the specimens kept at higher temperature or without additional dosage of ethanol. Among these sponges those kept with additional illumination showed the slowest degradation process.

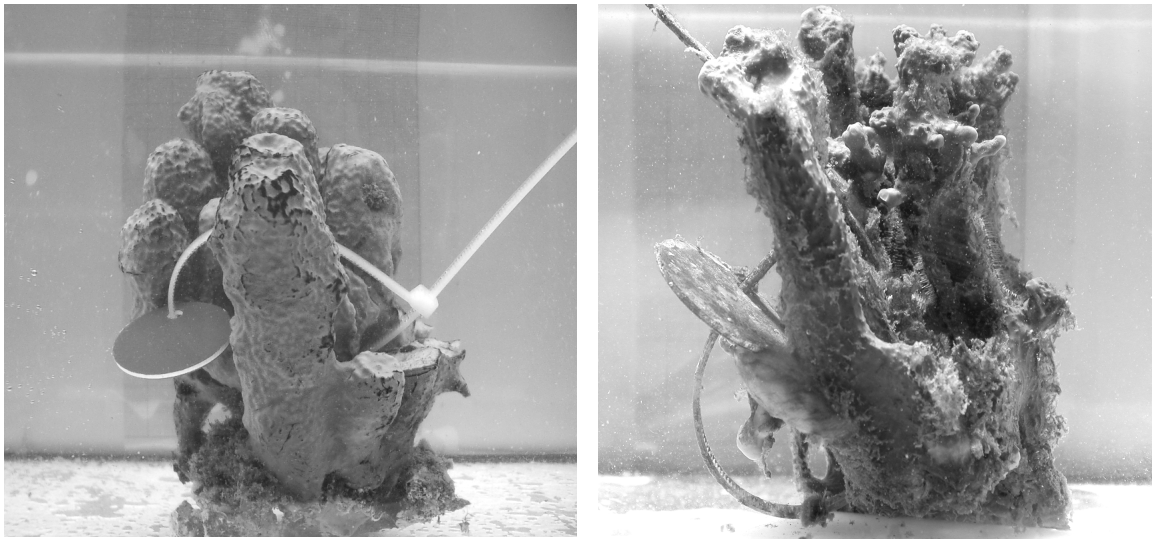
DGGE Analysis of Bacterial Communities

The results of DGGE analyses targeting bacteria in artificially long-term cultivated sponges are presented in Figures 2a to 2c. Since the DGGE analysis of the four individual sponges cultivated under the same conditions revealed very similar results, the DGGE gels are exemplarily presented for two of four specimens.

Altogether, the DGGE analyses resulted in a banding pattern composed of 16 to 27 bands per lane. The DGGE gel shown in Figure 2a comprises samples of the two specimens (numbers 49 and 50) cultivated at 20 ± 2°C without any feeding and additional

light. Lanes I to V of specimen 49 display 18 to 21 bands. Lanes VI to X of specimen 50 display 20 to 21 bands. An exception is lane X that displays 16 bands only. However, the DGGE analysis of the sponges kept at 20 ± 2°C and without additional feeding revealed a large number of permanent bands that remained unchanged throughout the experiment, as exemplified in Figure 2a.

a b

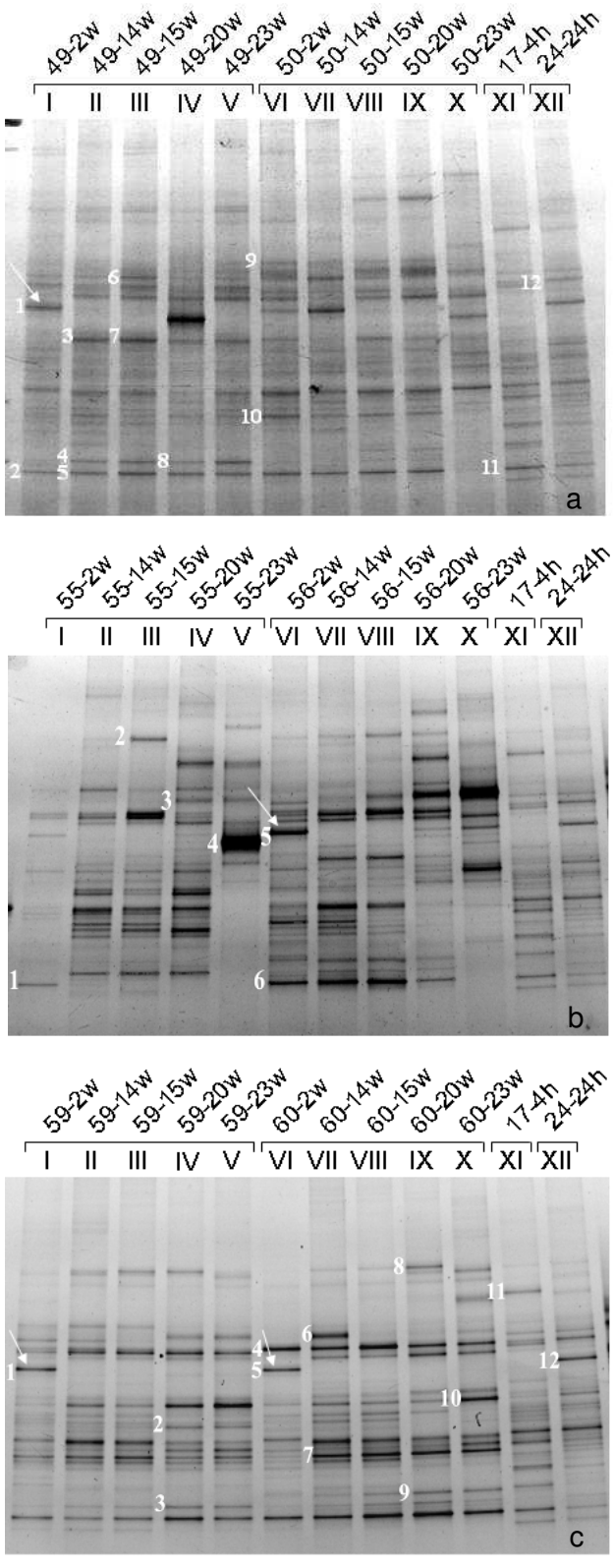


Figures 1a and b. Morphological changes of an individual *A. aerophoba* sponge following cultivation kept at a water temperature of 20 ± 2°C and with an additional dosage of ethanol and illumination. The appearance of the sponges in the beginning of the experiment showing their natural habitus is exemplarily shown by a photograph of specimen number 11 (a). Following cultivation the sponges showed characteristic morphological changes (b).

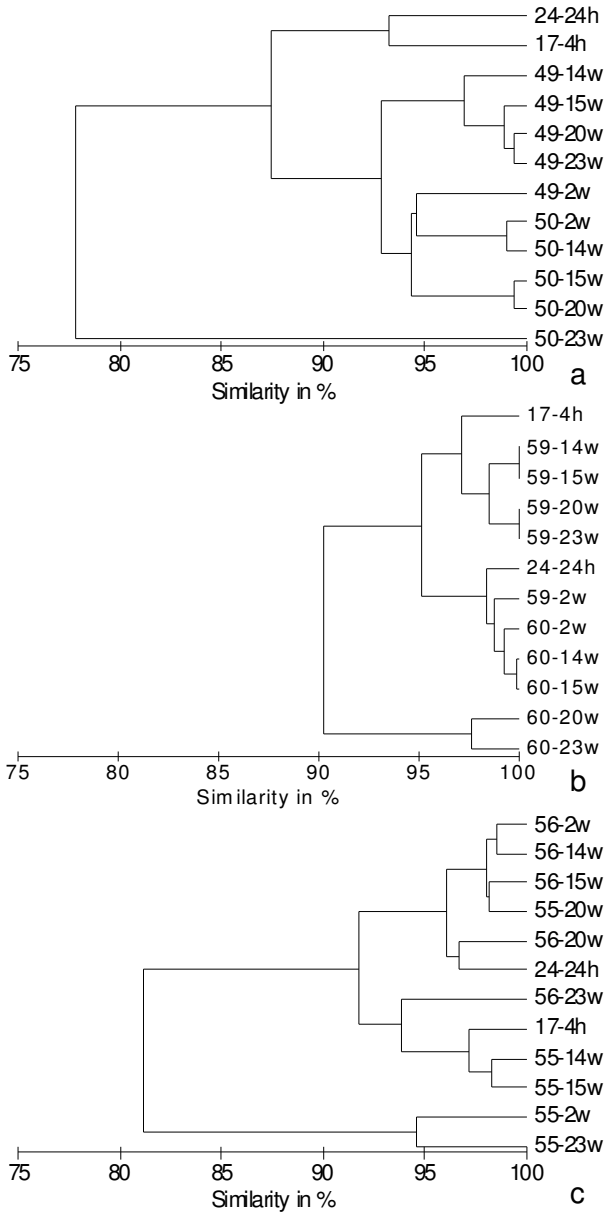
Method-dependent variations of the banding pattern between different DGGE runs of one sample become apparent when comparing reference samples (lanes XI and XII) in all DGGE gels (Figure 2). The DGGE analysis of the reference sponge number 17, lane XI, displays 21 to 23 bands in all three gels. Lane XII of sponge number 24 displays 22 to 25 bands. Thus, comparing the banding pattern of one sample separated in different DGGE gels, the method-dependent variation was two to three bands per lane.

By way of example, Figure 2b shows the DGGE analysis of one pair of sponges kept at 25 ± 2°C without feeding. Analysis was performed to assess possible influences of the water temperature on the bacterial community. Lanes I to V of specimen 55 display 16 to 25 bands. Lanes I and V reveal a lack of separation and weak bands.

Specimen 56 is represented in lanes VI to X with 23 to 27 bands. DGGE analysis of the sponges cultivated at a higher temperature revealed that the majority of bands remained unchanged throughout the experiment, as in the gel shown in Figure 2a.



Figures 2a, b, and c. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments from individual *A. aerophoba* specimens 49 and 50 (a), 55 and 56 (b), 59 and 60 (c) with the reference sponges 17 and 24 following cultivation under different conditions (Table 1). *Lane numbers I-V* comprise the banding pattern obtained for the sponge samples 49 (a), 55 (b), and 59 (c), *lane numbers VI-X* comprise the banding pattern obtained for the sponge samples 50 (a), 56 (b), and 60 (c) with different sampling times (*2w* 2 weeks, *14w* 14 weeks, *15w* 15 weeks, *23w* 23 weeks, *4h* 4 hours, and *24h* 24 hours after removal of the sponges from their habitat). *Lane numbers XI* and *XII* refer in all Figures (a-c) to the reference sponges 17 and 24. The Arabic numerals indicate DGGE bands, for which sequence information was obtained (Table 2). Variability in the occurrence of a single band in the DGGE analysis is marked by arrows in Figures 2.



Figures 3a, b, and c. Dendrograms of the cluster analysis showing the similarity of the DGGE banding patterns of the partial 16S rDNA sequences of sponge-associated microorganisms of the individual *A. aerophoba* specimens 49 and 50 (a), 55 and 56 (b), 59 and 60 (c), with the reference sponges 17 and 24 following cultivation under different conditions.

Figure 2c comprises the sponges kept at 25 ± 2°C with additional feeding. Lanes I to V refer to specimen 59, lanes V to X to specimen 60. The number of bands per lane was 22 to 25 and 20 to 26, respectively. DGGE analysis showed that the majority of bands are common to all samples. This reflects a bacterial community in different individuals of *A. aerophoba* sponges that stayed rather unchanged, independent of feeding conditions.

A main concern was whether the removal of the sponges from their habitat caused any changes in the bacterial community. Therefore, a DGGE analysis was performed with samples of freshly collected sponges. It is shown in lanes V to X of the gel in Figure 4. The effect of harvesting is obtained by comparing the banding patterns of these lanes. A number of 19 bands was obtained for specimen number 10 in lanes V to XII. Thus, no effect on bacterial community composition was apparent in this case. In lanes X to XIII

representing specimen 11, a slight decrease in the number of countable bands from 19 to 17 can be noticed. Lanes I to IV of the same gel comprise the reference samples of sponges 17 and 24 and show the same stability of the bacterial community as the previous results. Lanes XI to XIII exemplify the surrounding seawater banding pattern, with all displaying 10 bands. This DGGE analysis revealed that the bacteria represented by the dominant bands of the sponge samples are less prolific or absent in the seawater. The banding pattern of the seawater samples is clearly different from that of the sponge samples, but very similar compared to each other.

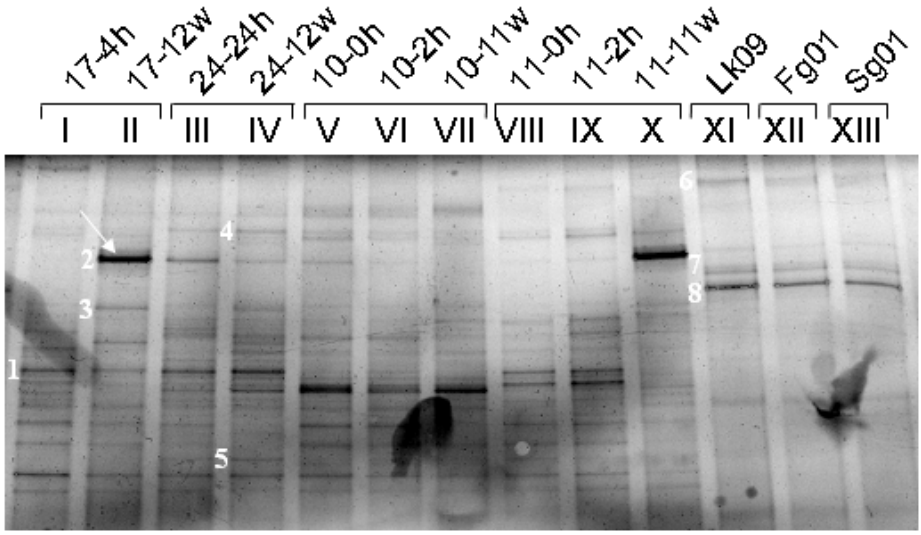


Figure 4. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments from individual *A. aerophoba* specimens 10, 11, 17, and 24 following cultivation under different conditions (Table 1) in comparison to samples of seawater from different places. *Lane numbers I-II* refer to the samples of sponge number 17, *lane numbers III-IV* refer to the samples of sponge 24, *lane numbers V-VII* refer to the samples of sponge 10, *lane numbers VIII-X* refer to the samples of sponge 11 with different sampling times (*4h* 4 hours, *12w* 12 weeks, *24h* 24 hours, *0h* 0 hours, *2h* 2 hours, *11w* 11 weeks after removal of the sponges from their habitat). *Lane number XI* refers to the banding pattern obtained from the seawater sampled in the Limski canal (Lk09), *lane number XII* refers to the banding pattern obtained from the seawater sampled near the Island Figarole (Fg01), *lane number XIII* refers to the banding pattern obtained from seawater sampled near the Island San Giovanni (Sg01). The Arabic numerals indicate DGGE bands, for which sequence information was obtained (Table 2). Variability in the occurrence of a single band in the DGGE analysis is marked by an arrow in Figure 4.

Comparison of the DGGE analyses of a given specimen reveals that the majority of bands are present throughout all lanes. The occurrence of these bands remains unchanged despite severe tissue degeneration of the respective sponge. Different water temperatures or feeding did not have any impact on the microbial composition in sponges. However, an unambiguous variability of the occurrence of a single band per lane in the DGGE analyses was observed in seven cases. This band is marked by arrows in Figures

2 and 4. The presence of this specific 16S rDNA band correlated with the light conditions of the culture tanks. If additional light was provided, the respective DNA band became more distinct. Otherwise, the band faded with time. Subsequent sequence analyses of the arrow-marked DNA band showed affiliation with the *Cyanobacteria* "*Aplysina aerophoba* clone TK09". As *Cyanobacteria* are photoautotrophs, dependence on the light conditions is explicable.

Cluster Analysis

The band profiles in Figure 2 were analyzed by cluster analysis to examine the relative similarities of bacterial communities of the aging sponges. All cluster analyses are illustrated by dendrograms (Figure 3). Each dendrogram comprises the result of the similarity analysis of the banding patterns of four specimens. Two of them are samples of specimens taken at different points in time, while the remaining two serve as references.

The dendrograms show a generally high overall similarity of at least 78% of the microbial communities of all examined samples. When the lanes with an insufficient separation of the bands are excluded, overall similarity increases to more than 87%. This also corresponds to the visual impression of the banding pattern in the DGGE gels.

Results of Sequencing the Most Dominant 16S rDNA Bands from DGGE Analyses

For further analysis of the microbial community the dominant 16S rDNA bands were sequenced for the determination of the taxonomical affiliation with the respective microorganisms. A total of 70 bands were excised from the DGGE gels for sequencing. Reliable bacterial 16s rDNA sequences were obtained from 38 of these bands. These sequences were used for analysis by database alignment. The sequences obtained from the rest of the bands showed either too many ambiguous nucleotides or the obtained sequences were too short to have meaningful affiliation with a bacterial phylum. The Arabic numerals in Figure 2 and Figure 4 indicate DGGE bands, for which sequence information was obtained. As expected, bands with the same position represent the same bacteria in all successful sequence analyses. An example is the band representing the bacterium "*Theonella swinhoei* clone JAWS6" (Figure 2a, bands 3 and 7). A summary of all the sequences obtained is given in Table 2. Generally, all sequences were related to bacteria of five phyla: *Actinobacteria, Cyanobacteria, α-Proteobacteria, γ-Proteobacteria,* and *Chloroflexi.* All 16S rDNA sequences obtained from the DGGE gel presented in Figure 2a could be assigned to sponge-associated bacteria (Table 2). Four of six sequences obtained from the DGGE gel presented in Figure 2b could be assigned to sponge-associated bacteria. The remaining two sequences showed high homology to an "uncultured *γ-Proteobacteria* clone UNHYB_07" originally isolated from marine bacterioplankton or to an "uncultured marine bacteria clone RS.Sph.012" originally isolated from the mucus of the coral *Fungia granulosa*. Ten out of the twelve sequences

obtained from the DGGE gel presented in Figure 2c could be assigned to sponge-associated bacteria. The remaining two sequences, "*Shewanella livingstonensis* clone SE86" and "uncultured bacteria clone P200-32", showed high homology to one arctic non-marine bacteria and one non-marine bacteria isolated from glacier ice. Generally, the most bacterial sequences obtained from the sponges kept under the different cultivation conditions could be assigned to sponge-associated bacteria.

Table 2. Denaturing gradient gel electrophoresis (DGGE) sequence analysis.

DGGE band	***Nearest sequence match in GenBank (BLAST)***	***Bacterial division***	***Homo-logy (%)***	***Accession Number***
Sequencing results referring to DGGE banding pattern of Figure 2a				
1	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	98,3	AJ347056
2	*Theonella swinhoei* clone RSWS12	*Actinobacteria*	98,3	AY434941
3	*Theonella swinhoei* clone JAWS6	*γ-Proteobacteria*	100	AF434983
4	*Theonella swinhoei* clone RSWS15	*Actinobacteria*	98,2	AF434943
5	*Theonella swinhoei* clone RSWS12	*Actinobacteria*	98,2	AY434941
6	*Aplysina aerophoba* clone TK57	*Chloroflexi*	98,2	AJ347069
7	*Theonella swinhoei* clone JAWS6	*γ-Proteobacteria*	95,0	AF434983
8	*Theonella swinhoei* clone RSWS15	*Actinobacteria*	98,3	AF434943
9	*Aplysina aerophoba* clone TK79	*Chloroflexi*	98,8	AJ347081
10	*Aplysina aerophoba* clone TK100	uncertain affiliation	97,8	AJ347071
11	*Theonella swinhoei* clone RSWS12	*Actinobacteria*	98,1	AY434941
12	*Aplysina aerophoba* clone TK79	*Chloroflexi*	100	AJ347081
Sequencing results referring to DGGE banding pattern of Figure 2b				
1	*Theonella swinhoei* clone RSWS12	*Actinobacteria*	97,9	AY434941
2	*Aplysina aerophoba* clone TK79	*Chloroflexi*	99,1	AJ347081
3	Uncultured *γ-Proteobacteria* clone UNHYB_07	*γ-Proteobacteria*	99,1	AJ630698
4	Uncultured marine bacteria clone RS.Sph.012	*α-Proteobacteria*	98,0	DQ097286
5	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	100	AJ347056
6	*Theonella swinhoei* clone RSWS12	*Actinobacteria*	96,4	AY434941
Sequencing results referring to DGGE banding pattern of Figure 2c				
1	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	100	AJ347056
2	*Theonella swinhoei* clone PAWS63	*Chloroflexi*	98,5	AF186450
3	*Theonella swinhoei* clone PAUC43f	uncertain affiliation	97,5	AF186415
4	*Aplysina aerophoba* clone TK79	*Chloroflexi*	100	AJ347081
5	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	100	AJ347056
6	*Shewanella livingstonensis* clone SE86	*γ-Proteobacteria*	98,0	AY771775
7	*Theonella swinhoei* clone JAWS4	*Chloroflexi*	96,5	AY897076

DGGE band	*Nearest sequence match in GenBank (BLAST)*	*Bacterial division*	*Homo-logy (%)*	*Accession Number*
Sequencing results referring to DGGE banding pattern of Figure 2c				
8	Uncultured bacteria clone DZ_H3	uncertain affiliation	97,0	AY702851
9	*Theonella swinhoei* clone RSWS27	*Actinobacteria*	94,5	AF434955
10	*Theonella swinhoei* clone JAWS6	*γ-Proteobacteria*	99,1	AF434983
11	Uncultured bacteria clone P200-32	uncertain affiliation	97,1	DQ076443
12	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	100	AJ347056
Sequencing results referring to DGGE banding pattern of Figure 4				
1	*Aplysina aerophoba* clone TK74	*Chloroflexi*	96,3	AJ347079
2	*Aplysina aerophoba* clone TK09	*Cyanobacteria*	100	AJ347056
3	Uncultured bacteria clone Cnuc17	uncertain affiliation	98,1	DQ079033
4	*Aplysina aerophoba* clone TK79	*Chloroflexi*	100	AJ347081
5	Uncultured bacteria clone Molly26PossActino	*Actinobacteria*	98,4	AY775500
6	Uncultured *γ-Proteobacteria* clone CONW15	*γ-Proteobacteria*	99,4	AY830050
7	Uncultured *α- Proteobacteria* clone FFW82	*α-Proteobacteria*	100	AY828394
8	Uncultured *α- Proteobacteria* clone CONW94	*α-Proteobacteria*	99,0	AY828397

Figure 4 presents the banding profile of the DGGE of sponge samples in contrast to seawater samples from different places. Four out of five sequences obtained from the sponge samples could be assigned to sponge-associated bacteria. The remaining one showed high homology to a non-marine *Actinobacteria* “uncultured bacteria clone Molly26PossActino”. Generally, two of the 16S rDNA sequences “*Aplysina aerophoba* clone TK09” and *“Aplysina aerophoba* clone TK79” were derived from all investigated sponges. The dominant bands in the DGGE gel of the seawater samples were all attributed to planktonic bacteria (Table 2).

Analysis of Metabolites

In order to investigate whether the long-term cultivation of *A. aerophoba* leads to changes in the metabolites pattern, the characteristic brominated alkaloids of this sponge species were analyzed by HPLC. This analysis revealed the typical main compounds aeroplysinin-1, aerophobin-1, aerophobin-2, and isofistularin-3 in all sponges.

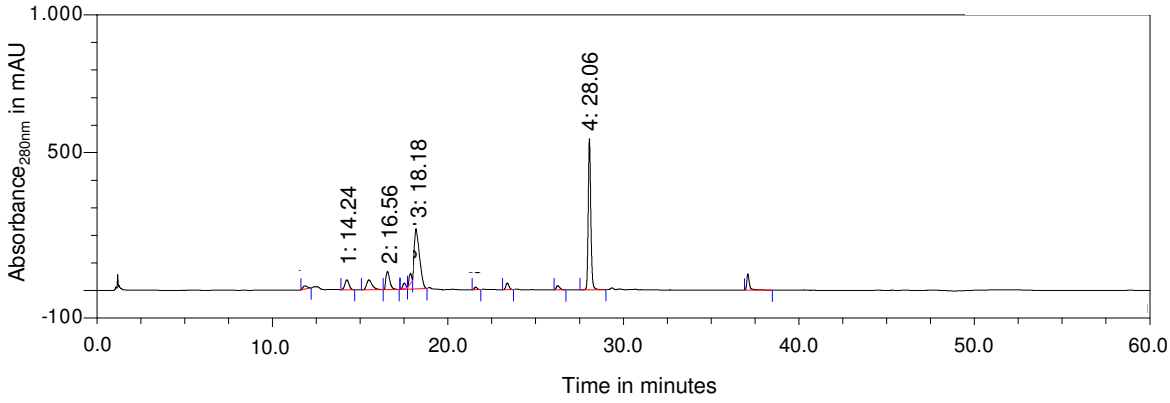


Figure 5. Representative high performance liquid chromatography (HPCL) chromatogram (UV 280 nm) of a methanolic extract of *A. aerophoba* showing the peaks of the four brominated alkaloids characteristic for *A. aerophoba*. Peak 1 refers to aeroplysinin-1, peak 2 to aerophobin-1, peak 3 to aerophobin-2, and, peak 4 to isofistularin-3.

Figure 5 shows a representative HPLC diagram. Following the retention times in minutes, peak 1 corresponds to aeroplysinin-1, peak 2 to aerophobin-1, peak 3 to aerophobin-2, and peak 4 to isofistularin-3. The signals of the latter two metabolites were dominant in all analyzed samples.

The concentrations of all four metabolites are summarized in Table 3. All analyzed samples contained aerophobin-2 and isofistularin-3 as major alkaloids and, thus, showed an alkaloid pattern characteristic of *A. aerophoba*. Generally, absolute concentration of the investigated metabolites increased. The mean value of aerophobin-2 in all analyzed samples increased in the course of the experiment from initially 120.01 ± 35.64 µmol g^{-1} dry matter (DM) to 197.35 ± 80.57 µmol g^{-1} DM. For isofistularin-3, an average concentration of initially 50.95 ± 12.99 µmol g^{-1} DM increased to 80.66 ± 22.59 µmol g^{-1} DM. The concentrations of the two minor components aerophobin-1 and aeroplysinin-1 were 29.85 ± 8.83 µmol g^{-1} DM and 24.17 ± 9.44 µmol g^{-1} DM, respectively. In the course of the experiment, these concentrations increased to 44.24 ± 17.36 µmol g^{-1} DM and 68.24 ± 35.14 µmol g^{-1} DM.

Table 3. Concentrations of metabolites during cultivation.

