Chemical micro-environments, ventilation behaviour & microbial processes in sponges

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I would like to thank my referees Prof. Dr. Bo Barker Jörgensen and Prof. Dr. Willhelm Hagen for carefully reviewing the thesis and for their availability.

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PREFACE

This cumulative doctoral thesis includes a summary (in German and English), a general introduction, two reviews (review on flow and review on sponge microbes) one of which is submitted, and three research articles, one of which is published. This work was supervised by Dr. Friederike Hoffmann Prof., Dr D. de Beer and Dr. B. Barker-Jörgensen. The field work for this study was conducted at the Mediterranean coast in France (Station de recherche d'Endoume) and Spain (CSIS Barcelona), in the laboratory in the Netherlands (University Waggeningen) and in Germany (Max-Planck-Institute for Marine Microbiology). This work was funded by the EU SPONGES project N^{o} 017800 and by Max-Planck-Institute for Marine Microbiology.

The articles presented are in the following chapters:

General introduction:

Chapter 1:
In Chapter 1 'General introduction' presents background knowledge about sponges and specificities of the two target species: *Dysidea avara* and *Chondrosia reniformis* including explanatory photos. The research questions and aims are outlined. This chapter is unpublished.

Reviews:

Chapter 2:
In Chapter 2 'Review: Water flow from and around sponges' background information is given about sponge structure and the importance of ambient flow to sponges for their growth, ability to capture food and as means to take away their waste products. A summary is given of how much we know at this point about the ability of sponge to create their own oscular flow and original data is presented. This chapter introduces in more details the topics treated in Chapter 4 and 5. The original data in this chapter will be submitted as a note.

Chapter 3:
In Chapter 3 'Sponges (Porifera) and sponge microbes (submitted)' presents a review of our actual state of knowledge about sponge-associated microbes. This chapter introduces in all details the topic treated in Chapter 6. This chapter has been submitted to Encyclopedia of Geobiology, Springer Verlag, Heidelberg, Germany, as the second of two authors. I contributed to this publication by bringing original data, by writing some of the document's sections and by editing the document.

Research results chapters:

Chapter 4:
In Chapter 4 'Oxygen dynamics and flow patterns of *Dysidea avara* (Porifera, Demospongiae)' the relationship between *Dysidea avara*'s pumping activity

and oxygen content in the tissues is explored. This chapter has been published as first author in the Journal of the Marine Biological Association of the U.K., vol. 87: 1677-1682. The co-authors of this publication gave me access to sponges and laboratory facilities or were my supervisors.. They corrected the manuscript. Han Røy provided guidance on Particle Tracking Velocimetry. Otherwise, I did all the data collection, analysis and writing up.

Chapter 5:
In Chapter 5 'Heterogeneous oxygenation resulting from active and passive flow in two Mediterranean sponges' the oxygenation of the two target species is described spatially and temporally both under captivity and in the field. The impact of ambient flow on sponge tissue oxygenation is assessed. This chapter is in preparation for publication as first author. Field data was obtained by M. Weber under my supervision. I did all the laboratory data collection and analysis. D. Mendola provided access to captive sponges and some of the equipment. I wrote the manuscript.

Chapter 6:
In Chapter 6 'Microbial abundance and processes in three Mediterranean sponges' I present how many microbes are found in which part of the two target sponges and explores which of the major microbial processes, such as nitrification, denitrification, anammox and sulfate reduction, take place in the target species. This chapter is in preparation for publication as first author. S. Schöttner provided the sponge microbes pictures, the cell counts and aquiferous system estimates. G. Lavik and M. Kuypers offered advice on experimental design and interpretation. I conducted the experiments, carried out the laboratory analyses, interpreted the data and wrote the manuscript.

Concluding chapter:

Chapter 7:
In Chapter 7 a general discussion and outlook are offered to sum up the thesis and propose follow-up research for the topics addressed in the thesis. This chapter is unpublished.

Zusammenfassung

Schwämme sind die ältesten vielzelligen Organismen der Erde. Diese sessilen Filtrierer beherbergen eine Vielzahl unterschiedlicher Mikroorganismen in ihrem Gewebe, über deren Funktion immer noch wenig bekannt ist. Schwämme (und/oder ihre assoziierten Mikroben) produzieren Sekundärmetabolite, die auch von großem Interesse für die pharmazeutische Industrie sind. Die Kultivierung von Schwämmen in Aquakultur ist jedoch schwierig, was unter anderem an unserem begrenzten Wissen über die Physiologie der Schwämme und ihrer Mikroorganismen liegt. Aus diesem Grund haben wir in dieser Studie Sauerstoffkonzentration und mikrobielle Schlüsselprozesse in zwei Mittelmeer-Schwämmen untersucht, die auch von kommerziellem Interesse sind: *Dysidea avara* produziert den Anti-Psoriasis-Wirkstoff Avarol, und *Chondrosia reniformis* besitzt Kollagenfasern, die für kosmetische Produkten genutzt werden können.

Das Vorhandensein oder Fehlen von Sauerstoff im Schwamm hat einen starken Einfluss auf die Mikrobengemeinschaft und die mikrobiellen Prozesse im Schwamm. Daher wurde der Zusammenhang zwischen der Sauerstoffkonzentration im Schwammgewebe und dem Pumpverhalten des Schwammes untersucht. Wie haben gezeigt, dass das Gewebe eines pumpenden Schwammes sauerstoffgesättigt ist, während ein nicht pumpender Schwamm anoxisch ist. Das Pumpen kann jedoch nur zum Teil die Sauerstoffverteilung im Schwammgewebe erklären; es muss also noch andere Ursachen für die Sauerstoffverteilung geben. Da Schwämme in Laborexperimenten bekanntermaßen schwächer pumpen als in der Natur, haben wir untersucht, ob Anoxia auch im Gewebe „ungestörter“ Schwämme auftritt, die sich in ihrer natürlichen

Umgebung befinden. Wir haben gezeigt, dass in ungestörten Schwämmen ebenfalls Perioden mit anoxischen Bedingungen auftreten, die bis zu einer Stunde anhalten konnten. Ferner konnten wir zeigen, dass die Versorgung mit Sauerstoff im Schwamm sehr heterogen war; es bestand jedoch eine Tendenz zu einer besseren Sauerstoffversorgung in der Nähe des Osculums. Unsere Resultate zeigen, dass möglicherweise eine Verzögerung zwischen Pumpaktivität und Sauerstoffversorgung besteht.

Zudem wurde gezeigt, dass die Strömungsgeschwindigkeit in der direkten Umgebung des Schwammes ebenfalls einen Einfluss auf die Sauerstoffversorgung des Schwammes hatte. Große Schwämme mit einem gut ausgebildeten Kanalsystem konnten ihr Pumpverhalten je nach An/Abwesenheit und Stärke der umgebenden Strömung variieren. Im Gegensatz dazu waren kleine Schwämme ohne Osculum (und wahrscheinlich auch ohne funktionierendes Kanalsystem) für die Sauerstoffversorgung eindeutig von der Umgebungsströmung abhängig.

Das Auffinden von anoxischen Zonen und die Tatsache, dass beide Schwammspezies mehr Mikroorgansimen besitzen als das umgebende Seewasser, eröffnet die Möglichkeit, dass auch anaerobe Prozesse in unseren Versuchs-Schwämme auftreten können. Wir haben die Anzahl der Mikroben in unseren Versuchs-Schwämmen nach einer neu entwickelten Methode kalkuliert und dies mit dem prozentualen Volumenanteil des Kanalsystems korreliert. Dabei war die Anzahl der Bakterien generell höher als bisher beschrieben; wir sind der Meinung, daß mit unserer neuen Methode auch Mikroben-

Nischen innerhalb eines Schwammes aufgespürt werden können, die eine andere Anzahl an Bakterien besitzen als das übrige Gewebe. Dies war der Fall in *C. reniformis*, wo Kollagen und Mesohyl eine signifikant unterschiedliche Anzahl an Bakterien besaßen. Sowohl in den Spezies mit hoher (*C. reniformis*) als auch in denen mit niedriger (*D. avara*) Anzahl von Mikroben konnten wir zwei mikrobielle Schlüsselprozesse nachweisen: Nitrifikation und Denitrifikation. Nitrifikation, ein aerober mikrobieller Prozess, treibt einen einfachen Stickstoffkreislauf in dieser Spezies an: Ammonium wird vom Schwamm im Überschuss produziert. Während 95 % des Ammoniums einfach durch Filtration entsorgt werden, werden die restlichen 5 % durch mikrobielle Ammonium- und Nitrit-Oxidation (Nitrifikation) zu Nitrat umgewandelt. Die Hälfte des dadurch produzierten Nitrats wird durch Denitirifzierung auf anaerobem Weg zu N2 reduziert und geht dadurch dem biologischen System verloren. Die Entdeckung von Denitifikation in *D. avara* und *C. reniformis* ist nach unserem Wissen der erste Nachweis der Denitrifikation in Mittelmeerschwämmen. Anammox und Sulfatreduktion waren in beiden Schwämmen unterhalb des Detektionslimits. In weiterführenden Untersuchungen wäre es sinnvoll herauszufinden, welche Faktoren das Pumpverhalten der Schwämme steuern und welche weiteren mikrobiellen Prozesse in Schwämmen auftreten. Zudem wäre es wichtig herauszufinden, ob ein Energietransfer zwischen Mikroben und Schwammzellen stattfindet.

Summary

Sponges are the first multi-cellular organisms on the tree of life and are sessile filter-feeders. They harbor a large variety and a high number of associated microbes within their body, the role of which is still poorly understood. Sponges (and/or their microbes) produce secondary metabolites which are of interest to the pharmaceutical industry, however, the aquaculture of sponges remains problematic and our knowledge of sponge physiology and of the role of sponge-associated microbes is scarce. For these reasons, we investigated the pattern of oxygen distribution and key microbial processes in two Mediterranean sponges of commercial interest: *Dysidea avara* which produced avarol an anti-psoriasis, and *Chondrosia reniformis* whose collagen fibers can be used for cosmetics.

Microbes and microbial processes will be strongly influenced by the presence or absence of oxygen within the sponge so we described the relationship between body oxygenation and ventilation. We showed that a ventilating sponge had well oxygenated tissue while a non-pumping sponge was anoxic. Pumping activity partially explained the oxygenation in the sponge body pointing clearly at other causes for oxygenation. Since it is well known that sponges will ventilate less in laboratory conditions, we established whether tissue anoxia could also be found in undisturbed sponges in their natural environment. We showed that field (undisturbed) sponges also had periods of anoxia lasting up to 1h and that oxygenation across the sponge was heterogeneous with a tendency towards better oxygenation near an osculum. Our results indicate the possible presence of a lag time between sponge ventilating activity and oxygen levels in the sponge.

Ambient flow was also shown to have an impact on sponge oxygenation. Large sponges with a functional aquiferous system were able to change the frequency of ventilation according to the presence or absence of ambient flow and according to the magnitude of ambient flow. In contrast, a small sponge without an osculum (and presumably without a functional aquiferous system) was unequivocally dependent on ambient flow for tissue oxygenation.

The discovery of anoxic zones together with the fact that both species harbor more microbes than seawater opened the possibility that anaerobic microbial processes exist in our target sponge species. We estimated the number of microbes in our target species using a new method together with the proportion of the aquiferous system in the sponges. We found that our bacterial counts are generally higher than other published records and we argue that our method allows detection of micro-niches within the sponge which may have a different number of microbes. This was the case in *C. reniformis* in which the collagen and the mesohyl had significantly different number of microbes. Both in the species with high (*C. reniformis*) and low (*D. avara*) number of microbes we found the presence of two key microbial processes: nitrification and denitrification. Nitrification, an aerobic microbial process reveals a simple nitrogen cycle in those species: ammonium is produced in excess. While 95 % of it is simply discarded through ventilation 5 % is converted into nitrate through microbial ammonium and nitrite oxidation (i.e. nitrification). Half of the nitrate thus produced is anaerobically lost to N_2 through the activity of denitrifiers. The discovery of denitrification in *D. avara* and *C. renformis* is the first report of denitrification in Mediterranean sponges. Anammox and sulfate

reduction were below detection limits in both species. We propose that meaningful ways to carry on with this research would be to find out which factors trigger sponge ventilation, to establish which other microbial processes occur in sponges and to ascertain whether an energy transfer exists from microbes to sponges.

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Chapter 1

1. General introduction

Sponges are the simplest multi-cellular organisms, are at the very base of the Metazoan tree of life and occur both in sea and freshwater (Ruppert and Barnes, 1994). Sponges appeared approximately 600 million years ago in the fossil record, in the Pre-Cambrian. Genetic studies showed that sponges belong undoubtedly to the Metazoa (Ax, 1995) (Müller, 1998 a, b). All living (about 6000 species) and extinct sponges are grouped into three classes: the exclusively marine sponges Calcarea (calcareous sponges) with calcareous skeletal elements (spicules), Hexactinellida (glass sponges) with siliceous spicules, and the Demospongiae (demosponges), a non-monophyletic class which comprise the majority (85%) of extant sponges with siliceous spicules, spongin (fibrous protein) or collagen fibers (Hooper and Van Soest, 2002).

Sponges lack true tissues*. The outer cell layer of sponges, the pinacoderm, defines the boundary between the outside and the inside of the sponge and is made up of pinacocyte cells. In some sponges it is very thick and is referred to as 'cortex'. The mesohyl, the inner body mass is composed of a gelatinous proteinaceous matrix which contains a limited number of cell types. The collencyte produce the spicules and the archeocytes are ameboid and totipotent and can transform themselves into any other sponge cell type. They wander freely between the skeletal elements (spicules, sponging fibers or collagen in demosponges, Figure 1) (Ruppert and Barnes, 1994).

* The word ‘tissue’ is nevertheless used throughout the thesis but should not be taken as a synonym of higher Metazoa tissue

Their exact function is still under debate but include particle digestion through phagocytosis. The choanocytes are flagellated cells with a collar (Figure 1). They are grouped in choanocytes chambers and through the beating of the flagella, create a pressure difference which draws the water trough the inhalant openings (ostia) via canals to the choanocyte chambers (Figure 1) where particle trapping takes place. The water passes further towards the exhalent canals which lead to the exhalent siphon, called the oscula or oscules (Figure 1) (see Chapter 3) (Ruppert and Barnes, 1994).

The water inlets (ostia) are approximately 5-50 μm in diameter (depending on the species) and the water outlet (oscula) have a diameter of 1 or more mm. The pumping mechanism driven by the choanocytes is very efficient and sponges have been shown to be able to pump many times their own body volume per hour (e.g. up to 1 L per hour and ml of body volume (Reiswig, 1971). Tropical cave sponges filter their own body volume 7.2 times per second (Kötter *et al.*, accepted). Sponges are predominantly filter feeders (Ruppert and Barnes, 1994) although some species are carnivorous (Vacelet and Boury-Esnault, 1995, Vacelet, 2008) and incorporate food items via the surface cells (Vacelet and Boury-Esnault, 1995).

Non-carnivorous sponges feed on a variety of particulate foods sources through phagocytosis of large food items (5-50 μm), for example, phytoplankton (Rupert & Barnes 1994). The choanocytes capture small food items (<1 – 4 μm) such as pico- and ultra-plankton (Kötter *et al.*, accepted). Particles are then transferred to the archeocytes where digestion occurs (Rupert & Barnes 1994). Sponges also have symbioses with eu- and prokaryotes (Chapter 3 and 6) and can either derive indirect nutritional benefit from their symbionts (e.g. products from photosynthetic activity) or directly used the micro-organisms growing within their body as food (see Chapter 3

for review). Sponges also take up dissolved organic carbon (DOC), the process being most likely mediated through their associated microbes (De Goeij *et al.*, 2008).

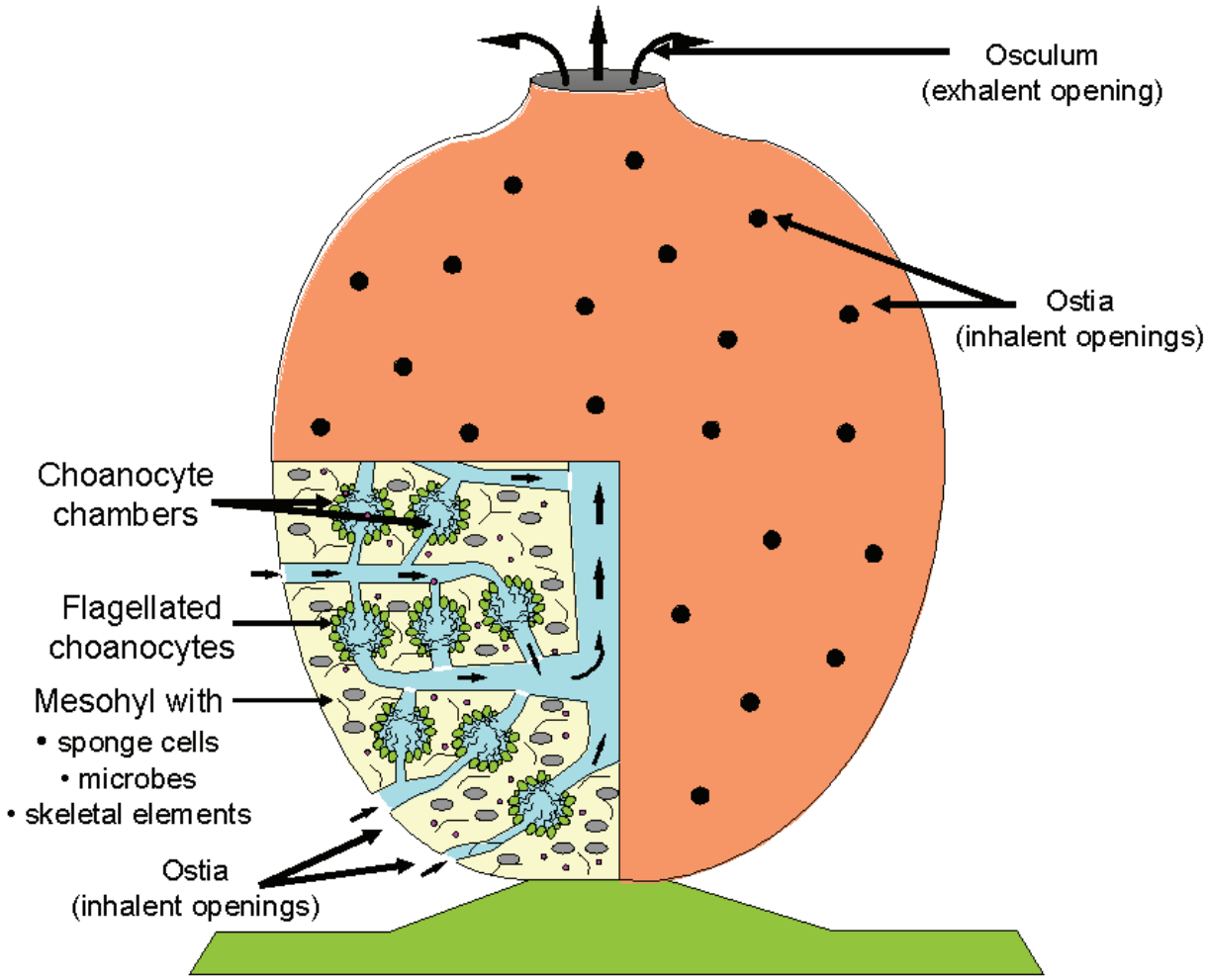

Figure 1: Schematic representation of an imaginary typical sponge showing exterior and internal morphological features. Note that the scale of the different elements is fictive. (Modified and reproduced with permission from original drawing of R. Osinga)

Sponges are sessile (i.e. attached to the substratum) invertebrates but are capable of some 'movement' through tissue growth towards advantageous locations or tissue retraction away from deleterious locations. Their have adapted to their sessile lifestyle and absence of locomotion by chemical defense and attack mechanisms. They are the

main producers of bio-active compounds, along with bryozoans and ascidians. Sponges produce numerous bio-active compounds to protect themselves from predation, biofouling and allow them to win competitive interactions with other sessile invertebrates through allelopathy (the release of a toxic substance to win a competitive interaction). The efficacy of these deterrent methods is such that sponge secondary metabolites are sought after for biotechnology (Thakur and Müller, 2004). The immense diversity and complex biochemistry of these chemical defense mechanisms is still hardly explored. Essential for the commercialization of these secondary metabolites is that sponges must be cultivated on industrial scale but our knowledge of their requirement in captivity is scant. It is thus imperative to better understand sponges and their associated microbes in order to succeed maintaining sponges in aquaculture.

1.1 Sponge research: past and present

Sponges have a very plastic morphology and members of one species can look very different making it very difficult to correctly identify a sponge by its growth form and color alone. Taxonomists have had to rely mostly on skeletal elements (Hooper and Van Soest, 2002) and the presence or absence of certain characteristic chemicals to identify sponges (Bergquist and Wells, 1983). Thus the taxonomy of sponges has been, and continues to be one of the major research goals for spongologists (Figure 2). A successful attempt was made by Hooper and Van Soest (2002) to gather all existing materials in a book, 'Systems Porifera', which constitutes the generally accepted reference on which future sponge taxonomy can be based. Modern taxonomists use molecular data (see Figure 2) for taxonomical purposes and this brought about the discovery of ‘cryptic’ species, those species that were considered to form one group

according to morphological evidence but were shown to be distinct groups genetically. Similarly, cosmopolitan species, which were thought to occur all over the world, are now often found to differ genetically although they share identical morphological characteristics. Consequently, the systematics of those species must be modified according to those new findings, keeping the taxonomy of sponges in a state of flux.

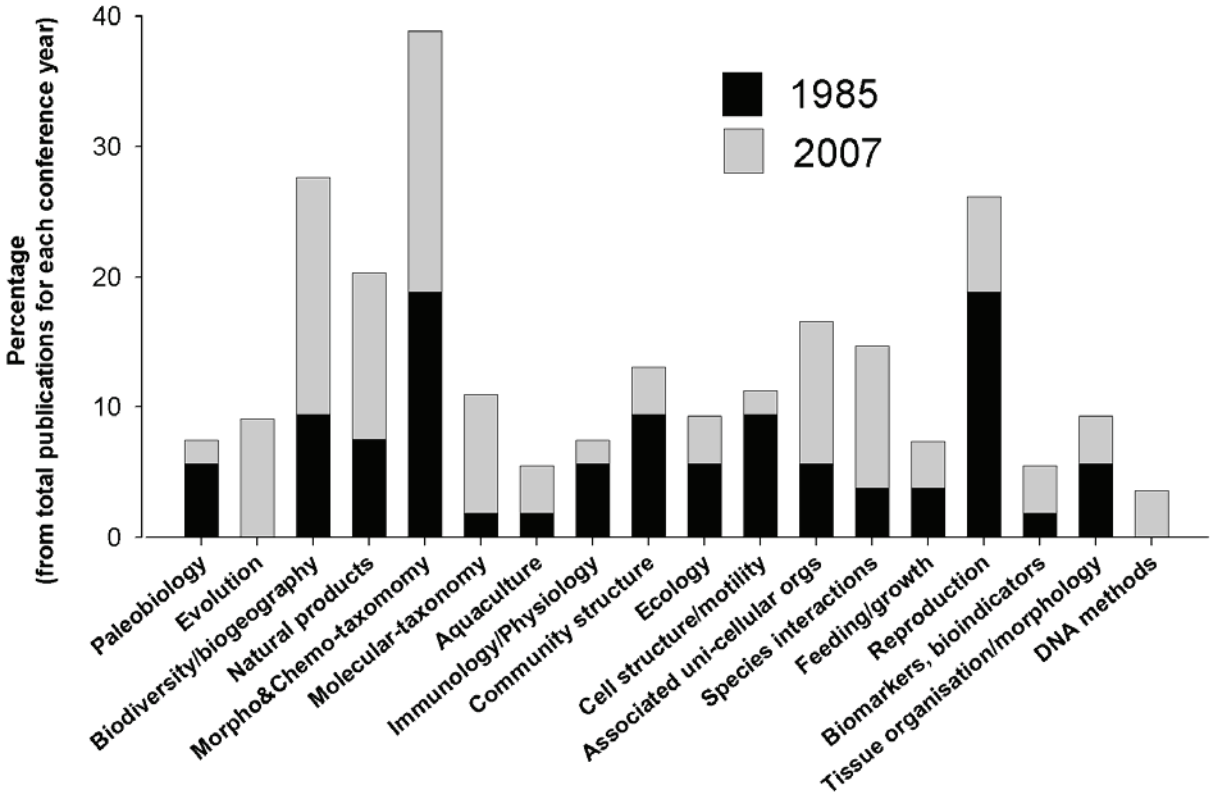

Figure 2: Percentage of publications presented at the 1985 and 2007 international sponge symposium by categories, representing the foci of sponge researchers

In recent years, sponges have raised the interest of pharmaceutical and cosmetic companies eager to commercialize some of their structural components or bioactive compounds (see natural products, Figure 2). Although other invertebrates such as bryozoans and coelenterates (corals) are also sources of bioactive compounds,

sponges are the most promising source (Figure 3). In the past, only bath sponges were traditionally harvested and/or cultivated. What makes them commercially interesting is their network of inter-connected fibers, the sponge skeleton, which has exceptional water retention properties. Other species are also sought after for their structural elements; for example, *Chondrosia reniformis* possesses an extensive network of collagen fibers (see *C. reniformis* description below). Bioactive compounds (antimicrobial, antifouling, anticancer and antiinflammatory) are interesting natural products in sponges (Figure 4).

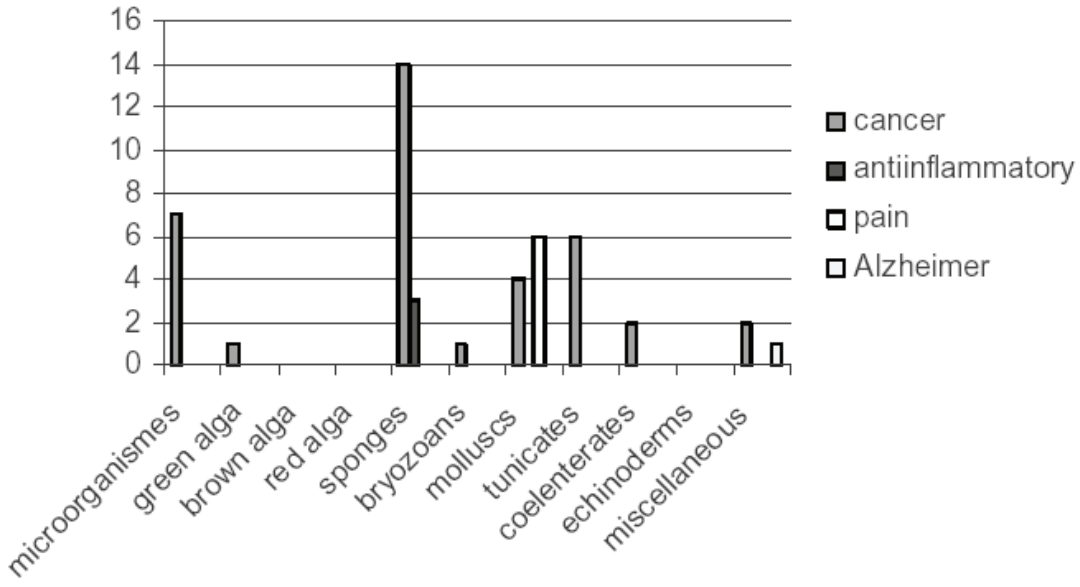

Figure 3: Sources of bio-active compounds amongst invertebrates, plants and microorganisms (Reproduced with authorization from C. Debitus)

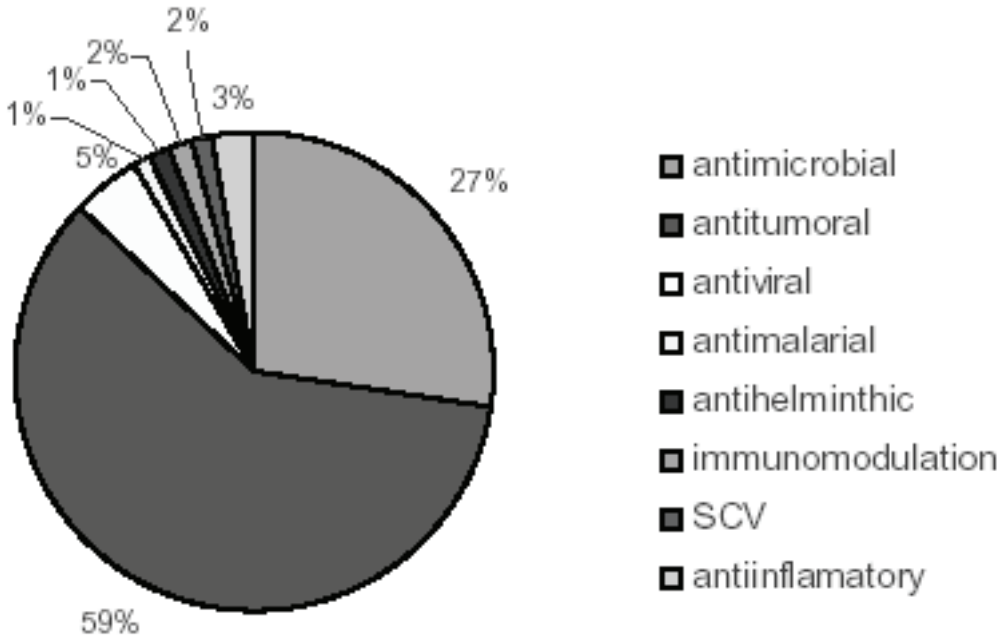

Figure 4: Effects of sponge bio-active compounds with antitumoral (59%), antimicrobial (27 %) and the antiviral (5 %) being the most widespread. (SCV = peptide with proven immune stimulating effects). (Reproduced with authorization from C. Debitus)

There is now a strong interest in the cultivation of sponge species whose bioactive compounds cannot be produced through chemical synthesis or when the cost of synthesis is too high. Thus, the cultivation of many species is now undertaken either *in situ* (on cultivation lines in the sea) and in aquarium, for example for *Dysidea avara* (see species description below). The research focus having been mostly on taxonomy (Figure 2), relatively little is known about sponge physiology or aquaculture (Figure 2) when compared to other invertebrates like corals. Keeping sponges healthy in aquarium is notoriously difficult, as the specific needs of sponges are still poorly understood. Only too often, individuals collected in the sea die after transport or survive in aquarium for a period of about 12 months but do not gain biomass and do not reproduce in captivity. Despite the close monitoring of water

quality and the advances made on which food to offer them, it is still a challenge to keep sponges in aquarium for any long period of time, let alone having them gain biomass for commercial purposes.