Concentrations of the metabolites during cultivation								
	Aerophobin-1 µmol g^{-1} DM		Aerophobin-2 µmol g^{-1} DM		Isofistularin-3 µmol g^{-1} DM		Aeroplysinin-1 µmol g^{-1} DM	
Specimen No.	2 weeks	23 weeks	2 weeks	23 weeks	2 weeks	23 weeks	2 weeks	23 weeks
49	29.03	21.08	94.80	26.03	44.85	39.00	9.75	17.48
50	24.30	29.78	110.78	144.98	46.20	61.65	29.33	32.63
51	27.83	39.15	95.78	162.90	36.15	64.95	24.68	103.35
52	36.45	28.80	164.03	138.98	63.98	66.60	19.95	69.38
mean	29.40 ± 5.11	29.70 ± 7.41	116.34 ± 32.62	118.22 ± 62.30	47.79 ± 11.67	58.05 ± 12.87	20.93 ± 8.38	55.71± 38.52
53	21.23	27.08	95.93	129.83	42.15	59.33	34.88	34.88
54	28.95	44.70	127.58	222.08	61.73	92.18	37.80	61.28
55	41.93	52.80	195.68	275.25	76.35	97.80	27.75	143.40
56	46.73	73.88	151.20	264.15	60.60	94.80	18.38	93.98
mean	34.71 ± 11.71	49.61 ± 19.42	142.59 ± 42.01	222.83 ± 66.10	60.21 ± 14.01	86.03 ± 17.95	29.70 ± 8.65	83.38 ± 46.75
57	21.53	48.15	100.50	242.18	45.83	102.98	33.30	91.28
58	29.85	56.18	129.45	293.85	55.58	111.83	18.83	65.03
59	34.13	73.13	106.73	284.03	47.48	102.60	27.30	49.20
60	16.28	36.15	67.65	183.98	30.53	74.25	8.10	57.08
mean	25.44 ± 8.05	53.40 ± 15.51	101.08 ± 25.53	251.01 ± 49.99	44.85 ± 10.46	97.91 ± 16.34	21.88 ± 10.94	65.64 ± 18.27
mean of specimens 49-60	29.85 ± 8.83	44.24 ± 17.36	120.01 ± 35.64	197.35 ± 80.57	50.95 ± 12.99	80.66 ± 22.59	24.17 ± 9.44	68.24 ± 35.14

Figure 6 summarizes the mean concentrations of the main brominated alkaloids of the 12 analyzed specimens in the beginning and at the end of the experiment. Despite a high standard variation due to the high individual variations of the absolute alkaloid contents, a clear increase of the respective concentrations is noticeable.

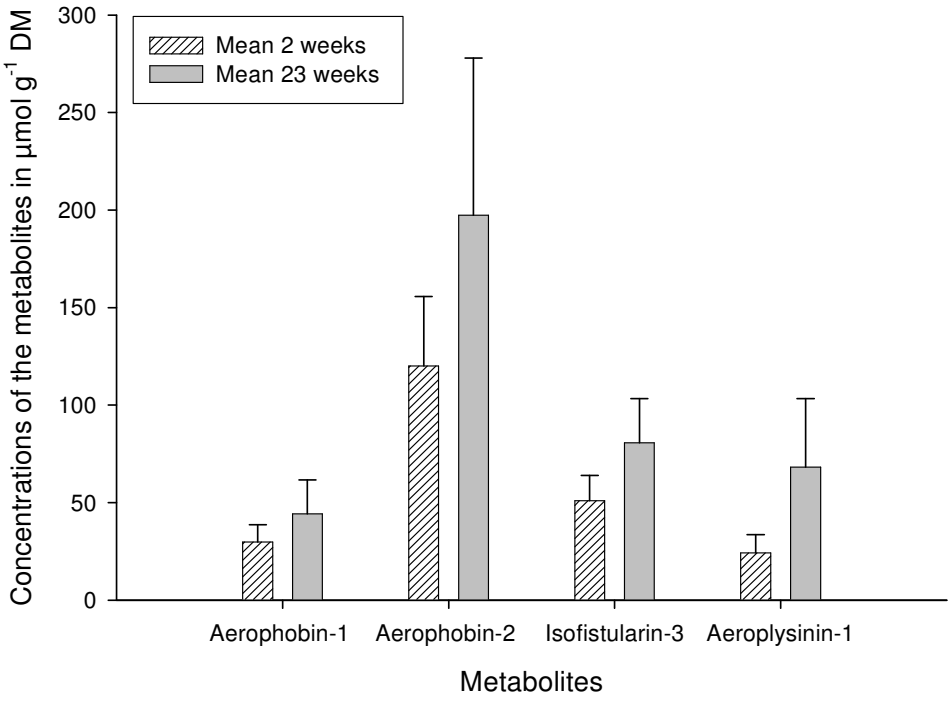


Figure 6. Concentrations changes of the brominated alkaloids characteristic for *A. aerophoba*. The shaded bars represent the mean concentrations of the respective metabolites in the beginning of the cultivation under artificial conditions, the gray-scale bars represent the average concentration in µmol g^{-1} dry matter (DM) of the respective metabolite at the end of the cultivation.

Discussion

Some sponges of the genus *Aplysina* are known to harbor a well-studied microbial association (Friedrich *et al.*, 1999; Hentschel *et al.*, 2001; Hentschel *et al.*, 2002; Thoms *et al.*, 2003). The association of sponges and microorganisms appears to be highly specific (Friedrich *et al.*, 2001; Hentschel *et al.*, 2002; Hill *et al.*, 2006; Thiel *et al.*, 2007a; Wilkinson *et al.*, 1981). A possible reason for the difficulty in cultivation of sponges is the displacement of essentially required bacteria. However, the principal conclusion drawn from the DGGE analysis is a highly stable microbial community despite the loss of host biomass. No adverse effect of any cultivation conditions on the bacterial community was found. The comparison of the banding profile of the reference samples used in every DGGE gel revealed that the separation of the same sample leads to a method-dependant variation of two to three bands in the total number between different gel runs. This reduces the possibility for a real comparison of the different gels obtained from the different cultivation conditions with each other. However, the banding profile of the different DGGE gels presented in Figure 2a-c revealed altogether a very similar banding pattern concerning the amount of the displayed bands. Indeed, none of the different cultivation conditions leads to a very apparent shift in the bacterial population. This points to a very stable bacterial community independent of the given cultivation condition. As the

sponges themselves displayed severe morphological changes in the course of time, they did not appear to perish as a result of the loss of essential bacterial symbionts. Friedrich *et al.* (2001), Hentschel *et al.* (2002), and Thoms *et al.* (2003) also investigated changes in the bacterial community of sponges of the genus *Aplysina* as a function of different study conditions. Altogether, these studies suggested a highly stable bacterial community in sponges of this genus. A very stable bacterial community in sponges of the species *G. baretti* were reported by Hoffmann *et al.* (2006). However, none of these studies was performed in an artificial seawater system with a laboratory cultivation setup. Interestingly, no successive changes in the microbial community were observed even without the continuous replenishment of the marine microorganisms. Cluster analysis also confirmed the stability of the given bacterial communities in *A. aerophoba*.

Generally, the majority of the sequencing results (30 out of the 35 sequences) obtained from the sponges kept under the different cultivation conditions belong to already described sponge-associated bacteria. The remaining five sequences obtained from the sponges show high homology to either two non sponge-associated marine bacteria or to three non-marine bacteria. The DGGE is a valuable method to describe complex bacteria population in an environmental sample. However, a limitation is that it displays only the most common abundant bacteria in a sample (Fromin *et al.*, 2002). From the DGGE analyses and the subsequent sequencing result the most dominant microorganisms in the investigated *A. aerophoba* sponges belong to bacteria phyla typically found in sponges. It is conceivable that the stability of this bacterial population in *A. aerophoba* arise from a highly evolutionary adapted association between the microorganisms and their hosts. A second limitation of the DGGE is that usually only short fragments with a length up to 500 bp can be separated (Myers *et al.*, 1985). This is only one third of the total length of the 16S rDNA, hampering a precise phylogenetic analysis of sequence information obtained from DGGE gels. Thus, the phylogenetic inference of the sequences derived from even shorter DGGE fragments is less refined. So far we cannot conclude whether the short sequence led to an improper affiliation with the three non-marine bacteria or if closely related bacteria are not described from marine samples yet. Short sequences derived from DGGE gels diminish precise phylogenetic analyses, but still allow a broad phylogenetic affiliation (Diez *et al.*, 2001). Sequence alignments of selected 16S rDNA amplicons from the DGGE gels resulted in five bacteria groups in cultured *A. aerophoba*: *Actinobacteria*, *Cyanobacteria*, *α-Proteobacteria*, *γ-Proteobacteria*, and *Chloroflexi*. This is in accordance with the findings of Hentschel *et al.* (2006), who reported that the most abundant bacterial phylotypes within the sponges are *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *α-*, *γ-*, and *δ-Proteobacteria*, and *Gemmatimonadetes*. The banding pattern of the seawater samples is clearly different from that of the sponge samples, but very similar compared to each other. The sequencing reveals that dominant groups of 16S rRNA gene sequences found in marine bacterioplankton belong to *α-* and *γ-Proteobacteria* in further accordance to Hentschel *et al.* (2006).

In contrast to the above mentioned findings concerning the stability of the microbial population of *A. aerophoba*, Mohamed *et al.* (2008a) reported that the dominance of α- and γ-*Proteobacteria* decreased and *Actinobacteria* were not detected in *Mycale laxissima* in aquaculture. The *Bacteroidetes* group was found to be enriched. In this study no variability in the occurrence of the α- and γ-*Proteobacteria* was observed. However, none of the sequences obtained from the DGGE gels were attributed to the phyla *Bacteroidetes*.

Webster *et al.* (2008a) report that in *R. odorabile* sponges the *Bacteroidetes* and Firmicutes where only present in specimens cultivated at 33°C. These specimens at the same time showed major necrosis in contrast to controls kept at 27°C. A shift in the bacterial community composition with the *Bacteroidetes* showing the greatest variability between healthy and diseased *A. aerophoba* sponges was reported by Webster *et al.* (2008b). Whether the dominant appearance of *Bacteroidetes* in these two studies has caused the disease process or whether the dominant appearance has been facilitated by the necrosis remains unclear.

As a conclusion, the changes in bacterial communities seem to be either sponge species-specific or associated with major tissue necrosis following temperature stress. When cultivating *A. aerophoba* under artificial conditions corresponding more to their natural habitat no changes in the bacterial community or massive tissue necrosis were noticeable. When examining the feasibility of growing sponges in aquaculture for the production of natural products in cases where microorganisms were involved in the producing process, it is important to monitor both morphological changes of the sponges and changes in bacterial communities.

The most considerable individual morphological changes of *A. aerophoba* during cultivation included the appearance of the spongin skeleton accompanied by a reduction of sponge biomass, sealing of the oscula and the formation of buds during cultivation. Little is known about the “budding” and triggers of this form of asexual reproduction. The formation of buds can be induced experimentally by injuring the freshwater sponge *Radiospongilla cerebellata* (Saller, 1990), thus, “budding” as a reaction of the sponge to stress is conceivable. The morphological changes of the sponges during cultivation such as the lost of biomass can only hardly be measured quantitatively, they are more of a qualitative nature. Generally, the sponges kept at 25 ± 2°C or without additional ethanol dosage showed the most rapid degeneration. The sponges kept at a lower water temperature with additional illumination and ethanol dosage exhibited much less tissue degradation at the same time. Morphological changes like the reduction of sponge tissue with the appearance of the spongin skeleton and the reduction of the oscula of *A. aerophoba* during cultivation were also described by Klöppel *et al.* (2008). Moreover, they reported on an increase of the cyanobacterial content in the light-exposed specimens. This is in accordance to the findings presented here. Despite the overall stability of the microbial community in *A. aerophoba*, an unambiguous variability was detected for the

Cyanobacteria "*Aplysina aerophoba* clone TK09". This variability was ascribed to the predominant light conditions. The sponges that showed an increasing thickness of the corresponding band of "*Aplysina aerophoba* clone TK09" appeared to maintain their original morphology for a longer period. Possibly, this is due to the known provision of nutrients by photoautotrophic *Cyanobacteria*. Thus, illumination seems to have a positive influence on the health conditions of sponges during cultivation. This is in accordance to the findings of Wilkinson and Vacelet (1979) who reported an enhanced growth of *A. aerophoba* sponges in light. Additionally the water temperature seems to have a large influence on the health of sponges as shown by Webster *et al.* (2008a). The extent to which the dosage of ethanol influences the health condition of sponges will have to be addressed in further studies.

Analysis of the characteristic brominated alkaloids of *A. aerophoba* revealed the typical natural product profile for these species (Ciminiello *et al*., 1997). Over a period of six months, under the given cultivation conditions, *A. aerophoba* specimens did not only maintain their typical main metabolites, aeroplysinin-1, aerophobin-1, aerophobin-2, and isofistularin-3, but, in contrast, the metabolite concentration seemed to increase during cultivation. In comparison to the very stable quantitative composition of the four secondary metabolites, the qualitative amounts showed a pronounced individual variability. This natural variability in *Aplysina* sponges aggravates investigations of artificially induced changes in the natural product profile, as large standard deviations are likely to obscure possible changes. On the individual level, however, an increase in the absolute concentrations of the different metabolites is clearly noticeable. The concentration range detected is in the same order of magnitude as published by Thoms *et al.* (2006) in fresh tissue of *A. aerophoba* collected at the Mediterranean coast of Rovinj. There was also an individual variability of the absolute alkaloid contents described. The concentration of aerophobin-2 in *A. aerophoba* collected at the Mediterranean coast of Banyuls-Sur-Mer, France is one order lower than it is reported for the specimen from Rovinj (Thoms *et al*., 2006). Even lower concentrations of about one to two orders of magnitude in *A. aerophoba* metabolites were reported by Klöppel *et al.* (2008).

The increase in the absolute concentrations of the different metabolites of *A. aerophoba* during the cultivation may be explained in two ways. First, the increase in the concentrations of the metabolites may be a reaction of the sponges to cultivation stress. Second, secondary metabolites were often reported to be localized in spherulous cells (Turon *et al*., 2000). Degradation of sponge tissue may possibly lead to an accumulation of these spherulous cells in the remaining tissue. However, no correlation between the occurrence of defined bacteria and the metabolites was found.

An encouraging result of this study, in view of natural products recovery, is the fact that the analyzed secondary metabolites concentrations increased over time. However, the cultivation of *Aplysina aerophoba* under completely artificial conditions resulted in tissue degradation of all individuals. Despite this degeneration, the overall bacteria

community was highly stable. Consequently, sponge viability cannot be correlated to the bacterial community and the diminishing of *A. aerophoba* cannot be attributed to changes in the microbial association.

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4.1.2 Supplementary results: Morphological and Bacterial Changes of *Aplysina aerophoba* upon Maintenance for 54 and 76 weeks

4.1.2.1 Morphological Changes of *Aplysina aerophoba*

In the study described in chapter 1 (section 4.1.1) concerning the cultivation of sponges under different artificial conditions 16 sponges of the species *A. aerophoba* (Specimen number 49–60, 10, 11, 17 and 24) have been investigated over a time period of six month. After six months of cultivation the specimens number 49 to 60 disintegrated entirely. In contrast, the specimens number 10, 11, 17, and 24 remained healthy for a much longer time. Thus, it was possible to investigate whether a prolonged cultivation of sponges for a time period of 54 and 76 weeks, respectively under artificial conditions led to changes of the sponge-associated communities. The cultivation conditions of these sponge specimens (10, 11, 17 and 24) remained same as described in section 4.1.1. They were all kept at a water temperature of 20 ± 2°C, and an additionally ethanol dosage of approximately 5 mL EtOH per week occurred. An additionally illumination occurred in the basin with the specimens 11 and 17 only. Comparing the health condition of the specimen number 10, 11, 17, and 24 with each other a different progress in the tissue degeneration in the course of time of cultivation was noticeable. The morphological appearance of the sponges at the beginning of the experiment corresponded to their natural habitus, as exemplarily shown by photographs of specimen number 17 and 24 in Figure 10a and Figure 11a. The morphological changes of cultivated sponges always showed the same characteristics. The morphological changes included an irreversible discoloration of the tissues, from the natural color to black in some areas. The loss of biomass was considerable and the spongin skeleton was widely apparent. The sponge body showed deformations in form of buds and no oscula were visible. Some sponges were overgrown by algae. However, the additionally illuminated sponges showed less tissue degradation compared at the same point of time, as exemplarily shown by photographs of specimen number 17 (illuminated) and 24 (without additional illumination) after 52 weeks of cultivation in Figure 10b and Figure 11b. Specimens number 10 and 24 showed a greater extend of the biomass loss in comparison to specimens number 11 and 17 at same point of time. After 54 weeks specimen number 10 and 24 disintegrated entirely and the cultivation of these sponges has been stopped. In contrast, the cultivation of specimens number 11 and 17 occurred for 76 weeks, as exemplarily shown by a photograph of specimen number 17 after 70 weeks of cultivation in Figure 11c. This result of the positive effect of illumination to *A. aerophoba* is is in accordance to the findings of Wilkinson and Vacelet (Wilkinson and Vacelet, 1979) who reported an enhanced growth of *A. aerophoba* sponges in light.

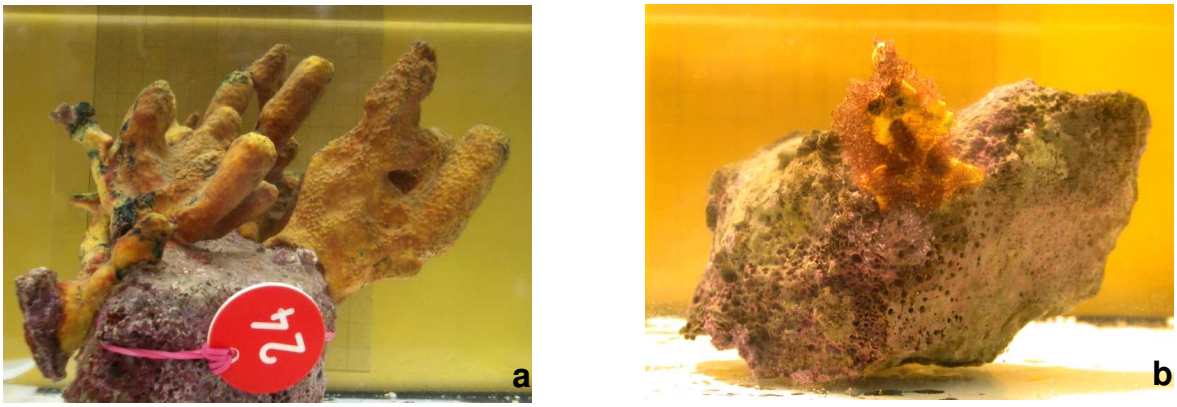

Figure 10a and b. *Aplysina aerophoba* specimen number 24 at the beginning of the experiment (a) and after 52 weeks of cultivation (b).

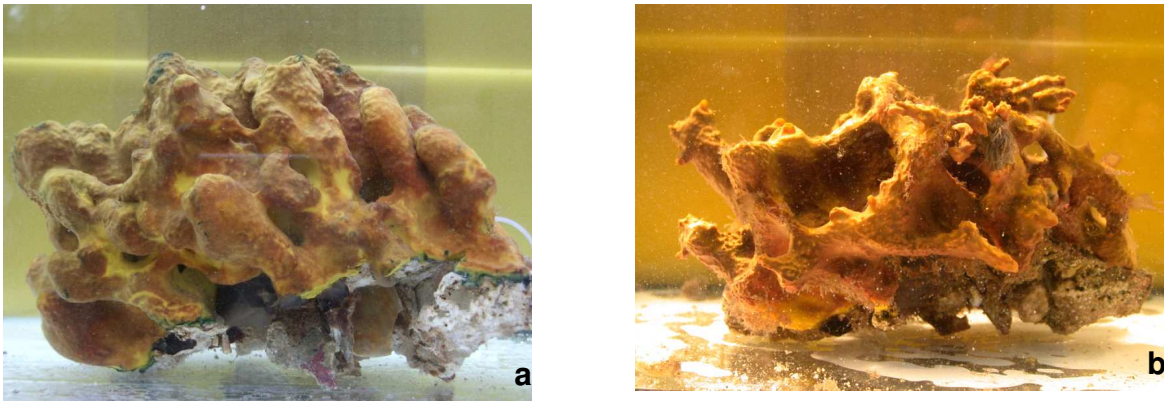

Figure 11a-c. *Aplysina aerophoba* specimen number 17 at the beginning of the experiment (a), after 52 weeks (b) and after 70 weeks (c) of cultivation.

4.1.2.2 Bacterial Changes of *Aplysina aerophoba*

The analysis of the bacterial community of cultivated sponges under artificial conditions over a time period of six months revealed a highly stable microbial community despite the loss of host biomass. No adverse effects of any cultivation conditions on the bacterial community were found (section 4.1.1). A main concern was whether cultivation over longer period of time led to changes of the sponge-associated bacterial community as reported by Mohamed *et al.* (2008b) for the sponge species *Mycale laxissima*. However, long-term cultivation of *A. aerophoba* over a time period of 52 and 76 weeks, respectively revealed similar results concerning the bacterial stability as previously presented (section 4.1.1). The analysis of the bacterial community by the use of DGGE of the sponge species *A. aerophoba* for a time period of 76 weeks (Figure 12) revealed no changes in the diversity of the sponge-associated bacteria. Comparing the samples of one specimen to different samples times a high similarity of the banding pattern is obvious. The majority of bands obtained from freshly collected specimens (lanes I, IV, VIII and XII) were retained in the samples of the respective specimens to different sample times.

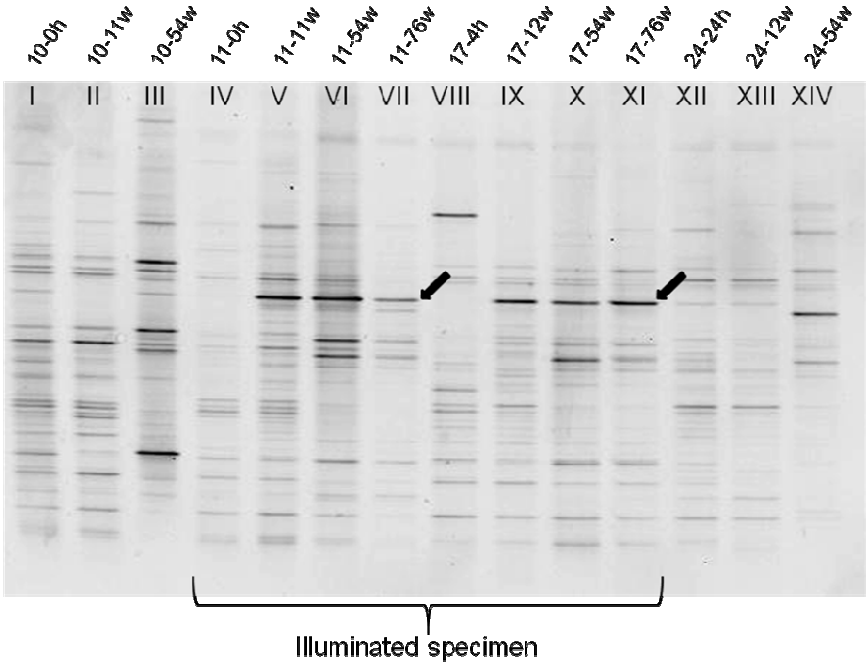

Figure 12. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments from individual *Aplysina aerophoba* specimens number 10, 11, 17 and 24 following cultivation under different conditions. *Lane numbers I–III* refer to the samples of specimen number 10, *lane numbers IV–VII* refer to the samples of specimen number 11, *lane numbers VIII–XI* refer to the samples of specimen number 17, *lane numbers XII–XIV* refer to the samples of specimen number 24 with different sampling times (*0h* 0 hours, *4h* 4 hours, *24h* 24 hours, *11w* 11 weeks, *12w* 12 weeks, *54w* 54 weeks and *76w* 76 weeks after removal of the sponges from their habitat). Variability in the occurrence of a single band in the DGGE analysis is marked by arrows.

However, a single bacterium showed a variable occurrence in the different samples of two sponge specimens (marked by arrows in Figure 12). These two sponges were kept under additional illumination and this respective bacterium achieves dominance during cultivation in those sponges. In contrast, in the sponges that were not additionally illuminated this bacterium showed no dominant occurrence over the time. No excision of bands of the DGGE gel occurred. Thus, the identity of this bacterium remained undetermined. However, taking the light-dependant occurrence of this bacterium into consideration an affiliation to photosynthetically active bacteria is assumable.

This assumption is supported by works of Klöppel *et al.* (2008) who reported a light-depending varying cyanobacterial content of *A. aerophoba* during different cultivation conditions. In that study, the e*x-situ* cultivation of *A. aerophoba* under light-exposed conditions led to an increase of the *Cyanobacteria* content. In contrast, keeping the sponges under cryptic–dark conditions led to a reorganization of the sponge tissue, accompanied by a decrease of the *Cyanobacteria* content.

Furthermore, illumination seems to have a positive influence on the health conditions of sponges during cultivation, since those sponges were able to maintain over a longer cultivation period. This is in accordance to the findings of Wilkinson and Vacelet (1979) who reported an enhanced growth of *A. aerophoba* sponges in light, presumably due to a transfer of photosynthetically fixed nutrients from associated bacteria to their hosts.

4.2 Chapter 2 Bacterial Communities from Different Sponge Species

Differences between surface- and tissue-associated bacterial communities of altogether twelve Mediterranean sponge species (*Agelas oroides*, *Chondrosia reniformis*, *Petrosia ficiformis*, *Geodia* sp., *Tethya* sp., *Axinella polypoides*, *Dysidea avara*, *Oscarella lobularis*, *Ircinia* sp., *Crambe crambe*, *Chondrilla nucula*, and *Aplysina aerophoba*) and eight different reference samples (bacterial communities obtained from seawater, the surfaces of inanimate substrata (two ceramic tiles placed at 5 m depth for 2 days and a bottle found at the seabed) as well as different animate surfaces, such as five invertebrates, a sea cucumber (*Holothuroidea* sp.), a seastar (*Echinaster sepositus*), two sea squirts (*Halocynthia papillosa* and an undetermined species from the family Ascidiae), a mussel (*Mytilus edulis*), and three macroalgae (*Codium bursa*, *Valonia* sp., and *Ulva rigida*) have been investigated in the second part (chapter 2) of this work. Out of these twelve sponge species eight sponge species have provide sufficient PCR products obtained from both the surface and the tissue samples. From the eight different reference samples six provided sufficient PCR products and thus, sufficient DGGE banding patterns. This eight sponge species and the six reference samples have been fully analyzed, including cluster analysis of the DGGE banding patterns and sequencing analysis of the 16S rDNA from members of the bacterial community. These results of surface and tissue bacterial communities of eight different sponge species and six reference samples were summarized in the first part of chapter 2 (section 4.2.1). The remaining four sponge species did not provide sufficient PCR products either from the surface or from the tissue samples, thus were excluded from a fully investigation. Also two of the originally eight reference samples were excluded from further investigations. The results of the remaining four sponge species and two reference samples will be presented in the second part of chapter 2 (section 4.2.2).

4.2.1 Manuscript 2: Differences between Bacterial Communities Associated with the Surface or Tissue of Mediterranean Sponge Species

Differences between Bacterial Communities Associated with the Surface or Tissue of Mediterranean Sponge Species

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Abstract

Bacterial communities associated with the surfaces of several Mediterranean sponge species (*Agelas oroides*, *Chondrosia reniformis*, *Petrosia ficiformis*, *Geodia* sp., *Tethya* sp., *Axinella polypoides*, *Dysidea avara* and *Oscarella lobularis*) were compared to those associated with the mesohyl of sponges and other animate or inanimate reference surfaces as well as with those from bulk seawater. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified bacterial 16S rRNA genes obtained from the surfaces and tissues of these sponges demonstrated that the bacterial communities were generally different from each other. The bacterial communities from sponges were different from those on reference surfaces or from bulk seawater. Additionally, clear distinctions in 16S rDNA fingerprint patterns between the bacterial communities from mesohyl samples of “high-microbial abundance (HMA) sponges” and “low-microbial abundance (LMA) sponges” were revealed by DGGE and cluster analysis. A dominant occurrence of particularly GC-rich 16S rDNA fragments was found only in the DGGE banding pattern obtained from the mesohyl of HMA sponges. Furthermore, sequencing analysis of 16S rDNA fragments obtained from mesohyl samples of HMA sponges revealed a dominant occurrence of sponge-associated bacteria. The bacterial communities within the mesohyl of HMA sponges showed a close relationship to each other and seem to be sponge-specific.

Introduction

Sponges (phylum Porifera) are among the most prolific sources of new natural products with bioactive properties (see Blunt *et al.*, 2010 and its proceeding versions). They are benthic, sessile and active filter feeders that feed on bacterial plankton and detritus (Bergquist, 1978). Thus, a certain fraction of microorganisms is generally expected to be detectable within sponges. However, it has been stated that sponge-bacteria symbioses have existed for approximately 600 million years (Wilkinson, 1984). This would date such associations back to the Precambrian. Such long-term associations occurring between microorganisms and sponges can result in the evolution of adaptations that strongly suggest a functional relationship between them. Thus, sponges offer an excellent model to investigate invertebrate-microorganism associations (Schmitt *et al.*, 2007a; Thakur and Muller, 2005) and in the past decade, the number of studies in this field increased steadily (reviewed in Taylor *et al.*, 2007).

Early microscopic studies from Vacelet and Donadey (1977) revealed that sponges can be placed into two different groups, termed “high-microbial abundance (HMA) sponges” and “low-microbial abundance (LMA) sponges” (Hentschel *et al.*, 2006). The concentrations of bacteria associated with LMA sponges reflect those of ambient seawater. While seawater in the northwestern Mediterranean Sea typically contains 5×10^5 cells per mL (Ribes *et al.*, 1999), the concentrations of associated microorganisms in HMA sponges exceed that of the surrounding seawater by two or three orders of magnitude (Friedrich *et al.*, 2001; Hentschel *et al.*, 2003; Webster and Hill, 2001). Weisz *et al.* (2008) reported tissue density and pumping rate differences between HMA and LMA sponges having demonstrable impacts on sponge life strategies. However, it remains unclear whether the presence of bacteria caused or is the result of these morphological and physiological differences between HMA and LMA sponges (Weisz *et al.*, 2008).

The hypothesis of a uniform bacterial signature in the mesohyl of phylogenetically and geographically distantly related sponges that is both sponge-specific and distinctly different from that of seawater, as advanced by Hentschel and colleagues (2002), has been supported by Hill *et al.* (2006), Lafi *et al.* (2005), and Fieseler *et al.* (2004). This suggests the existence of highly specific selective pressures occurring in sponges. However, the uniformity of bacterial communities from different sponge species is still debated. As reviewed by Taylor *et al.* (2007), it is unclear whether straightforward vertical transmission of ancient symbiotic microorganisms, perhaps combined with horizontal (environmental) transmission of microbes, leads to a common bacterial signature between different sponge species over space and time, or if the enormous filtration capacity of sponges leads to non-specific enrichment of rare seawater microbes leading to the sponge-specific bacterial community concept. However, specific bacterial communities in the mesohyl of sponges are highly stable, and neither transplanting sponges to foreign habitats (Thoms *et al.*, 2003), short-term cultivation of sponges in seawater aquaria under antibiotic exposure (Friedrich *et al.*, 1999) nor long-term cultivation of sponges under

artificial conditions (Gerçe *et al*., 2009) leads to significant shifts within the sponge-associated bacterial community, suggesting a highly integrated sponge–bacteria interaction.

In contrast to the well-investigated tissue-associated bacterial communities of sponges, much less is known to date about the ecology of bacterial communities on the surface of sponges. A seasonally variable surface bacterial community on *Ircinia fusca* has been reported by Thakur *et al.* (2004a). On the other hand, Lee *et al.* (2006a) showed a geographically and seasonally consistent bacterial surface community on *Mycale adhaerens*. Furthermore, the surface bacterial community on *M. adhaerens* has been reported as clearly distinguishable from that of a reference inanimate surface bacterial community (Lee and Qian, 2004; Lee *et al*., 2006b). These results are supported by reports from Dobretsov *et al.* (2005) for three other sponge species: *Haliclona cymaeformis*, *Haliclona* sp. and *Callyspongia* sp. Taken together, these studies suggest the occurrence of highly integrated sponge-surface bacteria interactions comparable to that occurring with sponges and their mesohyl-associated bacteria. However, in all these studies, no comparisons to bacterial mesohyl communities have been performed. Qualitative analysis of bacterial populations associated with the surface of different Mediterranean sponge species, for investigating the relationship between surface- and mesohyl-associated bacterial communities, has not been performed yet.

The aim of this study was to compare bacterial communities on the surfaces of eight Mediterranean sponge species (*Agelas oroides*, *Chondrosia reniformis*, *Petrosia ficiformis*, *Geodia* sp., *Tethya* sp., *Axinella polypoides*, *Dysidea avara* and *Oscarella lobularis*) in relation to their tissue bacterial communities by denaturing gradient gel electrophoresis (DGGE). Bacterial communities from the surfaces of inanimate substrata and seawater and different animate surfaces, such as two invertebrates *(Holothuroidea* sp. and *Echinaster sepositus*) and a macroalga (*Codium bursa*), served as references. Based on the assumption of sponge-specific bacterial communities, this study was expected to support the occurrence of highly similar intraspecific bacterial populations between communities of the surfaces versus the tissues of the sponges.

Methods

Sponge Collection and Sampling of Tissues and Surfaces

Eight different sponge species were collected by scuba diving in the Limski Channel near Rovinj (Croatia) or next to two offshore islands near Rovinj - Figarola Island and San Giovanni Island in the Adriatic Sea (Table 1). The sponges included in the study were grouped in HMA (*A. oroides*, *C. reniformis*, *P. ficiformis* and *Geodia* sp.) and LMA sponges (*A. polypoides*, *D. avara* and *O. lobularis*) referring to published literature. The previous classification in HMA sponges occurred either at the species level for *A. oroides*, *P. ficiformis* (Vacelet and Donadey, 1977) and *C. reniformis* (Schlaeppy *et al*., 2010), or at

the genus level for *Geodia* sp. (Hoffmann *et al.*, 2005). The three sponge species *A. polypoides*, *D. avara* and *O. lobularis* have been previously classified as LMA sponges (Schlaeppy *et al.*, 2010; Vacelet and Donadey, 1977). Although bacteria associated with sponges of the genus *Tethya* have been investigated (Sipkema and Blanch, 2010; Thiel *et al.*, 2007b), no quantitative analysis of the bacterial concentration has been performed in *Tethya* sp. yet. Thus, no assignment of *Tethya* sp. in HMA or LMA sponge was performed.

Sponges were collected from depths of 10 to 24 m, removed carefully with their substrate, and individually labeled. Individual specimens were placed separately into plastic buckets to avoid contact with air and brought to the surface. Before each sampling, specimens were carefully rinsed with 0.22-µm filtered seawater to remove superficially attached microorganisms. Afterwards, an area of 20 to 25 cm² of the surface of each sponge was swabbed with a sterile cotton tip (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) to collect the bacterial community. As a control, cotton tips of the used batch have been tested for occurrence of microbial 16S rDNA. No 16S rDNA was detected in these cotton tips. After sampling, each cotton tip was immediately frozen at -30°C. For extraction of DNA from sponge tissue, cube-shaped samples with an edge length of 0.5 to 1 cm were taken with an EtOH-sterilized scalpel blade. In order to ensure collection of communities from mesohyl only the tissues cortex from six sponge species (*C. reniformis*, *A. polypoides*, *Tethya* sp, *P. ficiformis*, *A. oroides* and *Geodia* sp.) was carefully removed by scalpel and discarded. Due to an encrusting form of growth for the remaining two sponges (*D. avara* and *O. lobularis*), separation of the mesohyl from the cortex was not feasible. Therefore, extraction of DNA occurred from the whole tissue and also contained DNA from surface bacteria, while in surface samples, no mesohyl bacteria were present. After sampling, the tissue samples were immediately frozen at -30°C until further processing.

Sampling of Seawater, Reference Samples, and DNA Extraction

Reference samples of seawater were taken next to Figarola Island, near Rovinj. An autoclaved modified glass bottle (Duran, Schott AG, Mainz, Germany) was filled with 500 mL of seawater at the sampling site by scuba diving. The water was cooled on ice until the return to the laboratory and immediately filtered through a membrane filter (ME 24/21 ST, ETO, 0.2 µm, Schleicher & Schuell, Whatman, Dassel, Germany). To evaluate differences between pioneer bacterial communities on inanimate surfaces and communities on animate surfaces, two ceramic tiles were placed at 5 m depth for 2 days. Furthermore, the surfaces of two invertebrates, namely the sea cucumber *Holothuroidea* sp. and the sea star *E. sepositus*, as well as a macroalga *C. bursa* were also sampled with a sterile cotton tip. The same sampling procedure as used for sampling the surfaces of the sponges was performed for sampling the reference surfaces. The filter and each cotton tip were immediately frozen and stored at -30°C until further processing.

Before DNA extraction from tissue samples, the tissue was ground using a sterile mortar and pestle submerged in liquid nitrogen. Genomic DNA was extracted either from approximately 100 mg tissue, from the cotton tip containing sponge or reference surface bacteria directly or from the filter containing seawater bacteria, using a commercial extraction kit (Fast DNA Spin Kit for soil, Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions, and stored at -30°C.

Denaturing Gradient Gel Electrophoresis (DGGE)

The universal primers 27F (5´-AGA GTT TGA TC(AC) TGG CTC AG-3´), with a degenerate base pair at one position with the GC-clamp (5´-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-5`) spanning *Escherichia coli* positions 8-27, and 518R (5´-ATT ACC GCG GCT GCT GG-3´) spanning *E.coli* positions 518-534, were used for amplification of a 566-bp fragment from bacterial 16S rDNA. All primers used were purchased from Biomers.net GmbH (Ulm, Germany). PCR was performed using a thermocycler (DNA Engine PTC-200, Bio-Rad Laboratories GmbH, Munich, Germany) as follows: one activation step (MolTaq 16S DNA-free polymerase, Molzym GmbH and Co. KG, Bremen, Germany) for 4 min at 94°C, 30 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 53°C and elongation for 1 min at 72°C, followed by a final elongation step for 10 min at 72°C. The PCR mix contained 5 µL DNA template with a concentration of ~10 ng/µL for sponge tissue samples or 1 ng/µL for sponge surface and reference samples, 2 U polymerase per reaction, 1 µL dNTPs (0.2 mM final concentration of each dNTP; GE Healthcare, Buckinghamshire, UK), 0.2 µL each primer (0.4 µM final concentration), and 5 µL of a 10x reaction buffer (1x final concentration; Molzym GmbH and Co. KG, Bremen, Germany) in a total volume of 50 µL.

Products from triplicate PCR reactions were combined, and DGGE was performed in a 6% (w/v) polyacrylamide gel in 1x TAE running buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5) with a 35% to 70% gradient of formamide and urea using a DGGE device (INGENY phorU System, Ingeny International BV, GP Goes, Netherlands). The 100% denaturant solution corresponded to 7 M urea and 40% (v/v) formamide. Gradient optimization was performed using first 0% to 100% formamide and urea to 35% to 70%. Conditions chosen for electrophoresis were 17 h at 62°C and a voltage of 100 V. The gels were stained for 45 min in DNA dye solution (SYBR® Gold, Invitrogen GmbH, Karlsruhe, Germany) at 1:10,000 dilution and visualized using a gel documentation device (Alpha Imager 2,200, Biozym Scientific GmbH, Oldendorf, Germany) connected with a UV trans-illuminator (UV trans-illuminator ECP-26.LMX, Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

Sequencing and Taxonomical Affiliation of Excised 16S rDNA Bands from DGGE Analysis

Bands were excised from DGGE gels and stored overnight in 50 µL water (DNA-free PCR water, Molzym GmbH and Co. KG, Bremen, Germany) at 4°C. PCR conditions were same as described before, with three modifications: concentration of polymerase was decreased to 0.75 U per reaction, the applied volume of eluted DNA was decreased to 1.6 µL and the primer pair 27F (without a GC clamp) and 518R were used for the second PCR. Sequencing of PCR products was performed (GATC Biotech AG, Konstanz, Germany) using the reverse primer 518R. The resulting sequences were compared to 16S ribosomal RNA (rRNA) gene sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLASTN) algorithm (Altschul *et al.*, 1990), with a word size of seven. The affiliation of the sequences obtained from the DGGE bands to different bacterial phyla was performed by the use of the Ribosomal Database Project (RDP) Classifier program (Wang *et al.*, 2007) using default parameters (confidence threshold 80%).

Cluster Analysis

To evaluate the DGGE gel, a binary matrix was constructed representing the presence and absence of every band in the banding pattern of each lane. A resemblance matrix was then generated using the Bray-Curtis index, followed by hierarchical cluster analysis of similarity among the bacterial communities (Clarke, 1993; Clarke and Warwick, 2001a) using cluster analysis software (Primer E Ltd., Plymouth, Version 5, UK). Cluster analysis was performed with a group average and without any standardization or transformation of the data. The similarity of samples is expressed as a percentage.

Results

Quantitative Evaluation of Bacterial Communities displayed in the DGGE Gel

The 16S rDNA banding patterns obtained from the bacterial communities of sponges and various reference samples are displayed in the DGGE gel shown in Figure 1. The abbreviations given in brackets indicate whether the bacterial communities were obtained from tissue (-T) or surface (-S) samples. The abbreviations used for the designation of the different samples are given in brackets for *A. oroides* (Ao), *C. reniformis* (Cr), *P. ficiformis* (Pf), *Geodia* sp. (Gsp), *Tethya* sp.(Tsp), *A. polypoides* (Ap), *D. avara* (Da), *O. lobularis* (Ol), seawater (Sw), two ceramic tiles (Pb and Pw), a sea cucumber *Holothuroidea* sp. (Hsp), a sea star *E. sepositus* (Es), and a macroalgae *C. bursa* (Cb). From left to right, the 16S rDNA banding patterns obtained from tissue and surface samples of HMA sponges (lanes I-VIII) are shown, then those of *Tethya* sp. (lanes IX and X), then those of LMA sponges (lanes XI-XVI) followed by the reference samples (lanes XVII-XXII).

In general, the DGGE analysis resulted in a banding pattern composed of 6 to 39 bands per lane (Table 1). In detail, the number of the bands in lanes from tissues (-T) and surfaces (-S) of HMA sponges were 17 and 16 bands for the sample from *A. oroides* (lanes I and II), *C. reniformis* 24 and 6 bands (lanes III and IV), *P. ficiformis* 25 and 14 bands (lanes V and VI), and *Geodia* sp. 39 and 10 bands (lanes VII and VIII), respectively. The number of bands in lanes from tissue and surfaces samples of *Tethya* sp. were 23 and 10 bands (lanes IX and X), respectively. Generally, in all cases tissue samples of the sponges displayed more DGGE bands than corresponding surface samples. The numbers of bands obtained from tissue samples of the HMA sponges *C. reniformis*, *P. ficiformis* and *Geodia* sp. (lanes III, V and VII) were actually 2- to 4-fold higher compared to the numbers of bands displayed by sponge surface samples (lanes IV, VI and VIII).

The number of bands in lanes from tissue and surface samples of LMA sponges were for the non-encrusting growing species *A. polypoides* 13 and 10 bands (lanes XI and XII) and for the encrusting growing species *D. avara* 20 and 10 bands (lanes XIII and XIV) and *O. lobularis* 16 and 14 bands (lanes XV and XVI), respectively.

The different organic and inorganic reference samples displayed 18 bands for seawater (lane XVII); 14 bands each for the two ceramic tiles (lanes XVIII and XIX); 13 bands each for the two invertebrates, a sea cucumber *Holothuroidea* sp. (lane XX) and a sea star (lane XXI) *E. sepositus* and finally for the algae *C. bursa* (lane XXII) 26 bands. Altogether, 111 different phylotypes have been counted and distinguished by their migration distances in the DGGE gel displayed in Figure 1.

Qualitative Evaluation of Bacterial Communities Displayed in the DGGE Gel

The bacterial 16S rDNA fragments obtained from mesohyl samples of the sponge species *A. oroides* (lane I), *C. reniformis* (lane III), *P. ficiformis* (lane V), *Geodia* sp. (lane VII), and to some extent *Tethya* sp. (lane IX) showed distinctive GC-rich 16S rDNA fragments, in comparison to the banding patterns obtained from the remaining samples (Figure 1). The differences in the GC content are characterized by the migration of the 16S rDNA fragments to the lower area of the DGGE gel. In contrast, the banding patterns of 16S rDNA fragments obtained from tissue samples of the sponge species *A. polypoides* (lane XI), *D. avara* (lane XIII), and *O. lobularis* (lane XV) and from the reference samples (lanes XVII-XXII) are dominated by sequences with a lower GC content. The banding pattern obtained from tissue of *Tethya* sp. (lane IX) corresponded more closely to the banding patterns of tissue samples from *A. oroides* (lane I), *C. reniformis* (lane III), *P. ficiformis* (lane V), and *Geodia* sp. (lane VII) with respect to the distribution of bands in the DGGE gel, but lacked the specific GC-rich fragments.

Table 1. Overview and origin of the sponge species and reference samples.

	Sample/abbreviation	***Origin***	***Number of bands in the DGGE gel obtained from***	
			the tissue samples	***the surface samples***
"High-microbial abundance sponges"	*Agelas oroides* (Ao)	Island San Giovanni	*17*	*16*
	Chondrosia reniformis (Cr)	Island Figarola	24	6
	Petrosia ficiformis (Pf)	Island San Giovanni	*25*	*14*
	Geodia sp. (Gsp)	Limski Channel	39	10
Uncertain bacterial density	*Tethya* sp. (Tsp)	Limski Channel	23	10
"Low-microbial abundance sponges"	*Axinella polypoides* (Ap)	Limski Channel	13	10
	Dysidea avara (Da)	Limski Channel	20	10
	Oscarella lobularis (Ol)	Limski Channel	16	14
Reference samples	seawater (Sw)	Island Figarola	-	18
	ceramic tile black (Pb)	Limski Channel	-	14
	ceramic tile white (Pw)	Limski Channel	-	14
	Holothuroidea sp. (Hsp)	Limski Channel	-	13
	Echinaster sepositus (Es)	Limski Channel	-	13
	Codium bursa (Cb)	Limski Channel	-	26

Comparing surface- and mesohyl-derived 16S rDNA banding patterns from *A. oroides* (lanes I and II), *C. reniformis* (lanes III and IV), *P. ficiformis* (lanes V and VI), *Geodia* sp. (lanes VII and VIII), and *Tethya* sp. (lanes IX and X), appreciable differences were obvious. Characteristic GC-rich fragments occurring in tissue samples were absent in surface samples from these sponge species. In contrast, the banding patterns of 16S rDNA fragments obtained from surface and tissue samples of the sponge species *A. polypoides* (lanes XI and XII), *D. avara* (lanes XIII and XIV), and *O. lobularis* (lanes XV and XVI) were all dominated by sequences with lower GC content. Comparing banding patterns obtained from surface and tissue samples of the encrusting growing sponges *D. avara* (lanes XIII and XIV) and *O. lobularis* (lanes XV and XVI), all bands obtained from surface samples were also present in banding patterns obtained from tissue samples, as was to be expected by the sampling procedure for these sponge species. In addition, the bacterial 16S rDNA fragments obtained from surface samples of all sponge species and from reference surface samples were dominated by the occurrence of GC-poor fragments. These samples also lack characteristic GC-rich fragments. The banding pattern obtained from the surface sample of *C. reniformis* (lane IV) was an exception because it displayed weak bands generally.

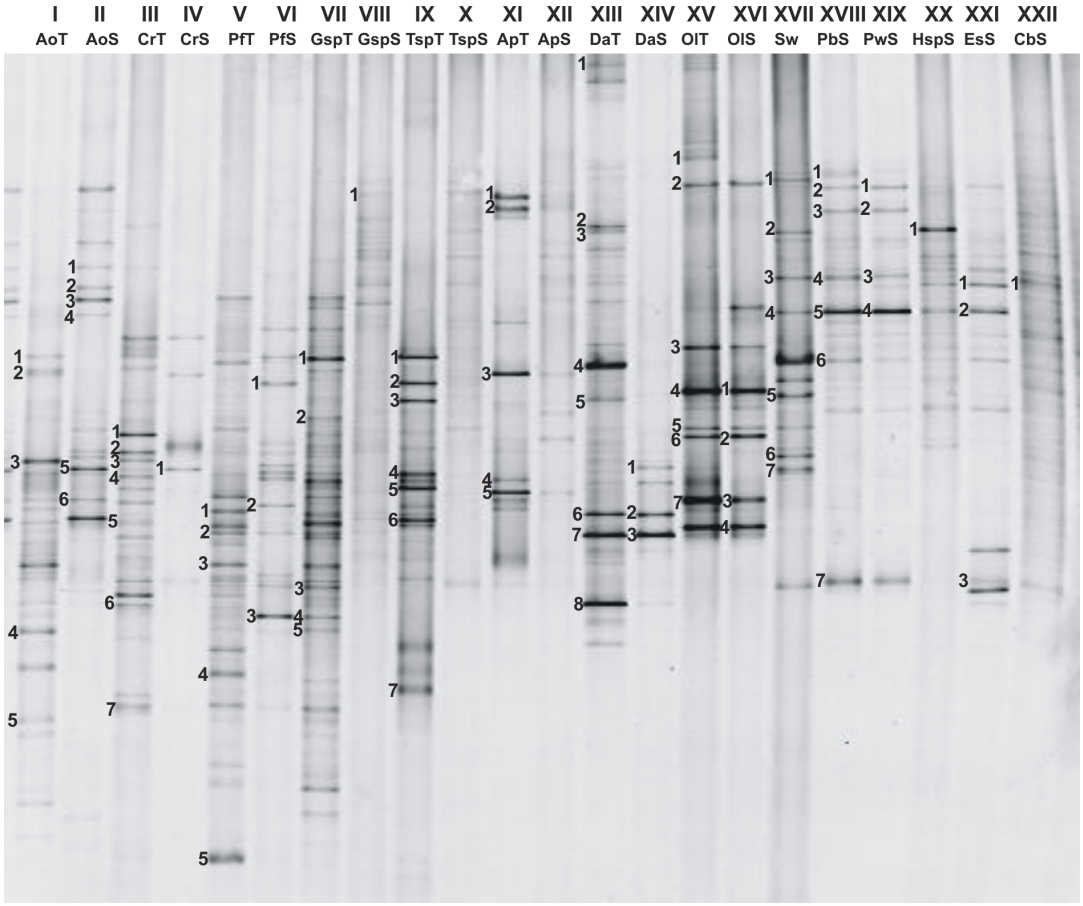

Figure 1. Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA fragments obtained from bacterial communities of eight different sponges species and six reference samples. The abbreviations given in brackets indicate whether the bacterial communities were obtained from tissue (-T) or surface (-S) samples. The abbreviations used for the designation of the samples are given in brackets. *Lanes I-II* display the banding pattern obtained from *A. oroides* (Ao), *lanes III–IV* from *C. reniformis* (Cr), *lanes V-VI* from *P. ficiformis* (Pf), *lanes VII-VIII* from *Geodia* sp. (Gsp), *lanes IX-X* from *Tethya* sp.(Tsp), *lanes XI-XII* from *A. polypoides* (Ap), *lanes XIII-XIV* from *D. avara* (Da), *lanes XV-XVI* from *O. lobularis* (Ol), *lane XVII* from seawater (Sw), *lanes XVII* and *XIX* from two ceramic tiles (Pb and Pw), *lane XX* from a sea cucumber *Holothuroidea* sp. (Hsp), *lane XXI* from a sea star *E. sepositus* (Es) and *lane XXII* from a macroalga *C. bursa* (Cb). Arabic numerals indicate DGGE bands for which sequence information was obtained (Table 2).

Cluster Analysis of Similarities among DGGE Gel Banding Patterns

Cluster analysis of banding patterns in the DGGE gel (Figure 1) is illustrated in Figure 2 by a dendrogram. In general, a high overall dissimilarity of bacterial communities from the different samples became obvious. The dendrogram of the cluster analysis corresponded to the visual impression of the banding patterns in the DGGE gel. In simplified terms, the similarities of the banding patterns obtained from all samples led to grouping into two clusters (clusters I and II in Figure 2).

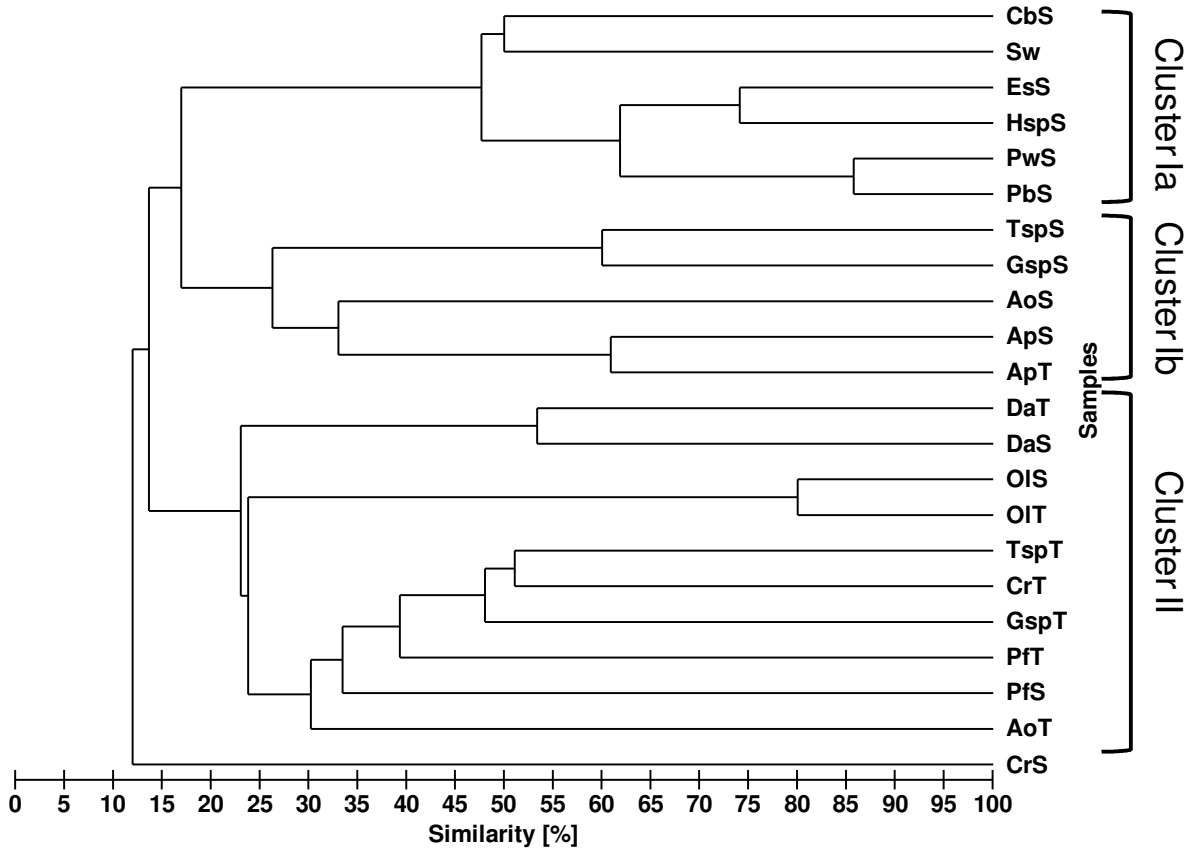

Figure 2. Dendrogram of cluster analysis showing similarities in percent of DGGE banding patterns for the partial 16S rDNA sequences obtained from sponge-associated microorganisms within tissues and on surfaces of eight different sponge species and from the bacterial communities on reference surface samples. The abbreviations given in brackets indicate whether the bacterial communities were obtained from tissue (-T) or surface (-S) samples. The abbreviations used for the designation of the samples are given in brackets for *A. oroides* (Ao), *C. reniformis* (Cr), *P. ficiformis* (Pf), *Geodia* sp. (Gsp), *Tethya* sp.(Tsp), *A. polypoides* (Ap), *D. avara* (Da), *O. lobularis* (Ol), seawater (Sw), two ceramic tiles (Pb and Pw), a sea cucumber *Holothuroidea* sp. (Hsp), a sea star *E. sepositus* (Es) and a macroalgae *C. bursa* (Cb).