1.2 Research questions and aims of the study

One of the hypotheses explaining the difficulty of growing sponges in aquaria is that the sponge-associated microbes may influence the well being of sponges in captivity or, that the complex, but so far poorly understood interactions between sponge metabolism and associated microbes may complicate cultivation requirements. We know that sponges can have up to 33 % of their tissues packed with micro-organisms (Vacelet, 1975) (cell densities 10^8-10^{10} cm^{-3} sponge) (Hentschel *et al.*, 2006) and that they belong to a wide variety of taxonomical groups with varied metabolisms (see Taylor (2007) for review and Chapter 3). We also know that the microbe population in sponges may change in number and diversity following a certain time in captivity (Hoffmann *et al.*, 2006a, b, Mohamed *et al.*, 2008) and that microbial processes of oxic and anoxic nature occur in some species, for example in *Geodia barretti* (Hoffmann *et al.*, 2005). Microbial processes will be strongly influenced by the presence of absence of oxygen in the sponge and thus several questions apply to the two study species, two Mediterranean sponges of commercial interest: what are the chemical micro-habitats such as oxygen within the sponge, how are they influenced by ventilation activity and what key microbial processes occur and at which rate?

Accordingly, the aims of this thesis are to:

1. characterize the pattern of oxygenation present in the target species using micro-electrodes and link them to sponge ventilation activity under a variety of conditions and to assess the importance of ambient flow on sponge oxygenation
2. characterize the presence and rates or the absence of key aerobic and anaerobic microbial processes in the target species

The research pertaining to these two aims is shown in research chapters 4 and 5 (for aim 1) and chapter 6 (for aim 2). Additionally, two review chapters one on flow around and from sponges (Chapter 2) and one on sponge-associated microbes (Chapter 3) are there to introduce the research chapters and also contain original data obtained during the 3 years and 1 week taken to complete this doctoral thesis.

1.3 Target species description

The two study species, *Dysidea avara* Schmidt 1862 (Demospongiae, Ceractinomorpha, Dictyoceratida, Dysideidae) and *Chondrosia reniformis* Nardo, 1847 (Demospongiae, Chondrosida, Chondrillidae) belong to the Demospongiae (Hooper and Van Soest, 2002). Both species are common Mediterranean sponges (Lazoski *et al.*, 2001, Hooper and Van Soest, 2002).

1.3.1 Dysidea avara

Dysidea avara is a common Mediterranean sponge (Uriz *et al.*, 1992) which grows in shallow water of the continental shelf (10 - 40 m) on hard substratum, between pebbles at the bottom, on overhangs, walls and at the entrance of caves. This genus occurs both in temperate and tropical regions (Cook and Bergquist 2002). It is approximately 5 to 15 cm in diameter, and can be pink, grayish or white in color (Figure 5). It is massive in morphology with distinct asperities (called conules) on its surface. This species harbors usually numerous and distinct oscules which are located in between the conules (Figure 6) and has a 'porous' body with an extensive aquiferous system in relation to the mesohyl (see Chapter 6). The sponge is elastic and easily compactable, yet tough. When the sponge is dying, the cells fall off the skeleton and the spongin fibers become visible. This often happens after transport or after the sponge has been kept several months in captivity (Figure 7).

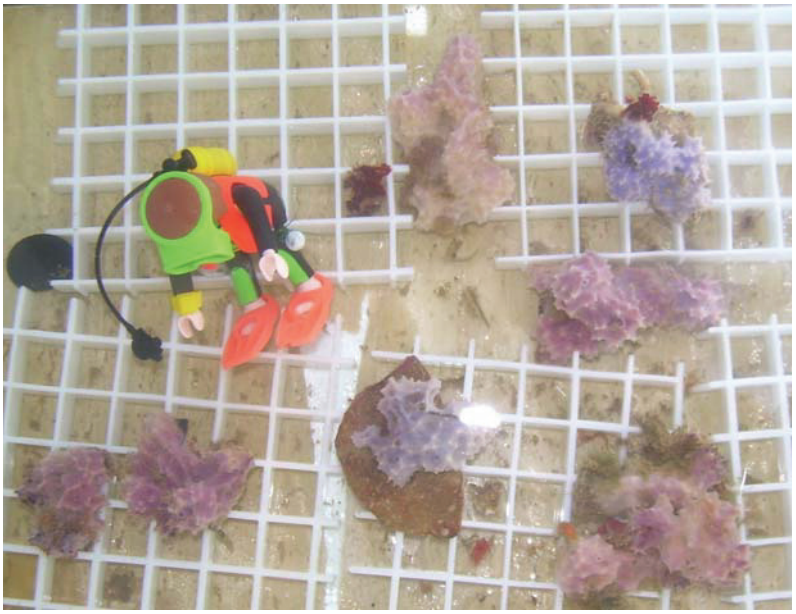

Figure 5: *Dysidea avara* individuals in holding aquarium

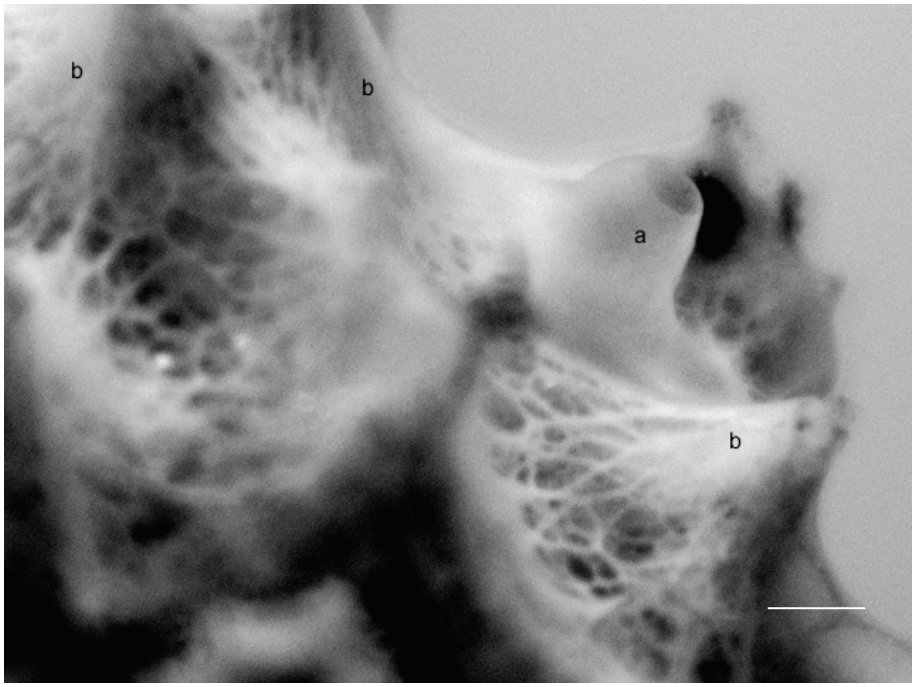

Figure 6: Close-up photo of *D. avara* showing a) an osculum b) conules made by the spongin skeleton. Scale bar = 1 mm

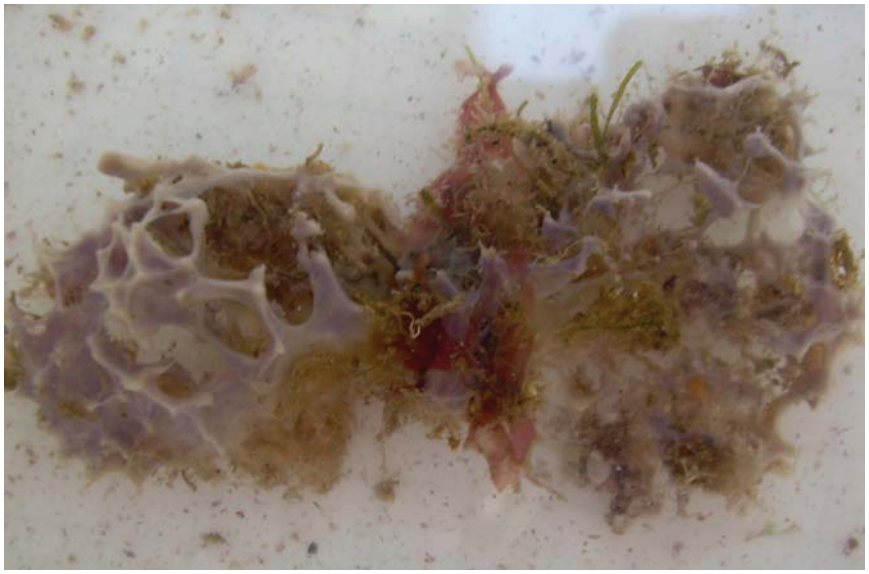

Figure 7: Dying *Dysidea avara* specimen, showing pink live cells on the spongin fiber skeleton. Notice the cells spread out around the sponge. Specimen = 4 cm long

This species lacks a mineral skeleton (spicules) but has spongin fibers, which remains connected in a network after the sponge has died (Figure 8) similarly to the common bath sponge. Foreign material (sedimentary inclusions) is incorporated into the sponging fibers and into the matrix thus rendering the sponge less pliable and more brittle (Figure 9).

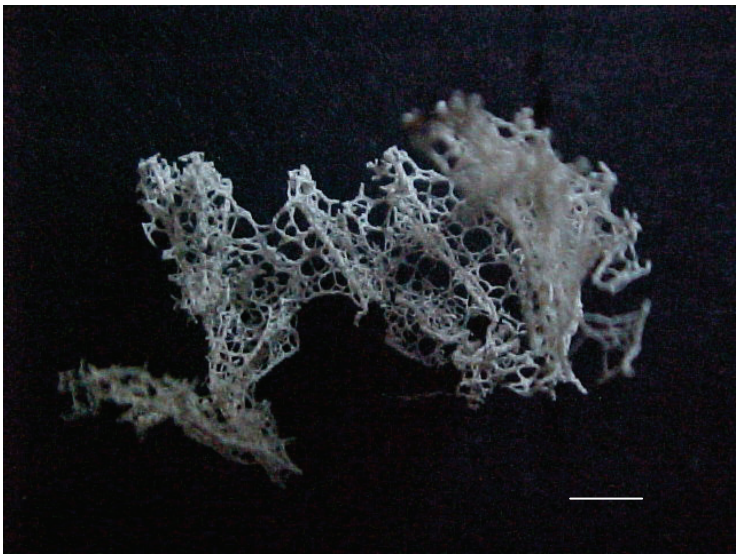

Figure 8: Close-up of a *Dysidea avara* skeleton spongin fibers. Scale bar = 5 mm

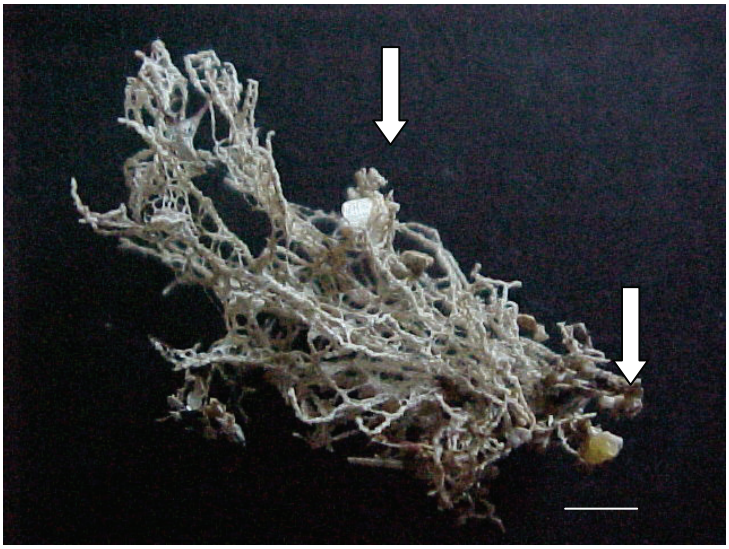

Figure 9: Close-up of a *Dysidea avara* skeleton showing the interconnecting spongin fibers, which create a network in which sand grains are embedded. Scale bar = 5 mm

D. avara has a range of secondary metabolites among which sesquiterpenes (Cook and Bergquist, 2002), avarol (Müller *et al.*, 1985b, Minale *et al.*, 1974) and avarone (Müller *et al.*, 1985a). Avarol is produced within the choanocytes that compose the choanocyte chambers (Uriz *et al.*, 1996) and is of interest for bio-medical companies due to its potent effect against psoriasis.

1.3.2 Chondrosia reniformis

Chondrosia reniformis is mottled brown and has a smooth surface (Figure 10). Small individuals often only have one osculum which services the whole sponge (Figure 11). This sponge occurs on shaded overhangs, cliffs and in caves in the shallow water (1-50 m) of the Mediterranean. The species is thought to be cosmopolitan (worldwide distribution) but might in fact comprise several species (see Lazoski *et al.* 2001). It harbors a relatively reduced aquiferous system (Chapter 6) and a mesohyl with very abundant symbiotic bacteria (Boury-Esnault (2002), and see Chapter 6). When the ribonucleid acid (RNA) was extracted from *C. reniformis*, it was found that two thirds

of it was of bacterial origin (Kuusksalu *et al.*, 2007). Its morphology is massive, often oblong and approximately 5-20 cm in diameter (Figure 10).

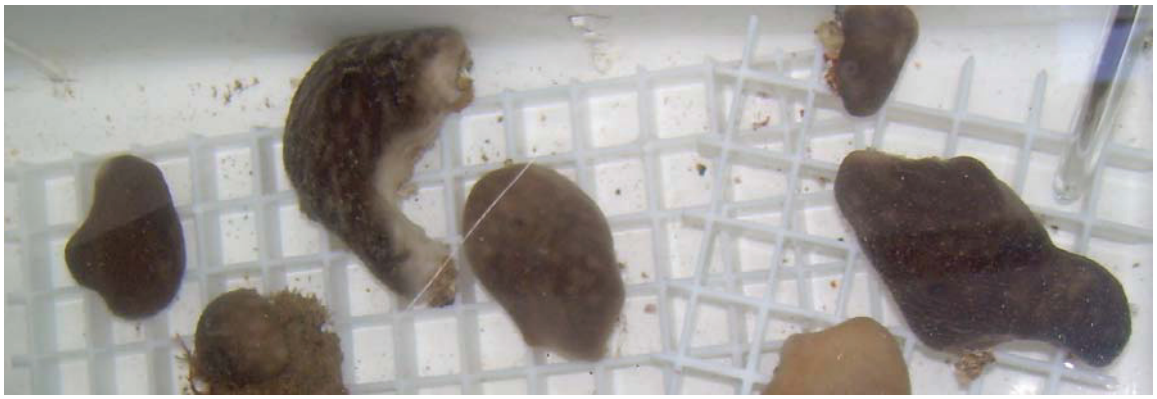

Figure 10: *Chondrosia reniformis* individuals in aquarium

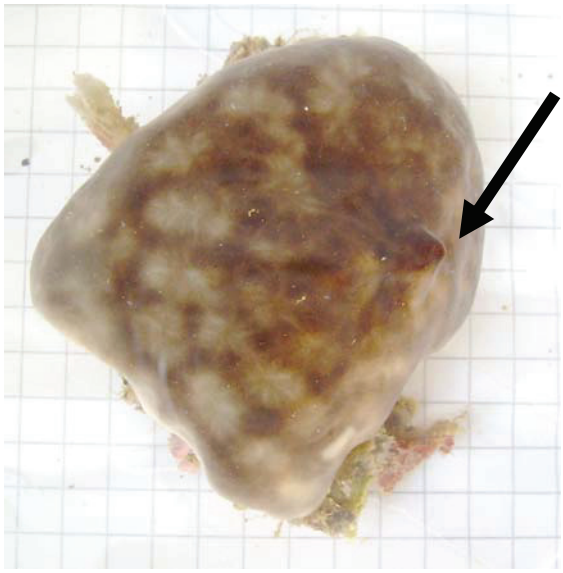

Figure 11: *Chondrosia reniformis* individual with one osculum (arrow). Specimen = 5 cm wide

Unlike other sponges, it lacks a structural skeleton of spicules or spongin fibers but has a high collagen content (Imhoff and Garrone, 1983, Garrone *et al.*, 1975). Particularly on the outside, where it forms a dense layer of collagen, called cortex, (Figure 12) with fibrillar collagen which is typical of the order and family (Boury-

Esnault, 2002). *Chondrosia reniformis*' high content in collagen makes it a sponge with good perspectives for biotechnology. The collagen can be used for creams, plasters against dry skin or insufficient sebum (Swatschek *et al.*, 2002a) and for the preparation of nano-particles (Swatschek *et al.* 2002b). However, the aquaculture of this species in aquarium tanks is a real challenge and often results in sponge death. The symptoms of sickness, often followed by death, are 1) a layer of slime covering the sponge (Figure 13) and containing bacteria and protists, (Hoffmann pers. com.) and 2) a strong sulfur smell. Black stains can usually be seen inside the sponge tissues but are usually invisible from outside.

Figure 12: Cup-up piece of *Chondrosia reniformis* showing cortical layer (arrows) and mesohyl (lighter colour)

The enzymatic activity of 2-5A synthetase in *C. reniformis* (Kuusksalu *et al.* 1995, Kelve *et al.*. 2003, Reintamm *et al.*. 2003) showed similarities between this sponges and mammals in which this enzyme is involved in the immune system. The cDNA

sequences of *C. reniformis* were highly similar to those of animals higher on the evolutionary tree, particularly those of vertebrates such as primates and humans (Kuusksalu *et al.* 2007).

Chondrosia reniformis has the ability to stiffen when touched. It is a remarkable property and it has been compared to the tissue of some echinoderms, which also harden upon touch (Bonasoro *et al.* 2001). When touched, the surface of the sponge changes from soft to stiff within seconds (Parma *et al.* 2007 p505 in book, and pers. obs.). This happens consistently during sponge specimen collection. However, unlike in echinoderms, where the interaction between collagen fibers is under nervous control, the tensile properties in the cortex of *C. reniformis* are under cellular control (Wilkie *et al.* 2006).

Chondrosia reniformis is able to disperse through the process of 'creeping' in which long outgrowths (10 cm to 3 m long) extend from the sponge body which eventually break off to create a new individual (Bonasoro *et al.*, 2001). This process has been interpreted as asexual reproduction, locomotory strategy or response to environmental changes (Parma *et al*. 2007) and is only possible due to the collagen nature *C. reniformis*' mesohyl. This creeping habit decreases with lower temperature and is accentuated by gravity. Large sponges were more dynamic and more likely to creep than small ones and the creeping motion is correlated to the likelihood of a substratum piece detaching itself from the matrix (Parma *et al.* 2007).

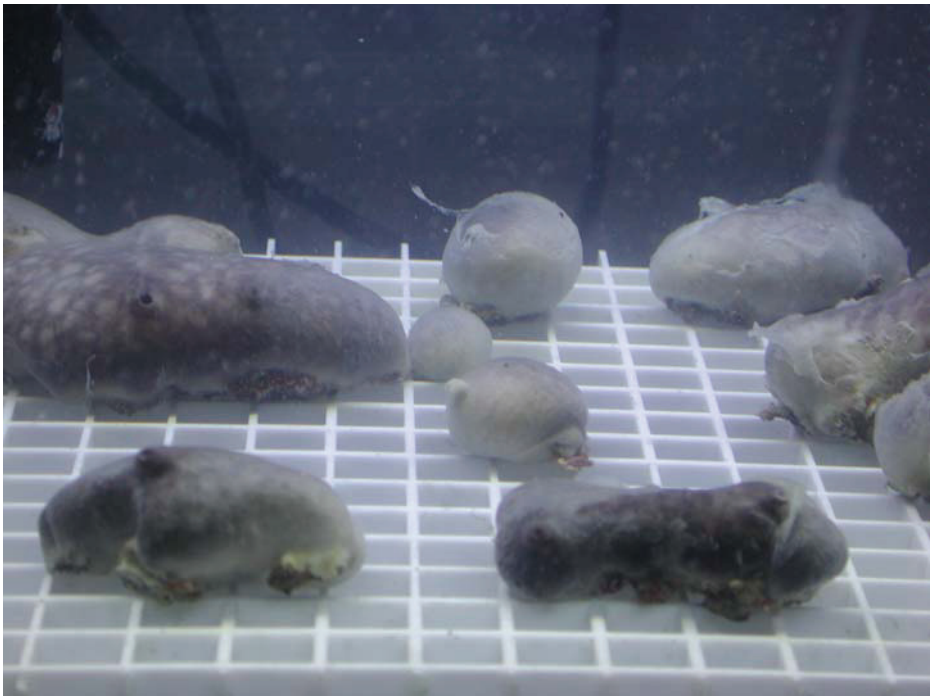

Figure 13: *Chondrosia reniformis* in an aquarium, showing a layer of slime over the sponges' surface which is usually a sign of sickness or imminent death

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Chapter 2: Flow review

2. Water flow around and from sponges

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This review contains original data which will be submitted as a note

This chapter is an introduction to chapter 4 and 5 and provides background information about the water flow around and from sponges.

2.1 Water flow around sponges – ambient flow

Sponges, like many other sessile invertebrates, depend greatly on the water flow around them. Sponges capture particles brought to them by the water flow and feed on them. The flow around sponges also takes their waste products far away from them to avoid recirculation of waste-rich and food-poor water. The nature and magnitude of water flow around sponges has several implications for sponges: it influences the sponges' growth form, presence or absence of oscules (Mendola 2008) and the sponge's shape. Furthermore, ambient flow has an impact on sponge tissue oxygenation and on the sponge's ability to capture food particles.

2.1.1 Sponge general morphology and oscule shape in relation to flow

Sponges' morphology is highly plastic. Sponge individuals within one species can have highly variable growth forms that change according to the habitat where they are found. In other words, the same species of sponge can look very different according to the depth and ambient flow regime where it lives (Hill and Hill, 2002). This has now been found to be true for several species of sponges in varied environments (Table 1). Most often, sponges which are exposed to high ambient flow have sturdier growth forms such as massive or encrusting and a larger number of spicules or skeletal elements while those exposed to low flow have more delicate growth forms, for example, arborescent or finger-like and have less spicules. Mendola (2008) reported that *D. avara*, one of our study organism (see Chapter 1 for species description), had different morphologies according to depth. It has a more pronounced 'chimney-like' morphology at deep sites (14.3 m) than at shallow sites (4.5 m).

The ability of *D. avara* to adapt its morphology to ambient flow was also noted in the laboratory and provides anecdotal evidence. *Dysidea avara* individuals were collected

in the field and brought to the laboratory. They had a massive morphology but after being exposed to ambient flow up to 10 cm s^{-1} in captivity (pers. obs), which was approximately twice the ambient flow found in their natural environment, they became very thin and encrusting. Once the flow regime in the culture tank was decreased to half, the specimens resumed their massive shape again within a few weeks. The ambient flow that those specimens had been subjected to was clearly unusual to them and elicited a physical response from the sponge to secure itself to the substratum.

Dysidea avara is also able to modify the width and the length of its oscular membrane in order for it to fulfill its purpose, namely, to project exhalent water far enough from itself . This is to avoid the risk of re-circulating old water which is devoid of particles, oxygen poor and waste product rich. Anecdotal evidence show how sensitive this mechanism can be: one of our experimental sponges was used to carry out oxygen microsensor measurements by placing the sensor inside the sponge osculum and leaving it overnight. The microsensor (20 μm tip) was placed in the middle of the osculum and did not come in contact with the sponge. In the morning, the microsensor had been engulfed by the sponge tissue, the sponge had closed the target osculum with the microsensor in the middle and had opened a new and functioning osculum 0.5 cm away from the original one. This shows how sensitive the exhalent system is: the sponge was able to determine that the osculum was not performing it function well enough and had opened a new one by remodeling its surface, and this within 12 h. Similar observations on the 'shutting off" of *D. avara*'s oscule has been reported by (Mendola, 2008).

Table 1: Evidence for ambient flow influencing sponge morphology

Main findings	**Species**	**Environment**	**Authors**
In high flow environment, massive and encrusting forms predominate, arborescent in low flow	Several		Bell and Barnes, 2000 Bell *et al*, 2002a Bell *et al*, 2002b Bell *et al*, 2006
Morphological differences attributed to depth and/or flow regime	*Spongia* spp.	Vertical cliff	Pronzato *et al.* 1998
Thicker and more spicules in sponge exposed to higher ambient flow	*Halicondria panicea*	Inlet in/out	Palumbi, 1984 Palumbi, 1986
Compact morphology follows gradient of current exposure	*Halicondria oculata*	Gradient of exposures to water movement	Kaandorp, 1999
Thick and encrusting forms dominant in oscillatory flow	*Haliclona panicea*	Oscillatory flow regime *versus* cave-like	Barthel, 1991
Larger bodies and more skeletal silica found in tide pools as outside, reproduced through transplantation	*Tetilla* sp.	Tide pools	Meroz-Fine *et al*, 2005
Increased spicules with increased flow and predation	*Anthosigmella varians*	Tropics	Hill and Hill, 2002

2.1.2 *Impact on sponge feeding ability*

Sponges without microalgal symbionts mostly depend on ambient flow for food availability (but see Chapter 3 for the role of non-photosynthetic microbes in sponge nutrition). It is the water flow and its residence time around the sponge that determines the amount of food the sponge has access to. Unidirectional flow may not be optimal for particle capture. Oscillating flow allows the passage of the same body of water several times over the individual sponge thus maximizing access to the food particles in that water mass. Since plankton is patchily distributed in time and space, oscillating flow might be the ambient flow regime allowing the largest food capture success. Mendola (2008) showed that regions of low velocity and high vorticity exist

around the sponge but are ephemeral and have low predictability. It is possible that the fine morphology of *D. avara*, with its conulose surface creates micro-hydrodynamic conditions which allows maximal food uptake. It has been hypothesized that the conules of *D. avara* produce water eddies at the back of which the water flow slows down, thus allowing food particles to pass through the inhalant pores of the sponge, the ostia. The external water movement forcing water through the filter feeding structure of suspension feeders is thought to increase the feeding rate and overall benefit of feeding.

To retain evolutionary fitness, all organisms must have a feeding budget in which the energy gained from feeding through assimilation is larger than the cost of capturing, digesting and expelling waste. It makes evolutionary sense that food capture be of as low cost as possible. For this reason, the impact of ambient flow on filter feeders has been investigated in several species of invertebrates, for example the ascidian *Pyura stonolifera* (Knott *et al*., 2004), in which passive flow was of obvious advantage to the animal. Riisgård *et al*. (1993) calculated the cost of filtration in *Halichondria panacea* and *Haliclona urceolus* and concluded that filtration in those sponges is of such low cost that they may not be benefiting from passive current-induced filtration to the same extent as other ciliary suspension feeders.

2.1.3 Impact on sponge oxygenation

Vogel (1974, 1977) studied the impact of ambient flow for 9 species of sponges. He showed that there was a positive correlation between augmented ambient flow and oscular flow both in actively pumping and non-pumping animals. The data presented

correlates external current speed and oscular flow in the range of seconds only. It is unclear whether the correlation also holds over longer times and whether the increased flow through the sponge results in either increasing feeding rate or increased oxygenation rates in the tissues. In Chapter 5, we explore the impact of changes in ambient flow on tissue oxygenation of large and small *Dysidea avara* and report on some novel data.

2.2 Sponge oscular flow

2.2.1 Nature and function of exhalant oscular flow

Oscular flow refers to the water which exits the sponge through its single or multiple oscula. Oscular flow can be measured by tracking particles (see Chapter 4) or dye exiting the osculum or by using flow-meters (see Chapter 5). We measured oscular flow using particle tracking velocimetry (PTV), a technique in which hollow glass particles are tracked from one photo to another and the distance they traveled over time calculated to estimate flow speed. In *D. avara*, oscular flow was perpendicular to the sponge surface (Figure 1) and highest in the middle of the osculum (Figure 2) which is the most efficient way to expel the waste-rich water far away enough from the sponge to avoid recirculation. However, the direction and speed of flow can vary according to how smooth the exhalent canals are which lead the water to the oscule. The *D. avara* specimen we used clearly had an internal structure obstructing flow at 12 mm across the oscule making the oscular flow lower at that particular spot (Figure 2). Even at low pumping velocities, the middle of the oscule remained the area with highest exiting flow velocity (Figure 2, empty circles). Oscular flow is often irregular with clear pluses of high speed followed by low speed (Figure 3). This pattern was

often observed when using the same or other methods of measuring oscular outflow (Chapter 4 and 5).