Cluster I consists of mainly surface samples (divided into two subclusters-cluster Ia and Ib) and cluster II of tissue samples. Both clusters (I and II) show a similarity to each other of about 12%. Subcluster Ia, comprising the non-sponge-derived reference surface samples, revealed similarity among the bacterial surface communities of over 48% to each other. Within this cluster, the similarity of bacterial communities from the two invertebrate surfaces of *E. sepositus* and *Holothuroidea* sp. (EsS and HspS) was clustered pairwise and had a similarity of nearly 75%, sharing 10 out of 13 bands. In addition, the banding patterns obtained from the two surfaces of the ceramic tiles (PbS and PwS) shared 12 out of 14 bands and were clustered pairwise showing the highest similarity in the dendrogram of 86%. The banding pattern displaying the bacterial community of the seawater sample (18 bands) showed the highest similarity of nearly 51% with the pattern from the surface sample of the macro algae *C. bursa* (26 bands),

having 11 bands in common. Furthermore, the banding patterns displaying bacterial communities on four sponge surfaces (*Tethya* sp., *Geodia* sp., *A. oroides* and *A. polypoides*) and additionally from tissue sample of *A. polypoides* were clustered together (subcluster Ib). These samples showed a similarity ranging from 26% to 61%. The highest similarity of nearly 60% (sharing 6 out of 10 bands) between two surface samples within subcluster Ib was present between the bacterial surface communities of *Tethya* sp. and *Geodia* sp. The banding pattern obtained from the surface bacterial community of *A. oroides* (16 bands) has five bands in common with the pattern obtained from the surface sample of *Geodia* sp. (10 bands) and shared two bands with the banding pattern of *Tethya* sp. (10 bands). In this cluster (subcluster Ib) comprised of mainly surface samples, a single exception was the tissue sample of the LMA, but not encrusting growing sponge species *A. polypoides*. This sample showed higher similarity (nearly 61%) with the surface sample of *A. polypoides*, sharing 7 out of 10 and 13 bands, respectively, than to banding patterns obtained from the remaining tissue samples. The banding patterns obtained from tissue samples from the remaining seven sponge species (DaT, OlT, TspT, CrT, GspT, PfT, and AoT) and the banding patterns of three sponge surface samples (DaS, OlS. and PfS) clustered together in a second cluster (cluster II) and showed a similarity between 23% and 80%. The banding pattern obtained from tissue samples from the sponge species *P. ficiformis* (PfT), *A. oroides* (AoT), *Tethya* sp. (TspT), *C. reniformis* (CrT), and *Geodia* sp. (GspT) showed a similarity of 30% to 51%. In addition, within this second cluster IIa pairwise clustering of tissue and surface samples from the encrusting growing LMA sponge species *D. avara* (DaT and DaS) and *O. lobularis* (OlT and OlS), respectively, occurred. The tissue sample of *O. lobularis* (OlT, 16 bands) showed the highest similarity of about 80% to the surface sample of *O. lobularis* (OlS, 14 bands), sharing 12 common bands. Accordingly, the bacterial community from both samples (tissue and surface) of *D. avara* (DaT, 20 bands and DaS, 10 bands) showed a similarity of about 53% (sharing 8 bands). The surface sample of *P. ficiformis* (PfS) constitutes an exception since it showed lower similarity to other bacterial surface communities and higher similarity to the bacterial community from the tissue samples of *A. oroides* (AoT) and *P. ficiformis* (PfT). The bacterial community on the surface of *C. reniformis* (CrS) showed the lowest similarity to all investigated samples (12%). Comparing the similarities between bacterial communities of the surface and mesohyl from each individual HMA sponge species, a high dissimilarity was observable except for *P. ficiformis*. For instance, the highest number of common bands shared between the banding pattern from the tissue sample of *A. oroides* (17 bands) and its surface sample (16 bands) was one single band. In contrast, the tissue (25 bands) and surface samples (14 bands) of *P. ficiformis* shared higher similarity and had seven bands in common. The surface and tissue bacterial communities from each individual LMA sponge species showed also high similarities. The mesohyl and surface samples of *A. polypoides* (13 and 10 bands, respectively) had seven bands in common. Due to the unfeasibility of separating mesohyl-associated bacteria from the surface bacteria for *D. avara* and *O. lobularis* they shared the highest amount of

common bands. For instance, the two samples of *O. lobularis* (16 and 14 bands, respectively) had 12 bands in common.

Results of Sequencing the Excised 16S rDNA Bands from DGGE Analysis

For further analysis of the bacterial communities, the dominant 16S rDNA bands apparent in the DGGE gel were excised and sequenced for determination of the taxonomical affiliation for the respective microorganisms. A total of 137 bands were excised from the DGGE gel for sequencing. Reliable bacterial 16S rDNA sequences were obtained from 90 of these bands. The sequences were deposited in Genbank (NCBI) with the accession numbers HM485597-HM485686. Furthermore, these sequences were used for analyses by database alignment. PCR-amplification of DNA eluted from the remaining 47 bands was either not successful or the sequences showed too many ambiguous nucleotides for a meaningful affiliation with a bacterial phylum. The Arabic numerals in Figure 1 indicate DGGE bands for which sequence information was obtained. Table 2 summarizes the affiliation of the 16S rDNA sequences obtained from the DGGE bands to the nearest sequence match in GenBank (BLASTN) with the percentage of homology. In addition, it summarizes the affiliations of the sequences obtained from the DGGE analysis to bacterial phyla by the use of Classifier (RDP). Generally, all 90 sequences were related to bacteria of five phyla: *Proteobacteria* (with further affiliation to the classes *α*- and *γ*-*Proteobacteria* in some cases), *Cyanobacteria*, *Actinobacteria*, *Acidobacteria* and *Bacteroidetes*. The bacterial division was denoted as “uncertain affiliation” in Table 2 when the similarity estimates by Classifier amounted less than 90%. All 22 sequences obtained from mesohyl samples of the HMA sponges *A. oroides* (AoT-1-5), *C. reniformis* (CrT-1--7), *P. ficiformis* (PfT-1-5) and *Geodia*. sp. (GspT-1-5) could be assigned to sponge-associated bacteria (Figure 1 and Table 2). In contrast, sequences obtained from all other remaining samples were assigned to bacteria isolated from a variety of sources. Of the 90 sequences, 81 showed the highest homology to bacteria of marine origin (e.g., bacterioplankton or marine sediment) and six to bacteria from terrestrial sources. Out of 90 sequences, 30 showed homology greater than 97%, enabling a taxonomical affiliation on the species level. Among these sequences showing homology higher than 97%, 18 sequences were obtained from reference samples. The remaining sequences showed homology less than 97% (down to 87%), enabling taxonomical affiliation only at the genus level or below.

Table 2. Denaturing gradient gel electrophoresis (DGGE) sequencing analysis results

Lane	*DGGE band*		*Nearest sequence match in GenBank (BLASTN)*	*Homo-logy (%)*	*Accession number*	*Bacterial division (similarity estimate by Classifier >90%, otherwise denoted as uncertain affiliation)*	*Host/ source*
I	AoT-	1	*Agelas dilatata* clone AD007	96	EF076127	Uncertain affiliation	Sponge
		2	*Agelas dilatata* clone AD057	93	EF076159.1	Uncertain affiliation	Sponge
		3	*Agelas dilatata* clone AD007	91	EF076127.1	Uncertain affiliation	Sponge
		4	*Xestospongia muta* clone XB1A10F	92	FJ481270.1	Uncertain affiliation	Sponge
		5	*Theonella swinhoei* clone PAUC43f	94	AF186415.1	Uncertain affiliation	Sponge
II	AoS-	1	*Desmacidon* sp. clone KspoB5	95	EU035934.2	*γ-Proteobacteria* (95%)	Sponge
		2	*Desmacidon* sp. clone KspoB5	96	EU035934.2	*γ-Proteobacteria* (96%)	Sponge
		3	*Desmacidon* sp. clone KspoB5	97	EU035934.2	*γ-Proteobacteria* (100%)	Sponge
		4	Uncult. bacterium clone B8S-132	91	EU652572.1	*γ-Proteobacteria* (97%)	Marine sediment
		5	*Agelas dilatata* clone AD026	91	EF076137.1	*γ-Proteobacteria* (91%)	Sponge
		6	*Aplysina aerophoba* clone TK79	95	AJ347081.1	Uncertain affiliation	Sponge
III	CrT-	1	*Agelas robusta* clone A16	93	GQ215668.1	Uncertain affiliation	Sponge
		2	*Svenzea zeai* clone A36	95	FJ529270.1	*α-Proteobacteria* (98%)	Sponge
		3	*Mycale laxissima* clone W01ML2H01 *Mycale laxissima* clone W01ML3D03	92	EF630125 EF630106	*γ-Proteobacteria* (98%)	Sponge
		4	*Corticium candelabrum* clone CC10	92	DQ247942.1	Uncertain affiliation	Sponge
		5	*Aplysina aerophoba* clone TK51	99	AJ347032.1	*α-Proteobacteria* (95%)	Sponge
		6	*Acanthostrongylophora* sp. clone OP408	93	EF513702.1	*Proteobacteria* (95%)	Sponge
		7	*Aplysina aerophoba* clone TK29	96	AJ347029.1	*Acidobacteria* (100%)	Sponge
IV	CrS-	1	*Montastraea annularis* clone CD207F09	95	DQ200625.1	*γ-Proteobacteria* (100%)	Coral
V	PfT-	1	*Acanthostrongylophora* sp. clones OP461, OP204, and OP103	93	FJ543140.1 EF513655.1 EF513643.1	*Bacteroidetes* (94%)	Sponge
		2	*Acanthostrongylophora* sp. clone OP441	97	EF513717.1	*Actinobacteria* (96%)	Sponge
		3	*Acanthostrongylophora* sp. clones OP462 and OP414	98	FJ543141.1/ FJ543130.1	Uncertain affiliation	Sponge

Lane	DGGE band		Nearest sequence match in GenBank (BLASTN)	Homo-logy (%)	Accession number	Bacterial division (similarity estimate by Classifier >90%, otherwise denoted as uncertain affiliation)	Host/ source
		4	*Acanthostrongylophora* sp. clone OP377	95	EF513688.1	Uncertain affiliation	Sponge
		5	*Acanthostrongylophora* sp. clone OP469	92	EF513731	Uncertain affiliation	Sponge
VI	PfS-	1	*Synechococcus* sp. CC9311	95	CP000435	*Cyanobacteria* (98%)	Bacterio-plankton
		2	*Svenzea zeai* clone A36	94	FJ529270.1	*α-Proteobacteria* (100%)	Sponge
		3	*Acanthostrongylophora* sp. clone OP408	97	EF513702.1	*α-Proteobacteria* (78%)	Sponge
VII	GspT-	1	*Aplysina aerophoba* clone HE11	98	EU495951	*γ-Proteobacteria* (99%)	Sponge
		2	*Plakortis* sp.clone PK002	96	EF076070.1	*γ-Proteobacteria* (100%)	Sponge
		3	*Aplysina aerophoba* clones TK74 and TK33	98 99	AJ347079.1 AJ347061.1	Uncertain affiliation	Sponge
		4	*Acanthostrongylophora* sp. clone OP408	95	EF513702.1	*Proteobacteria* (100%)	Sponge
		5	*Aplysina aerophoba* clone TK10	97	AJ347055.1	Uncertain affiliation	Sponge
VIII	GspS-	1	Uncult. *γ-Proteobacterium* clone SIMO-2256 Uncult. *γ-Proteobacterium* clone PI_4d4f	96	AY711622.1 AY580816.1	*γ-Proteobacteria* (100%)	Salt marsh sediment Bacterio-plankton
IX	TspT-	1	Uncult. *Proteobacterium* clone JJB320	98	GQ143791.1	*γ-Proteobacteria* (100%)	Marine sediment
		2	Uncult. bacterium clone Sd1-25	95	GQ246306.1	*γ-Proteobacteria* (95%)	Marine sediment
		3	Uncult. *γ-Proteobacterium* clone 3 T9d-oil	96	FM242300.1	*γ-Proteobacteria* (100%)	Marine sediment
		4	*Fungia* sp. clone FungiaD34clA10	96	EU636518.1	*α-Proteobacteria* (100%)	Coral
		5	Uncult. bacterium clone 1C227350	93	EU799714	Uncertain affiliation	Bacterio-plankton
		6	*Cymbastela concentrica* clones Cc007 and Cc006	96	AY942754.1 AY942753.1	*γ-Proteobacteria* (100%)	Sponge
		7	Uncult. bacterium clone FS266-92B-03	92	DQ513090.1	Uncertain affiliation	Marine sediment
XI	ApT-	1	Uncult. bacterium clone Crozet_s_787	95	FM214268.1	*γ-Proteobacteria* (99%)	Marine sediment
		2	Uncult. bacterium clone P0X3b5F08	90	EU491392.1	*γ-Proteobacteria* (99%)	Marine sediment
		3	Uncult. bacterium clone: WHB21-10	93	AB426357.1	*γ-Proteobacteria* (97%)	Marine sediment

Lane	*DGGE band*		*Nearest sequence match in GenBank (BLASTN)*	*Homo-logy (%)*	*Accession number*	*Bacterial division (similarity estimate by Classifier >90%, otherwise denoted as uncertain affiliation)*	*Host/ source*
		4	Uncult. *α-Proteobacterium* clone WN-FWB-150	91	DQ432407.1	*α-Proteobacteria* (98%)	Fresh-water
		5	Uncult. *α-Proteobacterium* clone WN-FWB-150	91	DQ432407.1	*α-Proteobacteria* (96%)	Fresh-water
XIII	DaT-	1	*Tethya aurantium* clone TAA-10-19	91	AM259864	Uncertain affiliation	sponge
		2	*Mycale laxissima* clone 6mML2E12	92	EF630135.1	*γ-Proteobacteria* (100%)	Sponge
		3	Uncult. bacterium clone bOHTK-93	97	FJ873329.1	*γ-Proteobacteria* (100%)	Marine sediment
		4	Uncult. *Synechococcus* sp. clone PEACE2006/178_P3	99	EU394578.1	*Cyanobacteria* (100%)	Bacterio-plankton
			Uncult. bacterium clone 2uA_G10		EU627881.1		Bacterio-plankton
		5	*Ircinia strobilina* clone W04IS4G07	96	EF629782.1	*Cyanobacteria* (100%)	Sponge
		6	*Erythropodium caribaeorum* clones EC113 and EC114	88	DQ889898.1 DQ889897.1	*Proteobacteria* (100%)	Coral
		7	*Erythropodium caribaeorum* clones EC113 and EC114	88	DQ889898.1 DQ889897.1	*Proteobacteria* (97%)	Coral
		8	*α-Proteobacterium* clone A1	90	DQ533534.1	*α-Proteobacteria* (95%)	Terrestrial source
			Uncult. *Rhodospirillaceae* bacterium clone DR938CH110701SACH19		DQ230958.1		Terrestrial source
XIV	DaS-	1	*Erythropodium caribaeorum* clones EC113 and EC114	88	DQ889898.1 DQ889897.1	*Proteobacteria* (100%)	Coral
		2	*Erythropodium caribaeorum* clones EC113 and EC114	87	DQ889898.1 DQ889897.1	*Proteobacteria* (99%)	Coral
		3	*Erythropodium caribaeorum* clones EC113 and EC114	88	DQ889898.1 DQ889897.1	*Proteobacteria* (100%)	Coral
XV	OlT-	1	Uncult. bacterium clone A64	92	GU066493.1	*γ-Proteobacteria* (99%)	Marine biofilm
		2	Uncult. *γ-Proteobacterium* clone ARTE9_99	97	GU230300.1	*γ-Proteobacteria* (100%)	Bacterio-plankton
		3	Uncult. bacterium clone A64	91	GU066493.1	*γ-Proteobacteria* (100%)	Marine biofilm
		4	Uncult. bacterium clone A64	90	GU066493.1	*γ-Proteobacteria* (99%)	Marine biofilm
		5	*Telmatospirillum siberiense* strain 26-4b1	90	AF524863.1	*α-Proteobacteria* (96%)	Terrestrial source
		6	*Telmatospirillum siberiense* strain 26-4b1	91	AF524863.1	*Proteobacteria* (99%)	Terrestrial source

Lane	DGGE band		Nearest sequence match in GenBank (BLASTN)	Homo-logy (%)	Accession number	Bacterial division (similarity estimate by Classifier >90%, otherwise denoted as uncertain affiliation)	Host/ source
		7	Uncult. *Lentisphaerae* bacterium clone HCM3MC91_8F_FL	92	EU373977.1	Uncertain affiliation	Marine sediment
XVI	OIS-	1	Uncult. bacterium clone A64	91	GU066493.1	*γ-Proteobacteria* (98%)	Marine biofilm
		2	*Telmatospirillum siberiense* strain 26-4b1	90	AF524863.1	*α-Proteobacteria* (92%)	Terrestrial source
		3	Uncult. *Lentisphaerae* bacterium clone HCM3MC91_8F_FL	92	EU373977.1	Uncertain affiliation	Marine sediment
		4	*Cymbastela concentrica* clone HERMI06	98	GQ160459.1	Uncertain affiliation	Sponge
XVII	Sw-	1	Uncult. bacterium clone 2C228195	99	EU800188.1	*γ-Proteobacteria* (98%)	Estuary
		2	Uncult. bacterium clone 4C229879	96	EU802537.1	*γ-Proteobacteria* (100%)	Bacterio-plankton
		3	*Alteromonas macleodii* 'Deep ecotype'	98	CP001103.1	*γ-Proteobacteria* (100%)	Bakterio-plankton
		4	*Pseudoalteromonas* sp. P17	95	EU880523.1	*γ-Proteobacteria* (100%)	Marine sediment
		5	*Ircinia strobilina* clone W04IS4G07	100	EF629782.1	*Cyanobacteria* (100%)	Sponge
		6	*Pocillopora meandrina* clone Pm_eggs_C10	98	FJ497115.1	*α-Proteobacteria* (93%)	Coral
		7	Uncult. bacterium clone S25_629	99	EF574285.1	*α-Proteobacteria* (95%)	Bacterio-plankton
XVIII	PbS-	1	Uncult. *Moraxella* sp. clone MJ28	98	GU212808	*γ-Proteobacteria* (100%)	Bacterio-plankton
		2	*Pseudoalteromonas* sp. MOLA 432 *Crassostrea gigas* isolate *Pseudoalteromonas* sp. 03/034	100	AM990699.1 AJ874351.1	*γ-Proteobacteria* (100%)	Bacterio-plankton Oyster
		3	*Crassostrea gigas* isolate *Pseudoalteromonas* sp. 01/121	99	AJ874345.1	*γ-Proteobacteria* (100%)	Oyster
		4	*Alteromonas macleodii* 'Deep ecotype	98	CP001103.1	*γ-Proteobacteria* (100%)	Bakterio-plankton
		5	*Pseudoalteromonas* sp. P17	100	EU880523	*γ-Proteobacteria* (100%)	Benthal sediment
		6	*Pseudoalteromonas* marina strain DHY3	99	GU198498.1	*γ-Proteobacteria* (100%)	Bacterio-plankton
		7	*Mycale laxissima* clone W04MLG11R	100	EF630271.1	*Actinobacteria* (100%)	Sponge

Lane	*DGGE band*		*Nearest sequence match in GenBank (BLASTN)*	*Homo-logy (%)*	*Accession number*	*Bacterial division (similarity estimate by Classifier >90%, otherwise denoted as uncertain affiliation)*	*Host/ source*
XIX	PwS-	1	*Pseudoalteromonas* sp. MOLA 432 *Crassostrea gigas* isolate *Pseudoalteromonas* sp. 03/034	100	AM990699.1 AJ874351.1	*γ-Proteobacteria* (100%)	Bacterio-plankton Oyster
		2	*Crassostrea gigas* isolate *Pseudoalteromonas* sp. 01/121	98	AJ874345.1	*γ-Proteobacteria* (100%)	Oyster
		3	*Siderastrea siderea* clone WA_08f *Vibrio tubiashii* strain Milford 74	95	EF123487.1 NR_026129.1	*γ-Proteobacteria* (100%)	Coral *Vibrio tubiashii*
		4	*Pseudoalteromonas* sp. P17	99	EU880523	*γ-Proteobacteria* (100%)	Marine sediment
XX	HspS-	1	Uncult. bacterium clone Ucs1554	98	AM997704.1	*γ-Proteobacteria* (97%)	Bacterio-plankton
XXI	EsS-	1	Uncult. *γ-Proteobacterium* NB1-d	93	AB013825.1	*γ-Proteobacteria* (100%)	Marine sediment
		2	*Pseudoalteromonas* sp. P17	99	EU880523	*γ-Proteobacteria* (100%)	Marine sediment
		3	*Maricaulis* sp. JL898	87	DQ985054.1	*α-Proteobacteria* (97%)	*Maricaulis* sp. JL898
XXII	CbS-	1	Uncult. *γ-Proteobacterium* clone ARTE12_253	97	GU230341	*γ-Proteobacteria* (100%)	Marine sediment

Discussion

Sponges are among the most prolific sources of newly discovered natural products with bioactive properties (see Blunt *et al.*, 2010 and its preceding versions). Furthermore, they offer an excellent model to investigate invertebrate-microorganism interactions (Schmitt *et al.*, 2007a; Thakur and Muller, 2005), and microbial communities associated with tissues of various sponge species are well investigated to date (see reviews Hentschel *et al.*, 2006; Taylor *et al.*, 2007). In contrast, knowledge about microbial communities directly associated with sponge surfaces is limited in comparison with bacterial communities associated with entire or mixed sponge tissue. Qualitative analysis of bacterial populations associated with surfaces of different Mediterranean sponge species and their phylogenetic relation to mesohyl-associated bacterial communities has not been performed yet, but this area is part of this study.

In general, two conclusions can be drawn from DGGE analysis of PCR-amplified bacterial 16S rRNA genes obtained from sponge and reference samples. First, DGGE

analysis of bacterial communities from surfaces of sponges demonstrated a high overall (intraspecific) dissimilarity to bacterial communities associated with the corresponding mesohyl of some sponge species (*A. oroides*, *C. reniformis*, *Geodia* sp. *Tethya* sp. and *P. ficiformis*). Second, the DGGE banding patterns of the bacterial communities within the mesohyl of those above-mentioned sponge species shared more (interspecific) resemblance to each other than to tissue-associated bacterial communities of the remaining sponge samples or to surface bacterial communities from the respective sponge species or from reference samples.

Regarding interspecific similarity, a notable characteristic of the 16S rDNA DGGE patterns obtained from tissue samples of *A. oroides*, *C. reniformis*, *P. ficiformis*, *Geodia* sp. and *Tethya* sp. was the dominant occurrence of GC-rich 16S rDNA fragments as compared to DGGE patterns obtained from tissue samples of *A. polypoides*, *D. avara* and *O. lobularis.* Four out of those five sponges displaying GC-rich 16S rDNA fragments were assigned to sponges that have previously been classified as (HMA) sponges. The classification occurred either at the species level for *A. oroides*, *P. ficiformis* (Vacelet and Donadey, 1977) and *C. reniformis* (Schlaeppy *et al.*, 2010), or at the genus level for *Geodia* sp. (Hoffmann *et al.*, 2005). No quantitative analysis of the bacterial concentration has been previously performed in *Tethya* spp.; thus, the assignment of *Tethya* sp. to either HMA or LMA sponges remains unresolved to date. The banding patterns obtained from tissue samples of *A. polypoides*, *D. avara* and *O. lobularis* differed clearly from those of HMA sponges. These banding patterns were dominated by GC-poor 16S rDNA fragments. These three sponge species (*A. polypoides*, *D. avara* and *O. lobularis*) were assigned to sponges that have been previously classified as LMA sponges (Schlaeppy *et al.*, 2010; Vacelet and Donadey, 1977). The GC content of nucleic acids is known to be correlated with the stability of their double helix, but the functional relevance of the GC content is still debated. Due to the higher stability of GC bonds, a correlation between higher genomic GC contents and optimal growth temperatures of bacteria adapted to elevated environmental conditions has been hypothesized. No correlation was found between the genomic GC content and the optimal growth temperature of bacteria (Galtier and Lobry, 1997; Hurst and Merchant, 2001), but a striking relationship between GC content of structural RNAs (tRNA, 5S-, 16S- and 23S rRNA) and growth temperature was reported by Galtier and Lobry (1997). However, the higher GC content displayed in the DGGE banding patterns of HMA sponges refers to the PCR-amplified 16S rDNA region between positions 8 and 534 (after *E. coli*) only. Whether the higher GC content of the 16S rDNA fragments of HMA sponges is related to a high GC content of the whole 16S rDNA and possesses a functional relevance, for example, a protective function, and thus leads to a higher occurrence of these bacteria in HMA sponges, has to be determined in further studies. Still the differences in GC content for the respective 16S rDNA fragments of sponge-associated bacteria in HMA and LMA sponges adds a further distinction between HMA and LMA sponges to the quantitative and morphological differences among the bacteria that were microscopically observed by Vacelet and Donadey (1977).

The distinction between bacterial communities of HMA and LMA sponges observable in the DGGE gel is confirmed by cluster analysis. Generally, two main clusters are apparent that broadly encompass surface samples of HMA sponges and reference samples (divided into two subclusters) and tissue samples of HMA sponges. The LMA sponge samples are distributed within those clusters. The similarities of bacterial communities associated with mesohyls from HMA sponges (*C. reniformis*, *Geodia* sp., *P. ficiformis,* and *A. oroides*) to each other that led to a clear cluster formation (cluster II) in the dendrogram containing these sponge species suggest a specific bacterial community in HMA sponges. In contrast, the LMA sponges (*A. polypoides*, *D. avara* and *O. lobularis*) harbor bacterial communities in their tissues that are clearly dissimilar to those from the mesohyl of HMA sponges. Furthermore, the bacterial communities associated with the mesohyl and surface of *A. polypoides* showed a similarity of nearly 61%. This result suggests that the bacterial community associated with the tissue of LMA sponges is rather a reflection of its surface community and probably mirrors a more transient bacteria community arising from sponge filter activity. The encrusting form of growth of both of the remaining LMA sponges *D. avara* and *O. lobularis* hampered separation of the sponge cortex from the mesohyl. Therefore, the bacterial communities associated with the mesohyl of *D. avara* and *O. lobularis* also contained all surface bacteria from the respective sponges. Thus, the high similarities between tissue and surface samples of these two LMA sponges cannot be used to confirm the assumption of unspecific and transient bacteria communities in LMA sponges.