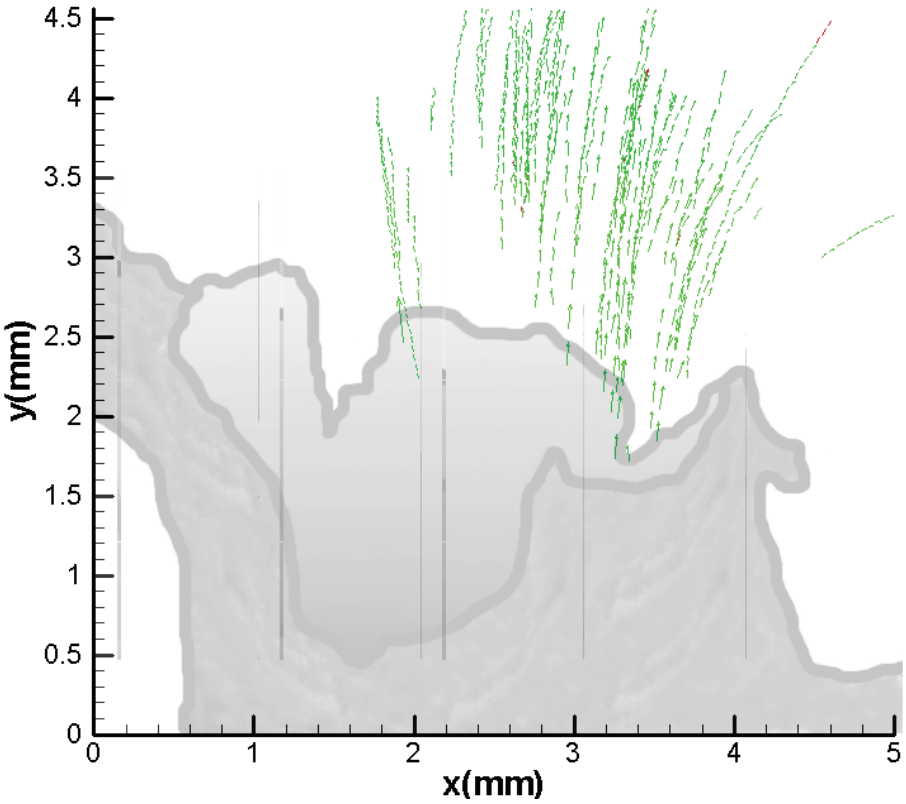

Figure 1: Overlay of a stylized sponge with vectors depicting the direction and velocity of hollow glass particles exiting the sponge osculum

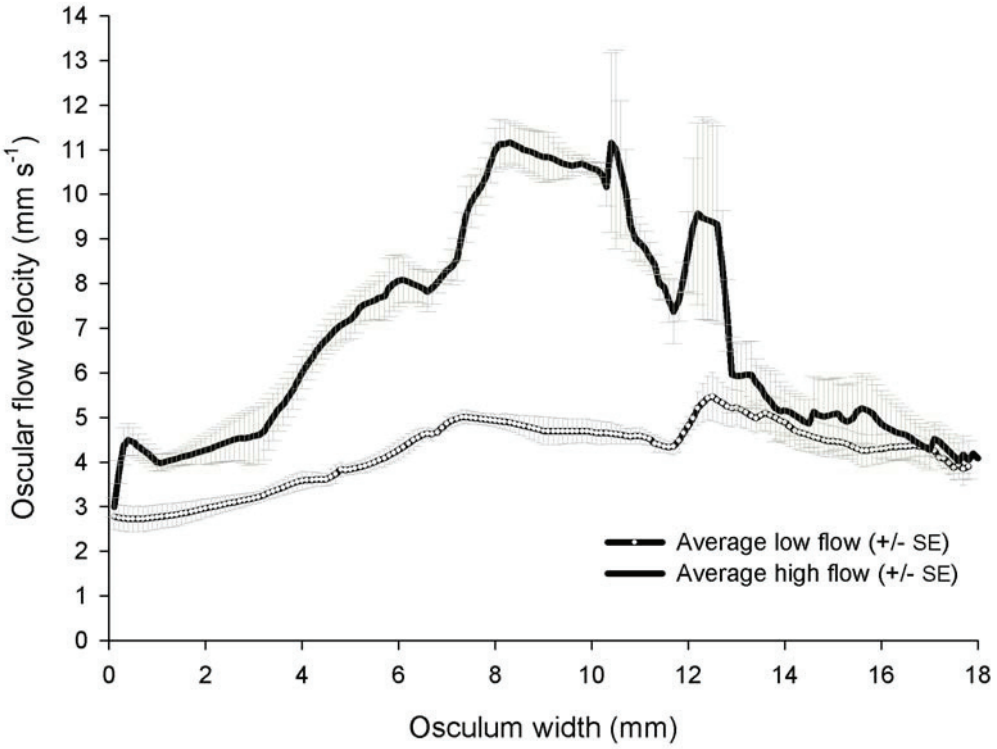

Figure 2: Theoretical curve made of average maximum and minimum speed of hollow spheres exiting the osculum of *D. avara*

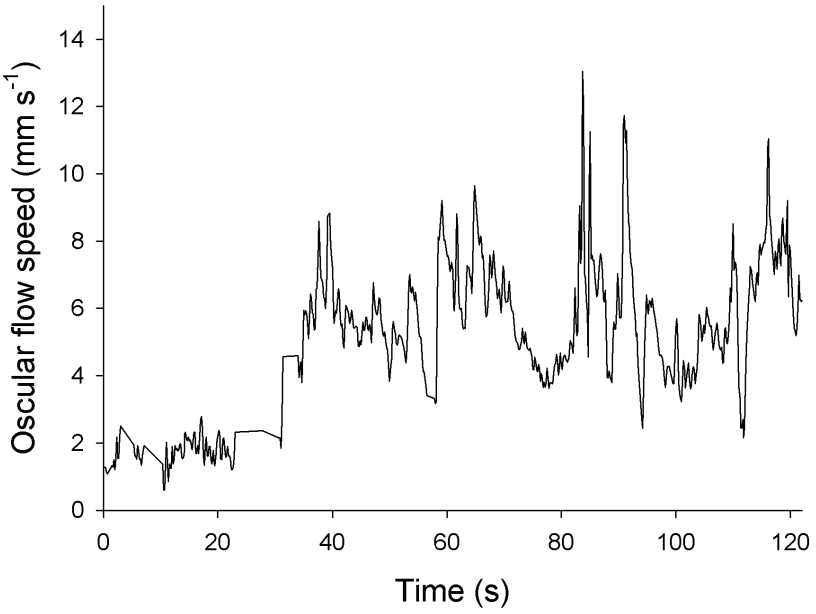

Figure 3: Oscular flow speed in *D. avara* measured by tracking illuminated particles on subsequent video images

The shape and position of the oscules on the sponge is variable, and can be changed by the sponge through cell relocation to adapt to new flow conditions. Aquaculture methods of *D. avara* yielded the best results when the sponge was firmly secured to the growing unit and not, for example, on rope cultures (Osinga pers. com.). The presumed reason is that *D. avara* places its oscules strategically according to the local ambient flow and when only loosely attached to an aquaculture structure it must invest copious amounts of energy to constantly relocate it oscules.

The presence and magnitude or absence of oscular flow in sponges has been researched in the 70s by Vogel and Reisweig. Since then, little has been done to deepen the understanding of why or how a sponge ventilates (but see Pile *et al.* (1996) where *Mycale lingua* had a mean velocity of exhalent current of 14 cm s^{-1}). The triggers to ventilating are also poorly understood and it is not even clear whether the active beating of the flagella in the choanocyte chambers is the default situation which would seem unnecessary for energetical reasons (see Jørgensen 1966). If so, ventilating is the normal state of a sponge and it stops due to some disturbance. Gerrodette and Flechsig (1979) have shown that sponges stop ventilating when the sediment load in the water is too high. Mendola (2008) showed that *D. avara* stops ventilating and shuts its oscula when the ambient water flow is too high. Reiswig, (1971) reported similar findings in Caribbean coral reef sponges. *Halichondria panacea* and *Haliclona urceolus* showed a 4.3 $\pm$ 2.3 time increase in filtration rate when exposed to water 6-12 degree warmer (Riisgård *et al*, 1993).

Periodicity was shown in the pumping activity of tropical marine sponge (Reiswig 1971). Other marine sponges and also some freshwater sponges show no periodicity

in ventilation patterns (see Reiswig, 1971, Gerrodette and Flechsig, 1979, Riisgård *et al*., 1993, and Savarese *et al*, 1997). Cessation of ventilation has been observed in several species, although the causes of it are unknown. The unexplained shut down of sponge ventilation has been reported in *Mycale lingua* (Pile *et al*, 1996), and *D. avara* (Mendola, 2008).

2.2.2 Relation between oscular flow and sponge tissue oxygenation

Tissue oxygenation is also thought to be linked to the magnitude of ventilation. In *Aplysina aerophoba* (Hoffmann *et al*., 2008) the relationship between ventilating activity and oxygenation was such that a pumping sponge had a well oxygenated body and a non-pumping sponge was anoxic (or close to it) everywhere except where oxygen could penetrate the sponge through diffusion across the animal's surface (Chapter 4, 5). However, no studies to date finely correlate the impact of ambient flow on sponge tissue oxygenation. New insights on this topic are given in Chapter 4 and 5.

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Chapter 3

3. Sponges (Porifera) and sponge microbes

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This review contains original data and provides background information for chapter 6 about the sponge-associated microbes

Synonyms

Sponge bacteria, sponge-associated bacteria, sponge-associated microbes, sponge-associated microbiota, sponge symbionts, sponge-microbe systems

Definitions

Sponges are sessile multi-cellular aquatic animals (Lat. Porifera = bearing pores) whose bodies have a typical body-plan which allows water to pass through the animal. Sponges are characterized by a skeleton made of calcareous, siliceous spicules or horny fibers.

Sponge microbes: Bacteria and Archaea that live in close and permanent association with sponges, usually in the mesohyl between sponge cells.

3.1 Introduction

Sponges are sedentary benthic organisms, found from shallow waters on tropical coral reefs to the Arctic and the deep sea. Some species however are restricted to fresh-water ecosystems. Sponges are characterized by a typical body plan built around a system of water canals and chambers (Figure 1). Within the chambers, flagellated cells called choanocytes produce a water current that enters the sponge body through surface pores (ostia), and leaves it through larger openings called oscula. Hence, sponges are filter feeders and incorporate particles from the water as energy and nutrient source. An external cell layer (pinacoderm) encloses the sponge mesohyl, a glycosidic matrix containing several cell types which perform a variety of functions. Sponge cells show a low degree of specialization and a high degree of independence so that the sponge body in some respect resembles a protozoan colony. However,

sponges are without any doubt placed as true members of the Metazoan (Ax, 1995; Müller, 1998 a, b).

Sponges have been grouped into three classes: Calcarea, Hexactinellida and Demospongiae. Calcarea are characterized by the presence of calcareous spicules. The upgrade of this class to the phylum level has recently been proposed by Borchiellini *et al.* (2001). Hexactinellida are characterized by siliceous spicules of hexactine structure and syncytial tissue organization. Demospongiae, the most numerous and diverse class is a non-monophyletic group (Boury-Esnault 2006). They generally have a mineral skeleton made of siliceous spicules, but several lineages, like the common bath sponge, have no mineral skeleton but a network of fibres instead. Modern sponge phylogeny is now based on a combination of molecular methods and sponge morphological features, but sponge spicules are still important features for species determination and identification of sponges in the fossil record.

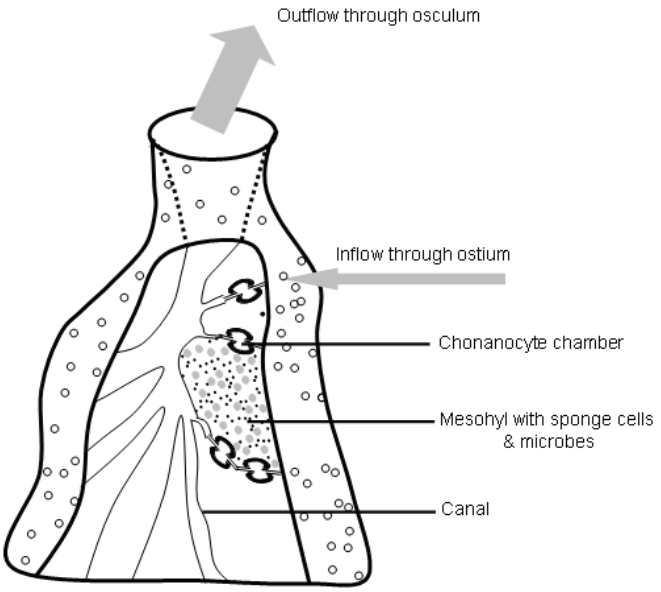

Figure 1: The sponge-microbe system: a schematic representation of a sponge with microbes in the mesohyl

Sponges form one of the deepest radiations of the Metazoa and can be regarded as the oldest animal phylum still alive today. The spicule record for sponges starts in the Late Proterozoic (Reitner and Wörheide, 2002), while chemofossil records even indicate the presence of sponges - or their direct ancestors - already in the Early Proterozoic: specific C_{30} steranes (24-isopropylcholestanes), which are unambiguous biomarkers for sponges, were found in 1800 Ma old stromatolites (McCaffrey *et al.*, 1994; Moldowan *et al.*, 1994).

Choanoflagellates, a monophyletic group of protists which can show a colonial lifestyle, are the closest sister group of the Metazoa (Medina *et al.*, 2001) and show a morphological resemblance to the flagellated choanocytes of sponges. It is therefore widely agreed that the "urmetazoa", the last common ancestors of all Metazoa, would have many traits found in sponges (see review in Gaidos *et al.*, 2007).

Numerous sponge species host vast amounts of microbes in their mesohyl matrix (Figure 2, 3) or sometimes in specialized cells, while others contain only few or no microorganisms at all. The former have been termed bacteriosponges (Reiswig, 1981) or "high-microbial-abundance sponges" (Hentschel *et al.*, 2003) while the latter are referred to as "low-microbial-abundance sponges". Bacterial population densities in bacteriosponges may reach $10^8 – 10^{10}$ microbes per gram of sponge wet weight, while those in low-microbial-abundance sponges are within the range of natural seawater.

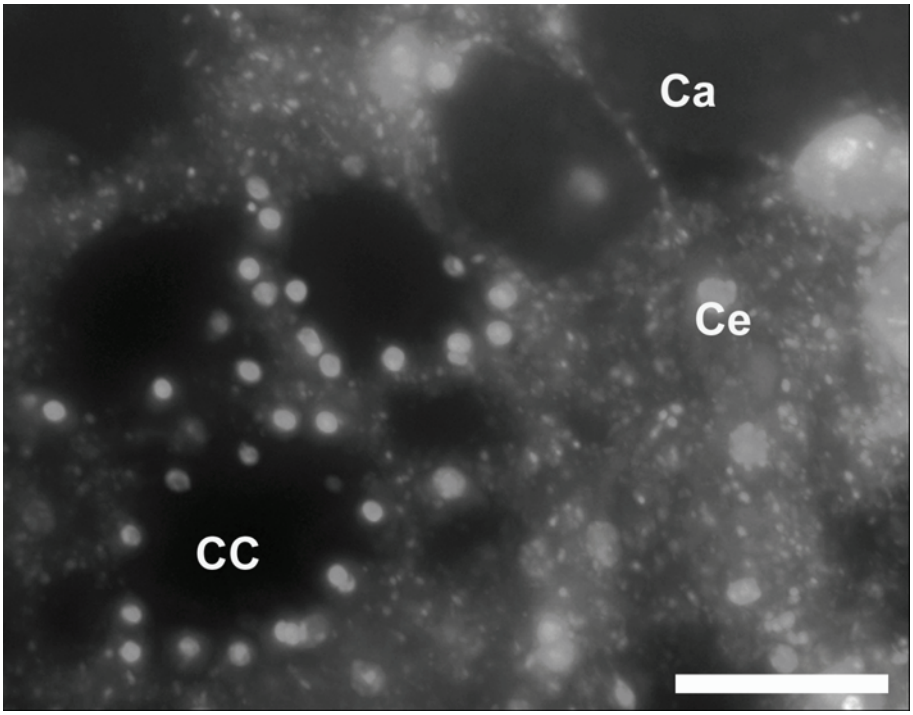

Figure 2: Fluorescence micrograph of a DAPI stained tissue section of the sponge *Geodia barretti*, CC = choanocyte chamber, Ca = canal, Ce = sponge cells. Large DAPI signals represent nuclei of sponge cells while small signals represent sponge microbes. Scale bar = 20 µm

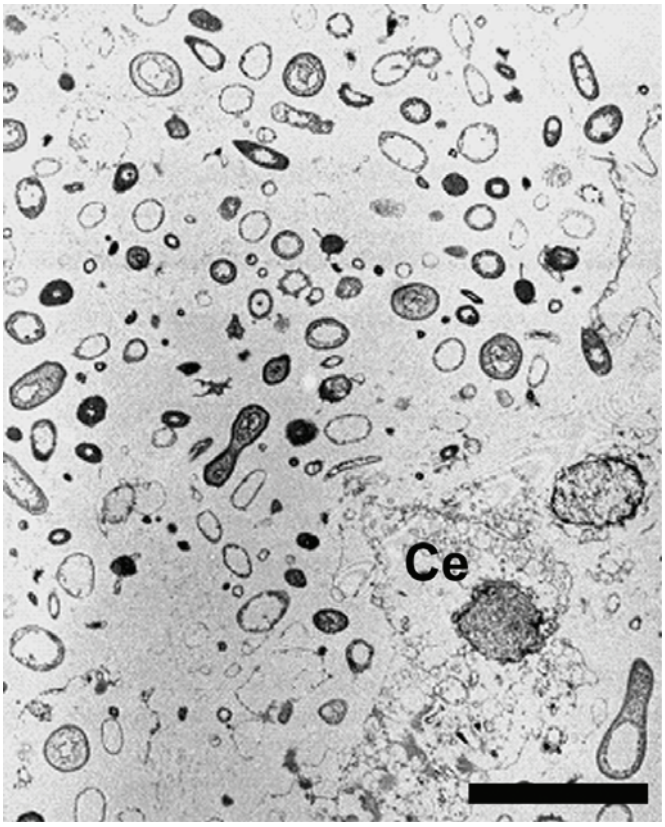

Figure 3: Transmission electron-microscope image of the sponge *Geodia barretti* showing sponge cells (lower right corner) and numerous, morphologically different sponge microbes. Scale bar = 5µm

The first studies exploring the microbial community of sponges were based on electron microscopy and isolation of microbes and yielded invaluable information about the micro-organism morphotypes and their specific location within the sponge (e.g. Wilkinson, 1978). Microbes were found to exist extra- and intracellularly (e.g. Vacelet and Donadey, 1977) and even the nucleus of sponge cells (Vacelet, 1970). More recently, the advent of molecular techniques have enabled a detailed phylogenetic description of the microorganisms associated with sponges (Hentschel *et al.*, 2006), whereas environmental genomics have given insights into their metabolic and physiological properties (Grozdanov and Hentschel, 2007).

Phylogenetically complex, yet highly sponge-specific microbial communities were identified in numerous species over large geographical ranges (Hentschel *et al.*, 2002). Sequences representing 16 bacterial phyla and both major archaeal lineages have been recovered to date (see review by Taylor *et al.*, 2007; Hentschel *et al.*, 2006; Hentschel *et al.*, 2003). One of these phyla, the Poribacteria, represents a new bacterial phylum, which is related to the Planctomycetales and has so far only been found in sponges (Fieseler *et al.*, 2004; Fieseler *et al.*, 2006). The other microbial phyla found in sponges are also found in other environments; however, many sponge microbes seem to form, within these phyla, monophyletic, sponge-specific 16S rRNA sequence clusters, which are absent in seawater (Taylor *et al.*, 2007; Hentschel *et al.*, 2006; and Hentschel *et al.*, 2003).

These recent review articles are an excellent summary of our current knowledge about the diversity and phylogenetic affiliation of sponge microbes and this topic will therefore not be addressed here in more detail. This article, in contrast, aims to focus

on the geobiological aspects of the sponge-microbe system: the question of a long-standing relationship between sponges and microbes; the consequences of fluctuating oxygen concentrations in sponge tissue for the sponge-microbe system and the nature and possible function of microbial processes within sponges.

3.2 Sponge microbes: secret passengers through evolutionary history?

Do sponges and their associated microbes have an ancient partnership which remained unchanged since the origin of sponges (Precambrian), or is it a more recent but stable association which pertains to date? In an extensive review of this topic Taylor *et al.* (2007) proposes three scenarios which may explain the occurrence of sponge-specific microbes. Firstly, it can be the product of a symbiosis of ancient origin perpetuated through the vertical (i.e. from adult to embryo) transmission of sponge-specific microorganisms. Secondly, the association can be the product of environmental acquisition through specific enrichment (immune system recognition, presence of favourable substrate, or bacterial protective capsule) or unspecific enrichment (rare sea water microbes get trapped and thus detectable only in the sponge). Finally, a combination of vertical and environmental transmission is also possible.

The strongest evidence for a long-standing, close symbiotic relationship between sponges and some microorganisms comes from the demonstration of co-evolution by comparative phylogenetic studies of sponges and their associated microbes. Erpenbeck *et al.* (2002) used a mitochondrial marker, cytochrome oxidase subunit 1

(CO1) and showed that the derived phylogenetic trees of four out of six symbionts were largely congruent with a tree containing sequences from the corresponding host sponges. This suggests that co-speciation had occurred (Erpenbeck *et al.*, 2002). A subsequent study indicated a high degree of host specificity between the filamentous cyanobacterium *Oscillatoria spongeliae* and various dictyoceratid sponges (Thacker and Starnes, 2003). When comparing the phylogeny of 23 geographically, morphologically, and environmentally distant sponge species (based on their 28S rDNA gene sequences) and their associated archaea (based on 16S rDNA) a genus-specific association of these sponges with Crenarchaeota of the Marine Group I was apparent (Holmes and Blanch, 2007). These results suggest close co-evolution and thus a long-term symbiosis between these archaea and their sponge hosts. Co-evolution requires that the host and symbiont maintain close association over evolutionary time. Vertical transmission, the mechanism through which co-evolution could occur, has been shown for numerous sponge species belonging to all three classes of Porifera (Ereskovsky *et al.*, 2005; Sharp *et al.*, 2007; Schmitt *et al.*, 2007; and references therein), including in species with highly varied reproductive strategies.

A pitfall for these comparative phylogenetic studies is that the phylogeny of sponges themselves is not fully resolved yet. This is particularly true for the Demosponges where phylogeny from the sub-class to the family level is still in a state of flux (Boury-Esnault, 2006). Accordingly, our understanding of symbiont evolution in sponges will continue to develop only in parallel with improvement of our knowledge of host phylogeny. A recently initiated CO1 sequencing project for taxonomically

diverse sponges (www.spongebarcoding.org) is a step in the right direction to achieve this goal.

Further support for a possible ancient origin of the sponge-microbe system comes from biomarker studies. Various fatty acids of probable microbial origin occur in a wide range of sponges irrespective of host phylogeny or geographic location (Thiel *et al.*, 1999; Thiel *et al.*, 2002). The apparent absence of some of these biomarkers from marine sediments and seawater led to the suggestion that the fatty acids and their microbial producers have been present in the sponges since ancient times.

The final type of evidence for ancient, close association between sponges and microorganisms comes from the fossil record. Reef mounds constructed by siliceous sponges and cyanobacterial mats in the early Cambrian shows that sponges and microbes closely coexisted hundreds of millions of years ago (Brunton and Dixon, 1994). Whether these microbes actually lived within the sponge tissue, as modern sponge microbes do, remain, at this stage of our knowledge, unclear.

In other words, Precambrian microorganism acquisition which started as a mere hypothesis is gaining support through studies of phylogeny, biomarkers and fossil record. Thus, there is increasing evidence for a long-standing co-evolution between sponges and certain microbial lineages, which may even date back to the roots of sponge evolution in the anaerobic, microbe-dominated world of the Proterozoic. There is evidence that a close association between sponges and bacteria may even have provided some of the genetic source material for Metazoan evolution (Lakshminarayan *et al.*, 2004).

The alternative scenario, environmental acquisition, can, however, not be ruled out for certain sponge microbes or, in some cases, may be the more likely explanation. In this scenario, it is assumed that sponge-specific microbes are in fact also present in sea water but that our techniques fail to detect them because of their rarity. In the sponge, specific enrichment of those rare microorganisms could occur as a result of a selection through the sponge immune system (Müller and Müller, 2003) or because those microbes which possess a capsule are protected from sponge cells phagocytosis and would increase in numbers within the sponge body, thus becoming detectable. Unspecific enrichment is also possible and could happen through the ability of sponge to filter large amounts of water through their body. The likelihood of rare microbes being detected by our current techniques is higher in a sponge than in seawater as they might accumulate and be present in high enough numbers to be found.

The third scenario proposed by Taylor *et al.* (2007) is a combination of both vertical and horizontal symbiont transmission (parental and environmental acquisition, respectively) which seems the most likely scenario to explain microbial diversity we find in sponges today (Hentschel *et al.*, 2002).

3.3 An anaerobic world in sponges

Until recently, sponge metabolism was viewed as being based on aerobic respiration, similarly to all Metazoa. Oxygen is usually supplied in excess to the sponge body through the water current created by the choanocytes (flagellated cells) (Reiswig, 1974). The remarkable ability of sponges to pump large amounts of water through

their body has led to the assumption that permanent oxygen saturation exist within the sponge body. However, the presence of anaerobic microbes in some sponge species hinted towards the presence of spatial or temporal anoxic niches (e.g. Webster *et al.*, 2001; Hoffmann *et al.*, 2005a).

Gatti *et al.* (2002) and Schönberg *et al.* (2004) were the first to use micro-sensors to investigate the oxygen distribution in the tissue of living sponges at a microscale. Gatti *et al.* (2002) used oxygen micro-optodes on sponges' primorphs (multi-cellular aggregates composed of previously dissociated single sponge cells) and described oxygen deficiency in *Suberites domuncula*. Clark-type oxygen micro-electrodes have since been used to explore the fine-scale oxygen dynamics in several sponge species from the Mediterranean and the North Atlantic. Anoxic regions have been detected in the cold-water sponge *Geodia barretti* (Hoffmann *et al.*, 2005a) and in its explants (Hoffmann *et al.*, 2005b), as well as in the Mediterranean sponges *Dysidea avara* (Schläppy *et al.*, 2007) and *Chondrosia reniformis* (Figure 2) kept in aquaria or cultivation tanks with re-circulating sea water.

Similarly, fluctuating oxygen concentrations leading to anoxia lasting up to one hour were also found in *D. avara* in the field when measured with an oxygen micro-electrode applied by SCUBA diving (Schläppy unpublished, Chapter 5). A positive correlation between pumping activity and oxygen content was described in the tissue and in the exhaled water of *D. avara* (Schläppy *et al.*, 2007) and *Aplysina aerophoba* (Hoffmann *et al.*, 2008). When the sponge was ventilating, its body was oxygen saturated. Figure 4a shows a typical profile of a pumping sponge, with oxygen

saturated water above the sponge surface and near saturated water within the sponge tissue (compare also Hoffmann *et al.*, 2007; Schläppy *et al.*, 2007) .

Oxygen concentrations decreased dramatically when pumping rates ceased. After 15 minutes without ventilation, the entire sponge body was anoxic with the exception of a 1 mm surface layer where oxygen penetrates due to molecular diffusion over the sponge surface (Hoffmann *et al.*, 2008).

Oxygen profiles are typically diffusive over the surface and into the tissue of a non-pumping sponge (Figure 4 b) (compare also Hoffmann *et al.*, 2005b; Schläppy *et al.*, 2007; Hoffmann *et al.*, 2008). *In situ*, many sponge species reduce or even stop their pumping activity for several hours at irregular intervals (Reiswig, 1971; Vogel, 1977; Pile *et al.*, 1997). It remains unclear whether the interruption of ventilation is caused by external events such as, for example, a high sediment load in the water (Gerodette and Flechsig, 1979; Leys *et al.*, 1999), decrease in salinity (Fell *et al.*, 1989) or by an intrinsic rhythm specific to a species (see Reiswig, 1971). In non-pumping sponges, molecular diffusion across a diffusive boundary layer at the sponge surface is the only source of oxygen.

The consequences of these findings are two-fold. Firstly, all sponge cells and microbes which lay deeper as 1 mm below the sponge surface need to be able to survive without direct and regular access to oxygen. Since a lack of ventilation causes tissue anoxia, the parts of the sponge body which are not affected by surface diffusion must be alternately exposed to oxic and anoxic conditions. Secondly, by modulating

the presence or absence and the magnitude of water flow through their body sponges have the possibility of actively switching between aerobic and anaerobic metabolism.

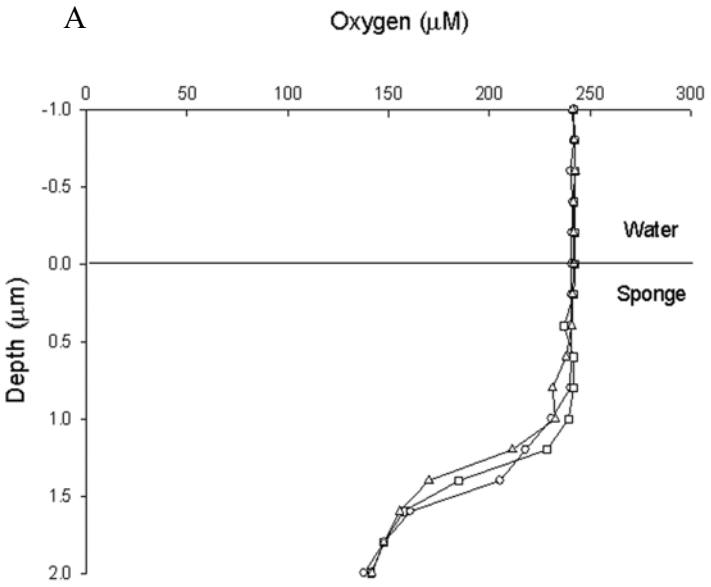

Figure 4a: Oxygen profiles above and within *Chondrosia reniformis* showing a pumping specimen with oxygen-saturated water above the sponge near saturated within the sponge. Profiles 1, 2 and 3 represented by circles, squares and triangles.

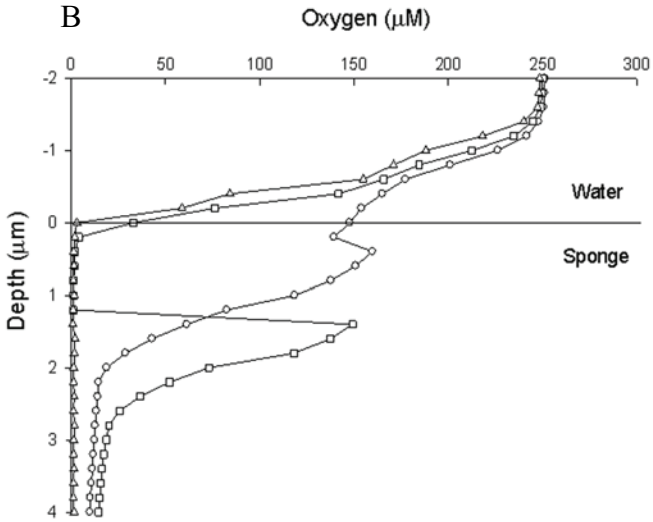

Figure 4 b: Oxygen profiles above and within *Chondrosia reniformis* showing a non-pumping specimen with oxygen-saturated water up to 1.5 cm above the sponge surface, a gradual decrease in the diffusive boundary layer and low oxygen concentration approaching anoxia from 2.5 cm into the sponge body. Note the different Y axes. Profiles 1, 2 and 3 represented by circles, squares and triangles

It follows that sponge-associated micro-organisms must be able to tolerate both situations and their metabolism may be activated or inactivated depending on the oxygen concentration in the sponge. This means that anaerobic microbial processes, like, sulfate reduction, for example, can take place (Hoffmann *et al.*, 2005a).