The deduction of a specific bacterial community associated with the mesohyl of HMA sponges is also supported by the sequencing results. These results, shown in Table 2, revealed that all sequences obtained from bacteria associated with the mesohyl of HMA sponges *A. oroides*, *C. reniformis*, *P. ficiformis* and *Geodia* sp. show the highest homology to bacteria previously found in sponges. The unexceptional affiliation of all sequences derived from HMA sponges mesohyl to bacteria previously found in sponge hosts points to the occurrence of a sponge-specific bacterial community in HMA sponges, as previously reported (Hentschel *et al.*, 2002). In contrast, sequences obtained from LMA sponge *A. polypoides* and the surface samples were assigned to bacteria originally isolated from a variety of sources. This result supports the assumption that the bacterial community associated with the tissue of the LMA sponges is rather a reflection of its surface and the environmental bacterial community. However, further investigations of other LMA sponges have to be performed to confirm whether the affiliations of LMA-derived sequences to non-sponge-associated bacteria result from a lower bacterial specificity in the mesohyl of LMA sponges or simply from a lack of studies in this field.

The different GC contents of the respective 16S rDNA fragments from tissue-associated bacteria of HMA and LMA sponges and the conspicuous affiliation of these sequences to either sponge- or non-sponge hosts/sources-associated bacteria, respectively, grouped the investigated eight sponge species in HMA and LMA sponges with the exception of *Tethya* sp.. While the distribution of the fragments in the banding

profiles obtained from *Tethya* sp. corresponds rather to those of the investigated HMA sponges (though lacking some of the particularly GC-rich fragments) than to those of LMA sponges, the affiliation of the *Tethya* sp.-derived sequences mainly to non-sponge-associated bacteria is a common feature with the LMA sponge *A. polypoides*. Although bacteria associated with sponges of the genus *Tethya* have been investigated (Sipkema and Blanch, 2010; Thiel *et al.*, 2007b), no quantitative analysis of the bacterial concentration has been performed in *Tethya* spp. yet. In some studies, sponges of the genus *Tethya* were regarded as LMA sponges (Bayer *et al.*, 2007; Weisz *et al.*, 2007). However, moderate numbers of different bacterial morphotypes within the inner tissue of *T. aurantium* have been reported by Thiel *et al.* (2007b), rather suggesting a belonging of the genus *Tethya* to HMA than to LMA sponges. In addition, a belonging of *T. crypta* to HMA sponges was reported by Weisz *et al.* (2008) by an approach of comparing HMA and LMA sponges per unit volume pumping rates. Altogether, the inconsistency in literature and the ambiguous DGGE and sequencing results concerning the grouping of *Tethya* sp. suggest the assumption that some sponge species show characteristics of HMA and LMA sponges. Further investigations of bacterial communities associated with sponges of the genus *Tethya* are necessary to determine whether *Tethya* sponges can be grouped to either HMA or LMA sponges or belong to a third group that combines features of both the HMA and LMA sponges.

Based on the specificity concept of the bacterial community associated with the mesohyl of sponges, it is worthwhile determining whether the same bacterial community occurs on the surface. This study demonstrated that such a (intraspecific) similarity could not be confirmed by DGGE and cluster analysis, particularly for HMA sponges. DGGE analysis revealed a generally high dissimilarity between bacterial communities of the mesohyl from HMA sponges and bacterial communities on the surfaces of these sponges. The missing clear dissociation of the surface-associated bacterial community from that of the mesohyl-associated of the HMA sponge *P. ficiformis* in the dendrogram of the cluster analysis may explained by the lack of a vertical transmission in this species (Maldonado, 2007), which is assumed to be one reason for the sponge-specific communities observed in other HMA sponge species (Taylor *et al.*, 2007).

Comparing the number of DGGE bands separated in the gel, all sponge surface samples displayed fewer DGGE bands than the corresponding tissue samples. It is commonly accepted that in DGGE analyses the main populations only (those representing more than 1% of the target organisms in terms of relative proportion) are displayed in the banding pattern (Muyzer *et al.*, 1993). As a result, the population present within a habitat is not fully represented on the DGGE banding pattern (Fromin *et al.*, 2002). Thus, differences in the number of DGGE bands (reflecting bacterial diversity) between surface and corresponding tissue samples may simply arise from different bacterial abundances. However, regarding the main bacterial species in the populations inhabiting the surface and mesohyl of sponges, a spatial distribution in diversity was revealed by the use of

DGGE. However, the reasons for the spatial bacterial distribution in sponges remain speculative at the present time and have to be resolved in further studies.

The cluster analysis of the similarities of the bacterial communities associated with surfaces of the sponge species *Tethya* sp., *Geodia* sp., and *A. oroides* showed a clear separation from those of the corresponding mesohyl-associated bacterial communities. This underscores that bacterial communities on sponge surfaces were different from each other and from those of the tissue samples. This result correlates with the findings of Dobretsov and colleagues (2005), who reported similarities of 48% to 70% among different sponge surface communities and 38% in comparison to reference sample. A high consistency of the bacterial community on the surface of *M. adhaerens* from different sites and seasons that is different from a reference bacterial community was reported by Lee *et al*., (2006a). Quantitative and qualitative differences between bacterial communities on other hosts and inanimate references surface were also reported for other invertebrates, such as Ascidiacea (Wahl *et al*., 1994), Asteroidea (Guenther *et al*., 2007), or macroalgae (Lachnit *et al*., 2009).

A distinctive feature of the inanimate reference samples was the finding of *γ-Proteobacteria* of the genus *Pseudoalteromonas* dominating the bacterial community. The surface of one ceramic tile plates that was submerged for only 2 days showed five out of seven sequence bands that were affiliated with *Pseudoalteromonas* species (PbS-2, PbS-3, PbS-4, PbS-5, and PbS-6). The deployment of the ceramic tiles for only 2 days under sea does not allow the formation of a mature biofilm. Jones *et al.* (Jones *et al*., 2007) reported that bacterial community structures of biofilms after 2 to 4 days of growth are different from that of older biofilms. Thus, the DGGE banding patterns of the ceramic tile samples submerged for 2 days displayed the first attaching bacteria only. Two subdivisions of *Proteobacteria* the *γ- and α-Proteobacteria* seem to be successively dominating the bacterial composition in the early stages of marine biofilm formation on submerged surfaces. While *γ-Proteobacteria* seem to be abundant in the first hours of biofilm formation, *α-Proteobacteria* became dominant after 24 hours (or a day) of biofilm formation (Dang *et al*., 2008; Lee *et al*., 2008). *Pseudoalteromonas* species have been identified in both studies. However, the bacterial composition seems to be influenced by the artificial surfaces used (Jones *et al*., 2007), possibly explaining the dominant occurrence of *Pseudoalteromonas* species on the submerged ceramic tiles even after 2 days. No affiliation to *Pseudoalteromonas* from sponge-derived sequences could be derived. However, bacteria from the genus *Pseudoalteromonas* have been found on the surface of different sponge species (Chelossi *et al*., 2004; Ivanova *et al*., 2002; Lau *et al*., 2005; Lee and Qian, 2003). Furthermore, it has been demonstrated that marine *Pseudoalteromonas* species generally are found in association with marine eukaryotes and produce biologically active compounds to prevent settlement by other fouling organisms (Holmstrom and Kjelleberg, 1999).

In summary, bacterial communities associated with mesohyls of HMA sponges analyzed here generally differ from those on surfaces of the sponges and from those on reference surfaces. Additionally, clear distinctions in the 16S rDNA molecular fingerprint pattern between the bacterial communities from tissue samples of HMA sponges and LMA sponges were revealed. Bacterial communities within the mesohyl of HMA sponges show a close relationship to each other and seem to be sponge-specific.

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4.2.2 Supplementary results: Surface and Tissue Bacterial Communities of Different Sponge Species

In the study described in the first part of chapter 2 (section 4.2.1) concerning differences of surface- and tissue-associated bacterial communities of eight Mediterranean sponge species originally twelve different sponge species (*Agelas oroides*, *Chondrosia reniformis*, *Petrosia ficiformis*, *Geodia* sp., *Tethya* sp., *Axinella polypoides*, *Dysidea avara*, *Oscarella lobularis*, *Ircinia* sp., *Crambe crambe*, *Chondrilla nucula* and *Aplysina aerophoba*) have been investigated. Bacterial communities obtained from seawater, the surfaces of inanimate substrata (two ceramic tiles placed at 5 m depth for 2 days and a bottle found at the seabed) and different animate surfaces, such as five invertebrates, a sea cucumber (*Holothuroidea* sp.), a seastar (*Echinaster sepositus*), two sea squirts (*Halocynthia papillosa* and an undetermined species from the family Ascidiae), a mussel (*Mytilus edulis*), and three macroalgae (*Codium bursa*, *Valonia* sp., and *Ulva rigida*) served as references.

In general, to reduce randomly happening biases typically occur when performing PCRs (Ishii and Fukui, 2001; Suzuki and Giovannoni, 1996) triple amplification of every single sample occurred. To reveal possible inequalities between these triplicate samples the separation of the three replicates of one sample has been performed by the use of DGGE. Figure 13a to c shows the triplet banding patterns obtained from the bacterial communities of the different tissue (a) and surface (b) samples of the sponges and of the references (c). Comparing the triplet samples the majority of bands within the banding patterns were represented in all three samples of one specimen/reference sample, revealing that little bias occurred during PCR only. However, a single band that was present in only one of the three lanes of one sample, is displayed in the banding pattern of the tissue samples of *A. aerophoba* and the surface sample of one the two ceramic tiles (marked by arrows in Figure 13a and c). DGGE gel revealed also inequalities in the surface samples of *A. polypoides*, *Tethya* sp. and *C. reniformis*. Comparing the lanes of the triplet samples of these three sponges one of the three lanes showed no or weak banding pattern (Figure 13b). The influence of random events during PCR for following DGGE analysis has been reduced by performing triplicate PCR amplifications of one sample and mixing them before further analyses occur as recommended by Kanagawa (2003).

Not every sample yielded a result in the amplification of the bacterial 16S rDNA. Thus, subsequent DGGE analysis failed in displaying a banding pattern of those samples. Out of the twelve originally investigated sponge species the amplification of the bacterial 16S rDNA obtained from both the surface and the tissue of eight sponge species (*A. oroides*, *C. reniformis*, *P. ficiformis*, *Geodia* sp., *Tethya* sp., *A. polypoides*, *D. avara* and *O. lobularis*) resulted in a sufficient PCR-product only and therewith in a DGGE banding pattern.

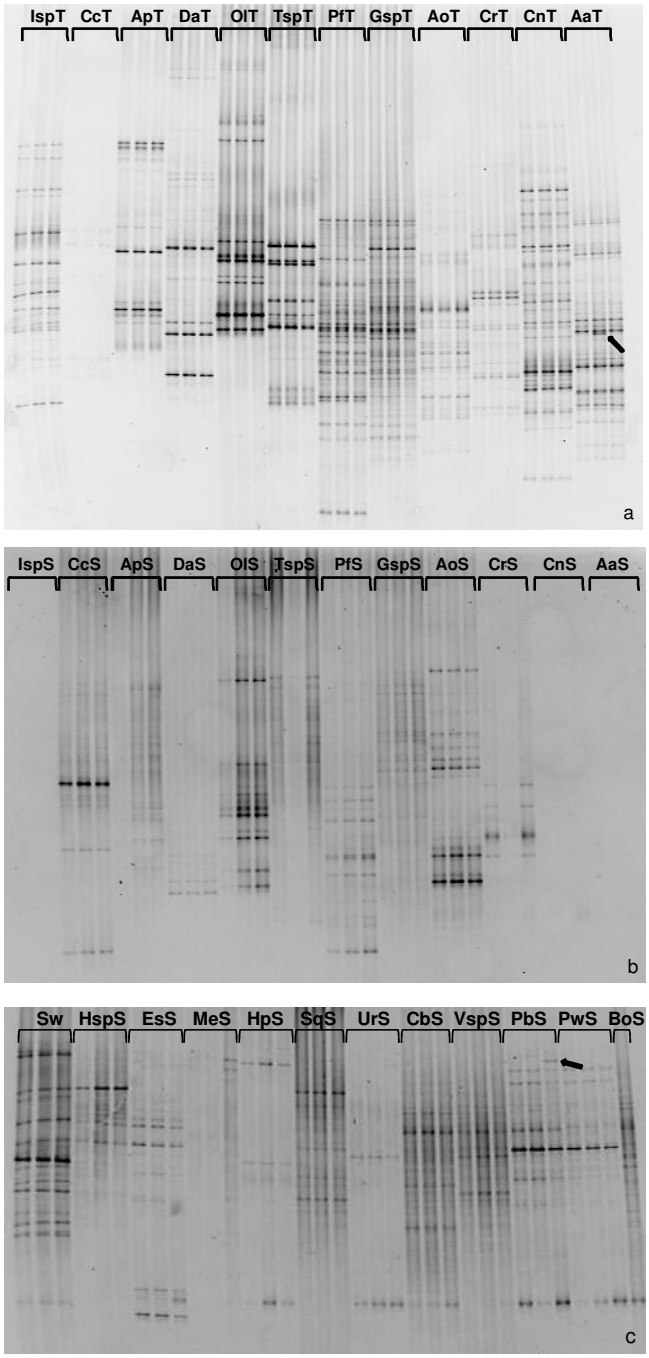

Figure 13a-c. Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA fragments obtained from tissue (a) and surface samples (b) of twelve different sponge species and twelve reference samples (c). Figure 13a and b display the triple banding pattern obtained from tissue (-T) and surface (-S) samples from *Ircinia* sp. (Isp), *C. crambe* (Cr), *A. polypoides* (Ap), *D. avara* (Da), *O. lobularis* (Ol), *P. ficiformis* (Pf), *Geodia* sp. (Gsp), *A. oroides* (Ao), *C. reniformis* (Cr), *C. nucula* (Cn) and *A. aerophoba* (Aa). Figure 13c displays the triple banding pattern obtained from reference surface (-S) samples from seawater (Sw), *Holothuroide*a sp. (Hsp), *E. sepositus* (Es), *M. edulis* (Me), *H. papillosa* (Hp), a sea squirt (Sq), *U. rigida* (Ur), *C. bursa* (Cb), *Valonia* sp. (Vsp), two ceramic tiles (Pb and Pw) and from a bottle (Bo). The arrows in Figure 13a and c mark bands that are only present in one of the triplicate lanes of one sample.

In contrast, the remaining four sponge species samples (C. crambe, *Ircinia* sp., *C. nucula* and *A. aerophoba*) did not provide sufficient PCR products either from the surface or from the tissue samples. A distinct DGGE banding pattern of the amplified 16S rDNA product has been obtained from the surface sample (CrS) but not from the tissue sample (CrT) of *C. crambe*. The surface sample of *C. crambe* displayed a very weak banding pattern composed of two to four bands per lane only. In contrast, DGGE banding patterns have been obtained from the tissue samples of *Ircinia* sp. (IspT), *C. nucula* (CnT) and *A. aerophoba* (AaT) but not from the respective surface samples of these sponges. All

reference surface samples displayed a DGGE banding pattern, however, some showed weak bands in the DGGE gel such the samples of *H. papillosa* (HpS), *M. edulis* (MeS) and *U. rigida* (UrS). Of the originally twelve sponge species and eight reference samples eight sponge samples and six reference samples were further investigated by cluster and sequencing analysis (as previously presented in the first part of chapter 2 (section 4.2.1).

However, whether the unsuccessful amplification of bacterial 16S rDNA obtained from the tissue sample of *C. crambe* or from the surface samples of *Ircinia* sp., *C. nucula* and *A. aerophoba* resulted from a sparsely bacterial occurrence in those samples only or simply by an erroneous PCR remains unclear. In general, it is well known that the efficacy of PCR may be dramatically reduced when applied directly to biological or environmental samples, due to the occurrence of inhibitory substances (Radstrom *et al*., 2004; Wilson, 1997). Optimization of the PCR protocol occurred, including dilution of the 16S rDNA templates to reduce possible inhibitory effects by inhibitory substances within the samples. However, it cannot excluded that secondary metabolites in the sponges samples still remained in concentrations that led to an insufficient amplification of the 16S rDNA by the use of PCR. Especially, the extraction of 16S rDNA from the tissue sample of *C. crambe* involves the possibility of an insufficient elimination of the secondary metabolites of the sponge samples during DNA extraction. A strong antimicrobial activity has been reported for crude tissue extracts from *C. crambe* (Becerro *et al*., 1994; Becerro, 1997). Thus, the occurrence of secondary metabolites in the sample of *C. crambe* could have led to the unsuccessful attempt to amplify the bacterial 16S rDNA. DGGE analysis of the 16S rDNA obtained from the surface sample of *C. crambe* revealed the occurrence of a bacterial community on the surface of this sponge species. However, only few bacteria were displayed in the DGGE from the tissue sample of *C. crambe*. The result of the distinct DGGE banding pattern obtained from the surface sample of *C. crambe* is in contrast to the findings of Becerro *et al.* (1994) who reported that no bacteria were found on the surface of *C. crambe* by microscopic investigation and only few isolates were obtained from the inner part of these sponge species. Furthermore, no DGGE banding pattern has been obtained from the surface samples of *Ircinia* sp., *C. nucula* and *A. aerophoba*. The absence of bacteria on the surface of *Ircinia* sp. is also inconsistent with the finding of Becerro *et al.* (1994) who found epibionts present on the surface of *Ircinia fasciculata*. No classification of the species of the specimen of the genus *Ircinia* has been performed. Thus, one possible reason for the inconsistency of the results in comparison to the findings of Becerro *et al.* (1994) could be that different species of same genera show different epibacterial fouling. However, to confirm an epibiotic free surface of the sponge species *Ircinia* sp., *C. nucula* and *A. aerophoba* microscopic analysis has to be performed in further studies.

5 Conclusions and Outlook

In this work it was shown that a biotechnologically *ex-situ* cultivation of sponges provides a promising approach to the supply of natural products from sponges, due to the high stability of the associated bacterial community in *A. aerophoba* and the maintenance of the secondary metabolites during cultivation. Based on the two hypotheses that were previously considered to be responsible for the difficulties in the establishment of enduring sponge cultivation, those of a loss of, for the sponges physiology and metabolism, essentially required sponge-associated microorganisms during sponge cultivation, could be excluded by this study.

As a result, when examining the feasibility of growing sponges in aquaculture for the production of natural products, the qualitative and quantitative aspects of abiotic cultivation conditions and the adequate supply of nutrients should be in the focus.

Furthermore, at academic level the gain of knowledge regarding bacterial communities in and on sponges can be summarized as follows:

- Bacterial communities on surfaces and mesohyls of HMA sponges investigated here generally differ from each other and from those on reference surfaces.
- Bacterial communities within the mesohyl of HMA sponges show a close relationship to each other. This supports the concept of a specific bacterial community associated with the mesohyl of HMA sponges.
- Bacterial communities on surfaces and in mesohyls of LMA sponges showed the most pronounced similarities. The bacterial community of LMA sponges seems to be less sponge-specific.
- Bacterial communities from the mesohyl of HMA sponges showed a dominant occurrence of bacteria with high GC content in 16S rDNA PCR amplicons in comparison to the mesohyl-associated bacterial communities of LMA sponges or to surface bacterial communities of HMA and LMA sponges.

Future works should focus on the development of a sponge cultivation setup that ensures a sufficient nutrient supply to the sponges without impairment of the water quality in order to obtain growing sponges. Subsequent analysis of the bacterial community can reveal whether the sponge-associated bacterial diversity will be maintained also in growing sponges. In addition, analysis of the secondary metabolites pattern in growing sponges would reveal whether the increase in the secondary metabolite concentrations is based on the accumulation of the spherulous cells in disintegrating sponges. Furthermore, investigations of mesohyl- and surface associated bacterial communities of LMA sponges should be performed to confirm dominant occurrence of bacteria with low GC content and lower sponge-specific bacteria associations in those sponges.

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www.springerlink.com

7 Appendices

7.1 Abbreviations

Ara-A: Adenine-arabinoside

Ara-C: Cytosine-arabinoside

ASW: Artificial seawater

AZT: Azidothymidine

BC: Before Christ

bp: base pair

DGGE: Denaturing gradient gel electrophoresis

DM: Dry matter

DNA: Deoxyribonucleic acid

dNTP: Desoxy nucleoside triphosphate

DTP: Developmental Therapeutics Program

EDTA: Ethylenediaminetetraacetic acid

et al.: Et alia

GC: Guanin-Cytosin

HIV/AIDS: Human immunodeficiency virus/acquired immune deficiency syndrome

KIT: Karlsruhe Institute of Technology

LMA: High-microbial abundance sponges

LMA: Low-microbial abundance sponges

Ma: Mega Annum (one million years)

NCI: United States National Cancer Institute

NIH: National Institutes of Health

PCR: Polymerase chain reaction

rDNA: Ribosomal deoxyribonucleic acid

rRNA: Ribosomal ribonucleic acid

sp.: Species

TAE: Tris-acetate-EDTA

Tris: Tris(hydroxymethyl)aminomethane

Tm: Trade mark

U: Unit

Uv: Ultraviolet

Units

cm: Centimetre

g: Gram

kg: Kilogram

L: Liter

mg: Milligram

mL: Milliliter

mM: Millimol

ng: Nanogram

pM: Picomol

s: Second

T: Ton

µm: Micrometer

µmol: Micromolar

Abbreviations of investigated samples

Cb: *Codium bursa*

Sw: Seawater

Es: *Echinaster sepositus*

Hsp: *Holothuroidea* sp.

Pw & Pb: Two ceramic tiles

Tsp: *Tethya sp.*

Gsp: *Geodia* sp.

Ao: *Agelas oroides*

Ap: *Axinella polypoides*

Da: *Dysidea avara*

Ol: *Oscarella lobularis*

Pf: *Petrosia ficiformis*

Cr: *Chondrosia reniformis*

7.2 16S rDNA Sequences of DGGE Analyses Presented in Chapter 1 and 2

7.2.1 16S rDNA Sequences of DGGE Analyses Presented in Chapter 1

16S rDNA Sequences of *A. aerophoba* Sponges number 49 and 50 (DGGE Analyses with Primer GC 341F/518R)

1 (49_50_518R)

GGTCTTCTTCCTTGTAgAAAAAGAGGTTTACAGCCCAAAGGCCTCCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTC
GCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

2 (49_50_518R)

GTCaATTTCGTCCCTGCTGAAAGGGGTTTACGACCCGAAGGCCTTCTTCCCCCACGCGGCGTCGCTGCGTCAGGCTTT
CGCCCATTGCGCAATATTCCCTCCTGCTGCCTCCCGTAGG

3 (49_50_518R)

CACTACTGAAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGCATTGCTGCATCAGGCTTGCGCCCATTGTG
CAATATTCCCCACTGCTGCCTCCCGTAGG

4 (49_50_518R)

TCAATTTCGTCCCTGCTTAAAGGGGTTTACAACCCGAGGGCCTTCATCCCCCACGCGGCGTTGCTGCGTCAGGCTTTC
GCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGG

5 (49_50_518R)

GTCATTTCGTCCCTGCTGAAAGGGGTTTACGACCCGAAGGCCTTCTTCCCCCACGCGGCGTCGCTGCGTCAGGCTTTC
GCCCATTGCGCAATATTCCCTCCTGCTGCCTCCCGTAGG

6 (49_50_518R)

CCCTACCNCGTCACATATAAAAGGACTTTACGATCCGAAAACCTTCTTCGCCCACGCGGCGTCGCTGCATCAGGCTTC
CGCCCATTGTGCAATATCCCTTACTGCTGCCTCCCGTAGG

7 (49_50_518R)

TAATAACNTCAGACCCGGGGGGTATTAACCCCAGGCTTTTCTTCaCtACTGAAAGTGCTTTACAACCCGCAGGCCTTCTTC
ACACACGCGGACATTGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAgG

8 (49_50_518R)

GTACCGTCATTTCGTCCCTGCTGAAAGGGGTTTACAACCCGAGGGCCTTCATCCCCCACGCGGCGTTGCTGCGTCAGG
CTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGG

9 (49_50_518R)

AACCNCCTACCTCGTCACATNATTAAAAGGNACTTTACNATCCCGAAAACCTTCTTCAcCCAcGCGGCGTCGCTGCATCA
GACTTCCGTCCATTGtGCAATATCCCTCACTGCTGCCTCCCGTAGG

10 (49_50_518R)

TCATGACGCATGCCCTGTTtGAACATGCGTTGTtCCTTCCNATCTGACAGTGGTTTACACCCCGAAAGGCTTCCTCCCAC
ACGCGGCGTCGCTGCGTCAGTCTTGTCGACCATTGCGCAATATTCCCTACTGCTGCCTCCCGTAGG

11 (49_50_518R)

AATTTCCGTGCCCTGCTGAAAGGGGTTTACGACCCGAAGGCCTTCTTCCCCCACGCGGCGTCGCTGCGTCAGGCTTTC
GCCCATTGCGCAATATTCCCTCCTGCTGCCTCCCGTAGG

12 (49_50_518R)

CCTCTNCNCGTCACATANTNAAAGGGCTTTACGATCCGAAAACCTTCTTCACCCACGCGGCGTCGCTGCATCAGACTTC
CGTCCATTGTGCAATATCCCTTACTGCTGCCTCCCGTAGG

16S rDNA Sequences of *A. aerophoba* Sponges number 55 and 56 (DGGE Analyses with Primer GC 341F/518R)

1 (55_56 518R)

CNGTGAAATNTAGGCNCGGNCGTCTTTTTTTCTGGCAGGTAACCCGTNCAATTTTCGTNCCCTGACTGNAAAGGGGTTT
ACGACCCGAAGGCCTTCTTCCCCCACGCGGCGTCGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCTCCTGCTG
CCTCCCGTAGG

2 (55_56 518R)

GATTAGCNCGAACTTATTNCCTATGCTACCGTNCCTTTCCTCGTCACATAGTAAAAGGGCTTTACGATCCGAAAACCTTC
TTCACCCACGCGGCGTCGCTGCATCAGACTTCCGTCCATTGTGCAATATCCCTTACTGCTGCCTCCCGTAGG

3 (55_56 518R)

CCGGAGTTTAGCCCGANGTCTTTTTCTGGCGANNANCGTTCCAGGTAACNAGGCTATTAACNTATNCCCTTTCCTCCTC
GCTGAaAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATGGcTGCATCAGGCTTTCGCGCATTGTGCAATA
TTCCCCACTGCTGCCTCCCGT

4 (55_56 518R)

CCGGAGATTAGCCGGGGTTTCTTTACcaGAAACTGTcaTTATcATCTCTGGCGAAAGAGCTTTACGACCCTAGGGCCTTC
ATCACTCACGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTCCAAGATTCCCCACTGCTGCCTCCCGTAGGC

5 (55_56 518R)

CGGAGNTANCCTGGCTTNTTTCTGAGTACCGTCAGGTCTTCTTCCTTGAgAAAAGAGGTTTACAGCCCAAAGGCCTCCA
TCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

6 (55_56 518R)

CNGTGATTNGCCGCGCTTCTTCTGCNGTACCGTCAATTTCGTCCCTGCTGAAAGGGGTTTACGACCCGAAGCCTTCTTC
CCCCACGCGGCGTCGCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCTCCTGCTGCCTCCCGTAGG

16S rDNA Sequences of *A. aerophoba* Sponges number 59 and 60 (DGGE Analyses with Primer GC 341F/518R)

1 (59_60_518R)