Another possible consequence of varying oxygen concentrations in sponge tissue is reported by (Müller *et al.*, 2004 a, b). Using *Suberites domuncula* and its alphaproteobacterial symbiont (SB2) as a model system, they showed that the expression of genes, which were essential for metabolic interaction of microbe and host, were maximal under aerated conditions. Coupled with the observed loss of SB2 cells from the sponge surface under low-oxygen conditions, it was concluded that the oxygen level is responsible for regulating the bacterial fauna in *S. domuncula*. Whether this type of mechanism is equally important in other sponge-microbe systems remains to be investigated.

Tissue anoxia in sponges may act as a controlling factor towards the number of anaerobic sponge-associated microbes and act as a regulator of the nature and extent of their metabolic activity. Alternatively, it may act as a mechanism through which the sponge can kill undesirable seawater microbes. Regardless of whether anoxia in sponges is intentional or inevitable: many sponges can survive long periods of tissue anoxia and either tolerate or even foster anaerobic microbes and corresponding anaerobic microbial activity within their body. A close association with a diverse microbial community including (facultative) anaerobic or microaerophilic microorganisms may have been particularly advantageous at a time when fluctuating oxygen conditions were present on earth.

3.4 Interactions between sponges and sponge microbes

While our knowledge about phylogeny and metabolic capacities of the sponge microbes continuously increases, the role that microbes play for the sponge host is still a matter of debate. Microbes undoubtedly benefit from the nutrient-rich and protected environment of the sponge mesohyl but the benefit to the sponge host remains unclear.

Sponge microbes may consume carbon sources which are not accessible to the sponge, and may be subsequently ingested by the sponge cells. This process is commonly known as "microbial farming" and is well described (Wilkinson and Garrone, 1980; Ilan and Abelson, 1995; Vacelet *et al.*, 1996 and own observation). Autotrophic symbionts transform inorganic carbon (DIC) into organic carbon which may then be transferred to the host. Energy transfer between symbiont and sponge is best described for phototrophic symbionts (e.g. cyanobacteria: Wilkinson, 1983). Chemolithotrophic processes, such as nitrification, are similar in principle. The energy which is needed for carbon fixation, however, is gained by a chemical process: the aerobic oxidation first of ammonium to nitrite and then of nitrite to nitrate. The presence of nitrifying microbes as well as the transformation of ammonium to nitrite and nitrate has been demonstrated in many sponge species (e.g. Diaz and Ward, 1997; Diaz *et al.*, 2004; Bayer *et al.*, 2007). In addition to carbon fixation, these microbes efficiently remove the sponge waste product ammonium and the toxic nitrite. Heterotrophic microbes consume simple compounds (dissolved organic carbon, DOC), while sponge cells prefer small particles like pelagic bacteria or phytoplankton (Willenz, 1980; Pile *et al.*, 1996; Witte *et al.*, 1997; Ribes *et al.*, 1999). Some sponges, however, have been identified as important DOC sinks (Yahel *et al.*, 2003;

de Goeij *et al.*, accepted). Interestingly, these sponges host high amounts of associated bacteria. This leads to the conclusion that DOC is consumed by the sponge microbes, which in turn are consumed by the sponge cells. A diverse community of associated microorganisms with high metabolic diversity is able to metabolize a wide array of simple molecules and to transfer them into biomass as a food resource for the sponge.

The second possibility for a mutual interaction between sponges and microbes lies within the production of secondary metabolites. Sponges produce a large variety of bioactive compounds (Blunt *et al.*, 2003; Paul and Puglisi, 2004), which act as defenses against surface fouling, infection, or predation. Sponge microbes, which are obviously not harmed by the antimicrobial metabolites, are in fact often the actual producers of these compounds (Hildebrand *et al.*, 2004; Piel, 2004; Piel, 2006). In addition to protecting the sponge, secondary metabolites act as a controlling factor in the host-microbe interaction. At least some groups of sponge microbes are clearly beneficial to the sponge host, while others may be commensals.

3.5 Conclusion and outlook

Sponges are the most ancient Metazoans on earth and are today distributed over a wide range of aquatic ecosystems. Phylogenetically complex, yet highly sponge-specific microbial communities live in close association with numerous sponge species. A combination of both vertical and horizontal symbiont transmission (parental and environmental acquisition, respectively) seems the most likely scenario to explain microbial diversity we find in sponges today. Tissue anoxia is a feature found in several sponges species and is related to sponge pumping activity. Sponge microbes with autotrophic, heterotrophic, aerobic or anaerobic metabolisms may contribute to the nutrition of the host. Other interactions between sponges and sponge

microbes involve the synthesis of secondary metabolites. Due to modulation of internal oxygen concentration and the production of secondary metabolites, sponges are able to control their microbial communities. While our knowledge about the phylogeny of sponge microbes is increasing rapidly, many questions still remain unanswered concerning the activity of sponge microbes and their interaction with the host sponge. Future challenges will involve linking microbial phylogeny to microbial function on a micro-scale within the sponge tissue, and to examine the complex interactions within the sponge-microbe system from the origin of Metazoan life until today.

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Chapter 4

4. Oxygen dynamics and flow patterns of *Dysidea avara* (Porifera, Demospongiae)

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4.1 Abstract

The present publication presents oxygen properties and pumping behaviour of *Dysidea avara*. Oxygen profiles were measured near and inside the atrial space of the osculum with a Clark-type micro-electrode. Pumping sponges had profiles with oxygen concentrations marginally lower than that of the aquarium water. In contrast, diffusive profiles, with a clear boundary layer above the sponge surface, and oxygen penetrating only 0.5 mm into the sponge tissue, were typically that of a sponge which was not pumping. Diffusive oxygen flux at the sponge surface was 4.2 μmol O_2 cm^{-2} d^{-1} and the calculated volumetric filtration rate was 0.3 cm^3 water cm^{-3} sponge min^{-1}. The oxygen concentration in the osculum was temporally fluctuating between 95 to 59 % saturation at a frequency of approximately once per minute. The combination of continuous oxygen micro-electrode measurements and particle tracking velocimetry (PTV) allowed us to simultaneously observe fine-scale oxygen fluxes and oscular flow patterns in active sponges, even at extremely low pumping rates. Oscular oxygen concentration and flow were correlated but not always synchronous to the second. Particle tracking velocimetry was used to visualize the flow field around the sponge and to distinguish sponge-generated flow from the unidirectional current in a flow-cell.

Keywords: Oxygen microsensor, particle tracking velocimetry, *Dysidea avara*, anoxia, pumping

4.2 Introduction

Sponges are filter-feeders and transport food particles, oxygen and waste products in the currents they generate and which pass through their bodies in characteristic aquiferous systems made of pores and canals. Understanding the fine spatial and temporal variability of oxygen conditions within sponges is of significant interest in sponge physiology and can be effectively measured using micro-sensors. For example, Gatti *et al*. (2002) used micro-optodes to study the oxygen supply in primmorphs (multi-cellular aggregates made from sponge dissociated single cells (Müller *et al*., 1999)) and adult specimens of *Suberites domuncula*. Oxygen micro-electrodes have also been successfully used to measure the photosynthetic activity of sponge symbionts in *Cliona nigricans* and *Cliona viridis* (Schönberg *et al*., 2005). Oxygen micro-electrodes have further been used to explore the nature of fine-scale oxygen dynamics in the cold water sponge *Geodia baretti* (Hoffmann *et al*., 2005a) and in its explants (Hoffmann *et al*., 2005b), where anoxic regions inside the sponge body were shown for the first time, throwing new light onto the internal micro-environments of sponges. A study of the oxygen dynamics of *Aplysina aerophoba* (Hoffmann *et al*., 2008) showed that a non-pumping sponge becomes anoxic after 15 minutes, and relies on diffusional processes for oxygenation. In contrast, pumping individuals of *A. aerophoba* were subject to advectional forces, making diffusion insignificant.

Sponges may switch to an anaerobic metabolism (Grieshaber *et al*., 1994), thus providing a temporary niche for anaerobic microorganisms. During such a switch, the presence and degree of activity in sponge-associated microbes are likely to be strongly controlled by oxygen. In this study, *Dysidea avara* Schmidt, 1862, a common

Mediterranean species, is used as representative of a sponge with a massive growth form. We used high resolution oxygen micro-electrodes to determine fine-scale oxygen dynamics, while simultaneously visualizing overall flow patterns and quantifying sponge exhalant flow speed with particle tracking velocimetry (PTV).

4.3 Material and Methods

4.3.1 Sampling

Dysidea avara is a small sponge with massive morphology and a conulose surface which allows easy recognition of oscula (Figure 1). This species is common, easily available and has desirable morphological characteristics for micro-electrode work such as a body mostly occupied by a well developed aquiferous system and a soft spongin skeleton (Galera *et al*., 2000) which did not impede the experimental methods.

Several specimens of *D. avara* ranging from 3 to 20 cm^3 were collected in 10-15 m water depth in Muntanya de Montgó, Punta del Romani at Cala Illa Mateua in the township of L'Escala, Girona, Spain (42º06.863' N 03º10.116' E), by SCUBA diving, at an ambient temperature of 20 ^oC and an ambient salinity of 37.4 parts per thousand (ppt). Sponges were kept in an 85 L aquarium for one month before experiments commenced at the University of Wageningen (The Netherlands). The holding aquarium was filled with 50 % natural seawater from the sampling site and 50 % artificial seawater made by mixing 37 g/L into reverse osmosis water using Reef Crystals salts (by Aquarium Systems, Sarrebourg, France). After the initial filling of the aquarium, 5 % were changed per week with the artificial seawater (first drain, then re-fill). Over time, the natural seawater was nearly completely diluted out of the

system. The total re-circulating volume was approximately 150 L. Temperature and salinity were adjusted to the ambient conditions at the sampling location: temperature was maintained at 20 ^{o}C (± 0.3 ^{o}C), and salinity at 37.4 ppt (± 0.2 - 0.8 ppt). Aeration was provided through air stones, airlifts and continuous water recirculation driven by a pump. The oxygen in the water within the aquarium was always maintained at close to air saturation levels. Sponges were fed twice daily with marine broths made from fish and shrimps, and/or manufactured shellfish diets (INVE, Belgium CAR-1), and three to four times weekly with cultured *Phaeodactylum tricornutum, Nannochloropsis* sp. (2-3 x 10-5 particles/ml).

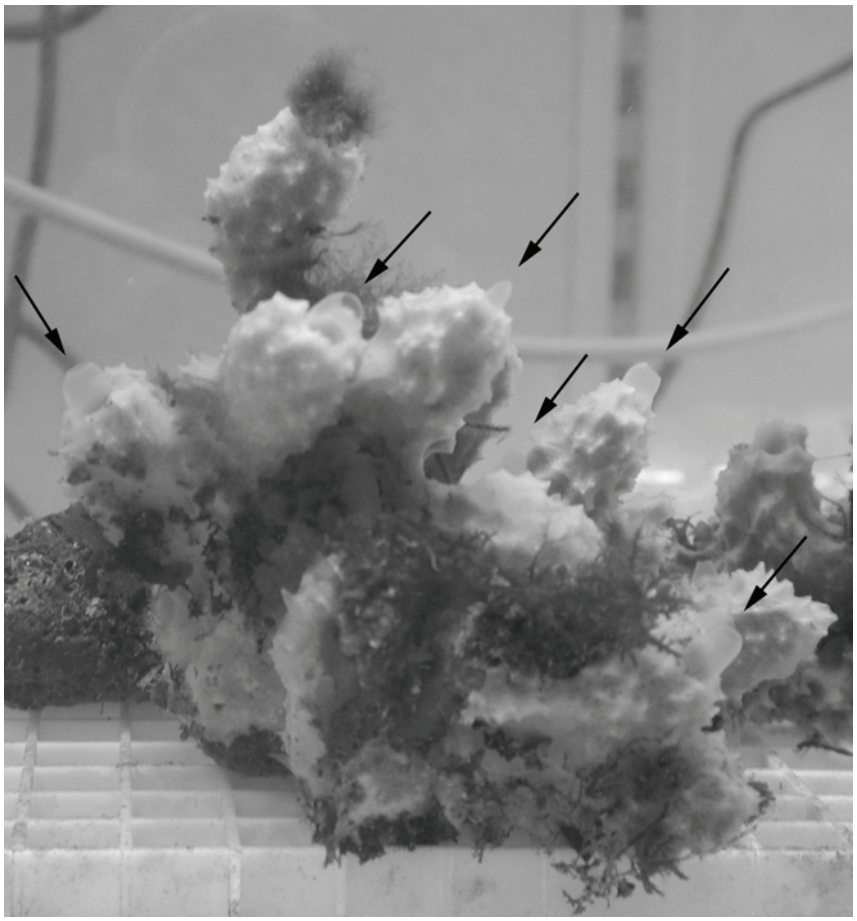

Figure 1: *Dysida avara* specimen in aquarium showing the prominent oscules (arrows)

4.3.2 Oxygen micro-electrode measurements

A Clark-type oxygen electrode (Revsbech 1989) with a 20 μm tip diameter was made at the Max-Planck-Institute for Marine Microbiology, Bremen, in Germany and used for all measurements in the present study. A two point calibration was made using oxygen saturated seawater from the holding tank and anoxic seawater made by addition of an oxygen scavenger (sodium sulfite). Care was taken to obtain the zero value as quickly as possible so as to avoid "poisoning" of the sensor by a protracted stay in the sodium sulfite solution.

4.3.2.1 Oxygen profiles

A total of 51 oxygen profiles in 0.2 mm increments were made from 9 sponges (3 replicate profiles per location and some individuals were measured at more than 1 location). The profiles of four individuals were selected for their representativeness of a range of *D. avara* tissue oxygenation. Profiles started at 2 - 1.5 mm above the sponge surface down to 2 - 3 mm depth in the sponge body. Before profiling commenced, a sponge specimen was transferred from the holding tank to the experimental 8 L flow-cell. Unidirectional flow was produced through a standard aquarium pump which recirculated the seawater originating from the sponge holding tank. The water in the flow-cell was aerated with an air stone, kept at 20 ^{o}C through periodical water replacement and no food was added. The micro-electrode was attached to a computer-controlled micro-manipulator and lowered three times within 20 minutes at the same location in the sponge body without delay between single profiles. The sensor penetrated the sponge easily so no pre-piercing of the body was necessary as is sometimes required in other sponge species (Schönberg *et al*., 2004). There were no recognizable signs of stress in the experimental animals. From

diffusive oxygen profiles over the sponge surface, oxygen fluxes were calculated based on Fick's 1st law of diffusion.

4.3.2.2 Static oxygen measurements

Another individual than those used for profiles was selected for continuous oxygen concentration measurements at a fixed location into the atrial space of an osculum. To assess the nature of sponge tissue oxygenation over time, oxygen concentration was recorded every second for 20 minutes. The oxygen micro-electrode was inserted 2 mm into an open osculum and was left in the atrium without ambient water flow, under stable salinity and temperature conditions.

4.3.3 Particle tracking velocimetry (PTV)

Particle tracking velocimetry (PTV) was carried out for 148 seconds at the same time as the static oxygen measurement (and on the same sponge) to enable quantitative estimation of oscular flow speed by following the trajectory of individual particles in the water over time. The particles used were neutrally buoyant hollow glass spheres of 10 μm diameter. The flow-cell water was periodically spiked with fresh seeding particles because as the sponge pumped, it retained a portion of the spheres, thus gradually decreasing their density in the flow-cell.

During PTV, the particles were illuminated with a diode laser (Lasiris LAS-670-30) equipped with line generating optics (LAS-1 line-20 TS). The 0.2 mm wide sheet of light was aligned perpendicular to the sponge surface, crossing the centre of the sponge osculum, and being oriented in parallel to the flow-cell unidirectional flow (Figure 2). The particles moving in the path of the laser sheet were recorded with a

charge-coupled device (CCD) camera (Sony XCD-X710) at 7 frames per second. An interrogation area was chosen adjacent to the osculum out of which particles were tracked over several picture frames to depict the spheres' movement near the sponge's surface and out of the exhalant current. Their velocity was determined by measuring the distance between the same particles on subsequent images over 148 seconds. Simultaneously with PTV, oxygen concentrations were measured by placing the micro-electrode in the atrium of the sponge osculum, as close as possible to the laser sheet. Moreover, once the static oxygen measurement was completed, the flow-cell flow was restored and PTV was used on a different sponge to enable qualitative visualization of two-dimensional sponge and ambient flow fields. From these images a mean oscular flow velocity was calculated from the 6 sets of 7 spheres (over 1 second) closest to the osculum.

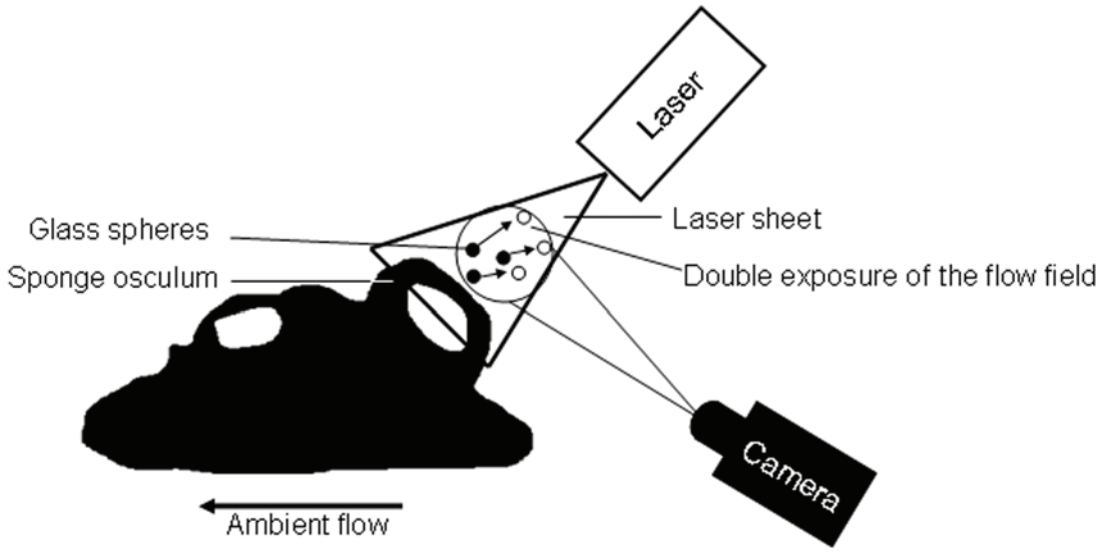

Figure 2: Sketch showing the location of the laser sheet in relation to the camera and the sponge. The distance between the spheres at time 1 (dark spheres) and at time 2 (white spheres) were calculated from two pictures taken out of the interrogation area

4. 4 Results

Four selected oxygen profiles describe a variety of tissue oxygenation in *Dysidea avara* (Figure 3 a - d). The oxygen concentration in the body of four *D. avara* specimen ranged from fully oxic (Figure 3a) through intermediate stages (Figure 3 b and 3 c) to anoxic (Figure 3 d). One sponge had well-oxygenated tissue (Figure 3 a) and, at 3 mm tissue depth, oxygen concentrations still reached 99.4 % of the fully saturated flow-cell water. The oxygen content in this sponge remained stable over the 20 minutes required to carry out the measurements, and yielded three nearly identical profiles (Figure 3 a).

In two other individuals, intermediate oxygen concentrations were present, and changed over the 20 minutes required to make the profiles. The oxygen concentration in the second individual increased over time, but retained the same characteristics, namely a sharp decrease of oxygen concentration just below the sponge surface down to 1 mm depth, followed by oxygen concentration approaching saturation at 2 and 3 cm depth (Figure 3 b). The oxygen profile in the third individual showed a mixture of increasing oxygen concentration over time in the first 1 mm of sponge tissue, whereas oxygen concentration in the tissue below 2 mm decreased, approaching anoxia, over the course or the 20 minutes required to make the profile (Figure 3 c)

In the fourth specimen, the water above the sponge was fully oxygen-saturated but oxygen concentrations then strongly and steadily decreased in the upper 0.5 mm above the sponge surface, showing the presence of a boundary layer. It continued to decrease with distance from the sponge surface and reached almost anoxic conditions in ≥ 0.5-2 mm tissue depth (Figure 1 d). In the hypothetical case of a *D. avara*

specimen being anoxic for 24h, this would translate into an oxygen flux over the sponge surface of 4.22 µmol O_2 cm^{-2} d^{-1}, and an oxygen consumption rate of 84 µmol cm^{-3} d^{-1}. The pumped volume, which would be necessary to balance this respiration rate to prevent tissue anoxia, was calculated to be 373 cm^3 water cm^{-3} sponge d^{-1} (0.26 cm^3 water cm^{-3} sponge min^{-1}).

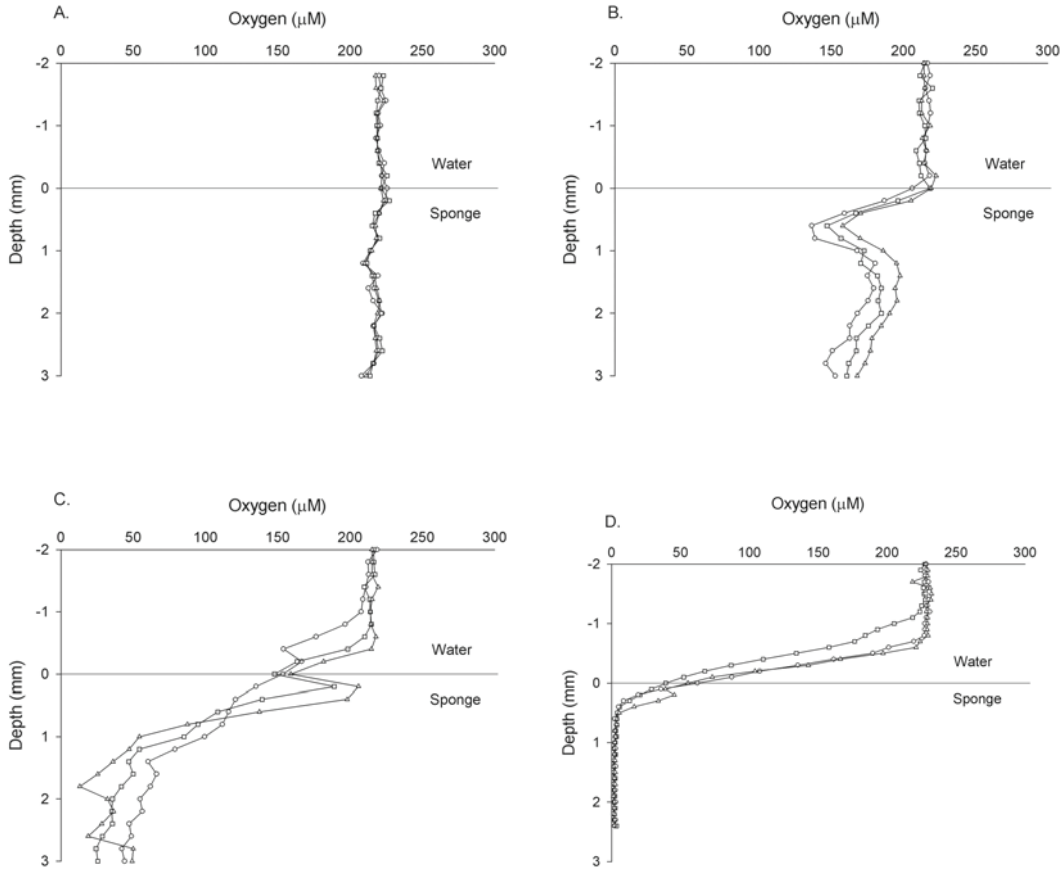

Figure 3: Oxygen profiles above and within *Dysidea avara* A) - Oxygen profile of a pumping specimen, showing oxygen-saturated water above and within the sponge B) Oxygen profile of a mildly pumping specimen C) Oxygen profile of a weakly pumping specimen D) - Oxygen profile of a non-pumping *D. avara*, showing oxygen saturated water above the sponge surface, a strong and gradual decrease in the diffusive boundary layer, and very low oxygen concentrations within the sponge tissue, reaching almost anoxic conditions at 0.5 mm. Circles represent the first profile (of the set of three), squares the second and triangles the third. Each profile is a unique data set showing oxygen saturation at a particular location within the sponge at a particular second. The error bars are not shown as they would only represent the accuracy of the micro-electrode measurement

Static oxygen measurements in the oscular lacunar space of the first sponge showed additional temporal variation in oxygen concentrations. When measured over 20 minutes without ambient flow, the atrial oxygen concentration oscillated between near fully oxygenated with 269 µmol L^{-1} ± 1.3 SE (95% saturated) to lower concentrations of 167 µmol L^{-1} oxygen ± 3.7 SE (59 % saturated), while the oxygen in the ambient water remained at 283 µmol L^{-1} (Figure 4). Deoxygenated water was expelled from the sponge osculum approximately 25 times in 20 minutes. Those flushing events occurred over durations of 4 - 28 seconds (average 13 ± 1 SE; see troughs on Figure 4), indicating a pumping rhythm interrupted by resting phases.

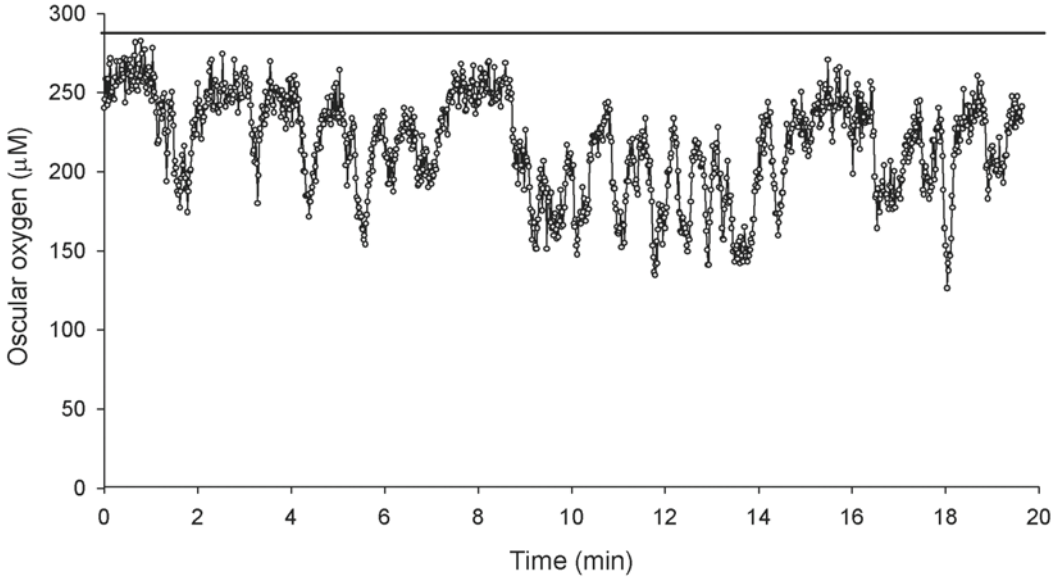

Figure 4: Continuous oxygen micro-sensor measurement in an osculum of *D. avara* over a time span of 20 minutes, showing temporal fluctuations and where each data point represents the oxygen saturation at that particular second. The concentration of oxygen in the overlaying water is shown as a straight line

Simultaneous oxygen micro-electrode and particle tracking velocimetry (PTV) measurements showed a correlation between internal oxygen concentrations and oscular flow velocity, although those variables were not always synchronous to the

second (Figure 5). The relationship between sponge pumping activity (particle speed mm s^{-1}) and oxygen concentration (μM) in the sponge tissue was statistically significant (regression with N=148, $r^2 = 0.17$, df =1 (degrees of freedom), F = 0.0001, P = 0.05). Flow velocity out of the osculum was between 0.12 and 0.99 mm s^{-1} with an average of 0.48 mm $^{-1}$ (± 0.04 SE).

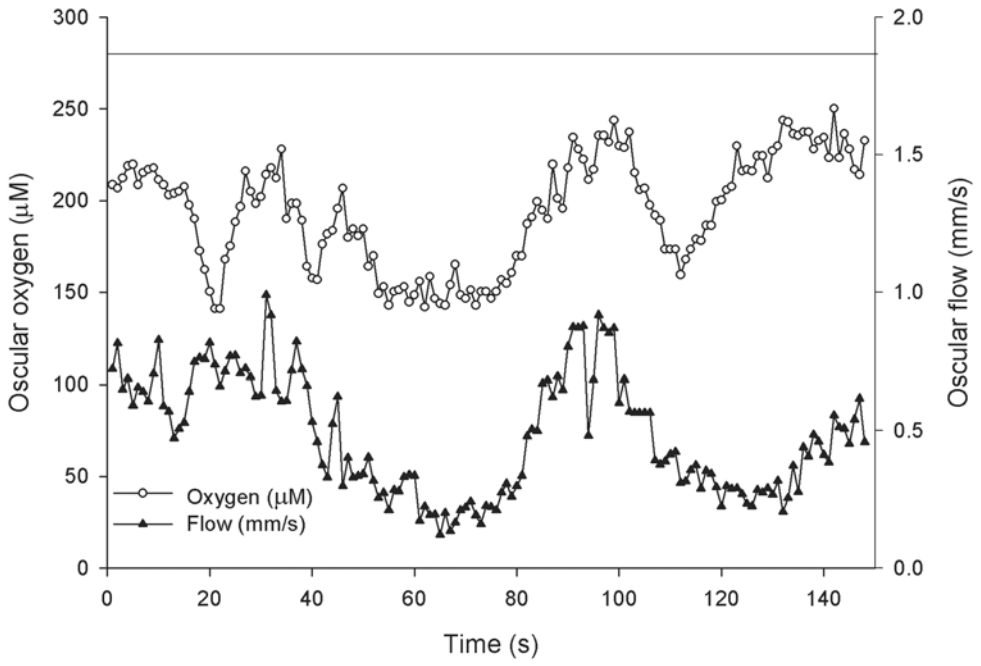

Figure 5: Oxygen and flow velocity measured simultaneously in the atrium of an osculum of *Dysidea avara*. Internal oxygen concentration and oscular flow velocity were occasionally decoupled, but mostly synchronous and approximately in accordance with one another (regression with N=148, $r^2 = 0.17$, df =1 (degrees of freedom), F = 0.0001, P = 0.05). The concentration of oxygen in the overlaying water is shown as a straight line

Particle tracking velocimetry allowed the two-dimensional visualization of sponge oscular and ambient flow a *D. avara* specimen kept in a flow-cell with unidirectional flow. Glass spheres carried in the cell's flow were deflected by the sponge-induced

exhalant current, which changed the direction of the sphere movement from horizontal and parallel to the sponge surface to nearly vertical (Figure 6). The calculated oscular flow speed for this specimen was 1.4 cm s^{-1} ($\pm$ 0.2 SE).