CCGGAGNTAGCCTGGTCTTATTTTTGAGGTACCgTCaGGTCTTCTTCCTTGAgAAAAGAGGTTTACAGCCCAAAGGCCTC
CATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

2 (59_60_518R)

CCGTGATTAGCCGACGNTNTTCCCGGGTACTGTCCTTTCTCATCCCCGGGAAAAGGGGTTTACAACTCGAGGgCCTTC
ATCCCCCACGCGGTGTCGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCTGCTGCTGCCTcCCGTAGG

3 (59_60_518R)

GGNTGGGCCGNTNNCCCCCNGGGGGNNCCCCCATTTCCNTCCCTGTTNNTTGGGGGNAANAANCCCCGGGCCTTCCT
CCCCCACGCGGCGTNGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCtCCCGTAGG

4 (59_60_518R)

GGNTGGGCCGNTNNCCCCCNGGGGGNNCCCCCATTTCCNTCCCTGTTNNTTGGGGGNAANAANCCCCGGGCCTTCCT
CCCCCACGCGGCGTNGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCtCCCGTAGG

5 (59_60_518R)

CGGGAGNTANCCTGGCTTATTcCTcAaGTACCGTCaGGTCTTCTTCCTTGAgAAAAGAGGTTTACAGCCCAAAGGCCTCC
ATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

6 (59_60_518R)

CNGGAGNTTANCCCGGATGTCTTTTTTTGTCGAGTAACGTCCCAGGTAACAAGGATATTAACATTATTTCCNTTTACCTC
CCTACGACTGTAAAGTGCTTTACAACGCCTAAGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTTCGCCGCA
TTGTGCAATATTCCCCACTGCTGCCTCCCGTAGG

7 (59_60_518R)

GGNTTGGCCGNNNTCCCCCTGGGGTACCNCCCTTTCCCTTCCCTTNTTCTTGGGGNTNANAACCCCGAAGGCCTTCGT
CCCCCACGCGACGTCGCTGGGTCAGGCTTTCGCCCATTGCCCAAGaATTCCCtGCTGCtGCCtCCCGTAGG

8 (59_60_518R)

CNTGATTAGCGGATNCTTATTTCGTAAGTTACCGTNCATATTCTTNACTAACAAAAGGCAGTTTACAATACCGAAGAACC
TTTCGTTCCTGTCACGCGGTGTCGCTCCGTCAGGCTTTCGCCCATTGCGGAGAATTCCTTACTGCTGCCTCCCGTAGG

9 (59_60_518R)

CNGTGATTGGCCGGGTCTTTTTCTGCNGTACCGTCATTTNCGTTCCCTGCTGAAAGGGGTTTACAACCCGAANGCCTTC
ATCCCCCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGG

10 (59_60_518R)

CNGGAGATTAGCCCGGTGTTTCNNCNTGTAGGCTANCNCCCAAGTACTCCGAGGTCTATTTATTTCCGCGGGACTATN
ANCCCCGCTACTGAAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGCATGTGCTGCATCAGGCTTGCGCCC
ATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGG

11 (59_60_518R)

CCGTATTANAGCCTTNTTNTTGGCTACTGTCGNATCTTCACCAATAAAAGCAGTTTACGATCCAAGAACCTTCATCCTGC
ACGCGGCGTTGCTCCATCAGGCTTGCGCCCATTGTGGAAAATTCCCTACTGCTGCCTCCCGTAGG

12 (59_60_518R)

CGGAGNTAGCCTGNTTTCNCTTTGAGGTACCNCCGGTCTTCTTCCTTGTNTNNAAGGAGGTTTACAGCCCAAAGGCCT
CCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

16S rDNA Sequences of *A. aerophoba* Sponges number 10, 11, 17 and 24 and Seawater (DGGE Analyses with Primer GC 341F/518R)

1 (10_11_17_24 and seawater 518R)

CCGTGATTAGCCTGGCCTTATTTCGCCGGGTACCGTCAAAATTCTTCCCCgGGTCAAAAGGGATTTACAACCCTAAGGC
CTTCATCACCCACGCGGCGTTGCTGCATCAGGCTTTCGCCCATTGTGCAAGAATTCCTCGCTGCTGCCTCCCGTAGG

2 (10_11_17_24 and seawater 518R)

TAGCNCTGGCTTATTCCTCAGTACCGTCAGGTCTTCTTCCTTGAgAAAAGAGGTTTACAGCCCAAAGGCCTCCATCCCT
CACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGG

3 (10_11_17_24 and seawater 518R)

CNGGAATTAGCCCGGGGCCTTACTTNTACGGTTACCCGTNCATTATNCTTNCACCGTTGAAAGAGTTTTACAACCCGAA
GGCCGTCTTCACTCACGCGGCATGGCTGGATCAGGGTTTCCCCCATTGTCCAAGATTCCTTACTGCTGCCTCCCGTAG
G

4 (10_11_17_24 and seawater 518R)

GNTTAGCCGAACTTNTTTCTATGGCTACCGTCCTCTCTCGTCACATAGAAAAGGGCTTTACGATCCGAAAACCTTCTTCA
CCCACGCGGCGTCGCTGCATCAGACTTCCGTCCATTGTGCAATATCCCTTACTGCTGCCTCCCGTAGG

5 (10_11_17_24 and seawater 518R)

CNNGATTAGCCGNGTTTTTTCTGCNGTACCGTCaATTTCGTTCCCTGACTGAAAGGGGTTTACAACCCGAAGGCCTTCA
TCCCCCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGG

6 (10_11_17_24 and seawater 518R)

CGGAAtTAGCCGGTGCTTAtTCTGCAGGTAACaTCAAGGATTATAAGGTATTAACTTATAACTTTTTCTCCCTGCTTAAAGT
GCTTTACAACCCgAAGGCCTTCTTCACACACGCGGTATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACT
GCTGCCTCCCGTAGG

7 (10_11_17_24 and seawater 518R)

AY828394CNGGAATTAGCCGNNCTTTTTNTTCGGGTACAgTcaTTTTCTTCCCCGACGAAAGAGCTTTACAACCCAAAGG
CCTTCTTCACTCACGCGGCATCGCTGCATCAGAGTTTCCTCCATTGTGCAAGATTCCCCACTGCTGCCTCCCGTAGG

8 (10_11_17_24 and seawater 518R)

ANTANCCGTTTTTCNTCTTCGGGTACCCCNTTTTCTTCCCCGTATTNTTTAGCTTTACAACCCACGGGCCTTCTTCACTC
ACGCGGCATCGCTGCATCAGAGTTTCCTCCATTGTGCAAGATTCCCCACTGCTGCCTCCCGTAGG

7.2.2 16S rDNA Sequences of DGGE Analysis Presented in Chapter 2

16S rDNA Sequences from Tissue Samples of Different Sponge Species (DGGE analyses with Primer GC 27F/518R)

>AoT-1 6086762 sequence exported from AoT-1.ab1

TCGACNNATAGAAAGGGCTTTACGATCCGAAAACCTTCTTCGCCCACGCGGCGTCGCTGCATCAGACTTGCGTCCATT
GTGCAATATCCCTCACTGCTGCCTCCCGCAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGGGGTACACCCTCTCA
GGCCCCCTACCCGTCATCGCCTTGGTGAGCCTTTACCTCACCAACCAGCTGATGGGATGCAACCTCATCCTGTAGCAC
CTCAGTCTTTTCTCACAGCCTTCTAATCACCGTGAGCTTAGTCGGTTTTAGCTGCTGTTTCCAGCAGTTATCCCGCTCTA
CAGGGCAGATCAGTTACTTGTTACTCACCCGTTCGCCACTCTCCACGTCCGAAGACGTTTCCCGTTCGACTTGCATGTG
TTAGGCACGCCGCCAACGTTCATCCTGAGCCATGATCAAACtct

>AoT-2 6086774 sequence exported from AoT-2.ab1

TCTTCNCNTNGAGAAAGAGGTTTACAACTCGAAAGCCGTCATCCCTCACGCGGCGTCGCGGCATCAGGCTTTCGCCCA
TTGTGCANGATTCCTTGCTGCTGCCTCCCGGAGGAGTGGGGGCCGTGTCTCAGTCCCCCTCTGACTGGTCATGCTCTC
ACACCAGCTACCCGTTTATGGCTTGGTGTGCCGTTACCACACCAACTACCTGATAGGACGCAGGCCCATCCTAGAGCG
ATAAATCTTTCCTCTACAAGCTCTTAGCAAGCAGAGTGTATCCGGTATTAGCAGCGATTTCTCGCTGTTATCCCGAACTC
AAGGGCAGGTTACCCACGTGTTACTCAGCCGTCGGCCACTAGGGAACTCACCCGAAGGTGAGACCTCGTTCGACTTG
CATGCATTAGGCGCGCCGCCAGCGTTCGTCCTGAGCCATGATCAAaCtct

>AoT-3 6086786 sequence exported from AoT-3.ab1

CGTCACACAGAAAGGGCTTTACGATCCGAAAACCTTCTTCGCCCACGCGGCGTCGCTGCATCAGACTTCCGTCCATTG
TGCAATATCCCTTGCTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGGGGAACGCCCTCTCAG
GCCCCCTACCCGTCATCGCCTTGGTGAGCCATTACCTCACCAACCAGCTGATGGGATGCAACCTCATCCTGTAGCGCC
TCAGCCTTTTCTCACGGGCTTCTAGTCACCGTGAGCTTAGTCGGTATTAGCTGCTGTTTCCAGCAGTTATCCCGTTCTA
CAGGGCAGATCAGTTACTTGTTACTCACCCGTTCGCCACTCTCCACGCCCGAAGGCGCTTCTCGTTCAACTTGCATGCA
TTAGGCACGCCGCCAACGTTCATCCTGAGCCNNNNNCAAACtct

>AoT-4 6086810 sequence exported from AoT-4.ab1

CTTCTTCCCCGTCAAAAGAGCTTTACAACCCGAAGGCCTTCGTCGCTCACGCGGCGTTGCTGCGTCAGGCTTTCGCCC
ATTGCGCAAGATTCCTCGCTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAGTCTGGCTGATCATCCTCT
CAGACCAGCTACCGATCGTCGCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATCGGACGCAGGCCCCTCCCAAGG
CGCCGGAGCTTTCCCCACCGCATACACGGTGGGCTCATGCGGTATTAGCTCGCCTTTCGACGAGTTGTTCCGCACCTC
GGGACAGGTCACCTACGCGTTACTCAGCCGTTTGCCACTCAGTCTCCCCACCCGAAGGCGGGGGCTTCGTTCGACTT
GCATGCGTAAGGCACGCCGCCAGCGTTTATCCTGAGCCATGAtcAAACtct

>AoT-5 6086834 sequence exported from AoT-5.ab1

GGAGAaTTGACCGACAAGTAGTTCGTCCCGCCTGACAGGggttTACAACCCTGAGGCCTTCCTCCCCCACGCGGCGTCG
CTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
ATGTGGCTGATCACGCTCTCACGTCAGCTACCCGTCATAGCCTTGGTAGGCCGTTACCCCACCAACAAGCTGATAGGC
CGCGAGCTCATCCGAAGGCGCCAGCATAGAAGAGGCCGACTTTCACGCCGGGCATCCAGTCCCGGCGCTGTACGCG
GTATTAGTCCAGGTTTCCCTGGGTTATCCCCCACCCTCGGGTAGATCGCTCACGTATTACTCACCCGTTCGCCGCTTTC
CCGATTCCCGAAGGAACCGTTCTCGCACGACTTGCATGTGTTAGGCaCgNCGCCAGCGTTCGTTCTGAGCCATGATCA
AACtct

>ApT-1 5992378 sequence exported from ApT-1.ab1

AAAGTGCTTTACAACCCACAGGCCTTCTTCACACACGCGACATCGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATTCTCTCAAACCAGCTACTGA
TCGTCGCCTTGGTGAGCCTTTACCTCACCAACAAGCTAATCAGACATAGGCTCATCCGATAGCACGAGGCCCGAAGGT
CCCCCGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCGTGCGTTTCCACACGTTATCCCCCGCTACTGGGTAGATTCC
TATGTATTACTCACCCGTCCGCCACTCGTCAGCACCAAGTTCACCCCGAAGGGATCACAGCGGTCTGTTACCGTTCGA
CTTGCATGTGTTAAGCATGCCGCTAGCGTTCAATCTGAGCCATGATCAAACTCT

>ApT-2 5992519 sequence exported from ApT-2.ab1

AAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGTGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACCAGCTACTGA
TCGTTGCCTTGGTAAGCCTTTACCTTACCAACAAGCTAATCAGACATAGACTCATCTGATAGTGCCAGGCCAAAAGGTC
CCCAGCTTTCTCCCGTAGGACGTATGCGGTATTAATCCGAGTTTCCCCGGGCTATCCCCCGCTATCAGGTAGATTTCTA
TGCATTACTCACCCGTCCGCCACTCGTCGGCGCAGAAGCAAGCTCCTGCCCGTTACCGTTCGACTTGCATGTGTTAAG
CATGCCACCAGCGTTCAATCTGAGCCATGATCAAACTCT

>ApT-3 5992532 sequence exported from ApT-3.ab1

AAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTATAGA
TCGTCGCCTTGGTAGGCCTTTACCCCACCAACAAGCTAATCTAACGCAGGCTCATCCGACAGCGACAGCTTTCAAGAA
GAGGCCATCTTTCCCCCGTAGGGTATATGCGGTATTAGCTCCGGTTTCCCGGAGTTGTCCCCCGCTGTCGGGCAGATT
CCTACGCGTTACTCACCCGTCCGCCACTCGTCAGCACAAAAGCAAGCTTCTGCCTGTTACCGTTCGACTTGCATGTGTT
AAGCATGCCGCCAGCGTTCAATCTGATCCATGATCAAACTCT

>ApT-4 6044823 sequence exported from ApT-4.ab1

GCTGGATCAGGGTTTCCCCCATTGTCCAAGATTCCTCACTGGTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCC
AGTGGGGCTGATCATCCTCTCAAACCAGCTATGGATCGTCGGCTTGGTGGGCTTTTACCCCACCAACAACCTAATCCAA
CGCGGGCCGATCTTTCGGCGATAAATCTTTCCCCCTGAGGGCGTATGCGGAATTAGCCTGAGTTTCCCCAGGTTGTTC
CCCACCGAAGGGCACGTTCCCACGCGTTACTCACCCGTTCGCCGCTCGGTCCGAAGACCGCGCTCGACTTGCATGTG
TTAAGCCTGCCGCCAGCGTTCGTTCTGAGCCGTGATCAAACTCT

>ApT-5 5964771 sequence exported from ApT-5.ab1

GANCTTTACAACCCTAAaGCCTTCTCAATCACGCGGCATGGCTGGATCAGGGTTTCCCCCATTGTCCAAGATTCCTCAC
TGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAACCAGCTATGGATCGT
CGGCTTGGTGGGCTTTTACCCCACCAACAACCTAATCCAACGCGGGCCGATCTTTCGGCGATAAATCTTTCCCCCTGA
GGGCGTATGCGGAATTAGCCTGAGTTTCCCCAGGTTGTTCCCCCACCGAAAGGCACGTTCCCACGCGTTACTCACCCGT
TCGCCGCTCGGTCCGAAGACCGCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCGTTCTGAGCCATGATCAA
ACTCT

>CrT-1 5964743 sequence exported from CrT-1.ab1

TCTTCACTCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCTCGCTGCTGCCTCCCGTAGGAGTC
TGGGCCGTTTCTCAGTCCCAGTCTGGCTGATCATCCTCTCAGACCAGCTATCGATCGTCGCCTTGGTGAGCCGTTACC
CCACCAACTAGCTAATCGAACGCAGGCCCCTCCCAAGGCGCCGGAGCTTTCCCCACCAGGCCGAACCGGTGGGCTCA
TGCGGTATTAGGCCGCCTTTCGACGGATTGTTCCGCACCTCGGGACAGGTCACCTACGCGTTACTCAGCCGTTTGCCA
CTCAGTCTCCCCACCCGAAGGCGGGGGCTTCGTTCGACTTGCATGCGTAAGGCACGCCGCCAGCGTTTATCCTGAGC
CATGATCAAACTCT

>CrT-2 5964744 sequence exported from CrT-2.ab1

GATGGCTGGATCATGGTTGCCCCCATTGTCCAAgATTCCCCACTGCTGCCTCCCGCAGGAGTCTGGGCCGTATCTCAG
TCCCAGTGTGGCTGATCATCCTCTCAAACCAGCTACTGATCGTAGGCTTGGTGAGCCGTTACCTCACCAACAACCTAAT
CAGACGCGGGCCGATCTTTCAGCGATAAATCTTTCCCCCGGAGGGCGTATGCGGTATTAATCCAAGTTTCCCTAGGCT
ATTCCGCACTGAAAGGCACGTTCCCACGCGTTACTCACCCGTTCGCCGCTAAGTCCGAAGACTTCGCTCGACTTGCAT
GTGTTAGGCCTGCCGCCAGCGTTCGTTCTGAGCCATGATCAAACTCT

>CrT-3 5964745 sequence exported from CrT-3.ab1

CCCGTCGGGCGATGGAGTTGTGGTTGACCGGAGCATGGCTGCATGCGGCTGCGGCGCCTTGNGCAATATTCCCCGCT
GCTGCCGCCCGTAAGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCACCTACGGATCGTT
GCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACGCATGCTCATCCAATAGTGCAAGGTTCCGAAGAGGCCC
CTGCTTTCCACCGCTCTCCTAAGAAAGCTAGTGCGTATGCGGTATTAATCCGGATTTCTCCGGGCTATCCCCCGCTACT
GGGTAGATTCCTACGTGTTACGCACCCGTCCGCCGCTCGTCAGCAACTAGCAAGCTAGTTCTGTTACCGCTCGACTTG
CATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCT

>CrT-4 5964746 sequence exported from CrT-4.ab1

CCCTGGGAACCCGTGGCTACTCCCCCGTAGTCTTGGCCGTATTGGAAACGGTTAAGTATAGCTCAGGCGCCTTGGGAA
GGGCTCGCGTTCGATTATCTGGTTGGTGGGGTAACGGCCTACCAAGGTGATGATCGATAGCTGGTCTGAGAGGATGAT
CAGCCACACTGGGACTGAGATACGGCCCAAACTCCTATGGGGGGCAGCAGCGAGGAATATTGGACAATGGGCGCAAG
CCTGATCCAGCAACGCCGCGTGGAGGAAGAAGGCCTTTGGTTGTAAACTCCTTTTGTGCGGGATGAGACAAGGACAGT
ACCGCACGAATAAGTCACGGCTAACTAGGTGCCAGCAGCCGCGGTAAT

>CrT-5 5964747 sequence exported from CrT-5.ab1

CCCTGAGGTACGGAATAACTGCTGGAAACGGCTGCTAATACCGTATGTGCACTACGGGGGAAAGATTTATCGCCTTGG
GACGGGCCCGCGTCGGATTAGCTTGTTGGTGGGGTAACGGCCCACCAAGGCTCCGATCCGTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGAACTGAGACACGGTCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGG
AAACCCTGACGCAGCCATGCCGCGTGAGTGAAGAAGGTCTTCGGATTGTAAAGCTCTTTCAACGGGGAAGATGATGAC
GGTACCCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAA

>CrT-6 empty228 sequence exported from CrT-6.ab1

ATTGCGTGTATCANGCGTTTCNCNCATTGTCCAATATTCCCCANTGNTGCCTCCCGTAAGANTCTGGGCCGTGTCTCAN
TCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTATCNATCATCGCCTCGGTGAGCCGTCACCTCACCGACAAGCTAA
TCGAACGCAGGACCCTCCGAAAGCGGCTGAANCCTTTCCCCCTTGGGGCGTATGCGGTATTAGCCGCCGTTTCCAGC
GGTTGTCCCCCACTTCCGGGCAGATTCCTACGCGTTACTCACCCGTCCGCCACTCCACATGCCGCCCGAAGGCGACA
GCGGCGTCCGACTTGCATGTGTTAGGCATGCCGCCAGCGTTCGTTCTGAGCCATGATCAAAcTCT

>CrT-7 5992307 sequence exported from CrT-7.ab1

CCGAAGGTCTTCATCCTTCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCANTATCCCGTACTGCTGCCCAC
CGTAGCGGTCTGGACCGTGTCTCAGTTCCAGTGTGGCCGATCACCCTCTCAGGCCGGCTACCGATCGTCGCCTTGGT
GAGCCATTACCCCACCAACTAGCTAATCGGACGCGGGCCCCTCCTGGAACGGCAGCTTTCAAGAATAGGCCACCTTTC
CTCGCCGGCGCCGAAGCGCCGGCGAGCTTATGCGGCATTAGCTCATGTTTCCCCAAGTTATTCCCCTTTCCGAGGCAG
GTTACCCACGTGTTACTCACCCGTTCGCCGCTCTACTCGCGGCCCGAAGGCCACTTTCGCGCTCGACTTGCATGTATC
ANGCACGCCNNCNNTGTTCATTCTGAACCATGATCAAACTCT

>DaT-1 6044824 sequence exported from DaT-1.ab1

ATAGAGAAGAGGTTTACAACCCACAGGCATTCTTCCCNNNNNNGGtATTGCTCCGTCAGGCTTGCGCCCATTGCGGAAA
ATTCCCCACTGCTGCCTCCCGTAGAAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCATCCTCTCAAACCAGCTA
CTGATCGTAGCCTTGGTAAACCTTTACTTCACCAACTAGCTAATCAGGCGTGAGCTCATCTTAAGGCAGATAAATCTATT
TCACTCGTAAGCATATGGGGCATTAGCAATCGTTTCCAATTGTTATTCCCCTCCTCAAGGTAGATTCTCACGTATTACTC
ACCCGTCCGCTACTAAAGCATAAGCTTTCGTTCAACTTGCATGTGTTAAGCATACCGCCAGCGTTCATCCTGAGCCATG
ATCAAACTct

>DaT-2 6086840 sequence exported from DaT-2.ab1

CTACTAACTTTTCCTCCCTACTGAAAGTGCTTTACAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGGATCAGGC
TTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGAT
CATCCTCTCAGACCAGCTATGGATCGTCGCCATGGTGAGCCATTACCTCACCATCTAGCTAATCCAACGCAGGCTCATC
CAATAGTGACCGGTCCGAAGATCCCGGCCTTTCCCCCGTGGGGCGTATGCGGTATTAGCTTAAGTTTCCCTAAGTTATC
CCCCGCTACTGGGTAGATTCCTACGCGTTACTCACCCGTCCGCCGCTATACTCGCACCGAAGTGCTTTCTCGCTCGAC
TTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGAtcAAACtct

>DaT-3 6086852 sequence exported from DaT-3.ab1

CATGGGTATTAACCATGAGCTTTTTTTCaCGATTGAAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGCATTG
CTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCTGATCGTCCTCTCAGACCAGCTACAGATCGTTGCCTTGGTAGGCCATTACCCCACCAACAAGCTAATCCGA
CATAGGCTCATCCAATAGTGCGAAGTCCGAAGATCCTCCGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTCGGATT
TCTCCGAGTTGTCCCCCGCTACTGGGCAGATTCCTATGTATTACTCACCCGTCCGCTACTCGTCGCCTAGCAAGCAAG
CTTGCTATCGTTACCGTTCAACTTGCATGTGTTAAGCATGCCGCCAGCGTTCAATCTGAGCCATGNANaAAcTct

>DaT-4 6086769 sequence exported from DaT-4.ab1

TTGATAAAAGAGGTTTACAGCCCAGAGGCCTTCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGA
AAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAG
CTACTGATCGATGCCTTGGTGAGCCATTACCCACACCAACTAGCTAATCAAACGCGAGCTCATCCTCAGGCGAAATTCA
TTTCACCTCGCGGCATATGGGGTATTAGCAGCCGTTTCCAGCTGTTATCCCCCTCCTGAGGGCAGATTCTCACGCGTTA
CTCACCCGTCCGCCACTAACCCGAAGGTTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATCCTGAGCCA
TGatCAAACtct

>DaT-5 6086781 sequence exported from DaT-5.ab1

CTTCTCTCCATGTGAGAAaAGAGgTTATACAGCCCTCAGAGgCCTTTCATCCCTCACGCGGCGGTGGTCCGTCAGGNTT
TCGCCCATTGCGGAAAATTCCCCAGTGCTGCCTCCCGTAgGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGGTGATCA
TCCTCTCAGACCAGCTACTGATCGATGCCTTGGAGAGCTCTTACCTCACCAACTAGaTAATCAGACGCGGGCTCATCCT
CAGGCGAAATTCATTTCCCCTCTCGGCATATGGGGTATTAGCGCCCGTTTCCAGCCGTTATCCCCCTCCTGAGGGcAGA
TTCCCACGCGTTACTCACCCGTCCGCCACTAACCCGAAGGTTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGT
TCATCCTGAGCCATGATCAAA

>DaT-6 6086793 sequence exported from DaT-6.ab1

AGGGTATTAGCCCGCGCCTTTTCTTCCCCCACGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATTG
CTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCGGACCGTGTCTCAGTTCCG
GTGTGACTGGCCATCCTCTCAGACCAGTTACCGATCGTCGCCTTGGTGGGCCGTTACCCCTCCAACAAGCTAATCGGA
CATAGGCCGCTCCGTAGGCGCGAGGCCCGAGGGTCCCCCGCTTTGCTCCTAAGAGATCATGCGGTATTAGCCCCAAT
TTCTTGGAGTTGTCCCCCACCTACGGACACGTTCCTATGCGCTACTCACCCGTCCGCCACTCGCCGCCAAGGAGCAAG
CTCCTCGCGCTGCCGTTCGACTTGCATGTGTAAGGCATGCCGCCAGCGTTCAATCTGAGCCatgatcAAACtt

>DaT-7 6086805 sequence exported from DaT-7.ab1

AGGGTATTAGCCCGCGCCTTTTCTTCCCCCACGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATTG
CTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCGGACCGTGTCTCAGTTCCG
GTGTGACTGGCCATCCTCTCAGACCAGTTACCGATCGTCGCCTTGGTGGGCCGTTACCCCTCCAACAAGCTAATCGGA
CATAGGCCGCTCCGTAGGCGCGAGGCCCGAGGGTCCCCCGCTTTGCTCCTGAGAGATCATGCGGTATTAGCCCCAAT
TTCTTGGAGTTGTCCCCCACCTACGGACACGTTCCTATGCGCTACTCACCCGTCCGCCACTCGCCGCCAAGGAGCAAG
CTCCTCGCGCTGCCGTTCGACTTGCATGTGTAAGGCATGCCGCCAGCGTTCAATCTGAGCCATGAtcAAACtct

>DaT-8 6086817 sequence exported from DaT-8.ab1

CTTCNCCGCTGAAGAGCTTTACAACCCTAAGGCCTTCTTCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTG
TCCAAGATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGA
CCAGCTACGGATCGCAGGCTTGGTGGGCCGTTACCCCGCCAACAACCTAATCCGACGCGGGCCCCTCCCGGAGCGAT
AAATCTTTCCCCCGCAGGGGACATGGGGCATTAGCCTCGGTTTCCCGAGGTTATTCCCCACTCCGGGACAGGTTCCCA
CGCGCTACTCACCCGTGCGCCGCTCGCCCCGAAGGGCGCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTC
GTTCTGAGCCatgatcAAACtct

>GspT-1 6086849 sequence exported from GspT-1.ab1

CAGGCTTCCGCCCATTGTCCAATATTCCCCAATGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGA
CTGGTCATCCTCTCAGACCAGCTACGGATCGTCGTCTTGGTAGGCCATTACCCCACCAACAAACTAATCCGACGCAGG
CTCATCCAACAGTGACAGGTCCGAAGATCCCCATCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTCGGATTTCTCCG
AGTTGTCCCCCGCTGCTGGGCAGATTCCTACGCGTTACTCACCCGTCCGCCACTCGTCAGCACAAGAGCAAGCTCTTG
CCTGTTACCGTTCGACTTGCATGTGTTAAGCATGCCGCCAGNANTCAATCTGAGCCATGATCAAACtct

>GspT-2 6086766 sequence exported from GspT-2.ab1

CCTCAGGCTTTTCTTCGCTACTGAAAGTGCtTTACAACCCGCAGGCCTTCTTCACACACGCGGAATTGcTGCNNCAGGC
TTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGCGCCGTGTCTCAGTCGCAGTGTGGCTGAT
CATCCTCTCAGACCAGCTACTGATCGTTGCCTTGGTAGGCCGTTACCCTACCAACAAGCTAATCAGACATAGGCTCATC
CAATAGCGCGAGGTCCGAAGATCCCCCGCTTTCCCCCGTAGGGCGNATGCGGTATTAGCTCCGGTTTCCCGGAGTTG
TCCCCCACTACTGGGCAGATTCCTATGCGTTACTCACCCGTCCGCCACTCGTCGCCCTTCATTACCCGAAGGTAATGAA
GTCGTTACCGTTCGACTTGCATGTGTTAGGCATGcCGccAGCGtTCAATctgagcCATGATcaAACTCt

>GspT-3 6086767 sequence exported from GspT-3.ab1

ATTCTTCcCCGGCAAAGGGATTTACAACCCTAAGGCCTTCATCACCCACGCGGCGTTGCTGCATCAGGCTTTCGCCCAT
TGTGCAAGATTCCTCGCTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTCTGGCTGATCATCCTCTCA
GACCAGCTACCGATCGCTGCCTTGGTAGGCCGTTACCCCACCAACTAGCTAATCGGACGCAGGCTCCTCCCAAGGCG
CCGGAACATTTCCCCACCGGGCTGAACCGGTGGGCGTATGGGGCATTAGCTCGCGTTTCCACGAGTTGTTCCCCTCCT
CGGGACAGATCACCTACGCGTTACTCAGCCGTTTGCCACTCAGTCACCGACCCGAAGGCCGGGCTTCGTTCGACTTGC
ATGCGTAAGGCACGCCGCCAGCGTTTATCCTGAGCCatgatgAAACtct

>GspT-4 6086779 sequence exported from GspT-4.ab1

TTTACAACCCTAAAGNCTTCGTGCACaCGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCANNNNNTCCCCACTGCT
GCCTCCCGTAGGAGTCTGGGCCGGGTCTCAGTCCCAGTGTGGGTGATCATCCTCTCAGACCAGCTATCGATCGTCGC
CTCGGTGAGCCGTCACCTCACCGACTAGCTAATCGAACGCAGGACCCTCCCAAAGCGGCCGAAGCCTTTCCCCCTTG
GGGCGTATGCGGTATTAGCCGCCGTTTCCAGCGGTTGTCCCCCACTTCCGGGCAGGTTCCTACGCGTTACTCACCCGT
CCGCCACTCCACATGCCCCCCGAAGGCGACTGCGGCGTCCGACTTGCATGTGTTAGGCATGCCGCCAGCGTTCGTTC
TGAGCCATGATCAAACTct

>GspT-5 6086791 sequence exported from GspT-5.ab1

TTCTTCCCTCGcAAAAGCGGTTTACAACCCGAAGGCCTTCCTCCCGCACGCGGTGTTGCTGCGTCAGGCTTTCGCCCA
TTGCGCANNNTTCTTAGCTGCTGCCTCCCGTAGGAGTCGGGGCCGTTCTCAGTCCCCGTGTGGCTGATCATCCTCTCA
GACCAGCTACCGATCGTCGCCTTGGTGGGCCGTTACCTCACCAACTAGCTAATCGGCCGCGGGCCCCTCCCGGAGCA
CCGGAGTTTTCACCACCCGATCTCTCATCGGGGGTCGTATGCGGTATTAGCCCGGCTTTCGCCGGGTTATCCCCCACT
CCAGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTCTCCACCAGCCCGAAGGCCGGCTTCTCGCTCGACTT
GCATGCCTAATACACACCGCCAGCGTTTGTCCTGAGCCATGAtCAAACtct

>OlT-1 6044825 sequence exported from OlT-1.ab1

CGGTGTTTCTTCACCACTGAAAGTGNNTTACAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTTC
GCCCATTGTGCAATATTCCCTACTGCTGCCAACCGTAGTTGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCGTCC
TCTCAGACCAGCTATGGATCGTCGCCTTGGTGGGCCATTACCCCACCAACTAGCTAATCCAACGTAGGCTCCTCTAATA
GCGCGAGGTCCGAAGATCCCCCGCTTTCCTCCTTGGAGATTATGCGGTATTAGCTCGAGTTTCCCCGAGTTGTCCCCC
ACTACAAGGTAGATTCCTACGCCTTACTCACCCGTCCGCCACTGTACTCAGTCCCGAAGGACCTTTCTCGTTCGACTTG
CATGTATGAGGCCTGCCGCCAACGTTCAATCTGAGCCATGATCAAACTCt

>OlT-2 6086770 sequence exported from OlT-2.ab1

TACCTGCTTTTTCTTCCTTACTGAAAGTGCTTTACAACCCTAGGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGG
TTTCCCCCATTGTGCAATATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATC
GTCCTCTCAGACCAGCTATGGATCGTCGCCTTGGTGGGCTTTTACCCCACCAACAAGCTAATCCAACGCAGGCTCCTC
CAATAGTGACAGGTCTCCGAAGAGATCCCCGCCTTTCCCCCATAGGGCGTATGCGGTATTAATCCGAGTTTCCCCGGG
CTATCCCCCGCTACTGGGTAGATTCCTACGCGTTACTCACCCGTCCGCCGCTCGTCAGCATCAAGTTCACCCCGAAGG
GATCANagCGATCTGTTACCGCTCGACTTGCATGTATTAGGCCTGCCGCCAACGTTCAATCTGAGCCATGATCAAACTCt

>OlT-3 6086782 sequence exported from OlT-3.ab1

CTCGAGTGTTTCTTCACCACTGAAAGTGCTTTACAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCT
TTCGCCCATTGTGCAATATTCCCTACTGCTGCCAACCGTAGTTGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCG
TCCTCTCGGACCAGCTATGGATCGTCGCCTTGGTGGGCCATTACCCCACCAACAAGCTAATCCAACGTAGGCTCCTCT
AGTAGCGCGAGGTCCGAAGATCCCCCGCTTTCCTCCTTGGAGATTATGCGGTATTAGCTCGAGTTTCCCCGAGTTGTC
CCCCACTACAAGGTAGATTCCTACGCCTTACTCACCCGTCCGCCACTGTAATCAGTCCCGAAGGACCTTTCTCGTTCGA
CTTGCATGTATGAGGCCTGCCGCCAACGTTCAATCTGAGCCATGATCAAACTct

>OlT-4 6086794 sequence exported from OlT-4.ab1

ATTATACACCGGTGTTTCTTCACCACTGAAAGTGCTTTACAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGCAT
CAGGCTTTCGCCCATTGTGCAATATTCCCTACTGCTGCCAACCGTAGTTGTCTGGACCGTGTCTCAGTTCCAGTGTGGC
TGATCGTCCTCTCAGACCAGCTATGGATCGTCGCCTTGGTGGGCCATTACCCCACCAACTAGCTAATCCAACGTAGGC
TCCTCTAATAGCGCGAGGTCCGAAGATCCCCCGCTTTCCTCCTTGGAGATTATGCGGTATTAGCTCGAGTTTCCCCGAG
TTGTCCCCCACTACAAGGTAGATTCCTACGCCTTACTCACCCGTCCGCCACTGTACTCAGTCCCGAAGGACCTTTCTCG
TTCGACTTGCATGTATGAGGCCTNNNNNNAACGTTCAATCTGAGCCATGNNCAAACTct

>OlT-5 6086806 sequence exported from OlT-5.ab1

AGTTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCAC
TGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTCCCAGTGTGGCTGTTCATCCTCTCAGACCAGCTACGGATCAC
AGGCTTGGTGAGCTTTTACCTCACCAACAACCTAATCCGACGCGGGCTCATCTCAAAGCGAAAAACTTTCCCCCGCAG
GGGGTATGGGGTATTAGCCACCGTTTCCGTTGGTTATTCCCCACTTTGAGGCAGATTCCCACGCGTTACTCACCCGTTC
GCCACTCGCACCGAAGTGCTCGTTCGACTTGCATGTGTTAGGCATGCCGCCAGCGTTCGTTCTGAGCCATGATCAAACt
ct

>OlT-6 6086818 sequence exported from OlT-6.ab1

AGTTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCAC
TGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGTTCATCCTCTCAGACCAGCTACGGATCAC
AGGCTTGGTGAGCTTTTACCTCACCAACAACCTAATCCGACGCGGGCTCATCTCAAAGCGAAAAACTTTCCCCCGCAG
GGGGTATGGGGTATTAGCCACCGTTTCCGTTGGTTATTCCCCACTTTGAGGCAGATTCCCACGCGTTACTCACCCGTTC
GCCACTCGCACCGAAGTGCTCGTTCGACTTGCATGTGTTAGGCATGCCGCCAGCGTTCGTTCTGAGCCATGATCAAAC
Tct

>OlT-7 5964775 sequence exported from OlT-7.ab1

TCGTTCCGGGTGACagAGGTTTACGACCCGAAAGCCTTCATCCCTCACGCGGCATCGCTCCGTCAAGCTTGCGCTCATT
GCGGAAGATTCTCGACTGCAGCCTCCCGTAGGAGTCTGGGCAGTGTCTCAGTCCCAGTGTGGGTGACCATCCTCTCA
GACCACCTACCTGTCGTAGCCTTGGTGGGCCATTACCCCACCAACCAGCTAATAGGACGCGAGTCCATCCTAAGGCGG
ATCACTCCTTTAGCAATTTTTCCTTGCAGCAAAACGCCACATTCGGTATTAATCCCACTTTCGCGGGGCTATCCCCAACC
TCAGGGCAGGTTACTCACGCGTTACTCACCCCTTCGCCACTATCCTGCGAGCAGGACCGTTCGACTTGCATGCCTAAT
CCATGCCGCCAGCGTTCATTCTGAGCCATGATCAAACTCT

>PfT-1 6086764 sequence exported from PfT-1.ab1

GCGTTCTTCCCGTACAAAAGGAGTTTACGCCCCATAGGGGTGTCTTCCTCCACGCGGCGTGGCTGGGTCAGGCTTGC
GCCCATTGCCCAAGATTCCCTACTGCTGCCTCCCGTAGAAGTCTGGCCCGTGTCTCAGTGCCAGTGTGACTGGTCGTC
CTCTCAGACCAGCTACGGATCGTAGCCTAGGTAGGCCGTTACCCTACCTACCAGCGTAATCCGACGCAGGCCCATCCT
CAAGCGCCAATACTTTCATCCTGTGCCATGCAGCACAGGACTTCATGCGGTATTAGCTACCGTTTCCGATAGTTATTCC
CCACTTGAGGGCAGGTTGCCTACGCGTTACTCACCCGTGCGCCACTGTACTCGCGCCCCGAAGGACGCTTTCTCGTTC
GACTTGCATGTATGAAGCCCGCCGCCAGCGTTCGTCCTGAGCCATGATCAAACtct

>PfT-2 6086788 sequence exported from PfT-2.ab1

TGAAGAGGTTTACAACCCGAAAGCCGTCATCCCTCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAATATT
CCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTACC
CGTCGATGCCTTGGTAGGCCGTTACCCCACCAACAAGCTGATAGGCCGCGAGATCCTCCTAGAGCACCTGAGCATTTT
CTCGCTCGGCCATGCGACCGGGCGGGGGTATCCGGTATTAGCCATCGTTTCCGATGGTTGTCCCGGTCTCTAGGACA
GATTTCTCACGTGTTACTCACCCGTTCGCCACTTTCCACCGGAGCAAGCTCCGGTTTCTCGTTCGACTTGCATGTGTTA
AGCACGCCGCCAGCGTTCGTNNNNNNCCATGATCAAACTct

>PfT-3 6086800 sequence exported from PfT-3.ab1

TCTTCCCCGAGAAAGAGGTTTACGACCCGCAGGCCTTCTTCCCTCACGCGGCGTCGCTGCGTCAGGCTTTCGCCCATT
GCGCAAGATTCCCTGCTGCTGCCTCCCGTAGGAGTGGGGGCCGTGTCTCAGTCCCCCTCTGGCCGGTCGTGCTCTCA
CACCGGCTACCCGTCGATGGCTTGGTGGGCCGTTACCTCACCAACTACCTGATGGGACGCAAGCCCATCCACTGGTG
GCCGAAGCCTTTGGTTACCCGCTATCGGCAGGCAACCACATGCGGTATTAGCTCGGATTTCTCCGGGTTGTCCCCCGC
CAGTGGGTAGGTTGCTTACGCGTTACTCAGCCGTCTGCCACTAACTCGCCCCCGAAGGGGTTCGCTCGTTCGACTTGC
ATGCATTAGGCGCGCCGCCAGCGTTCGTCCTGAGCCATGATCAAACTCT

>PfT-4 6086824 sequence exported from PfT-4.ab1

ACGAGCGTNGGTTCGTTCCCACCTGACAgTGgTTTACACCCCGAGGNTTCCTCCCACTANCGGCTAcGCTGCGTCAGTC
TCTCGACCATTGCGCAATATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGACGGAT
CgACCTCTCAGACCCGCTACGCGTCGTCGCCTTGGTAGGCCGTTACCCCACCAACAAGCTGATACGCCGCGACCTCCT
CCCTGGGCGAGAGCTTACAAGAAGAGGCTCCCTTTGGTGCCGCCAACATGCGATGGCCGCACATAATGTGGTATTAGC
CCGGGTTTCCCCAGGTTGTCCCTCTCCCAAGGGCAGATTGgtCACGTGTTACTCACCCGTTCGCCACTCGGCTCCGGG
TGCAAGCACCCGGTCCTCGTTCGACTTGCATGTATAAAGCACGCCGCCAGCGTTCGTCCTGAGCCATGATCAAACtct

>PfT-5 6086848 sequence exported from PfT-5.ab1

CGGCGTCGCTGCNTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTC
TCAGTCCCAATGTGGCTGATCACGCTCTCACGTCAGCTACCCGTCATAGCCTTGGNGGGCCGTTACCCCACCAACAAG
CTGATAGGCCGCGAGCCCGTCCGAAGGCGCCAGCTTTCAAGAAGAGGCCAGCTTTCACGCCGGGGCTCCACTCCCAG
CGCTGCATGCGGTATTAATCCAGGTTTCCCTGGGCTATCCCCCGCCCTCGGGTAGGTCGCTCACGTGTTACTCACCCG
TTCGCCGCTTTACTGAGCCCCCGAAGGGGCCGTTCTCGCACGACTTGCATGTGTTAGGCACGCCGCCAGCGTTCGTC
CTGAGNCATGATCAAACtct

>TspT-1 6086815 sequence exported from TspT-1.ab1

ATTAACCGGTTGCTTTTCTACCCAATTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATTGATGGATC
AGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGC
TGATCATCCTCTCAGACCAGCTATAGATCGTCGCCTTGGTAGGCCATTACCCCACCAACAAGCTAATCTAACGCAGGCT
CATCTGGCAGTGACAGGTCCGAAGATCCCCATCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTCCGGTTTCCCGGAG
TTGTCCCCCGCTGCCAGGCAGATTCCTACGCGTTACTCACCCGTCCGCCACTCGTCAGCGCCAAGGTCACCCCGAAG
GGATCCCAGCGGCCTGTTACCGTTCGACTTGCATGTGTTAAGCATGCCGCCAGCGTTCAATCTGAGCCATGATCAAACt
ct

>TspT-2 6086827 sequence exported from TspT-2.ab1

GAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGCATTGATGCATCAGGCTTGCGCCCATTGTGCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTACTGA
TCGTCGCCTTGGTGGGCCTTTACCCCACCAACAAGCTAATCAGACATAGGCTCCTCCAATAGTGCGAGGCCCGAAGGT
CCCCCGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTCGGGTTTCCCCGAGTTGTCCCCCGCTACTGGGCAGATTCC
TATGCATTACTCACCCGTTCGCCACTCGTCGCCCAGGAACGCACCCGAAGGATTGTTCCCGTCGTTACCGTTCGACTT
GCATGTGTTAAGNATGNNGCCAGCGTTCAATCTGAGCCATGANCAAACTct

>TspT-3 6086839 sequence exported from TspT-3.ab1

TANTANAGTGCTTTACAACCCAAAGGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTTCGCCCATTGTGCAAT
ATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCGTCCTCTCAGACCAGCT
ATGGATCGTAGCCTTGGTAGGCCTTTACCCTACCAACAAGCTAATCCAACGCAGGCTCCTCCAATAGTGAGAGCTTTCA
AGAAGAGGCCCCCTTTCCCCCGTAGGGCGTATGCGGTATTAATCCGAGTTTCCCCGGGCTATCCCCCGCTACTGGGTA
GATTCCTACGCGTTACTCACCCGTCCGCCACTCGTCAGCATCAAAGTCACCCCGAAGGGATCCTAGCGATCTGTTACC
GTTCGACTTGCATGTATNNANCCTGCCGCCAACGTTCAATCTGAGCCATGATCAAACTct

>TspT-4 6086851 sequence exported from TspT-4.ab1

CTTCACAGCTGAAGAGCTTTACGACCcAtAGGGCCTTCGTCACTCACGCGGCATGGCTGGATCAGGGTTGCCCCCATTG
TCCAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAA
CCAGCTATGGATCGTAGGCTTGGTAGGCCGTTACCCCACCAACTACCTAATCCAACGCGGGCTAATCCGACACCGATA
AATCTTTCCCCCGAAGGGCGTATACGGTATTACTCTCAGTTTCCCGAGGCTATTCCGTAGTGTCGGGCATATTCCCACG

CGTTACTCACCCGTCCGCCGCTCACCCGAAGGTGCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCGTTCTG
AGCCATGAtCAAACTct

>TspT-5 6086780 sequence exported from TspT-5.ab1

TCGTCATCACTGAAAGTGGTTTACAACCCNGAAAGCTGTCATCCCACACGCGGCGTTGCTGCGTCAGGGTTGCCCCCA
TTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCTGATCGTCCTCTC
AGACCAGCTACCCGTCGATGCCTTGGTAGGCCGTTACCCCACCAACTAGCTGATAGGCCGCGAGACCCTCCCGGAGC
GCCGGAGCCTTTCTTCGCTCGGTCATGCGACCAAGCGGAGGCATCTGGTATTAGCCATCGTTTCCAATGGTTGTCCCA
GTCTCCGGGGCAGGTTTCTCACGTGTTACTCACCCGTTCGCCACTGTCCCTTGGGCCGAAGCCCTAGTTCTCGTTCGA
CTTGCATGTGTTAGGCACGCCGCCAGCGTTCGTCCTGAGCCatgatCAAACtct

>TspT-6 6086792 sequence exported from TspT-6.ab1

CGGAGGTTTCGCCCCGGGAtTTTCctcCCCACTGAAAGTGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGCATTGC
TGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG
TGTGGCTGATCGTCCTCTCAGACCAGCTACAGATCGTTGCCTTGGTAGGCCATTACCCCACCAACAAACTAATCTGACA
TGGGCTCATCCAACGGCGCGAGGTCCGAAGATCCCCCGCTTTCCCCCGTAGGGCTTATGCGGTATTAGCCCGAGTTTC
CCCGGGTTATTCCCCACCAATGGGCAGATTCCCATGCATTACTCACCCGTCCGCCACTTTACTCGCCTCCGAAGAGAC
TTTCTCGTTCGACTTGcaTGTGTTAAGCATGCCGCCAGCGtNcaATCTGAGCCATGATCAAACTct

>TspT-7 6086816 sequence exported from TspT-7.ab1

TCCCTGCTGaaGGGGTTTACAACCTCAGAAGGCCGTCTTCCCCCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTG
CGCAAGATTCCCTACTGCTGCCCCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCGTCCTCTCAGA
CCAGCTACCCGTCGATGCCTTGGTGAGCCACTACCTCACCAACTAGCTGATAGGCCGCGAGACCGTCCCGAAGCGCC
GGAACCTTTGCTGATGGCGCCATGCGGCGCCGTCAGAGTATTGGGTATTAGCCCAGGTTTCCCTGGGTTATCCCCATC
TCCGGGGGTTGGTTTCTCACGTGTTACGCACCCGTTCGCCACTCTCTCATATGGGGCCGAAGCCCCGAGATCGTTCGAC
TTGCATGTATGAAGCACGCCGCCAGCGTTCGTCCTGAGCCATGATCAAAcTct

16S rDNA Sequences from Surface Samples of Different Sponge Species and Reference Samples (DGGE analyses with Primer GC 27F/518R)

>AoS-1 5964738 sequence exported from AoS-1.ab1

TTTAAAGTCTTTACAACCCTAAGGCCTTCTTCACAAACGCGATATTCTGGATCAGGTTTTCGCCATTGTCCAATATTCCC
CACTGCTGCCTCCCGTAGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACCGATC
GTCGCCTTGGTGAGCCATTACCTCACCAACAAGCTAATCGGCCGCGGCTCATCTGATAGCGTGAGGTCCGAAGATCCC
CCACTTTACCCCGTAGTGTATGCGGTATTAGCCCGGGTTTCCCCGGTTGTCCCCCACTACCAGGCAGATTCCCACGTG
TTACTCACCCGTCCGCCACTCGCCGCCCAGGAGCAAGCTCCCTGCGCTGCCGTTCGACTTGCATGTGTTAAGCATGCC
GCCAGCGTTCAATCTGAGCCATGATCAAACTCT

>AoS-2 5985916 sequence exported from AoS-2.ab1

AACCCTAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCT
CCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACCGATCGTCGCCTTGG
TGAGCCATTACCTCACCAACAAGCTAATCGGCCGCGGGCTCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTAC
CCCGTAGGGTGCATGCGGTATTANCCCGGGTTTCCCCGGGTTATCCCCCACTACCAAGCAGATTCCCACGTGTTACTC
ACCCGTCCGCCACTCGCCGCCCGGGAGCAAGCTCCCTGCGCTGCCGTTCGACTTGCATGT

>AoS-3 6086763 sequence exported from AoS-3.ab1

TTGACACTCTGCTTTTCTTCCTCACTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATTGCTGGATCA
GGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCT
GATCATCCTCTCAGACCAGCTACCGATCGTCGCCTTGGTGAGCCATTACCTCACCAACAAGCTAATCGGCCGCGGGCT
CATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTACCCCGTAGGGTGTATGCGGTATTAGCCCGGGTTTCCCCGGG
TTATCCCCCACTACCAAGCAGATTCCCACGTGTTACTCACCCGTCCGCCACTCGCCGCCCGGGAGCAAGCTCCCTGCG
CTGCCGTTCGACTTGCATGTGTTAAGCATGCCGCCAGCGTTCAATCTGAGCCATGATCAAACtct

>AoS-4 6086775 sequence exported from AoS-4.ab1

TATTAACACTCTGCTTTTCTTCCCCACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGAT
CAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGG
CTGATCATCCTCTCAGACCAGCTACCGATCGTCGCCTTGGTAGGCCGTTACCTCACCAACAAGCTAATCGGACATAGG
CTCATCCAATAGCGCGAGGCCTGAATGGTCCCCCGCTTTCCCCCCAATCAATATGAGAGGGGGCGTATGCGGTATTAG
CCTGATTTTCACCAGGTTGTCCCCCGCTACTGGGCAGATTCCTATGCATTACTCACCCGTCCGCCACTCGCCGCCAGG
GAGCAAGCTCCCCGCGCTGCCGTTCGACTTGCATGTGTTAAGCATGCCGCCAGCGTTCAATCTGAGCCATGATCAAACt
ct

>AoS-5 6086787 sequence exported from AoS-5.ab1

ATTAACCGGAGGCTTTTCTTCGCCACTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATGGCTGTGT
CAGGCTTTCGCCCATTGCACAATATTCCCTACTGCTGCCGACCGTAGCCGTCTGGGCCGTGTCTCAGTCCCAGTGTGG
CTGATCGTCCTCTCAGACCAGCTATGGATCGTAGCCTTGGTGAGCCTTTACCTCACCAACAAGCTAATCCAACGCAGGC
TCCTCCGATAGCGTGAGGCCGCGCTCCGGCCGAAGCCAGTGCGTTGAGGTCCCCCACTTTCATCCGTGGATAGTATG
CGGTATTAGCCCGAGTTTCCTCGGGTTGTCCCCCACTATCGGGTAGATTCCTACGCGTTACTCACCCGTCCGCCACTTT
ACTCGCCCCCGAAGGGGTTTTCTCGTTCGACTTGCATGTATGAGGACTGCCGCCAACGTTCAATCTGAGAcATGATCAA
ACTct

>AoS-6 6086799 sequence exported from AoS-6.ab1

ATAGAAaAGGGCTTTACGATCCGAAAACCTTCTTCACCCACGCGGCGTCGCTGCATCAGACTTCCGATCCATTGTGCAA
TATCCCTTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGGGGGCCACCCTCTCAGGTCCC
CTAACCGTCATCGCCTTGGTAAGCCTTTACCTCACCAACTAGCTGATGGTACGCAACCTCATCCCTCAGCGCCTAAGCC
TTTCCTCACGGCTCTCTAACTACCGCGAGAACATCCGGTATTAGCCTCTGTTTCCAAAGGTTATCCCTGTCTGAAGGGC
AGATCAGTTACGTGTTACTCACCCGTGCGCCACTCTCCAGTCCCCCGAAAGGAACCTTCCCGTTCGACTTGCATGTATT
AGGCACGCCGCCAACGTTCATCCTGAGCCATGATCAAACtct

>CbS-1 6086845 sequence exported from CbS-1.ab1

ATTAACCCTagGCTTTTCTTCTCTACTGACAGCGCTTTACAACCCGCAGGCCTTCTTCACACACGCGGTAttGcTGGATCA
GGCTTGCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCT
GATCGTCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCGACTTAGGCTC
ATCCAATAGTGcGAGGTCCGAAGATCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCtCGAGTTTCCACGAGTT
GTCCCCCGCTACTGGGtAGATTCCTAAGTATTACTCACCCGTCCGCCACTCGACGCCTGGgAGCAAGCTCCCATCGTTt
CCGTTCTANTTGCATGTGTNAGGCATGCCGCCAGCGTtcAATCTGAGCCATGATcAAAACTct

>CrS-1 6044822 sequence exported from CrS-1.ab1

TAACGTACAACCTTTCCTCCTCGCTGAAAGTGCTTtaCAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGCATCAG
GCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTG
ACCATCCTCTCAGATCAGCTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACAAGCTAATCCGACGCAGGCTC
ATCCAGTAGCGCAAGGTTCCGAAGAAGCCCCTGCTTTCCCCCTTAGGGCGTATGCGGTATTAATCCGGATTTCTCCGG
GCTATCCCCCACTACCGGGCAGATTCCTACGTGTTACGCACCCGTCCGCCGCTCGTCATCTTCCAGCAAGCTGGAAAT
GTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTct