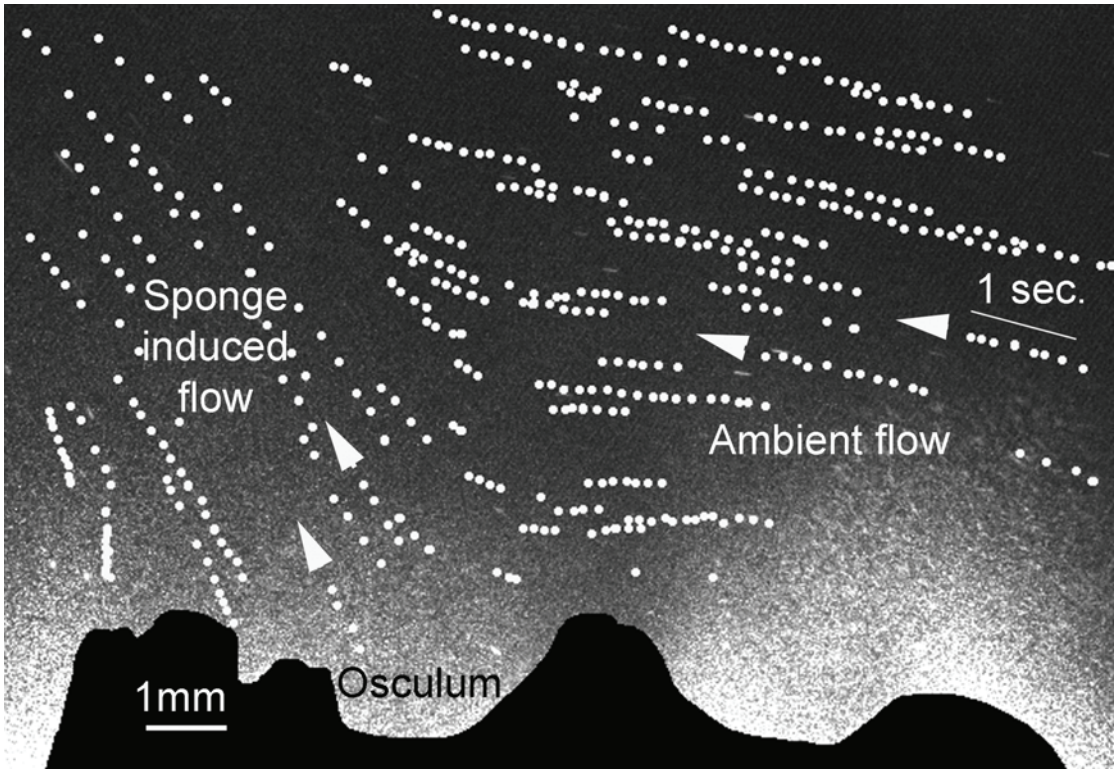

Figure 6: Multiple image overlay showing typical particle velocimetry above the surface of a *Dysidea avara* specimen and near the sponge's osculum, over the duration of several seconds. Ambient flow is almost horizontal from right to left while sponge induced flow is more vertical. 7 spheres in a line = 1 second

4.5 Discussion

Four set of oxygen profiles illustrated a range of oxygen concentrations in *Dysidea avara*. The profiles of the well-oxygenated *D. avara* specimen were characteristic of a pumping sponge (Hoffmann *et al*., 2008, Hoffmann *et al*., 2007) as the oxygen within the tissue was only marginally lower than that of the aquarium water (Figure 3 a). This shows that the water passed through the sponge rapidly, that the residence time was short, and that the water was only slightly oxygen depleted through the respiration of sponge cells and sponge-associated microbes.

In two intermediate sets of profiles (Figure 3 b, c) sponge tissue oxygenation lay between fully oxygenated and anoxic and showed some temporal variations. Sometimes, the sponge increased its pumping during the measurement of replicate profiles (Figure 3 b). At other times, it switched from opening-up to shutting-down during profile measurements (Figure 3 c).

In contrast, the anoxic specimen of *D. avara* was undoubtedly not pumping, because the profiles displayed a pronounced decrease in oxygen concentration from the fully oxygenated aquarium water to the sponge surface, and into the tissue (Figure 3 d). Only the uppermost 0.5 mm of sponge tissue remained oxygenated, while the rest was anoxic. The presence of an unmistakable boundary layer also showed that the sponge tissue oxygenation was solely diffusional and typical for diffusively determined profiles (Hoffmann *et al*., 2005a; Hoffmann *et al*., 2008). In that specimen, the interfacial flux was in the range of those found in other studies on non-pumping sponges, for example by Hoffmann *et al*. (2008) in *Aplysina aerophoba*, which had an average surface flux of 4.6 μmol O_2 cm^{-2} d^{-1}. *Geodia barretti* explants showed a

surface flux of 2.3 μmol O_2 cm^{-2} d^{-1} (Hoffmann *et al*., 2005b). The fact that the oxygen surface flux in the two Mediterranean sponges (*D. avara* and *A. aerophoba*) is higher (4.2 and 4.6 μmol O_2 cm^{-2} d^{-1}, respectively) than in the cold water sponge, *G. barretti*, may be explained by the water temperature in which the experiments were carried out, namely 20 ^{o}C for *D. avara*, 18 ^{o}C for *A. aerophoba* and 10 ^{o}C for *G. barretti*.

The four sets of oxygen profiles in *D. avara* show that oxygenation patterns close to the sponge surface can either be the product of advection or diffusion. Similar profiles have been made in other species of sponges (Hoffmann *et al*., 2008; Hoffmann *et al*., 2005b). This suggests that oxygenation near the surface may be more a reflection of both the animal's pumping behaviour and cell and associated-microbes respiration than of different morphology, size, or body density.

The estimated volumetric pumping rate of 0.26 cm^3 water cm^{-3} sponge min^{-1} which is necessary to maintain sufficient oxygen levels in *D. avara*'s body compares well to similar findings in *A. aerophoba*, where the minimum volumetric pumping rate was 0.2 cm^3 water cm^{-3} sponge min^{-1} (Hoffmann *et al*., 2008). Thus, only low pumping rates are needed to maintain adequate oxygen supply in the upper layer of the sponge body. It is therefore unlikely that oxygenation be the main reason for the sponge pumping behaviour. Obtaining food through filtration or the need for dilution of internal waste products is more likely to be the reason for pumping unless the sponge has an intrinsic rhythm (e.g. *Tethya crypta*, Reiswig, 1974). Pumping behaviour for other reasons than acquiring oxygen is also demonstrated in the static oxygen measurement. In stagnant water, the sponge pumped approximately once per minute

(Figure 4) although the water inside the sponge was far from anoxic and contained at least 59 % of oxygen compared to saturated water. This sponge may have been trying to feed (although no food was added in the flow-cell) or to dilute its metabolic products through pumping.

Oxygen concentration in the osculum was partially correlated to flow velocity (measured through PTV), which explained 17 % of the oxygen variability (Figure 5). The oscular flow velocity of our *D. avara* specimen was extremely low (0.48 mm s^{-1}). Whether the insertion of the micro-electrode, the handling of the experimental sponge or the prolonged stay in a holding aquarium may be responsible for such a low pumping activity is difficult to ascertain, but we have no evidence that the sponge was stressed. Although the oscular flow calculated from the specimen used for flow field images (Figure 6) was higher (1.4 cm s^{-1}), the values for *D. avara* in this study are much lower than the reported oscular velocity of other sponges that were studied *in situ*. For example, the Jamaican sponges *Mycale* sp., *Verongia gigantea* and *Tethya crypta* generated an average exhalant current velocity of 7.9, 12.8 and 17.3 cm s^{-1}, respectively (Reiswig 1974). Pile at al. (1996) found that boreal *Mycale lingua* in the Gulf of Maine reached pumping velocities of 14 cm s^{-1}. Vogel (1977) reports pumping rates of 7.5 to 22 cm s^{-1} in several Caribbean sponge species studied in the field.

The differences in oscular velocity between *D. avara* and those sponges may be explained by the sponge captivity, by the amount of food in the water or by intrinsic rhythms. The triggers responsible for the onset or discontinuation of pumping activity are still poorly understood and are likely to vary with species, environmental

conditions, sponge morphology, and oscula size. Oscular flow speeds have also been reported to vary within (Savarese *et al*., 1997) and among (Gerodette & Flechsig, 1979) individual sponges and between locations (Pile *et al*., 1996).

The various oxygen profiles, the static oxygen measurements and oscular flow velocities of this study show that the study sponges had means of finely regulating their pumping activity which was, in turn, partially reflected in the oxygen concentrations in the sponge. The effects of the most likely triggers for pumping activity (feeding, dilution of waste products and intrinsic rhythms) on oxygen concentrations within this species remain to be explored. Particle tracking velocimetry enabled the direct measurement of oscular flow in aquarium even at very low speed. Coupled with microsensor measurements, the non-invasive PTV could be used to further explore the effect of food availability on flow and oxygenation of sponges in captivity. In this respect, our data represent a stimulating starting point for further investigations in the physiology of sponges in *ex-situ* situations.

4.6 Acknowledgments

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Chapter 5

5. Heterogeneous oxygenation resulting from active and passive flow in two Mediterranean sponges

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5.1 Abstract

The oxygen dynamics and ventilation behaviour in *Dysidea avara* and *Chondrosia reniformis* (Porifera, Demospongiae) were investigated using oxygen micro-electrodes hot-bead thermistors. Both field and laboratory experiments proved the occurrences of anoxia in the sponge tissue that lasted up to approximately 1 h. Before and after the anoxic events, the sponge body was well aerated and contained similar oxygen concentrations as the ambient water. The onset of anoxia was not caused by the insertion of the micro-electrode into the sponge's body but started at various times after insertion (1 min to 6 h). Strong temporal and spatial heterogeneity of oxygen concentrations was observed with replicate oxygen profile series across the sponge surface, though tissue close to an osculum was generally better oxygenated. The oscular outflow velocities in *Dysidea avara* were in the range of 0 – 0.6 mm s^{-1}. The state of oxygenation of sponge tissue could only be partially attributed to its ventilating activity. Complex oxygenation patterns indicate a mosaic of different ventilation activities in the sponge. Ambient flow also influenced oxygenation patterns of sponges. Individuals with a functional aquiferous system regulated their pumping activity according to the ambient flow regime, while a small individual without osculum was passively influenced by ambient flow and became anoxic 39 minutes after ambient flow was stopped. Our findings show that temporal and spatial anoxic niches are found within sponges both in captivity and in the field, and are regulated by active (pumping) and passive (ambient flow) ventilation events. The variable oxygen concentrations inside the sponges will have great consequences for sponge metabolism, as well as for community composition and processes of sponge-associated microbes.

5.2 Introduction

Sponges are sessile marine filter-feeders. They possess an aquiferous system, which allows them to draw water into their body, filter food particles from the water and expel waste water through their oscula, their outflow siphon. Sponges in nature have been shown to have various ventilating behaviors. Some species, such as *Mycale* sp., ventilates continuously whereas others sponges, such as *Verongia gigantean*, periodically stop pumping at random intervals (Reiswig, 1971), possibly following an endogenous rhythm. Some species respond to physical disturbances. For example, *Verongia lacunose* reduces its ventilating activity to avoid damage to its aquiferous system in the presence of high sediment loads in the water (Gerrodette and Flechsig, 1979). *Microciona prolifera* explants ceased ventilating in response to lower salinities and their aquiferous system was reduced (Fell, 1998).

Through the ability of sponges to pump large amounts of water through their bodies, it has been assumed that the water within the body is oxygen saturated or close to it. However, anoxia has now been reported in several sponge species kept in captivity. Examples include *Geodia baretti*, a cold water bacteriosponge (Hoffmann *et al*., 2005 a, b), its explants (Hoffmann *et al*., 2005a), *Aplysina aerophoba* (Hoffmann *et al*., 2008), *Dysidea avara* (Schläppy *et al*., 2007) and *Chondrosia reniformis* (Hoffmann and Schläppy, submitted).

Oxygen is supplied to the sponge tissue by active ventilation and by advection driven by seawater currents (ambient flow), as the aquiferous system gives the tissue a certain permeability. This passive flow is driven by pressure differences and will be influenced by the sponge topography and of water current velocity. Passive

ventilation is of zero metabolic cost to the sponge (Vogel, 1974). Pile *et al*. (1997) found that *Baikalospongia bacillifera* ventilated less when ambient current was high. In the absence of ventilation or passive flow, diffusion is the only oxygen transport mechanism. Anoxia in sponges is probably due to several factors, most of which are still unknown. Absence of ventilation and low ambient flow are presumably important factors. Intuitively, the sponge should become anoxic if the combined demand of sponge cells and sponge-associated microbes' respiration exceeds the input of oxygen through active ventilation, diffusion and passive flow (see Hoffmann *et al*. 2008).

The presence of anoxic niches within a sponge is likely to influence microbial community structure and processes within sponges. The oxygen levels are a strong regulator for anaerobic processes. Typically, organic matter is degraded by the energetically most favorable process, which is aerobic respiration. The use of alternative electron acceptors, such as nitrate and sulfate occurs usually only under anoxic conditions. However, nitrate reducers and many sulfate reducers can nevertheless be active at low oxygen concentrations (Canfield and Marais, 1991). Facultative anaerobic microbes and obligate anaerobic microbes have been found in several species of sponges (Schumann-Kindel *et al*. 1997, Webster *et al.*, 2001), suggesting the presence of either temporal or spatial anoxic niches within sponges. Fermenting bacteria were found in *Ceratoporella nicholosni* (Santavy *et al*., 1990), sulfate reducing bacteria in tetractinellid sponges (Schumann-Kindel *et al*., 1997), and methanogenic euryarchaeotes may also be present (Webster *et al*., 2001).

Ventilation in sponges has been described in several species in the field or in laboratory conditions but how the temporal and spatial oxygen distribution in sponges is influenced by ventilation and ambient flow has not yet been documented. Microelectrode measurements of oxygen on sponges have been, to date, restricted to laboratory experiments. Therefore, we investigated the oxygen dynamics and ventilation behaviour of *Dysidea avara* and *Chondrosia reniformis* both in the laboratory and in the field, and in the presence or absence of ambient flow.

5.3 Material and Methods

5.3.1 Methodologies

5.3.1.1 Microsensors

Clark-type oxygen electrodes (Revsbech, 1989) with a 20 µm tip diameter were made at the Max-Planck-Institute for Marine Microbiology, Bremen, Germany and calibrated with a two point calibrations using oxygen-saturated seawater and oxygen depleted seawater made anoxic by the addition (to saturation) of an oxygen scavenger (sodium sulfite). Care was taken to obtain the zero value as quickly as possible as a protracted stay in the anoxic solution would have 'poisoned' the sensor, making it unusable.

For laboratory measurements the sensors were mounted on a motorized micromanipulator (Figure 1). The oxygen microsensor penetrated the surface of *D. avara*'s body easily, but the cortex of *C. reniformis* had to occasionally be pre-pierced before a microsensor could be inserted. Thermistor and oxygen measurement were carried out simultaneously and in a range of conditions: in an

aquarium with flow-through natural seawater and in a well-aerated 8 L flow-cell, at a temperature of 20 ^{o}C. For static measurements (not profiles) unidirectional flow was produced using a standard aquarium pump which re-circulated the seawater. The water in the flow-cell was aerated with an air stone, kept at a constant temperature. The sponges were not fed during the experiments. The micro-electrode was attached to a computer-controlled micro-manipulator (Figure 1). Static oxygen measurements were performed either in the tissue or in the osculum while profiles were always carried out in the tissue. A laminar flow-cell with a re-circulating mixture of artificial and natural seawater was used for experiments involving the manipulation of ambient flow.

For field measurements, a diver operable setup microsensor equipment was used as described in Weber *et al*. (2007). In both laboratory and in the field 4 sponges were used to make oxygen profiles by lowering the sensor 3 times to 3 mm inside the target sponges. Those profiles were performed along a transect going from a haphazardly chosen osculum and away from it in 2 mm steps (i.e. first set of 3 profiles was made at 2 mm from osculum, the 2nd and 3rd set at 4 and 6 mm from the osculum, respectively). Additionally, static oxygen measurements were conducted within the chosen osculum of the same individuals used for the oxygen profiles and the microsensor was left overnight. Only a selection of oxygen measurements are shown here. They were chosen for their representativeness of a range of tissue oxygenation.

5.3.1.2 Thermistor

A hot bead thermistor (temperature sensitive electrical resistor) and recorder were built at the Max-Planck-Institute for Marine Microbiology according to LaBarbera and Vogel (1976). Its design allowed measurement of water flow speed in sponges' osculum. The non-directional flow probe comprised a 1 mm diameter glass-bead-covered thermistor and an additional temperature compensation thermistor was sealed onto the distal end of a 2 mm diameter stainless steel tube in which the electrical connection led to the recorder. The apparatus functions according to the following principle: the bead is heated electrically and the heat is removed by the water flow created by the sponge. The energy needed to keep the bead heated is measured by the recorder and stored in the computer. To calibrate the thermistor, a cylindrical PVC block with a 10 cm deep circular grove (30 cm long) was made to rotate at a known speed with a motor. The grove was filled with seawater and the thermistor was held stationary inside the groove while the block moved in circles. Error was estimated to range between 2 and 15 %.

5.3.2 Sampling sites

Sponge specimens of *Dysidea avara* and *Chondrosia reniformis* were collected at several sites by SCUBA diving:

1. from a coralliferous community (20 m depth) on the Montgri Coast (Catalan coast; NW Mediterranean Sea, 42º 3' N, 3º 13' E, Spain).
2. at 10-15 m depth in Cala Montgo (Catalan coast, West Mediterranean Sea, 42º06.863' N 03º10.116' E Spain). The microsensor applications in the field also took place at this site.

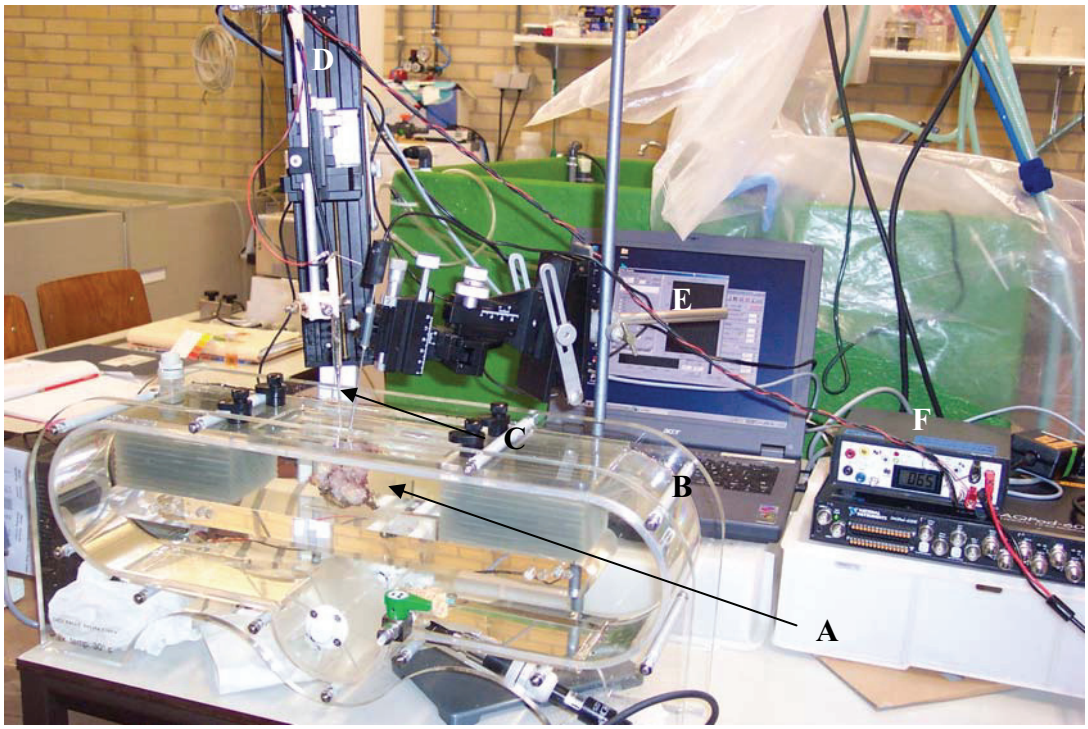

Figure 1: Microsensor and flow cell set up, showing a specimen of *D. avara* (A) in the flow-cell (B) and an oxygen microsensor (C) controlled by a micromanipulator (D) connected to a computer (E) and a pico-ampere meter (F)

5.3.3 Study species

Dysidea avara is a small sponge with well defined conuli and inter-dispersed oscula (exhalant siphons) (Figure 2). This species is a common Mediterranean sponge (Uriz *et al*., 1992), easily available (in shallow water of 4 – 40 m) and has desirable morphological characteristics for micro-electrode work such as a body made of a soft spongin skeleton (Galera *et al*., 2000). This species is of commercial interest due to its ability to produce avaraol and anti-inflammatory compounds (see Chapter 1). *Chondrosia reniformis* is a Mediterranean sponge commonly found in shallow water and has a cushion-like appearance, with a smooth body surface and few oscula (Figure 3). The body of the sponge lacks spicules but has high amounts of collagen fibers (Boury-Esnault, 2002) which make the species of interest for

commercial production of collagen for cosmetics or as nano-particle carrier (Swatschek *et al*., 2002) (see Chapter 1). A dense cortex sometimes precluded the insertion of the micro-electrode directly into the sponge body so a hole was pre-pierced in the sponge's surface.

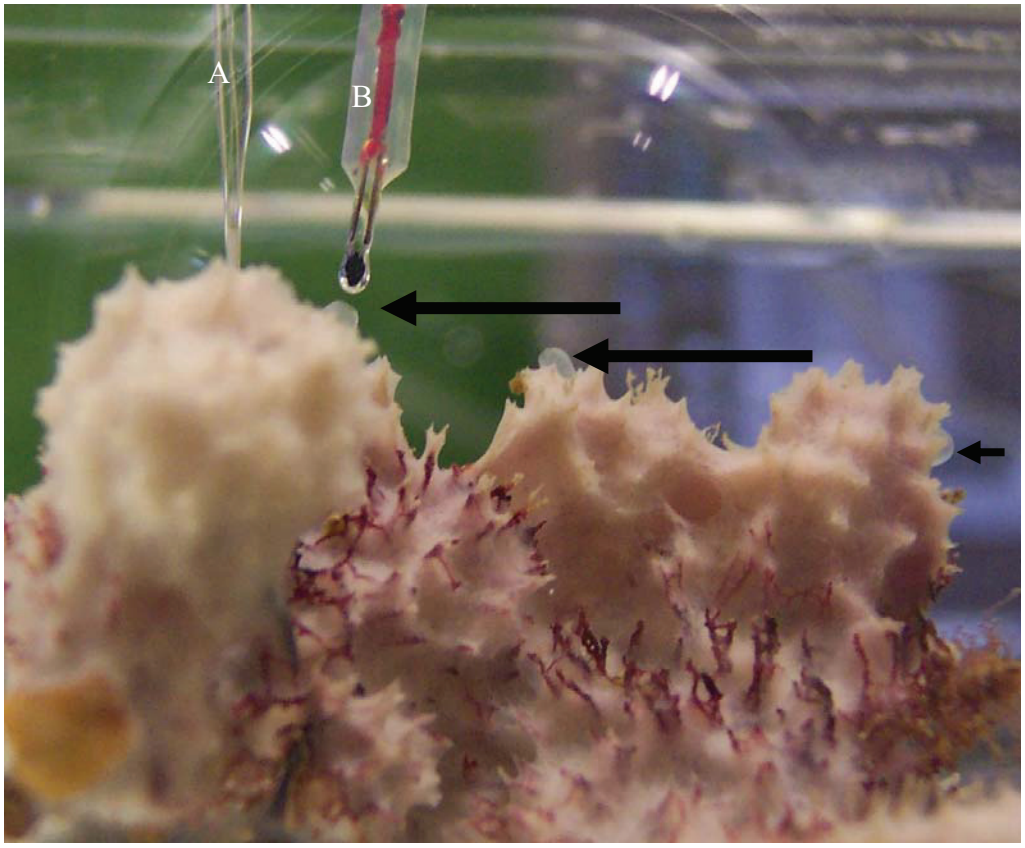

Figure 2: *Dysidea avara* with oxygen microsensor (A) in its body and a hot bead thermistor (B) above one of its oscula. The arrows point to the oscules

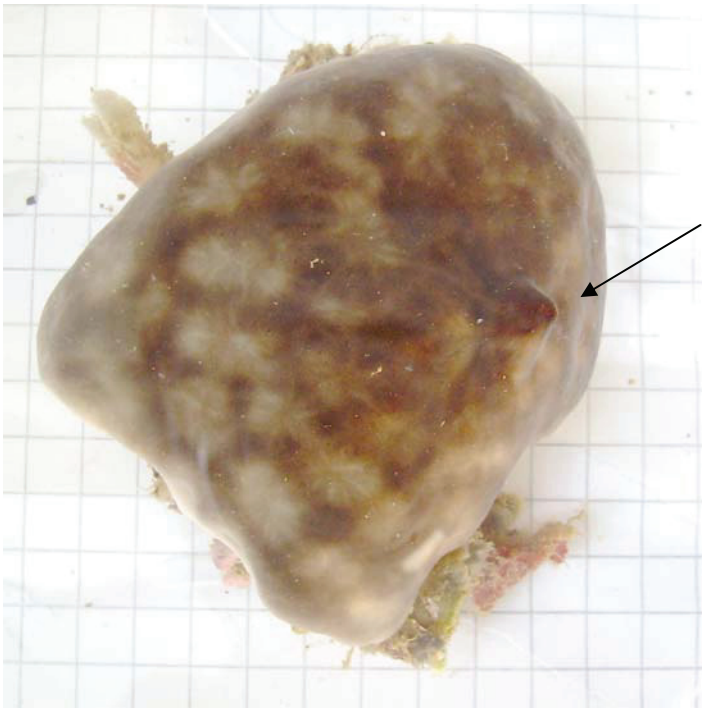

Figure 3: *Chondrosia reniformis*, specimen with one (semi-closed) oscule (arrow)

5.4 Results

5.4.1 Anoxia

The field measurements of oxygen dynamics in the osculum of *D. avara*, using a SCUBA diver-operated micro-electrode system, showed periods of tissue anoxia of 1 h (Figure 4 a) and of 6 min (Figure 4 b) in 2 out of the 4 studied specimens. Before and after the anoxic events the water exiting the osculum was close to saturated between 200-250 µM (Figure 4 a, b). For 2 other *D. avara* specimens in the field, oxygen in water stream exiting the osculum fluctuated between 120 and 225 µM and never approached anoxia (Figure 4 c, d).

Under laboratory conditions tissue anoxia was found in 4 out of 12 specimens of *D. avara* and in 2 out of 10 specimens of *C. reniformis* kept in captivity under a variety

of conditions. In *D. avara*, anoxic conditions were observed for 6 min (Figure 5 a) and 41 min periods (Figure 5 b). In another two specimens, suddenly decreasing oxygen levels followed by quickly increasing oxygen levels were also observed (Figure 5 c, d). These sharp changes in oxygen concentration where of ephemeral nature but occurred several times over 7 h (Figure 5 c) and 17 h (Figure 5 d). In *C. reniformis* anoxic events were observed for 1 hour and 42 minutes (Figure 5 e, f). The rest of the time the water exiting *C. reniformis*' osculum remained stable (150 – 200 µM). Anoxia started at random times after insertion of the sensor (1 min – 6 h).

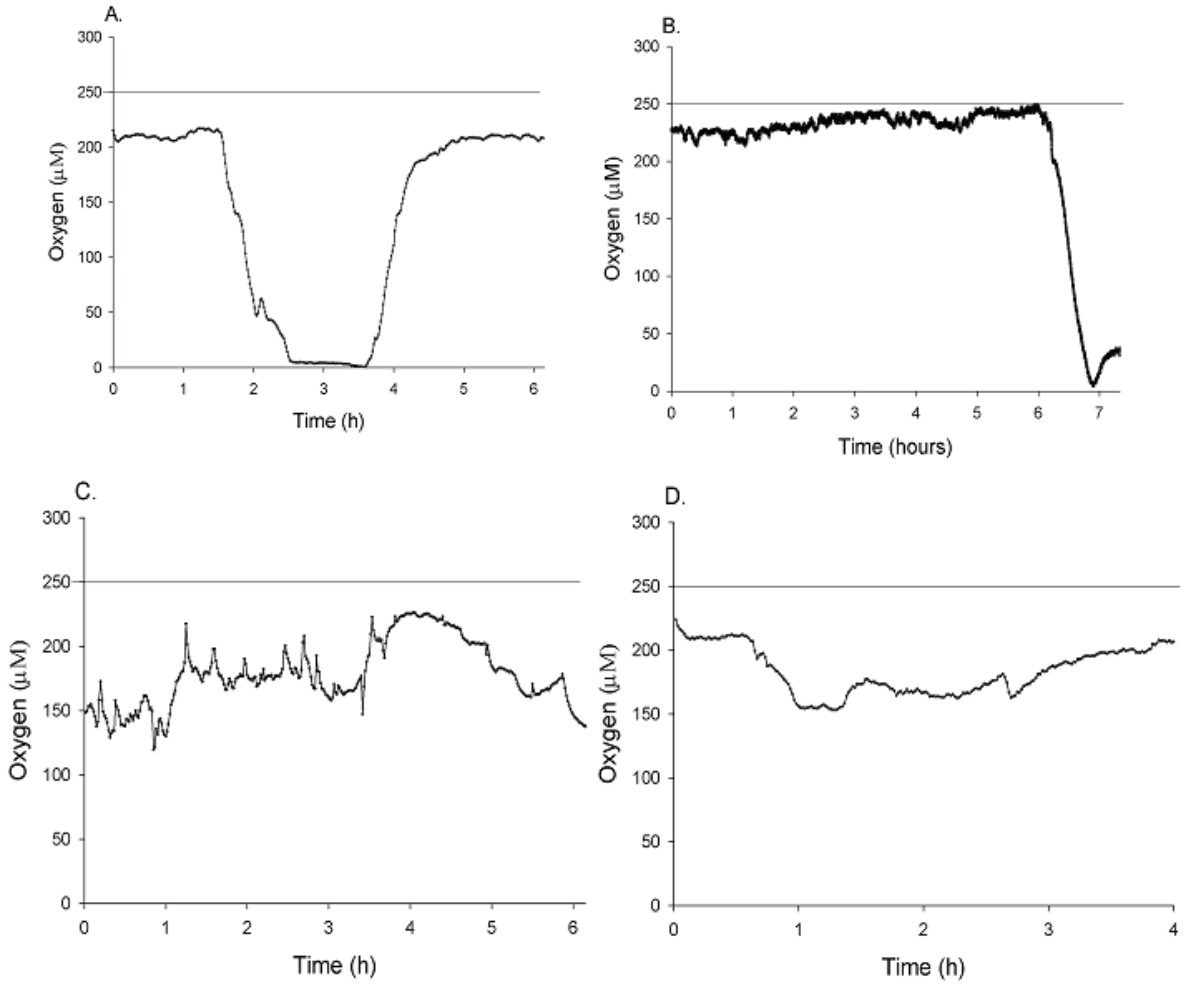

Fig. 4: Field static oxygen micro-electrode measurement in the osculum of four different *D. avara* individuals. The upper line indicates the oxygen content in the ambient water. Each data point represents a minute. A) showing anoxia for 1 h 04 minutes B) showing anoxia for 6 minutes C, D) showing fluctuating oxygen concentrations but without anoxia

When profiles where carried out on captive *C. reniformis* (Figure 6 a, b) and *D. avara* (Figure 6 c, d) a clear differentiation was observed between well-aerated (Figure 6 a, c) and poorly-aerated (Figure 6 b, d) individuals. Poorly-aerated sponges showed diffusive oxygen concentration profiles with a boundary layer of 0.5 – 1 mm, and an oxygen penetration of 1 and 2 mm into the sponge's body (Figure 6 b, d). Conversely, well aerated sponges had no boundary layer and the sponge tissue oxygenation was close to those levels found in ambient water up to 1 mm into the sponge tissue. At 3 mm, oxygen was approximately 200 μM in both species (Figure 6 a, c).

5.4.2 Spatial oxygen heterogeneity

5.4.2.1 Oxygenation and distance from target osculum

Spatially heterogeneous oxygenation patterns were found in field measurements made on 4 *D. avara* individuals (only 2 shown here, Figure 7). These replicate profiles showed some variability in the oxygen levels in the sponge, within the 3 - 6 min required to measure a single profile. Replicate profiles on the same location on the same sponge were sometime highly variable (Figure 7 a, c). Nonetheless, a pattern emerged which showed that in proximity of the osculum the tissue was better oxygenated than in areas far from the target osculum (Figure 7).

Under laboratory conditions, the high temporal and spatial variability in oxygen levels was even more pronounced than in the field (Figure 8). Particularly near the osculum the variability was prevailing, whereas in areas remote from the osculum more reproducible oxygen profiles were measured (Figure 8). High oscular flow speed (0.6 mm s^{-1}) did not always correlate with profiles of well-oxygenated sponge

tissue (Figure 8 c, e). The reverse was also true: at low oscular flow speeds the profiles were not always approaching anoxia (Figure 8 a, f).

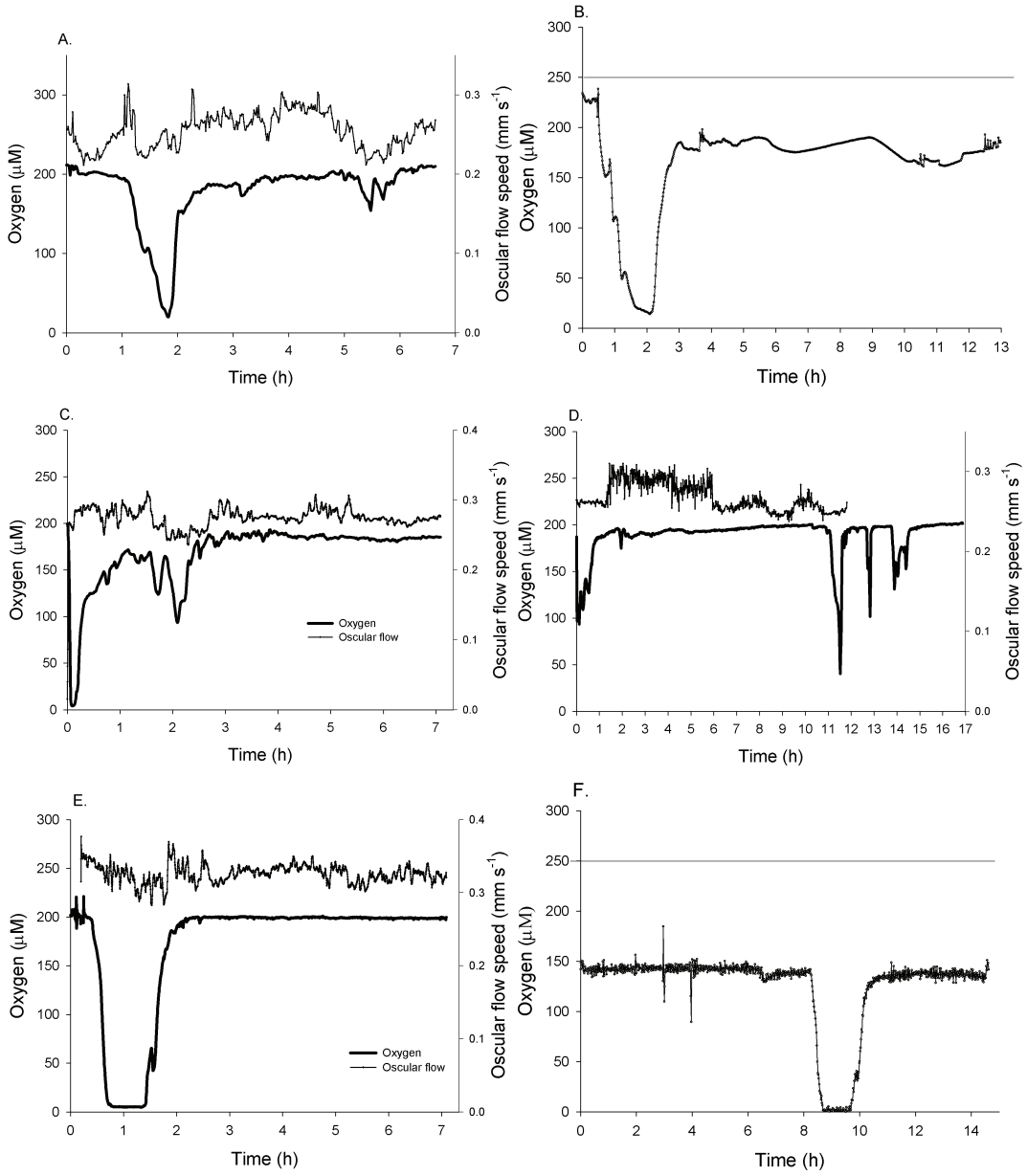

Fig. 5: Laboratory static oxygen micro-electrode measurement in the osculum of *D. avara* (A, B, C, D) *and C. reniformis* (E, F). Each data point represents a minute. The upper line indicates the oxygen content in the ambient water. A, B) *D. avara* showing conditions close to anoxia for 6 and 41 min, respectively B, C) *D. avara* showing repeatedly fluctuating oxygen conditions approaching anoxia E, F) in two freshly sampled *C. reniformis* individuals, showing anoxia for 60, 42 minutes, respectively

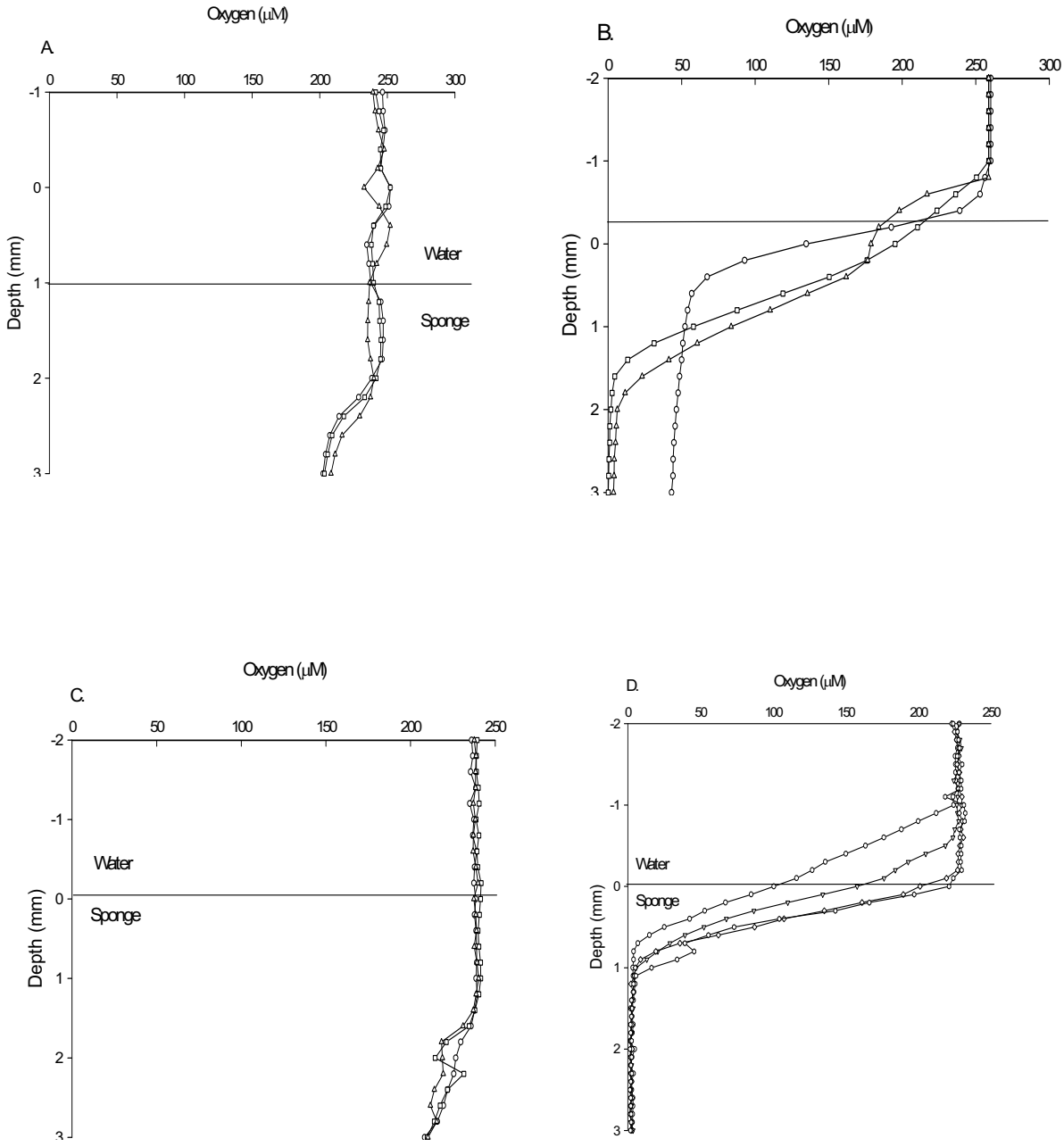

Fig. 6: Oxygen profiles (replicated 3x) in the body of *C. reniformis* A) when the sponge was well oxygenated B) when the sponge was anoxic. In the body of *D. avara* C) when it was well oxygenated D) when the sponge was anoxic

At a distance of 4 mm (midway) from the osculum, the most oxygen depleted tissue was found (Figure 8 b, e). At this location, a steep diffusion boundary layer was occasionally observed (Figure 8 b). In two sets of profiles, a lag-time between the oscular flow speed and the oxygenation occurred (Figure 8 b, f). When the first profile midway from the osculum was made (Figure 8 b), the sponge had already stopped pumping (oscular flow 0 mm s^{-1}), but the sponge had still had oxygen for about 6 minutes, the time required to carry out all three profiles. In another case, the oscular flow was high during the first two profiles near the osculum (Figure 8 f), but the effect of strong ventilation on oxygenation was only shown 2 minutes later in the last profile, which showed highly oxygenated sponge tissues.

5.4.2.2 Oxygenation and ambient flow

Oxygen concentration in the osculum of a *D. avara* specimen decreased at lower ambient flow (Figure 9). Exposed to unidirectional flow, the average oxygen content of the sponge oscular water was 5 μM (± 0.37 SEM) below the seawater concentration (227 μM). Approximately once every two minutes, stronger deoxygenated water (150 μM) exited the osculum during approximately 30 seconds (see Figure 9). Without ambient flow, the frequency of pulses of deoxygenated water increased to approximately one per minute, and the oxygen content of the sponge oscular water fluctuated in peaks between 105 and 218 μM (i.e. 9 – 122 μM below seawater) (Figure 9). Thus ambient flow increased the oxygen supply in the sponge. In an experiment with another specimen (*D. avara*) and when the flow velocity in the flow-cell was increased, the oxygenation remained in the same range as originally but upon decreasing ambient flow, oxygenation in the sponge decreased until ambient flow was stopped (Figure 10). At that point, oxygen

concentrations in the sponge showed fluctuations but returned to approximately in the range of original levels (Figure 10).

When the influence of ambient flow on tissue oxygenation of a small individual was repeated, another result was apparent. This individual lacked an osculum (and presumably a functional aquiferous system) and became completely anoxic in stagnant water. Upon switching off the flow the tissue became anoxic in 39 minutes. When flow was re-established, the tissue oxygenation recovered in 15 minutes to steady state levels (50 - 100 μM). When this was repeated several times with various ambient flow levels it became apparent that the flow velocities above 0.9 cm s^{-1} did not further increase the oxygen level in the tissue (Figure 11).

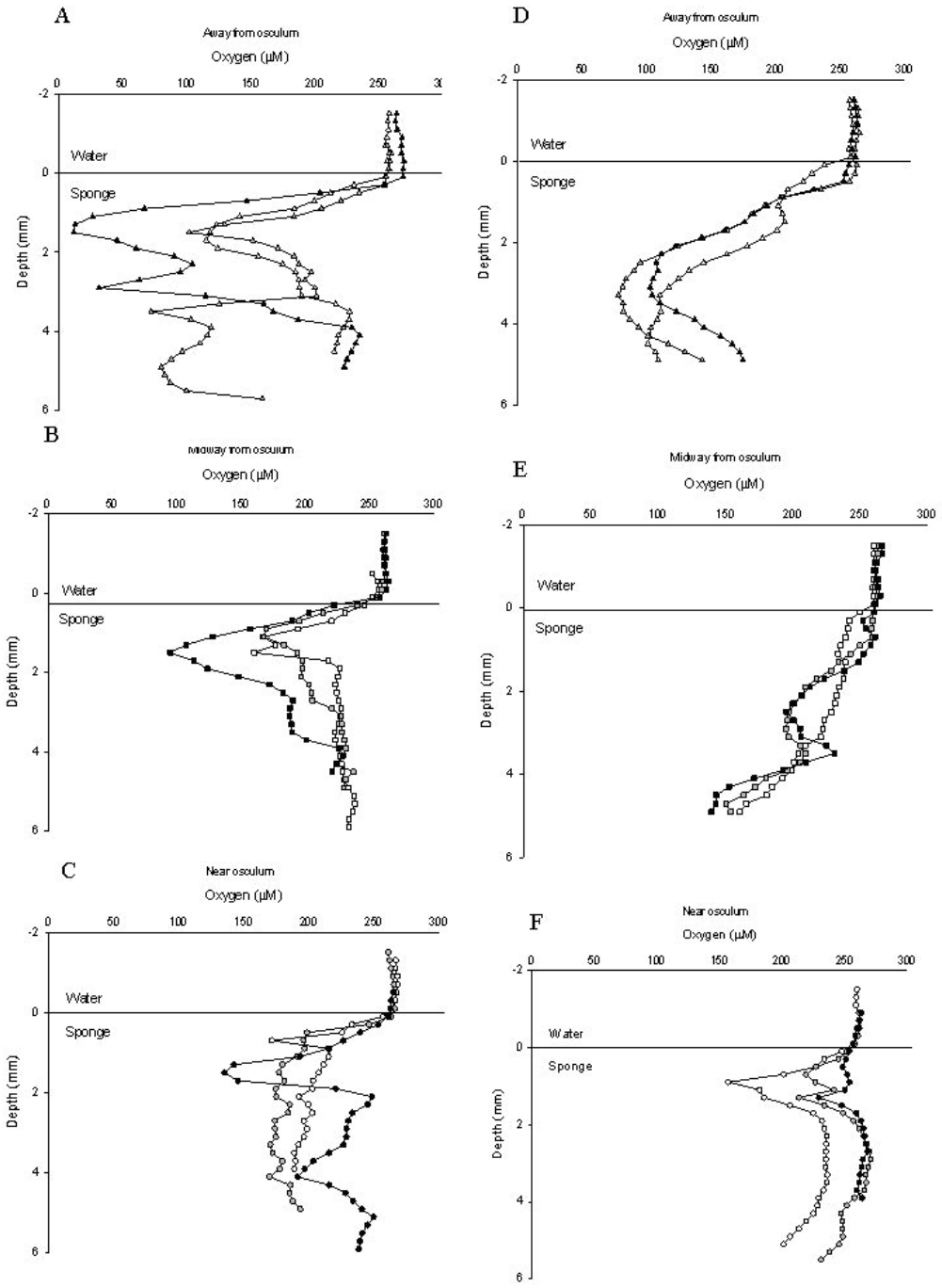

Fig. 7: Field profiles in two *D. avara* individuals (Individual 1 = A, B, C; Individual 2 = D, E, F). A, D) away, B, E) midway and C, F) near an osculum. Open symbols = 1st profile; grey symbols = 2nd profile and black symbols = 3rd profile

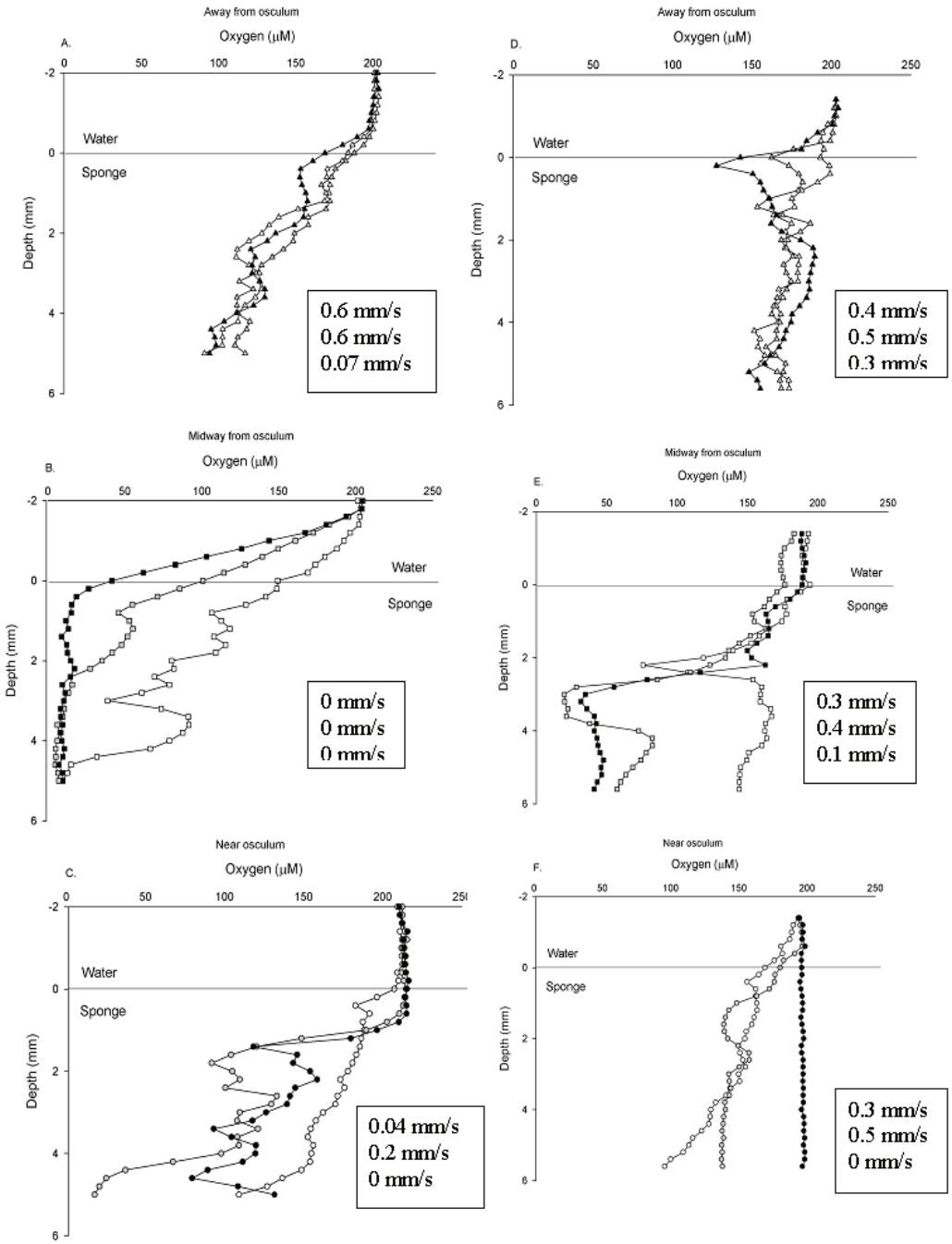

Fig. 8: Laboratory profiles in two *D. avara* individuals (Individual 1 = A, B, C; Individual 2 = D, E, F). A, D) away, B, E) midway and C, F) near an osculum. Open symbols = 1st profile, grey symbols = 2nd profile and black symbols = 3rd profile. The values in the box represent the oscular flow measured at the closest osculum to the micro-electrode. The first value correspond to the first profile, second value to second profile and third value to the third profile

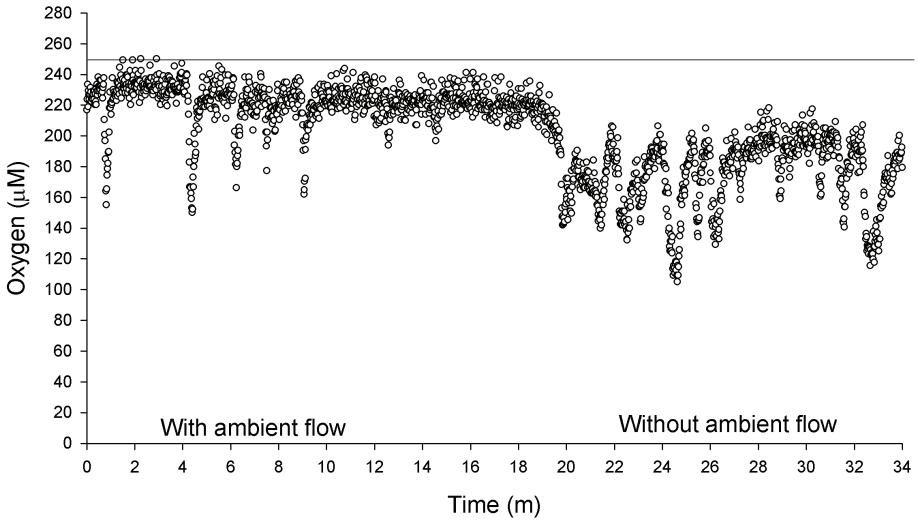

Fig. 9: Oxygen micro-electrode measurement in the osculum of *D. avara* in a flow-cell with re-circulating seawater, with the presence and absence of ambient flow. The vertical bar indicates oxygen content in the water

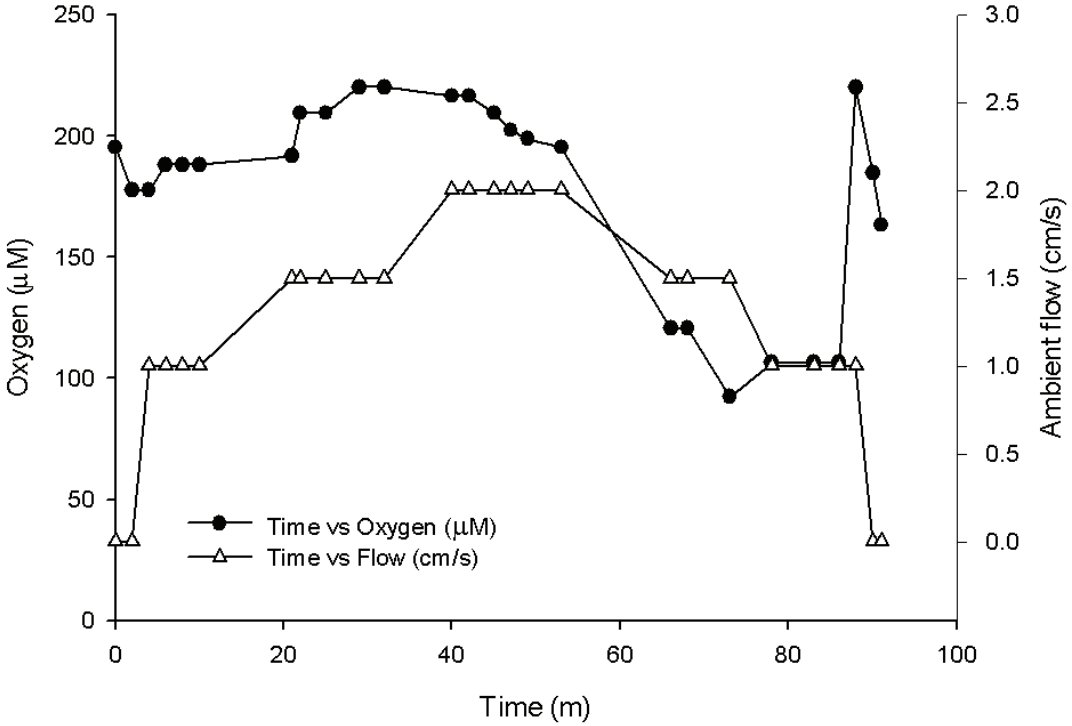

Fig. 10: Oxygen micro-electrode measurement in sponge in relation to ambient flow velocities in a large *D. avara* with oscula

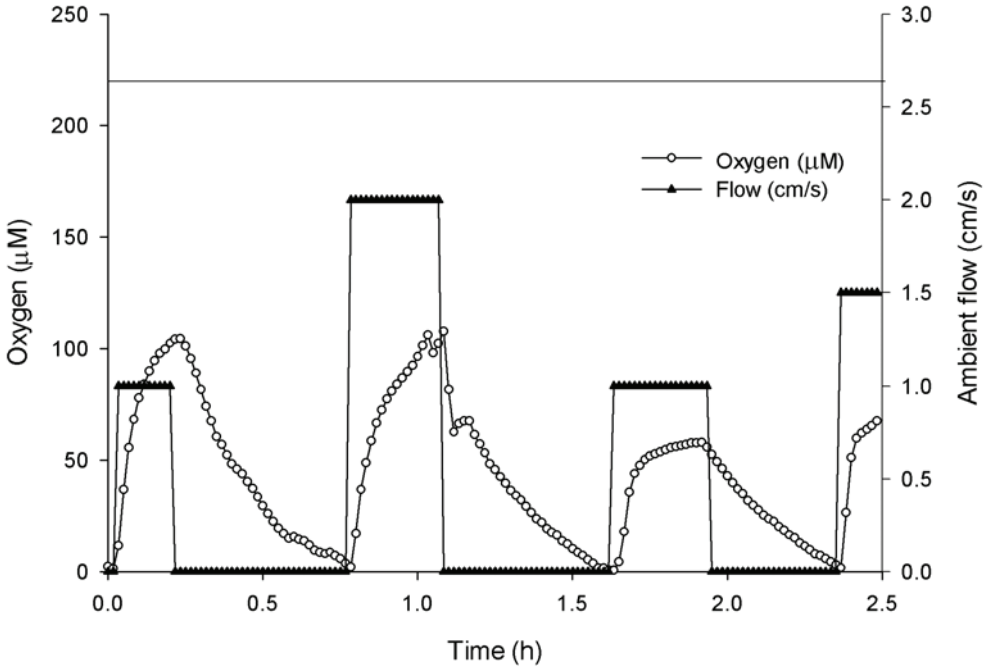

Fig. 11: Oxygen micro-electrode measurement in sponge in relation to ambient flow velocities in a small *D. avara* without oscula. The straight line indicated oxygen concentration in the seawater

5.5 Discussion

5.5.1 Anoxia

Field and laboratory static oxygen measurements within the tissue of both *D. avara* and *C. reniformis* specimens revealed that those sponges underwent periods of anoxia. The anoxia was therefore not a consequence of potentially unfavorable sponge laboratory conditions, as *D. avara* showed the same pattern of oxygen dynamics and oxygen distributions in both the laboratory and in undisturbed field conditions (Figure 4, 5). The onset of anoxia was not induced by the sensor measurements, but occurred random times after insertion (Figure 4, 5). For these reasons, we feel confident that our results show a realistic picture of the occurrence

of anoxia in both target species. These times of low oxygenation seem to occur commonly and appear to be part of the sponge functioning.