>DaS-1 6086829 sequence exported from DaS-1.ab1

GGGTATTAGCCCGCGCCTTTTCTTCCCCCACGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATTGCT
GGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCGGACCGTGTCTCAGTTCCGGT
GTGACTGGCCATCCTCTCAGACCAGTTACCGATCGTCGCCTTGGTGGGCCGTTACCCCTCCAACAAGCTAATCGGACA
TAGGCCGCTCCGTAGGCGCGAGGCCCGAGGGTCCCCCGCTTTGCTCCTAAGAGATCATGCGGTATTAGCCCCAATTT
CTTGGAGTTGTCCCCCACCTACGGACACGTTCCTATGCGCTACTCACCCGTCCGCCACTCGCCGCCAAGGAGCAAGCT
CCTCGCGCTGCCGTTCGACTTGCATGTGTAAGGCATGCCGCCAGCGTTCAATCTGAGCCATGatCAAACtct

>DaS-2 6086841 sequence exported from DaS-2.ab1

AGGGTATTAGCCCGCACCTTTTCTTCCCCCACGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATTGC
TGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCGGACCGTGTCTCAGTTCCGG
TGTGACTGGCCATCCTCTCAGACCAGTTACCGATCGTCGCCTTGGTGGGCCGTTACCCCTCCAACAAGCTAATCGGAC
ATAGGCCGCTCCGTAGGCGCGAGGCCCGAGGGTCCCCCGCTTTGCTCCTAAGAGATCATGCGGTATTAGCCCCAATTT
CTTGGAGTTGTCCCCCACCTACGGACACGTTCCTATGCGCTACTCACCCGTCCGCCACTCGCCGCCAAGGAGCAAGCT
CCTCGCGCTGCCGTTCGACTTGCATGTGTAAGGCATGCCGCCAGCGTTCAATCTGAGCCATGAtCAAACtct

>DaS-3 6086853 sequence exported from DaS-3.ab1

TGGGCGCAAAGGGTATTAGCCCGCGCCTTTTCTTCCCCCACGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCAC
GCGGCATTGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCCGGACCGTGTC
TCAGTTCCGGTGTGACTGGCCATCCTCTCAGACCAGTTACCGATCGTCGCCTTGGTGGGCCGTTACCCCTCCAACAAG
CTAATCGGACATAGGCCGCTCCGTAGGCGCGAGGCCCGAGGGTCCCCCGCTTTGCTCCTAAGAGATCATGCGGTATT
AGCCCCAATTTCTTGGAGTTGTCCCCCACCTACGGACACGTTCCTATGCGCTACTCACCCGTCCGCCACTCGCCGCCA
AGGAGCAAGCTCCTCGCGCTGCCGTTCGACTTGCATGTGTAAGGCATGCCGCCAGCGTTCAATCTGAGCCATGAtCAA
ACtct

>EsS-1 6086797 sequence exported from EsS-1.ab1

CAGTTATTAACTACTAACCTTTCCTCCTCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCT
GCATCAGAGTTTCCTCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTG
TGgcNgaTCATacTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCCACTTGG
GCACATCCAATCGCGAAAGGCTCCGAAGAGCCCCCTCCTTTCCCCCGtAGGGcGTATGCGGTATTAGCCATCGTTTCCa
ATGNTTGCCCCCCACGAGTGGNCAGTTTCCCAAGCATTACTCACCCGTCCGCCGCTCGACACCTCAGGAGCAAGCTCC
CTTGTGTTTCCGCTCgACTTGCATGTGATAgGCCTGCCGCCAGCTTTCAATCTGAgccATGAtcAA

>EsS-2 6086809 sequence exported from EsS-2.ab1

TAGCAGGTATTAACTACTAACCTTTCCTCCTGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCAT
GGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCC
CAGTGTGGCTGATCATCCTCTCAAACCAGCTAGGGATCGTTGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCC
ACTTGGGCCAATCTAAAGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAA
CTGTTGTCCCCCACCTCAAGGCATGTTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGCTACT
NTCTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCatgatcAAAcTct

>EsS-3 6086821 sequence exported from EsS-3.ab1

CTTCCNGGGAAGGATTTTACAACCCTAGGGCCGTCATCATCCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTC
CAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACC
AGCTATGGATCGTCGGCTTGGTGGGCCTTTACCCCACCAACTACCTAATCCACCGCGGGCCGGTCCTTCGGCGATAAA
TCTTTCCCCCTCACAAGGAGGGGCGCATCCGGTATTACAcccagtTTCCCGGGGCTATTCCGAACCGAAGGATACGTTCC
CACGTGTTACTCACCCGTCTGCCACTCCGTGTGCTCGGGGCCCGAGGGCACAGAGCACACGGCGTTCGACTTGCATG
TGTTAAGCCTGCCGCCAGCGTTCGTTCTGAGCCatgatcAAACtct

>GspS-1 5985920 sequence exported from GspS-1.ab1

AAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGTATTGCTGGATCAGGCTTGCCCCCATTGTCCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAACCAGCTATGGA
TCGTCGCCTTGGTGAGCCTTTACCCCACCAACAAGCTAATCCAACGCGGGCTCATCCAATAGCGTGAGGTCCGAAGAT
CCCCCGCTTTGCCCCGTANAGAGTATGCGGTATTAATCCGGATTTCTCCGGGCTGTCCCCCACTACAAGGTAGATTCC
CACGCGTTACTCACCCGTCCGCCACTC

>HspS-1 6086773 sequence exported from HspS-1.ab1

CGAGGTTATTAACCTCAGGCTTTTCGTCTCTATTGAAAGTGCTTTACAACCCTCAGGCCTTCTTCACACACGCGGTATTG
CTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCTGATCATCCTCTCAAACCAGCTATAGATCGTTGCCTTGGTGAGCCATTACCTCACCAACAAGCTAATCTAAC
GCGGGCTCATCTAATAGCGTGAGGTCCGAAGATCCCCCACTTTGCTCCGTAGAGATTATGCGGTATTAATCCGGATTTC
TCCGGGCTATCCCCCCACTATAAGGTAGATTCCCACGCGTTACGCACCCGTCCGCCACTAATCAATTCCAGCAAGCTGG
AATTTCATCGTTCGACTTGCATGTGTTAAGCATACCGCCAGCGTTCAATCTGAGCCATGATCAAACtct

>OlS-1 6086842 sequence exported from OlS-1.ab1

TTACTCACCGGTGTTTCTTCACCACTGAAAGTGCTTTACAACCCTAGAGCCTTCTTCACACACGCGGCATGGCTGCATC
AGGCTTTCGCCCATTGTGCAATATTCCCTACTGCTGCCAACCGTAGTTGTCTGGACCGTGCCTCAGTTCCAGTGTGGCT
GATCGTCCTCTCAGACCAGCTATGGATCGCCGCCTTGGTGGGCCATTACCCCACCAACTAGCTAATCCAACGTAGGCT
CCTCTAATAGCGCGAGGTCCGAAGATCCCCCGCTTTCCTCCTTGGAGATTATGCGGTATTAGCTCGAGTTTCCCCGAGT
TGTCCCCCACTACAAGGTAGATTCCTACGCCTTACTCACCCGTCCGCCACTGTACTCAGTCCCGAAGGACCTTTCTCGT
TCGACTTGCATGTATGAggNCtgcNgccAACGTTCAATCTGAGCCATGAtCAAACtct

>OlS-2 6086854 sequence exported from OlS-2.ab1

TCTTCCCCGCCGAAAGAGTTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGGTTTCCCCCATT
GTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGTTCATCCTCTCAG
ACCAGCTACGGATCACAGGCTTGGTGAGCTTTTACCTCACCAACAACCTAATCCGACGCGGGCTCATCTCAAAGCGAA
AAACTTTCCCCCGCAGGGGGTATGGGGTATTAGCCACCGTTTCCGTTGGTTATTCCCCACTTTGAGGCAGATTCCCAC
GCGTTACTCACCCGTTCGCCACTCGCACCGAAGTGCTCGTTCGACTTGCATGTGTTAGGCATGCCGCCAGCGTTCGTT
CTGAGCCATGATCAAACtct

>6086771 sequence exported from OlS-3.ab1

ATTATATTCGTTCCGGGTGACAGAGGTTTACGACCCGAAAGCCTTCATCCCTCACGCGGCATCGCTCCGTCAAGCTTG
CGCTCATTGCGGAAGATTCTCGACTGCAGCCTCCCGTAGGAGTCTGGGCAGTGTCTCAGTCCCAGTGTGGGTGACCAT
CCTCTCAGACCACCTACCTGTCGTAGCCTTGGTGGGCCATTACCCCACCAACCAGCTAATAGGACGCGAGTCCATCCT
AAGGCGGATCACTCCTTTAGCAATTTTTCCTTGCAGCAAAACGCCACATTCGGTATTAATCCCACTTTCGCGGGGCTAT
CCCCAACCTCAGGGCAGGTTACTCACGCGTTACTCACCCCTTCGCCACTATCCTGCGAGCAGGACCGTTCGACTTGCA
TGCCTAATCCATGCCGCCAGCGTTCATTCTGAGCCatgatcAAACtct

>OlS-4 6086783 sequence exported from OlS-4.ab1

CGCCCGGTGAAGAGTTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATGGCTGGATCAGGGTTTCCCCCATTGTC
CAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGGGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACC
AGCTACCGATCGCGGGCTTGGTGAGCCGTTACCTCACCAACTACCTAATCGGTCGCGGGTCCATCTTTCGGCGATAAA
TCTTTCCCCCGAAGGGCTTATTCGGNATTAGCCACGGTTTCCCGAGGTTATTCCGAACCAAAAGGTAGGTCCCCACGC
GTTACTCACCCGTTCGCCACTAGGTGTTCCAACCGAAGTCGGGCACCCCGTTCGACTTGCATGTGTTAAGCCTGCCGC
CAGCGTTCGTCCTGAGCCATGATcAAACTCT

>PbS-1 6086831 sequence exported from PbS-1.ab1

ATTAACCATTAGCCTCTCCTCCCTCGCTGTAAAGTGCTTTACAACCaAAAGGCCTTCTTCACACACGCGGCATGGCTGG
ATCAGGGTTGCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCcGGACCGTGTCTCAGTCCCGGTGT
GaCTGATCATCCTCTCAGACCAGTTACAGATCGTCGCCTTGGTGGGCCTTTACCCCACCAACTAGCTAATCTGATTTAG
GCTCATCTAATAGCAAGAGCTTGCGCCCCCTTTCACCCGTAGGCCGTATGCGGTATTAATTCGAGTTTCCCCGAGCTAT
CCCCCACTACTAGGCAGATTCCTAAATGTTACTCACCCGTCCGCCACTAATCATCTCTAGCAAGCTAGAGAATCATCGT
TCGACTTGCATGTGTTAAGCCTGCCGCCAGCGtTCAATCTGAGCCATGATCAAACTct

>PbS-2 6086843 sequence exported from PbS-2.ab1

TATTAACTACTAACCTTTCCTCCTTACTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATGGCTGCAT
CAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGG
CTGATCATCCTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACTTGGGC
TAATCTAAGGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACGTTATGCGGTATTAGCAGTCGTTTCCAACTGTTGTCC
CCCACCCTAAGGCATATTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCATCTTCTAGCAAGCTAGAAATGTTACCGC
TCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACtct

>PbS-3 6086855 sequence exported from PbS-3.ab1

TATTAACACTTAACCTTTCCTCCTTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCAT
CAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGG
CTGATCATCCTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACTTGGGC
TAATCTTATGGCGTGAGGCCCGAAGGTCCCCCACTTTGGTCCGTAGACATCATGCGGTATTAGCAGTCGTTTCCAACTG
TTGTCCCCCACCATAAGGCATATTCCCAAGCATTACTCACCCGTCCGCCGCTCGACGCCAGAGGAGCAAGCTCCTCTT
CGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTct

>PbS-4 6086772 sequence exported from PbS-4.ab1

CTAGCAGGAATTAACTACTAACTTTTCCTCACAGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGC
ATGGCTGCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTT
CCAGTGTGGCTGATCTTCCTCTCAGAACAGCTAGAGATCGTTGCCTTGGTAAGCCTTTACCTTACCAACTAGCTAATCT
CACTTGGGCCTCTCTTTGCGCCGGAGCCTAAGCCCCGTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAA
CTGTTATCCCCCTCGCAAAGGCAAGTTCCCAAGCATTACTCACCCGTCCGCCACTCGTCAGCGANTAGCAAGCTATTCC
TGTTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAAcTct

>PbS-5 6086784 sequence exported from PbS-5.ab1

CAGGTATTAACTACTAACCTTTCCTCCTGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGC
TGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG
TGTGGCTGATCATCCTCTCAAACCAGCTAGGGATCGTTGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACT
TGGGCCAATCTAAAGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAACTG
TTGTCCCCCACCTCAAGGCATGTTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGCTACTtTCT
GTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGAtCAAAcTct

>PbS-6 6086796 sequence exported from PbS-6.ab1

ATTAACTCNCACCCTTTCCTCCTGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCAT
CAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGG
CTGATCATCCTCTCAAACCAGCTAGGGATCGTTGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACTTGGGC
CAATCTAAAGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAACTGTTGTCC
CCCACCTCAAGGCATGTTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGCTACTTTCTGTTACC
GCTCGACTTGcATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAActct

>PbS-7 6086808 sequence exported from PbS-7.ab1

CTTCGTCACAGGCGAAAGCGGTTTACAACCCGAAGGCCGTCATCCCGCACGCGGCGTTGCTGCATCAGGCTTCCGCC
CATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCACCCTC
TCAGGCCGGCTACCCGTCAAAGCCTTGGTAAGCCACTACCCCACCAACAAGCTGATAAGCCGCGAGTCCATCCCCAAC
CGCCGAAACTTTCCAACCCCCACCATGCAGCAGGAGCTCCTATCCGGTATTAGCCCCAGTTTCCTGAAGTTATCCCAAA
GTCAAGGGCAGGTTACTCACGTGTTACTCACCCGTTCGCCACTCGAGCACCCCACAAAAGCAGGGCCTTTCCGTTCGA
CTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCcTgagcCatgatcAAACtct

>PfS-1 6086765 sequence exported from PfS-1.ab1

GAGAANAGGTTTACAGCCCAAAGGCCTTCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAAT
TCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCGTCCTCTCAGACCAGCTAC
TGATCGATGCCTTGGTGAGCATTTACCCCACCAACTAGCTAATCAGACGCGGGCTCATCCCCAGGCGAAAATTCATTTC
AGCTCTCGCCATATGGGGTATTAGCGGCCGTTTCCAACCGTTGTCCCCCTCCTAGGGCCAGATTCCCACGCGTTACTC
ACCCGTCCGCCACTCACCCGAAGGTGCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATCCTGAGCCATGA
TCAAACtct

>PfS-2 6086801 sequence exported from PfS-2.ab1

TGAAGAGCTTTACAACCCTAGGGCCTTCTTCACTCACGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTCCAAGATT
CCCCACTGCTGCCTCCCGTAGGAATCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAACCAGCTACT
GATCGTAGGCTTGGTGGGCCATTACCCCACCAACAACCTAATCAGACGCGGGCCGCTCTCTCAGCGATAAATCTTTCC
CCCGAAGGGCGTATGCGGTATTAATCCGGGTTTCCCCAGGCTATTCCGTACTGAGAGGCACGTTCCCACGCGTTACTC
ACCCGTTCGCCGCTAAGTCCAAGGACTTCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCGTTCTGAGCCAT
GATCAAACtct

>PfS-3 6086813 sequence exported from PfS-3.ab1

TCCTACGCGGGCGAGAGTGCTTTACAANCCCTAAGGCCTTCGTCACACACGCGGCATTGCTGGATCAGGCTTTCGCCC
ATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTC
AGACCAGCTATCGATCGTCGCCTCGGTGAGCCGTCACCTCACCGACTAGCTAATCGAACGCAGGACCCTCCGAAAGC
GGCCGAAGCCTTTCCCCCTTGGGGCGTATGCGGTATTAGCCGCCGTTTCCAGCGGTTGTTCCCCACTTCCGGGTAGGT
TCCTACGCGTTACTCACCCGTCCGCCACTCCACATGCCGCCCGAAGGCGACTGCGGCGTCCGACTTGCATGTGTTAG
GCATGCCGCCAGCGTTCGTTCTGAGCCATGATCAAACtct

>PwS-1 6086820 sequence exported from PwS-1.ab1

GTATTAACTACTAACCTTTCCTCCTTACTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACACGCGGCATGGCTGCA
TCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTG
GCTGATCATCCTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACTTGGG
CTAATCTAAGGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACGTTATGCGGTATTAGCAGTCGTTTCCAACTGTTGTC
CCCCACCCTAAGGCATATTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCATCTTCTAGCAAGCTAGAAATGTTACCG
CTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGAtCAAACtct

>PwS-2 6086832 sequence exported from PwS-2.ab1

ACTAAGTGCTATTAACACTTAACCTTTCCTCCTTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCA
TGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCTGATCATCCTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC
CACTTGGGCTAATCTTATGGCGTGAGGCCCGAAGGTCCCCCACTTTGGTCCGTAGACATCATGCGGTATTAGCAGTCG
TTTCCAACTGTTGTCCCCCACCATAAGGCATATTCCCAAGCATTACTCACCCGTCCGCCGCTCGNCGCCAGAGGAGCA
AGCTACTCTNNGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCaAACTct

>PwS-3 6086844 sequence exported from PwS-3.ab1

ACCTTCCTCaCTGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCANACACGCGGCATGGCTGCATCAGGCTTGCGC
CCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCATCCTC
TCAGACCAGCTAGGGATCGTCGCCTTGGTGAGCCCTTACCTCACCAACTAGCTAATCCCACTTGGGCCTATCTTGACG
CGAGAGgCCAAAACGCCCTNGTCTTTGAGCCGAAACTATTATGTGGTATTAGCCTTCGATTNcAATGCCTATCCCCCACA
TCAGGGCAATTTCCCAGGNATTACTCACCCGtCCGCCGCTCGACGCCGtTAACGTTCCCCGAAGGTTCAGCTAACTCGT
TtCCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATcTgagcCATGAtCAAACTCT

>PwS-4 6086856 sequence exported from PwS-4.ab1

CTAGCAGGTATTAACTACTAACCTTTCCTCCTGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCA
TGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCTGATCATCCTCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC
CACTTGGGCCAATCTAAAGGCGAGAGCCGAAGCCCCCTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCA
ACTGTTGTCCCCCACCTCAAGGCATGTTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGCTAC
TCTCTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGAtcAAAct

>Sw-1 6044826 sequence exported from Sw-1.ab1

TATTAACATACTGAGCACTTCCTCACTACTAAAAGTGCTTTACAACCCTAGGGCCTTCTTCACACAAGCGGCATGGCTG
GATCAGGGTTTCCCCCATTGTCCAATATTCCCGACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGT
GACTGATCATCCTCTCAGACCAGCTAAAGATCGTCGCCTTGGTGGGCCATTACCCCACCAACAAGCTAATCTTACGCAG
GCTCATCTAATAGCGAGAGCTTCAAAGAGAGGCCCCCTTTCATCCTTAGATCGTATGCGGTATTACCCAAAGTTTCCCT
TGGCTATCCCCCACTACTAGGTAGATTCCTACGCGTTACTCACCCGTCCGCCACTCGTCGACCACTAGCAAGCTAGTGT
CGTTACCGTTCGACTTGCATGTCTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCT

>Sw-2 6044827 sequence exported from Sw-2.ab1

AAGGATATtAACcNATTAGCTATTTCCTCCCAATTGAAAGTGCTTTACAACCCTAGGGCCTTCTTCACACAAGCGGCATG
GCTGCGTCAGGCTTTCGCCCATTGCGCAATATTCCCGACTGCTGCCTCCCGTAGGAGTCCGGGCCGTGTCTCAGTCCC
GGTGTGGCTGATCATCCTCTCAGACCAGCTAAAGATCGTCGCCTTGGTAGGCCATTACCCCACCAACTAGCTAATCTTA
CGCAGGCTCATCTAATAGCACGAGGTCCGAAGATCCCCCGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTCGCGTT
TCCACGAGTTGTCCCCCACTACTAGGTAGATTCCTACGCGTTACTCACCCGTCCGCCGCTGTACTCACCCCGAAGGGC
TTTCTCGCTCGACTTGCATGTCTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCT

>Sw-3 6044828 sequence exported from Sw-3.ab1

ATTAACTACTANCTTTTCCTCACAACTGAAAGTgctttaNAACCCGAAGGCCTTCTTCACACAAGCGGCATGGCTGCATCAG
GGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTG
ATCTTCCTCTCAGAACAGCTAGAGATCGTTGCCTTGGTAAGCCTTTACCTTACCAACTAGCTAATCTCACTTGGGCCTCT
CTTTGCGCCGGAGCCTAAGCCCCGTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAACTGTTATCCCCCT
CGCAAAGGCAAGTTCCCAAGCATTACTCACCCGTCCGCCACTCGTCAGCGAATAGCAAGCTCTTCCTGTTACCGTTCG
ACTTGcATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCt

>Sw-4 5985922 sequence exported from Sw-4.ab1

TGCTTTACAACCNGANNGCCTTCTTCATCACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGCGCAATATTCCCCACT
GCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCACTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTATGGATCCTT
GCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCAAACTTGGGCCCATCTAAAGGCGAGAGGCGAANCCCCCTTTG
GTCCGAAGACATTATGCGGTATTAGGAGTCGTTTCCAACTGTTGTCCCCCACCTCAAGGCATGTTCCAAAGCATTACTC
ACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGCTACTTTCTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCA
GCGTTCAATCTGAGCCATGATCAAACTCT

>Sw-5 6044831 sequence exported from Sw-5.ab1

TTCATTCCTTGAGAAAAGAGGTTTACAGCCCAGAGGCCTTCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCC
ATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCT
CAGACCAGCTACTGATCGATGCCTTGGTGAGCTCTTACCTCACCAACTAGCTAATCAGACGCGGGCTCATCCTCAGGC
GAAATTCATTTCACCTCTCGGCATATGGGGTATTAGCGGCCGTTTCCAGCCGTTATCCCCCTCCTGAGGGCAGATTCCC
ACGCGTTACTCACCCGTCCGCCACTAACCCGAAGGTTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATC
CTGAGCCATGAtCAAACTCt

>Sw-6 6086807 sequence exported from Sw-6.ab1

TCTTCACCAGTGAAAGTGCTTTACAACCCTAAGGCCTTCATCACACACGCGGCATTGCTGGATCAGGGTTGCCCCCATT
GTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAG
ACCAGCTACTGATCGTAGCCTTGGTAGGCCATTACCCTACCAACAAGCTAATCAGACGCGGGCTCCTCCATCTGCGGC
GGACCTTTCCCCCGAAGGGCGTATCCGGTATTAGCAGCCGTTTCCAGCTGTTATCCCAGGCAGATGGGCAGATTCCCA
CGCGTTACTCACCCGTGCGCCACTCTATAAAATAGCGTTCGACTTGCATGTGTTAAGCATGCCGCCAGCGTTCGTTCTG
AGCCATGATCAAACtct

>Sw-7 6086819 sequence exported from Sw-7.ab1

ATCTTCACCAGTGAAAGTGCTTTACAACCCTAAGGCCTTCATCACACACGCGGCATTGCTGGATCAGGGTTGCCCCCAT
TGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCA
GACCAGCTACTGATCGTCGCCTTGGTAGGCCGTTACCCTACCAACAAGCTAATCAGACGCGGGCCCCTCTTTCTCCGG
CGGACCTTTCCCCCGTAGGGCGTATGCGGTATTAGCGGTCGTTTCCAACCGTTATCCCGCAGAAAAAGGCAGGTTCCC
ACGCGTTACTCACCCGTGCGCCACTCTATAAAAAAcgTTCGACTTGNATGTGTTAgGNATGCCGCCAGCGTTCGTTCTGA
gCCATGAtCAAAcTct

8 Curriculum Vitae

Berna Gerçe

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Dissertation

Since 09. 2006	PhD at the Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Faculty of Chemical and Process Engineering, Karlsruhe Institute of Technology (KIT). *Examiner: Prof. Dr. C. Syldatk*

Title of the Dissertation: „Bacterial communities of different Mediterranean sponge species - Basic investigations of biotechnological sponge cultivation “

University Education

04. 2003 - 09. 2006	Course of biology (Diploma), University of Karlsruhe, Germany, final grade: 1.3 (grade system: 1 = very good, 2 = good, etc.)

Title of the Diploma thesis: „Untersuchung auf Veränderung der Zusammensetzung der mikrobiologischen Gemeinschaft durch Kultivierung am Beispiel *Aplysina aerophoba “* at the Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Faculty of Chemical and Process Engineering, KIT, (former University of Karlsruhe (TH)). *Examiner: Prof. Dr. C. Syldatk*

10. 1999 - 03. 2003	Course of biology, University of Kaiserslautern, Germany
04. 1999 - 09. 1999	Course of Sinology, University of Heidelberg, Germany
09. 1998 - 03. 1999	Stay abroad and course of Chinese language, Center of Chinese for foreign Students, Yunnan University, VR China

Formal education

09. 1996 - 06. 1998	Apprenticeship in biological technical assistent, Berufsbildende Schule Naturwissenschaften, Ludwigshafen am Rhein, Germany
06. 1996	A-level, Goethe-Gymnasium, Karlsruhe, Germany

Professional Experience

09. 2006- 10. 2003	Student assistant at the Water Technology Center, Karlsruhe, Germany
08.2001 – 12. 1999	Student assistant at the Institute of Biotechnology and Drug Research, Kaiserslautern Germany

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9 Publications

Articles:

Gerçe B, Schwartz T, Voigt M, Ruhle S, Kirchen S, Putz A, Proksch P, Obst U, Syldatk C, Hausmann R (2009) Morphological, Bacterial, and Secondary Metabolite Changes of *Aplysina aerophoba* upon Long-Term Maintenance Under Artificial Conditions. Microb Ecol 58 (4): 865-878

Gerçe B, Schwartz T, Syldatk C, Hausmann R (2011) Differences between Bacterial Communities Associated with the Surface or Tissue of Mediterranean Sponge Species. Microb Ecol 61 (4): 769-782

Poster

Gerçe, B., Rühle, S., Voigt, M., Putz, A., Proksch, P., Obst, U., Schwartz, T., Syldatk, C. & Hausmann, R., Effects of long-term cultivation of *Aplysina aerophoba* on the associated microorganism, Symposium “European BioPerspective”, 30. 05 - 01. 06. 2007, Cologne, Germany

Gerçe, B., Schwartz, T., Voigt, M., Rühle, S., Kirchen, S., Putz, A., Proksch, P., Obst, U., Syldatk, C. & Hausmann, R., The Effects of long-term cultivation of *Aplysina aerophoba* under artificial conditions on the associated bacteria, Symposium “European BioPerspective”, 07. - 09. 10. 2008, Hanover, Germany

Gerçe, B., Schwartz, T., Voigt, M., Rühle, S., Kirchen, S., Putz, A., Proksch, P., Obst, U., Syldatk, C. & Hausmann, R., The Effects of long-term cultivation of *Aplysina aerophoba* under artificial conditions on the associated bacteria, 12th International Symposium on Microbial Ecology (ISME 12), 17. - 22. 08. 2008, Cairns, Australia