That sponges can have anoxia has been observed previously in a variety of species (Hoffmann *et al.* 2005a, b, Schläppy *et al.* 2007, Hoffmann, 2008). However, our data shows that oxygenation is heterogeneous across the sponge, that oxygenation is most intense near an osculum, so that more remote areas are not as well oxygenated and must even rely on diffusion. Ventilation is the main oxygenation process and seems to be alternately active in different zones of the sponge. It is possible that the set of choanocyte chambers and canals leading to an osculum function as an independent unit (as an autonomous module). Observations made in the field (Mendola, unpublished data) and in the laboratory (Schläppy, unpublished data) showed that one osculum can be totally inactive while the neighboring osculum has a high oscular flow rate. This also supports the idea of modular functioning of *D. avara*. Our data indicate that oxic and anoxic zones can be present at various locations across the sponges and that the change from aerobic to anaerobic and vice-versa is occurring abruptly and not gradually. This means that within the deeper layers in sponges a highly dynamic oxygen regime is present, to which the bacterial populations must be adapted in order to pertain.

5.5.2 Oxygenation and distance from target osculum

Oxygen profiles carried out away, midway and close to an osculum showed highly variable tissue oxygenation patterns in space and time (Figure 7, 8). The expectation of oxygenation being a function of the distance to a target osculum was met in the field (Figure 7) but less so in the laboratory (Figure 8). Oxygenation

patterns were not, as might have been expected, the most oxygen depleted away from the osculum (Figure 8). If *D. avara* really has a 'modular' ventilation patterns, then it is possible that our measurements were made in different units and are so variable for this reason.

Oscular flow speed measured in the laboratory was sometimes related to how well the sponge was oxygenated, but not always. The presence of a lag time between ventilation and oxygenation is possible and would explain why ventilation and oxygenation data do not always match. In *Aplysina aerophoba*, the situation is very different and Hoffman *et al.* (2008) showed a clear relation between sponge tissue oxygenation and pumping activity. However, this species has a simple chimney-like architecture unlike *D. avara* which is more massive, often with many oscules, making it difficult to predict the architecture of the canal system. The tissue sampled with the electrode may have only been partially linked to the target osculum where the exhalent flow was measured.

5.5.3 Oxygenation and ambient flow

Another factor which influenced oxygenation patterns in sponges was the level of ambient flow and sponge ventilation behaviour. The larger specimens in this study showed the ability to modify their pumping behaviour according to the presence or absence of ambient flow (Figure 9), and according to ambient flow speed (Figure 10). This implies that the sponges were able to detect the presence or absence of ambient flow and modified their ventilating behavior accordingly, although we have no direct evidence as to the method of detection. Furthermore, the sponges reacted rapidly (within minutes) to a change of ambient flow magnitude by changing their

ventilation. When exposed to varying regimes of ambient flow, the oxygenation within the body of a *D. avara* specimen fluctuated little (approx. 180 – 200 μM), as long as the ambient flow was above 1 cm s^{-1} (Figure 10). However, when ambient flow was stopped, the sponge maintained oxygen concentration above 100μM within its tissues and did not become anoxic, suggesting a compensatory mechanism through increased ventilation.

When a similar experiment was conducted with a small *D. avara* individual which lacked an osculum (and thus probably a functional aquiferous system), ambient flow had a much larger impact on the oxygen levels within the sponge. The cessation of ambient flow, in this case, resulted in an immediate decrease of oxygen within the sponge. Sponge cell respiration and probably the respiration of sponge-associated microbes at the location of measurement must have exceeded the supply of oxygen through diffusion, so residual oxygen within the sponge tissues was depleted within 39 minutes. Varying levels of ambient flow did not make a substantial change in the maximum level of oxygen content within the sponge tissue (Figure 11), suggesting that, above a certain minimum ambient flow, the benefits of increased flow are not proportional. Our results support Vogel's findings (1975, 1977) that sponges can take advantage of ambient flow, which comes at a low cost to them. These results point to the importance of ambient flow to small sponges, for example explants (sponge pieces) often used for sponge aquaculture.

5.5.4 Consequences of sponge tissue anoxia

The consequences of temporal or spatial anoxic niches within the study species are several-fold. Firstly, it allows the presence of obligate anaerobic or facultative anaerobic micro-organisms in the sponge body. Such microbes and their processes have been found in several species of sponges such as *Rhopaloeides odorabile* (Webster *et al.* 2001, Hoffmann 2005b). Secondly, anoxic niches allow anaerobic microbes to become active. Whether anaerobic conditions last long enough for anaerobic microbial processes such as denitrification, anammox or sulfate reduction to occur remains to be investigated. Finally, the sponge-associated microbes must be able to tolerate and be active even under rapidly changing oxygen conditions.

By varying the internal oxygen condition within its body, a sponge may be able to maintain a diverse population of associated bacteria which can potentially be used as a food source (bacterial farming) or as a means to reduce the prevalence of the sponge cell metabolic waste products, such as ammonium. Alternatively, sponge-associated microbes may simply take advantage of an inherent behaviour - the periodic shutting down of the ventilating activity. In order to investigate this further, the presence or absence of key anaerobic microbial processes should be assessed in these two species of sponges and the mechanisms triggering ventilation understood. Only then could the interactions between the oxygenation of the sponge host and the metabolic activity of its associated microbes be elucidated.

5.6 Acknowledgments

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Chapter 6

6. Microbial abundance and microbial processes in three Mediterranean sponges

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6.1 Abstract

Sponges are known to harbor a large number of microbes with diverse metabolisms. The microbial abundance and the prevalence of aquiferous system in three Mediterranean sponges were quantified by direct cell counts on histological sections stained with DAPI. In *Chondrosia reniformis* the average number of microbes was 2.4 $\pm$ 0.27 * 10^{10} cm^{-3}, in *Aplysina aerophoba* 3.3 $\pm$ 0.09 * 10^{10} cm^{-3}, and in *Dysidea avara* 0.7 $\pm$ 0.04 * 10^{10} cm^{-3}. The aquiferous system amounted to 47, 21 and 6 % of the choanosome in *D. avara*, *A. aerophoba* and *C. reniformis*, respectively. Only in *Chondrosia reniformis* a separate habitat for the microbes was found: the collagen which harbored less microbes (0.94 $\pm$ 0.05 SEM *10^{10} cells cm^{-3} sponge) than the mesohyl (2.7 $\pm$ 0.27 SEM *10^{10} cells cm^{-3} sponge). These findings are consistent with the idea that sponges with reduced aquiferous system have more microbes than those with a large aquiferous system. Moreover, the reason for the high abundance of microbes is not due to the presence of more mesohyl volume per sponge volume (in *C. reniformis* and *A. aerophoba*) but due to the fact that the mesohyl is more densely populated in sponges with a reduced aquiferous system. Recent studies have shown the presence of anoxia in these sponges and the possibility of anaerobic microbial activity. In this study, microbial processes of aerobic and anaerobic nature were investigated in *D. avara* and *C. reniformis*. Their presence and rates were established through microsensor measurements of product accumulation and isotope tracer experiments. As a surprise, in *D. avara*, the sponge with lower microbial counts and a less dense tissue structure, the same microbial processes were found as in *C. reniformis*, with even higher rates. Total net nitrification occurred at 8.8 and 14.7 nmol cm-^{3} sponge h^{-1} for *C. reniformis* and *D. avara*, respectively. Rates of denitrification, an anaerobic microbial process, were 10 nmol N consumed cm^{-3} wet

weight sponge h^{-1} in *C. reniformis* and 14.9 nmol N cm^{-3} wet weight sponge h^{-1} in *D. avara* while anaerobic ammonium oxidation (anammox) and sulfate reduction were below detectable limits in both species. Our rates of denitrification are very close to the nitrification rate indicating that approximately half the nitrate produced by the sponges is reused for denitrification while about 5 % of the ammonium produced by sponges is converted into nitrate. This provides evidence that not only species of sponges with high microbial abundance host active microbes. The benefit of this association for the sponges is discussed.

6.2 Introduction

Sponges are evolutionary ancient Metazoans, the first multi-cellular organisms on the tree of life. They harbor great amounts and a large variety of microbes within their bodies (see Taylor *et al.*, 2007 for review). These microorganisms are sponge-specific and differ from those in the water column in number and nature (Hentschel *et al.*, 2003). Microscopical studies showed that, in some species, 33 % of the space in the sponge tissue is occupied by microbes (Vacelet and Donadey, 1977). These species have been termed 'bacteriosponge' (Reiswig, 1981) or 'high-microbial abundance' (HMA) sponges (Hentschel *et al.*, 2003) and can have as many as 10^8-10^{10} cells g^{-1} of sponge wet weight which is 2 - 4 orders of magnitude higher than bacterial concentration in seawater. In contrast, 'Low microbial abundance' (LMA) sponges have $\leq 10^6$ microbes g^{-1} of sponge wet weight (Hentschel *et al.* 2003). Micro-organisms can be present in the sponge matrix (mesohyl) between sponge cells, within sponge cells (Vacelet and Donadey 1977) and even within the nucleus (Friedrich *et al.*, 1999, Vacelet, 1975).

Molecular studies uncovered the large variety of sponge-associated microbes which belong to 14 different families (Taylor *et al.* 2007). Sponge-associated microorganisms are very similar across sponge species and within species across latitudinal gradients (Hentschel *et al.* 2002). Unfortunately, the ever-increasing knowledge of the taxonomic nature of sponge-associated microbes infers little about their metabolic function, activity and benefit to the sponge. Thus, microbially mediated biochemical processes must be uncovered if the nature of the sponge-microbe association is to be understood. To date, several microbial processes have been found in sponges: nitrification (Bayer *et al.*, 2007, Corredor *et al.*, 1988, Diaz and Ward, 1997, Jiménez and Ribes, 2007, Southwell *et al.*, 2008), nitrogen fixation (Wilkinson and Fay, 1979), methane oxidation (Vacelet *et al.*, 1996) and sulfate reduction (Hoffmann *et al*. 2005a).

Until recently, sponges were assumed to have an aerobic metabolism, similarly to many invertebrates. Their enormous capacity to filtrate water (e.g. Reiswig, 1971) was thought to provide adequate oxygenation across the whole sponge at all times. The discovery of anaerobic microbes within sponges led to the proposition of anoxic niches within the sponge body (Webster *et al.*, 2001). When oxygen concentrations were measured with microelectrodes inside the body of sponges, it became evident that sponges which do not ventilate become quickly anoxic. This is the case for *Aplysina arerophoba* (Hoffmann *et al.*, 2008), *Geodia barretti* explants (Hoffmann *et al.*, 2005a), *Dysidea avara* (Schläppy *et al.*, 2007), *Chondrosia reniformis* (Hoffmann and Schläppy, submitted) and *Cliona orientalis* (Schönberg *et al.*, 2004). The spatial and/or temporal anoxic micro-niches within the tissues of those species could result in the activation of the anaerobic microbes present in the sponge. The link between

anoxia in sponges and anaerobic microbial processes was recently made by Hoffmann *et al.* (2005b) who reported the simultaneous occurrence of anoxic zones in sponge tissues and of sulfate reduction, an anaerobic microbial process, in *Geodia barretti*, a cold water sponge.

Vacelet and Donadey (1977) observed that sponges with a dense mesohyl and a reduced aquiferous system host large amount of bacteria while species with a looser mesohyl and well developed aquiferous system host less bacteria. Recently, Weisz *et al.* (2008) provided supporting evidence toward this original observation and established that HMA sponges are denser (heavier) and have a lower pumping rate as the LMA species. In this study, we aim to test the hypothesis that HMA sponges (*Chondrosia reniformis* and *Aplysina aerophoba*) have a higher abundance of bacteria and a less developed aquiferous system by determining the microbial abundance using a new quantification method and we aim to quantify the prevalence of the aquiferous system in three Mediterranean sponges. Furthermore, we intend to establish whether dense tissue and a high number of associated microbes lead to more diverse microbial processes, including anaerobic processes. For this, we chose a LMA sponge, *Dysidea avara*, and a HMA sponge *Chondrosia reniformis*. Additionally, we included *Aplysina aerophoba* for which microbial cell counts using the classical sponge slurry approach were already made (Friedrich *et al.* 1999) and for which microbial processes have already been described (Bayer *et al.*, 2008, Jiménez and Ribes, 2007).

6.3 Material and Methods

6.3.1 Microbial counts and quantification of the aquiferous system

The *Dysidea avara* specimens came from two different locations: Adriatic Sea, Limski Canal, Croatia close to Rovinj, Croatia (44°67.50'N 13°37.0' Sec.), Northern Adriatic Sea Muntanya de Montgó, Punta del Romani at Cala Illa Mateua in the township of L'Escala, Girona, Spain (42º06.863' N 03º10.116' E), Northern Mediterranean. *Aplysina aerophoba* and *Chondrosia reniformis* specimen only came from the Croatian site. Sponges were immediately fixed in 2 % formalin after collection, dehydrated in a 30, 50, 70 % ethanol series and stored in 70 % ethanol. Three tissue blocks were cut from each sponge, at different zones of the body (apex, middle, and basis). Each block contained the continuum from sponge surface to sponge core. After saturation with liquid cryomedium, (Jung Tissue Freeze Medium ®, Leica Microsystems, Nussloch) for 12 hours at 4 ^{o}C, the block were trimmed into 1x0.5 cm pieces. They were embedded in base molds with fresh cryomedium and left to harden for 12 h at -80 ^{o}C. For histological analysis, 5 μm longitudinal sections were made using a cryostat microtome (HM 505E, Microm, Walldorf, Germany) at -35 ^{o}C. All sponge sections were mounted on gelatinized glass slides and prepared for microscopic analysis by using 0.2 % 4,6-diamino-2-phenyindole (DAPI, Sigma). Since auto-fluorescence was expected to occur on the sections stained with DAPI and thus give a spurious DAPI signal, we used a second stain to confirm that the DAPI signals really represented microbes. Fluorescence *in situ* hybridization (FISH) was performed with some of the sections of each species using the Cy3-labeled oligonucleotide probe EUB I-III (Daims *et al.*, 1999). Formamide concentrations in the hybridization solution and washing buffer were 35 % v.:v. After rinsing and air-drying, the sections were mounted in Citiflour.

Microscopical analysis was first carried out (at 100 x magnification) with a Zeiss Axiophot microscope equipped with Zeiss filters (for DAPI and Cy3) on the entire tissue section to obtain an overview and train visual perception. Colour micrographs were taken using a Zeiss Axiolmager M1 microscope with an AxiCam MRc camera system. Digital image processing was performed using AxioVision 4.4 software.

An ocular with a 122 x 122 μm counting grid and scale bar was used for determining the proportion of different tissue types (e.g. cortex, mesohyl) and for counting of DAPI- or FISH-stained bacterial cells (at 1000 x magnification). Thirty visual fields were inspected per specimen on a transect from the sponge's surface to its core to determine microbial abundance, counting 150 grid-cells per block. A percentage estimation of the aquiferous system (proportion of choanocyte chamber and canals to mesohyl in %) was carried out for each grid and averaged for each species.

6.2.2. Microbial processes in D. avara *and* C. reniformis

Twelve specimens of *D. avara* and *C. reniformis* were collected in 10-15 m water depth in Muntanya de Montgó, Punta del Romani at Cala Illa Mateua in the township of L'Escala, Girona, Spain (42º06.863' N 03º10.116' E), by SCUBA diving. The specimens were allowed to acclimatize for one day before the start of the experiments in a 20 L aquarium with freshly sampled seawater, an air stone and re-circulating water at 15^{o}C. Three specimen of each species were cut into explants of 0.2 – 0.5 g^{-1} prior to starting the denitrification and anammox incubation experiments and used directly for the experiment. All specimens looked healthy at the start of the experiments.

6.2.2.1 Nitrification

The presence of nitrification (the process of NH_4^+ transformation to NO_2^- and from NO_2^- to NO_3^-) was tested by conducting an incubation experiment in which 3 specimens of *C. reniformis* and 3 of *D. avara* which were kept in 1 L beakers with fresh marine seawater, with an air stone and a magnetic stirrer. The 3 controls beakers were set up identically but without sponges. The water was kept at constant temperature and in the dark. Additional ammonium (10 μM) was added to each beaker. Water aliquots (10 ml) were taken over 24 h and immediately frozen at -20 ^oC until analyses. Ammonium was processed in an auto-analyzer while nitrite and nitrate were determined with a $NO\text{-}NO_2\text{-}NO_x$ analyzer (Thermo Environmental Instruments Inc. USA). Aliquots of 200 μ L were injected in a glass chamber in which a reduction solution (nitrite: 7.5 g Na I mixed with 2.5 g H_3 PO_4 99 %; NO_x: 0.1 M Va (III) Cl). The reduction solutions were made anew for each day of measurement. After passing through 2M NaOH, the NO gas was freed from the sample, and NO_X was measured by passing through the ozonator. The integrated area under the curve (using the ECW 2000 Integration Package) and not the peak was used to calculate the NO_x and NO_2^- content of the samples. Nitrates were calculated: $NO_x - NO_2^- = NO_3^-$. The rate of production or consumption was calculated as the average of all 3 sponge replicates minus the average of the 3 controls.

6.2.2.2. Denitrification and anammox

Denitrification occurs through the combination of two mols of NO_3 to one mol of N_2. Depending on the residual ^{14}N in the system (either through the seawater or due to sponge microbe nitrification) and when the $^{15}NO_3^-$ tracer is added, denitrification can

produce N_2 of any of the three combinations through random pairing: $^{14}N^{14}N$, $^{14}N^{15}N$ or $^{15}N^{15}N$ (Nielsen 1992). Anammox (anaerobic ammonium oxidation) occurs when one mol of NO_2^- combines with one mol of NH_4^+ ion and produces N^2. We specifically check for anammox by using $^{15}NH_4^+$ and $^{14}NO_2^-$ as labels. Thus, the random ion pairing can yield $^{14}N^{14}N$ or $^{14}N^{15}N$ but not $^{15}N^{15}N$ when the incubation is done with $^{14}NO_2^-$ (Thamdrup and Dalsgaard, 2002).

Sponge explants of *C. reniformis* (0.3 – 0.9 g wet weight) and *D. avara* (0.02 – 0.4 g wet weight) were incubated in 12.5 ml gas-tight exetainers filled with natural seawater spiked with 10 µM $^{15}NO_3^-$ for the denitrification experiment and with 10 µM $^{15}NH_4^+$ and 2 µM $^{14}NO_2^-$ for the anammox experiment. The exetainers were free of air bubbles. We estimated a respiration rate of 9 µmol O_2 cm^{-3} sponge day^{-1} based on Hoffmann *et al.* (2005b) and estimated that 90 % of the oxygen in the exetainers should be depleted within 24 h (but residual oxygen may have been present). Microbial processes were stopped at 0, 2, 3, 6, 12 and 24 h for denitrification and 0, 1, 3, 6, 12 and 24 h for anammox by injecting 150 µL of saturated $HgCl_2$ solution in the exetainers which were gently shaken for good repartition of the chemical and stored up-side-down (to avoid gas leakage) after a head space of 2 ml of He gas was added in which the N^2 gas could escape from the water phase and remain until analyses. Samples were stored at room temperature until analyzed. Gas chromatography-isotopic ratio mass spectrometry was carried out to determine the isotope ratio ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) of the N_2 gas trapped in the exetainers' head space by injecting 250 µL. The concentration of the different isotopic combinations was determined and the N_2 production rates were calculated from the slope of the N_2 increase over time (Nielsen 1993).

6.2.2.3 Sulfate reduction

Both sulfide microelectrodes and radioactive isotopes were used in this study to detect sulfate reduction in *D. avara* and *C. reniformis*. The presence of sulfide was tested by use of a sulfide microsensor with a 20 micron tip and a 1 µM detection limit. Additionally, an incubation experiment of sponge explants was performed with $^{35}SO_4^{2-}$ using a modified method of the whole core method (Jörgensen, 1978). Incubation of sponge pieces was carried out in 5 ml syringes filled with 4.5 ml seawater for 0, 10, 30, and 60 minutes. The short incubation times were chosen as immediate reoxidation of the tracer masked sulfate reduction rates in the tissue of other sponges (Hoffmann *et al.*, 2005b). The incubations were performed with air (oxic), with argon (anoxic), and with argon and an extra carbon source which was made of a mixture of acetate, lactate, propionate and butyrate. 50 µL of a 25 mM stock solution of $^{35}SO_4^{2-}$ of 600 KBq adjusted to pH 8 with NaOH and was used to inject into the syringe containing the sponge. Sodium sulfate (10 mM) was also added as carrier for the tracer (Fossing and Jörgensen, 1989). The reaction was stopped after 0, 10, 30, and 60 minutes by adding 20 % zinc acetate. Reduced sulfur species were analyzed with the chromium reduction method (Fossing and Jörgensen, 1989), with the two-step method.

6. 4 Results

6.4.1 Microbial counts and prevalence of the aquiferous system

Visual training allowed the clear distinction between microbial or sponge cells. Bacteria were visible as < 2 µm speckles at 1000 x magnification and as characteristic FISH signals while the sponge cells were approximately 5 µm. Choanocyte chambers

were characterized by the arrangement of the choanocyte nuclei lining a lacuna whereas canals were areas devoid of cells or any other structures (Figure 1 - 3).

Chondrosia reniformis' body was heterogeneous and very dense (Table 1) with few visible canals (Figure 1 a). Two microbial niches were found: the collagen where the bacterial cells were mainly rods, vibrio-shaped and very small (2 - 0.2 μm) and the mesohyl which was characterized by high abundances of microbes, mostly rods of about 2 μm (Figure 1 b). Microbes were not as numerous in the collagen as in the choanosome and, exhibited in the choanosome, distinct clusters or chains of microorganisms of 4 - 16 μm in size (Figure 1 c, d) which were absent in the collagen (Figure 1 e). Thus, bacterial counts were carried out separately for the mesohyl (2.7 $*10^{10}$ cells cm^{-3} sponge) and collagen (0.97 $*10^{10}$ cells cm^{-3} sponge) and proved to be significantly different (chi-square, $p = 0.04$, $\acute{\alpha} = 0.05$, $df = 1$, with Yate's correction). Water canals were absent in the collagen and only in low numbers in the choanosome.

Aplysina aerophoba showed homogenous tissue composition and high tissue density (Figure 2 a, b, Table 1). Sponge cell nuclei were evenly distributed in the mesohyl but less so in the cortex and in inclusions. The mesohyl had canals of various sizes, the choanocyte chambers (4 -20 μm across, average 9-10 μm) (Figure 3 c) and sponge cells were uniformly scattered over the whole area. Sponge associated microbes were densely packed in the mesohyl (Figure 2 d) and were mostly rod-shaped (1 - 1.5 μm), but cocooid and filaments were also present.

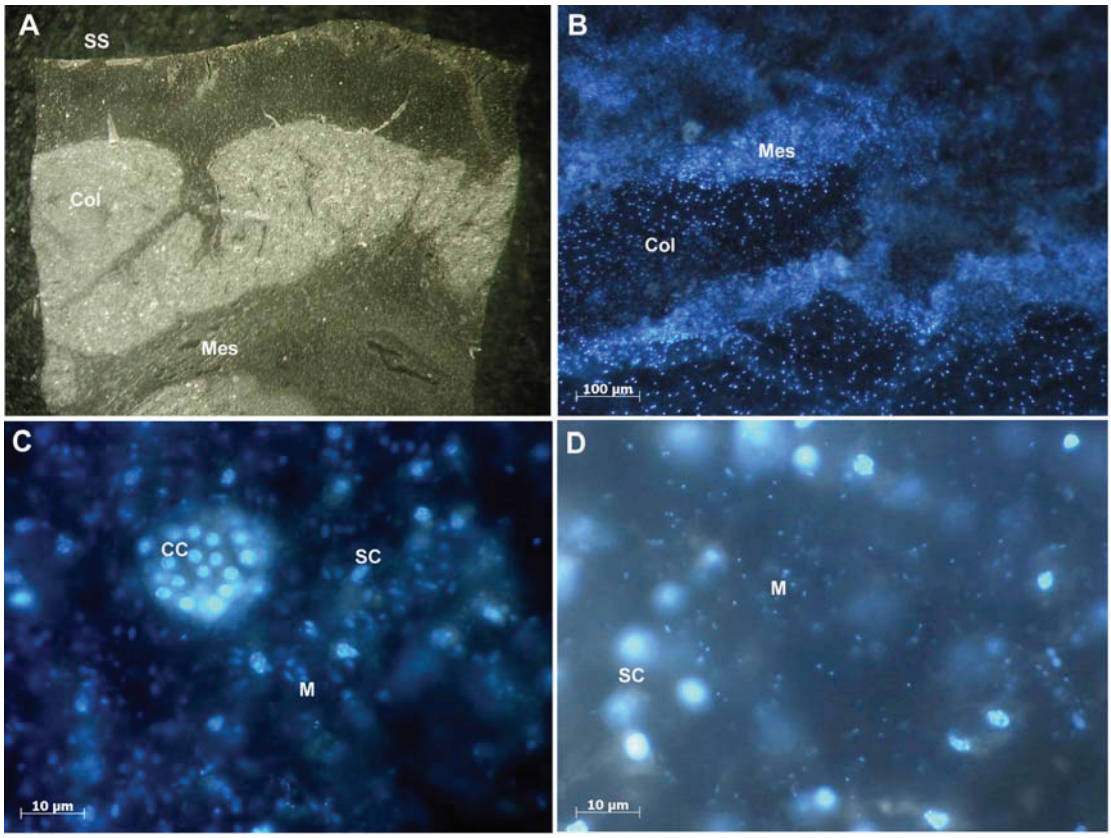

Figure 1: *Chondrosia reniformis* cryo-sections A) general overview, 3.2 x B) mesohyl and collagen overview, DAPI, 100x C) mesohyl detail with choaonocyte chamber, DAPI, 1000x D) mesohyl detail with sponge associated microbes and sponge cell nuclei, DAPI, 1000x. Col = collagen SS = sponge surface Mes = mesohyl. CC = choanocyte chamber SC = sponge nucleus M = microbe

Dysidea avara had a loose tissue composition (Figure 3 a). The choanosome was composed of choanocytes forming a giant complex of associated chambers (Figure 3 b). The bacteria were < 1µm, had a variety of morphologies and were arranged in clumps (Figure 3 c, d). The aquiferous system was most developed in *D. avara* followed by *A. aerophoba* and finally *C. reniformis*, which had densest body (Table 1).

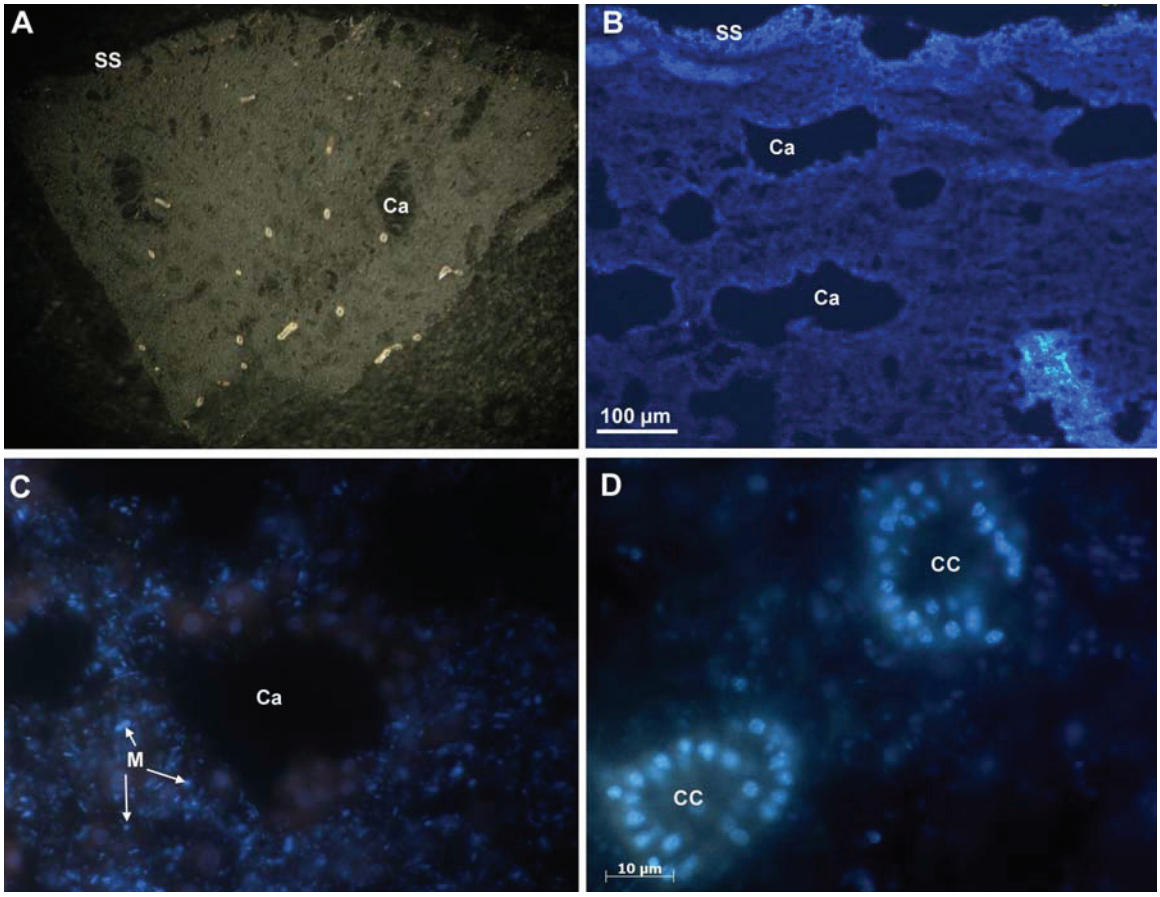

Figure 2: *Aplysina aerophoba* cryo-section a) general overview, 2.5 x b) Overview of the cortex region and of the mesohyl, DAPI, 100 x c) detail of the mesohyl with sponge-associated microbes, sponge cell nuclei and canals, DAPI, 1000x d) detail of the mesohyl with choanocyte chambers, DAPI, 1000x. Col = collagen. SS = sponge surface Mes = mesohyl. CC = choanocyte chamber SC = sponge nucleus M = microbe

Table 1: Average aquiferous system in % of the whole sponge and average number of microbes $*10^{10}$ cm^{-3} sponge ± SEM

	A. aerophoba	*C. Reniformis*	*D. avara*
n =	3	2	4
Aquiferous system % of whole body ± SEM	21 ± 0.7	6 ± 1	47 ± 1
Average number of microbes $*10^{10}$ cm^{-3} sponge ± SEM	3.3 ± 0.09	2.4 ± 0.003	0.09 ± 0.05

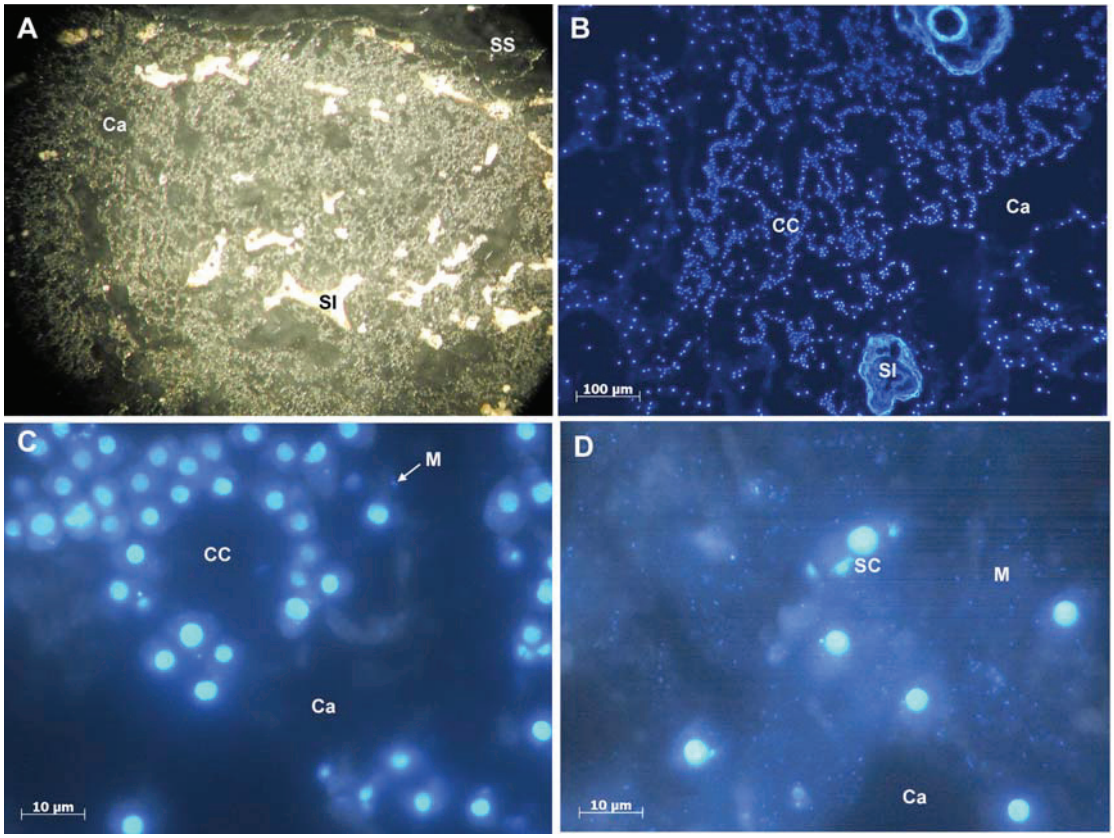

Figure 3: *Dysidea avara* cryo-sections A) general overview, 3.2x B) choanosome with sedimentary inclusions, DAPI, 100x C) choanosome detail with choanocyte chamber, DAPI, 1000x D) mesohyl detail with sponge-associated microbes and sponge cell nuclei. Col = collagen. SS = sponge surface Mes = mesohyl. CC = choanocyte chamber SC = sponge nucleus M = microbe SI = sedimentary inclusion

When the prevalence of the aquiferous system ('porosity') of the sponges was related to the number of microbes in the whole sponges (averages in Table 1), *C. reniformis* and *A. aerophoba* clustered separately but yet in the vicinity of each other while *D. avara* was separated from the HMA sponges (Figure 5 a). The same trend was observed when the porosity of the sponge taken under consideration; consequently, this resulted in higher microbial cell counts per volume (Figure 5 b). There was no direct correlation between porosity and microbe counts but rather two groups; one of HMA and one of LMA species (Figure 5 a, b).

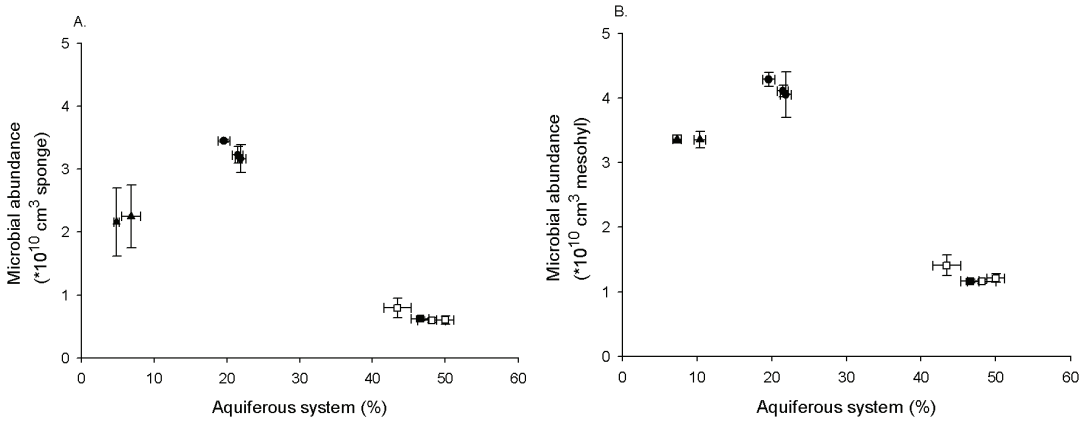

Figure 5: Relation between aquiferous system and number of microbes * 10^{10} a) in the total sponge. Note: the error bars are large because those counts include both microbial niches: collagen and mesohyl b) in the mesohyl. Circles = *A. aerophoba*, Triangles= *C. reniformis*, Squares= *D. avara*. White= Rovinj, Black= Cala Montgo

6.4.2 Microbial processes

The microbial processes of nitrification and denitrification were found in both *D. avara* and *C. reniformis* (Table 2), while anammox and sulfate reduction were not detected. Ammonium, which fueled nitrification, was produced in both study species at very similar rates (Table 2). At the same time, nitrite was excreted by both sponge species but at very low rates (Table 2), which corresponded only to 0.3 and 0.4 % of the ammonium excreted. Nitrate was produced in both species (Figure 6) indicating net nitrification. The rates were about ten times higher than nitrite (Table 2) and corresponded to 4.2 and 5.1 % of the ammonium excreted in *C. reniformis* and *D.avara*, respectively.

Table 2: microbial processes and rates in *Chondrosia reniformis* and *Dysidea avara*

	nmol N cm^{-3} sponge h^{-1}				nmol N cm^{-3} sponge h^{-1}		
	NH_4^+	NO_2^-	NO_3^-	Total net nitrification	$^{29}N_2$	$^{30}N_2$	Total net denitrification
C. reniformis	195.8	0.8	8.0	8.8	4.9	4.9	10
D. avara	241.7	0.8	13.9	14.7	4.9	10	14.9

Anammox being undetectable, a valid way of calculating denitrification rates was to take the slope of the N_2 increase over time of the $^{15}NO_3^-$ incubation experiment (Nielsen, 1993). Denitrification occurred in both study species and two types of N_2 were produced: $^{29}N_2$, resulting from the pairing of ^{14}N+ ^{15}N and $^{30}N_2$, resulting from the pairing of ^{15}N+ ^{15}N (Table 2) (Figure 6). The rates of production of $^{29}N_2$ and $^{30}N_2$ are shown in Table 1. Using random isotope pairing and taking both denitrification and combined nitrification-denitrification into account, the total denitrification rate was 10 and 14.9 nmol N_2 g^{-1} wet weight sponge h^{-1} in *C. reniformis* and *D. avara*, respectively. The net production of N_2 mostly occurred after 4 h of incubation (Figure 7).

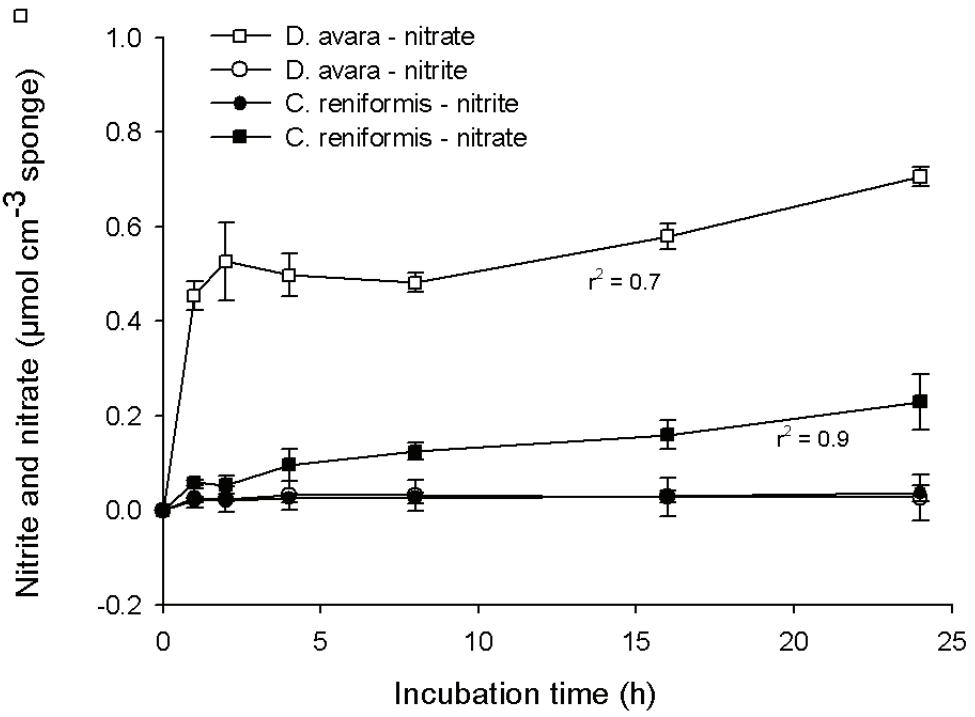

Figure 6: Nitrite and nitrate production over time for *C. reniformis* and *D. avara* when 10 µM $^{15}NO_3^-$ were added at the start of the incubation experiment, with standard error of the mean

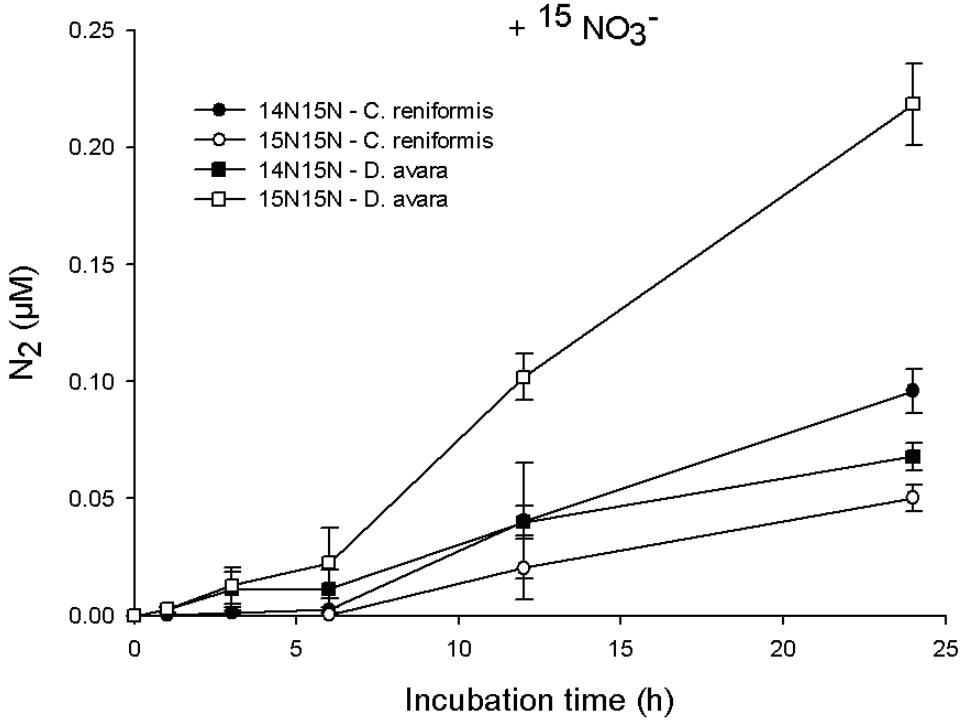

Figure 7: Denitrification in *C. reniformis* and *D. avara* when 10 µM $^{15}NO_3^-$ was added with standard error of the mean

6.5 Discussion

6.5.1 Microbial abundance and prevalence of the aquiferous system

The body of *C. reniformis* had two clearly visible components: collagen and mesohyl which differed enough in their microbial composition to be analyzed separately. *Chondrosia reniformis*' collagen and mesohyl had significantly different amounts of microbes pointing clearly to the existence of different microbial niches within the body of this species. This distinction did not arise in *A. aerophoba* and *D. avara* where the whole body was undoubtedly one single habitat. The occurrence of microbes in the collagen of *C. reniformis* was unexpected and the presence of these two well defined sponge environments holds various implications. From a purely methodological point of view, it is clear that the standard use of 'slurries' to assess microbial abundance in sponges may obscure tissue heterogeneity and would have, in the case of *C. reniformis*, yielded lower estimates. A microbial diversity analysis at the molecular level would possibly yield radically different results according to whether the tissue sample was made of collagen or mesohyl.

Our new method of assessing microbial abundance yields substantially higher counts than the often used 'slurry' method. Friedrich *et al.* (2001) reported microbial abundance of $6.4 \pm 4.6 \times 10^8$ g^{-1} sponge tissue in *Aplysina aerophoba* while we found an average of 3.3 ± 0.09 SEM $\times 10^{10}$. Further, *D. avara* has been qualified of being 'virtually free of bacteria symbionts' in a study done with a transmission electron microscope (Turon *et al.*, 1997) but our results show *D. avara* as having microbes 3 orders of magnitude higher (10^9 cm^{-3} sponge) than seawater (approximately 10^6) and line with sediments (4×10^9 cm^{-3}, Llobet-Brossa *et al*. 1998).

From an ecological point of view, these different tissue types may harbor different microbial communities due to the possible difference in the availability of nutrients, oxygen and space. In *C. reniformis*, the size and the morphotype of microbes was very different in the collagen (0.2 μm, rods, vibrio-shaped) than in the choanosome (rods, 2 μm) and suggest strongly that the two different tissue types have different abilities in the uptake and maintenance of sponge-associated microbes. In view of our results (and using visual training with both FISH and DAPI stains to recognize typical microbes), we feel confident that our method not only yields representative counts of microbes in sponges but also allows the detection of potential microbial niches (i.e. collagen *versus* mesohyl) within sponge tissues.

The inspection of tissue sections of three Mediterranean sponges, *A. aerophoba*, *C. reniformis* and *D. avara* revealed that high microbial abundance (HMA) sponges (*A. aerophoba* and *C. reniformis*) have a considerably smaller aquiferous system than low microbial abundance (LMA) sponges and also denser tissues. *D. avara* (a LMA sponge) in contrast, had a highly developed aquiferous system and far fewer microbes than the two other species (Figure 5 a). These findings are consistent with previous observations and support the idea that two different life strategies exist in sponges (e.g. Vacelet and Donadey, 1977). Our data, moreover, clearly shows that the reason for the high abundance of microbes is not only related to habitat availability (i.e. more mesohyl volume per sponge volume in species where the aquiferous system is reduced) but that the habitat present (sponge mesohyl) is also more densely populated in sponges with a reduced aquiferous system. Within the HMA sponges, however, the relationship is not entirely consistent because *A. aerophoba* had the larger aquiferous

system of the two HMA and the most microbes in the mesohyl. Other unknown factors must be playing a role in determining the number of microbes than the sponge tissue density alone.

The link between anatomy and microbial load has been attributed to the nutritional strategy of the sponge (Vacelet and Donadey, 1977). Species with a less pronounced aquiferous system are thought to farm microbes and subsequently use them as a food source while those species with a well developed aquiferous system (Vacelet and Donadey, 1977), large choanocyte chambers (Boury-Esnault *et al.*, 1990) and higher pumping rates (Weisz *et al.*, 2008) are thought to rely predominantly on capture of particulate organic matter. Our observation of higher microbial counts cm^{-3} mesohyl in HMA species supports the idea of an active accumulation of microbes by the sponge rather than a mere commensal settlement by external microbes. This issue could probably be resolved if the rate of consumption of its associated microbes by the sponge was established but only indirect evidence is available to date.

6.5.2 Microbial processes

We postulated earlier that higher count of microbes in dense sponges could result in the presence of a larger variety of key microbial processes and, if the microbes are active, of higher rates. In this study, we found that both *C. reniformis* (a HMA species) and *D. avara* (a LMA species) both have nitrification and denitrification rates while anammox and sulfate reduction were not detected. Furthermore, nitrification, denitrification and ammonium production were highest in *D. avara*, the LMA species (Table 2).

The absence of sulfate reduction was surprising. Anoxic conditions are known to occur in both *C. reniformis* (Hoffmann and Schläppy submitted) and in *D. avara* (Schläppy *et al.*, 2007) and sulfate reducers were found through the use of a specific FISH probe in *C. reniformis* (Schumann-Kindel *et al.*, 1996). One possible explanation is the rapid re-oxidation of the reduced sulfide which has been shown to occur in another sponge species (Hoffmann *et al.*, 2005b). Alternatively, sulfate reduction may be inhibited through the production of nitrite and nitrate by the nitrifiers, which fuel high rates of denitrification (as measured in this study) and out-compete sulfate reducers. Finally, it is possible that sponges can specifically inhibit sulfate reduction to prevent accumulation of toxic sulfide.

Nitrite and Nitrate were produced in both *C. reniformis* and *D. avara* indicating that the nitrifiers were active. The two nitrification steps (NH_4^- to NO_2^- to NO_3^-) were present and reveal the presence and activity of both ammonium oxidizing and nitrite oxidizing microbes (only very little nitrite was detected). Nitrification has been shown by Bayer *et al.* (2007) for *D. avara* and *C. reniformis* but denitrification (which occurred in partially oxic seawater) is shown here for the first time in Mediterranean sponges. The only other evidence of denitrification in sponges is in *Geodia barretti*, a cold water species (Hoffmann unpublished data), with 100 nmol cm^{-3} day, equals 4 nmol cm^{-3} h^{-1}. Our rates are double those found in *G. barretti*, with 10 and 14.9 cm^{-3} nmol h^{-1}. The percentage of nitrate which is reused for denitrification is 1/2 in our species while it is only 1/6 in *G. barretti* (Hoffmann, pers. com.). While we can not totally exclude that the sponge pieces used for the denitrification experiment were dying, and thus gave a false positive denitrification rate, we feel confident that it was

not the case. Had it been so, we would then have found sulfate reduction rates in the sulfate reducing experiment, which used a very similar incubation method.

The total net nitrification rates reported in this study are in the range of those reported by other authors for our target species although there are highly variable (Table 3). Those results do not take into account the anaerobic loss of nitrate through denitrification, are only indicative of net nitrification and are definitely an underestimate of total nitrification in these sponges. The presence of denitrification may explain some the variability in the nitrification results. Our rates of denitrification are very close to the nitrification rate which indicates that approximately half the nitrate produced by the sponge is reused for denitrification. The nitrifiers are removing only a small portion (about 5 %) of the ammonium produced, the rest being presumably expelled trough ventilation by the sponge. The rather large ammonium production may not only be a product of sponge respiration but also of fermentation processes in times of anoxia.

Table 3: Original net nitrification rates converted into other units for comparison purposes assuming dry weight is 10 % of wet weight and assuming 1.2 g wet weight = 1 cm^{-3}

Target spp.	This study (µmol g^{-1} wet weight sponge day^{-1})	Bayer *et al*. 2007 (µmol g^{-1} wet weight sponge day^{-1})	This study (nmol g^{-1} dry weight- h^{-1})	Jiménez *et al*. 2007 (nmol g^{-1} dry weight- h^{-1})	Bayer et *al*. 2008 (nmol g^{-1} dry weight- h^{-1})
C. reniformis	0.176	0	73.33	702	*A. aerophoba* 89-344
D. avara	0.294	approx. 0.3	122.5	0	

The fact that both nitrification and denitrification occur simultaneously was apparent in the comparatively high rate of $^{29}N_2$ produced. It could only stem from unlabeled NO_3^-, the source of which can either originate from the seawater in which the sponge

were incubated in or, more likely, from the nitrification activity of sponge-associated microbes.

This co-occurrence of two microbial processes may be similar in the other species of sponges for which nitrification rates have been reported. For example, *Aplysina aerophoba* produces a range of nitrate concentrations (Bayer *et al.*, 2008) and definitely has anoxic zones within its tissue when not pumping (Hoffmann *et al.*, 2008). Temporal and spatial occurrence of oxic and anoxic zones within the sponge would favor both the nitrifiers and the denitrifiers, in turn. The occurrence of nitrification and denitrification suggests strongly that the microbial community inside our study species is able to deal with shifts from aerobic to anaerobic conditions inside the sponge. Furthermore, the co-occurrence of nitrification and denitrification might explain the variations in NO_3^- production seen in some species (Bayer *et al.*, 2008).

We can interpret our results in several ways. Both HMA and LMA sponges host an active community of microbes with various physiologies, including anaerobic microbial processes. Total rates of microbial processes in LMA sponge can be even higher than in HMA sponges. The main benefit to the sponge of having such a guild of microbes may reside in the fact that they efficiently get rid of ammonium and nitrite (which could be deleterious to the sponge) and that nitrification may inhibit sulfate reducing bacteria. The sponge, by controlling its pumping activity, has a means of controlling the activity of both the nitrifiers and denitrifiers populations. It is also possible that the presence of heterotrophic denitrifiers provides the sponge with a mechanism for food storage, if it actually does feed on their associated microbe

(which is still to be proven). Our results show that regardless of whether a sponge hosts many or few microbes, they are active and metabolically diverse. Possibly, the HMA sponges may be able to derive a larger benefit from their associated microbes than LMA sponges.

6.6 Acknowledgments

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Chapter 7

7. General discussion

Sponges are ancient animals, the first multi-cellular organisms on the tree of life (Ruppert and Barnes, 1994) and are simultaneously extremely simple and very complex. Much of their biology and physiology is yet to be understood because research focus was put elsewhere (Chapter 1). The interest in their bio-active compounds has given rise to the need for sustainable sponge aquaculture but our knowledge of sponge biology and physiology is still insufficient to successfully breed sponges in captivity.

In this context and to clarify some of the still puzzling aspects of sponge biology, the focus of this thesis was 1) to characterize the micro-chemical environments (oxygen) and ventilation behavior present in the target species using micro-electrodes and 2) to assess the presence of key aerobic and anaerobic microbial processes in the target species. The knowledge gained through these investigations increases our limited understanding of sponges, of their associated microbes and can be used for optimizing sponge aquaculture systems.

Sponges are filter-feeders and have the ability of pumping enormous amounts of water through their body. The knowledge that sponges are able to pump large amounts of water (see Chapter 1) has led to the assumption that their body is well oxygenated at all times. Our findings show that sponges which were pumping displayed tissue oxygenation which was close to those of the oxygen saturated water while those specimens which had

stopped ventilating had a clear boundary layer at their surface and oxygen penetrated only through diffusion up to 0.5 mm into the sponge (Chapter 4 and 5). Further, we showed that pumping activity explained only 17 % of the oxygenation in the sponge body (Chapter 4) pointing clearly at other causes for oxygenation (Chapter 5). These results might have been a pure artifact of the sponges being in captivity as it is a known fact that they have reduced pumping activity in laboratory conditions (see Parker (1910), Jørgensen (1955)). It was thus essential to establish whether tissue anoxia could also be found in undisturbed sponges in their natural environment and this was clearly demonstrated in Chapter 5. When oxygen profiles across the sponges where carried out in the lab and in the field a very heterogeneous picture emerged but with a tendency for better oxygenation close to the target osculum (Chapter 5). We also showed that a small sponge without an osculum (and presumably without a functional aquiferous system) was unequivocally dependent on ambient flow for tissue oxygenation while the oxygenation of larger sponges with several oscula changed only little in presence of varying ambient flow (Chapter 5), probably due to its ability to control ventilation.

The discovery of anoxic zones together with the fact that both species harbor more microbes than seawater (Chapter 6) opened the possibility that anaerobic microbial processes exist in our target sponge species. This was confirmed by the discovery of denitrification in *D. avara* and *C. renformis*. This is the first report of denitrification in Mediterranean sponges. This together with proof of nitrification (Chapter 6), an aerobic microbial process, reveals a simple nitrogen cycle in those species: ammonium is produced in excess. While 95 % of it is simply discarded through ventilation 5 % is

converted into nitrate through microbial ammonium and nitrite oxidation (i.e. nitrification). Half of the nitrate thus produced is anaerobically lost to N_2 through the activity of denitrifiers.

7.1 Conclusion

The findings derived from this work brings novel findings to sponge research, namely a clear link between the sponges' body oxygenation in the field and in the laboratory and their microbial processes (Figure 1). The spatially and temporally heterogeneous patterns of oxygenation in the two study species together with their large amount of associated microbes (Chapter 6) have several implications, some of which may be of use to sponge aquaculturists. Firstly, oxygenation in the target species is complex and heterogeneous in space and time. Small sponges or explants (often used in sponge culture) and which depend on ambient flow must be kept in an appropriate flow regime to avoid permanent anoxia in their tissue. For larger sponges this plays only a smaller role and fluctuating ambient flow can be compensated by the sponge ability to modulate its ventilation.

Secondly, the changing oxygenation conditions allow these sponge species to conserve both anaerobic and aerobic microbes which can perform 'services'. For example, nitrifiers remove a portion of the ammonium produced by the sponge and also nitrate produced by the ammonium oxidizers. Our target species most probably benefit from the sulfate reducers being inhibited by the activity of the nitrifiers.

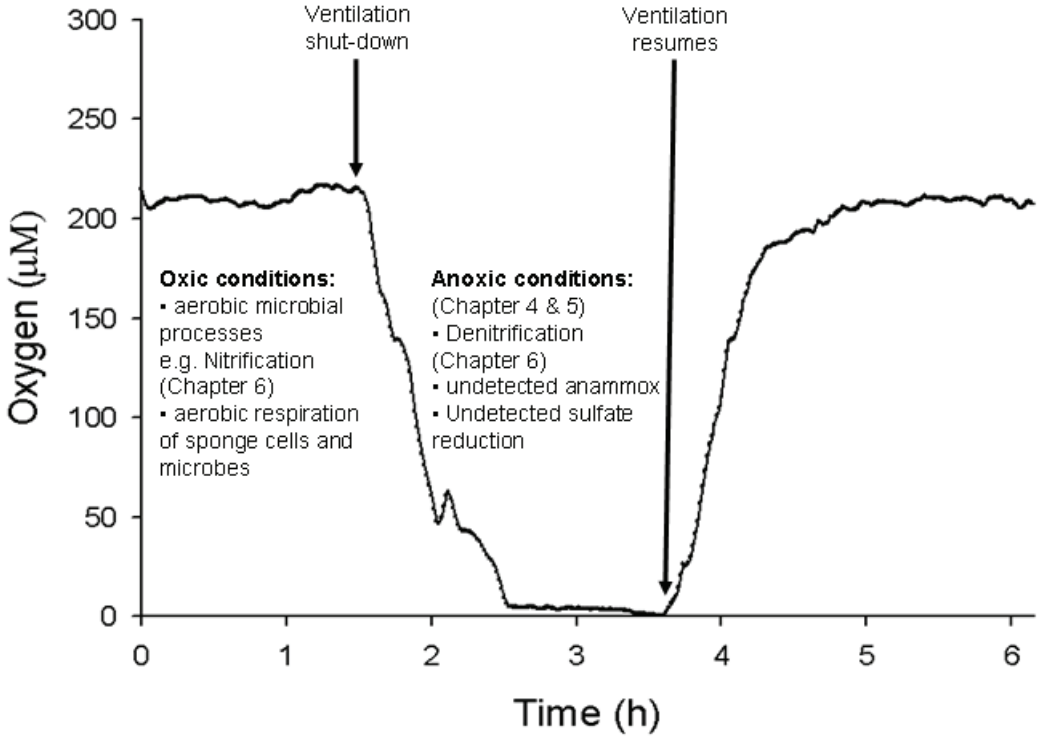

Figure 1: Schematic diagram showing the main findings of this research and how they articulate together

The denitrifiers remove nitrate and may also take up dissolved compounds originating from the sponge or from the seawater and transform them into microbial biomass. Subsequently, they may serve as a food source for the sponge. A similar mechanism has been proposed by Hoffmann *et al*. (2005) but this still needs to be ascertained for our study species. Our rates of nitrification and denitrification (Chapter 6) unequivocally prove that active populations of ammonium and nitrate oxidizers are present and this not only in the high microbial abundance species (*C. reniformis*). Under unpredictably changing oxygenation conditions, those populations can co-exist within the sponge

(Chapter 6). Additionally, the periods of tissue anoxia may allow the sponge to control (unwanted) microbes acquired by filtration (see this topic in Chapter 3).

7.2 Outlook

While this work constitute a good start in explaining the relationship between ventilation behavior and oxygenation of sponge tissue, a systematic investigation of the triggers to pumping is still needed (Figure 1, see arrows). The reasons for stopping or resuming to ventilate are unidentified and whether the aerobic and anaerobic microbial processes have an influence on the ventilation is unknown. Furthermore, the microbial processes discovered in those two sponge species may not be representative of what happens in other sponges, so a larger number and variety of sponges should be selected and their aerobic and anaerobic microbial processes studied. Finally, the direct link between the sponge and its microbes can still only be speculated on. A meaningful way to continue this research would be to establish whether or not sponges use their associated microbes for additional nutrition.

For all these reasons, I propose three meaningful ways to continue this research:

1. To establish which factors trigger the shutting down or restoration of ventilation
2. To establish which are the microbial processes in other species of sponges
3. To find out whether sponge derive additional nutrition from their associated microbes

These new research questions, if resolved, would bring us one step further towards understanding whether the sponge-microbe relationship is simply a matter of opportunity

or if it is a symbiosis with design. It would also allow us to design better aquaculture systems for the large production of sponge biomass for pharmaceutical purposes. This work is a stimulating starting point for further research on these ancient yet enigmatic animals.

7.3 References

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